

A Proteomic Study of Innate Immune Protection in the
Tammar wallaby (*Macropus eugenii*)

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Comparative Biochemistry and Physiology, Part D: (2006) 1, 283- 291.

Kiran S. Ambatipudi, Julie M. Old, Michael Guilhaus, Mark Raftery, Lyn Hinds and Elizabeth M. Deane

Chapter 3

In search of neutrophil granule proteins of the tammar wallaby (*Macropus eugenii*).

Molecular Immunology: (2008) 45, 690-700

Kiran S. Ambatipudi and Elizabeth M. Deane.

Chapter 4

A proteomic approach to analysis of antimicrobial activity in marsupial pouch secretions.

Developmental and Comparative Immunology: (2008) 32, 108-120

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Comparative Biochemistry and Physiology, Part D: (2007) 2, 322-331

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Summary

This study has taken a proteomics approach to investigating two aspects of innate immune protection in a model marsupial the tammar wallaby, *Macropus eugenii*. The proteins of neutrophils and their granules have been documented using two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS). The first step in this project required development of protocols for the effective isolation of neutrophils and their granules. Fifty three abundant proteins were initially identified from neutrophils and subsequently a range of protocols including stimulation with PMA, Ionomycin and calcium as well as differential centrifugation and cell lysis were used to isolate granule proteins. Five antimicrobial proteins of granule origin were identified along with a number of proteins associated with the process of exocytosis. The identification of these proteins from the neutrophils in the tammar wallaby clearly shows the degree of conservation of such proteins across different mammal species.

The second portion of this project was aimed at examining the unique nature of the marsupial pouch in protecting the young immunologically incompetent animal. Pouch secretions collected at major stages of the reproductive cycle showed varying levels of antimicrobial activity primarily against Gram negative *E. coli* but not against the Gram positive *S. aureus*. Greatest antimicrobial activity was observed in samples collected at oestrus, the anticipated time of birth of the young animal. Subsequent proteomic analysis, using 2DE and LC-MS/MS, led to confident identification of a range of peptides matched to β -lactoglobulin. As the likely origin of β -lactoglobulin could be mammary gland or

digested products from the gut of the pouch young, samples from these sources were also analyzed. In parallel with this portion of the study the changes in the skin proteome (the secretome) of the pouch were investigated through proteomic analysis of secretions from pouch skin of immature, mature reproductively active and post-reproductive females and from a non-pouch skin site. A limited number of proteins could be reliably identified although clear differences in the patterns of secretion were observed at these different life stages. Of the proteins that could be identified, globins were present at all stages with dermcidin, a known potent antimicrobial identified in an opportunistic sample collected from a common wombat, *Vombatus ursinus*. Limited though the successful identification of proteins secreted into the pouch has been, this project has clearly shown that (i) secretions from this site are unique (ii) the types of proteins secreted vary dependent on the reproductive maturity of the female and (iii) there is demonstrable antimicrobial activity against Gram negative organisms although the active component could not be specifically identified.

Declaration

The work presented in this thesis, to the best of my knowledge, is original except where acknowledged in the text. I hereby declare that I have not submitted this material, in whole or in part, for a higher degree at this or any other University or institution.

Kiran S Ambatipudi
June 2007

Contribution of Authors

Ambatipudi is the lead author and undertook all the experimental protocols and extensive planning. Training in use of the instrumentation and advice on analysis of MS data was provided by Raftery and Guilhaus. Hinds provided help, access and advice on marsupials as did supervisors to this project, Old and Deane. For the two papers on which Joss is an author Paper 3, Joss undertook the milk and gut proteomics (Gels +MS) and for Paper 4 – the wombat skin gel.

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*A small thing in return to my parents for sacrificing
so much about which they knew very little*

Chapter 1

Introduction – Literature Review and Project Rationale

1. Why study marsupial mammals?

Mammals are believed to have originally evolved around 300 million years ago from a branch of reptiles called Synapsids (Musser, 2003). During the Mesozoic era, early mammals underwent divergence and developed into the three groups of mammals alive today - the Prototheria (monotremes), the Metatheria (marsupials) and the Eutheria (commonly called placental mammals). Marsupials are distinguished from other 2 groups primarily by differences in their reproductive anatomy and associated reproductive strategies, the latter reflecting of an increasing level of maternal investment in gestation. Prototheria have a reptilian-like dorsal outlet for the uteri and lay eggs, metatheria have two uteri and lateral vaginae and give birth to altricial young, while eutheria have one uterus and one vagina and give birth to relatively mature young (Tyndale-Biscoe, 1973).

Marsupials, the focus of this study, are generally born after a short gestation period ranging from 11 days in the striped faced dunnart (*Sminthopsis macroura*) to 28 days in the tammar wallaby (*Macropus eugenii*) with the longest gestation period of 35 days observed in the koala (*Phascolarctos cinereus*) (Tyndale-Biscoe and Renfree, 1987). As a consequence of this short gestation, a number of the major organ systems of the newborn marsupial are underdeveloped and the major period of development and maturation of these systems occurs in the external maternal pouch (the *marsupium*) (Old and Deane, 2000). This feature of marsupial biology offers a unique opportunity for accessible developmental studies which is not readily available in eutherian mammals. Of particular interest is the development of the immune system of the young animal. In contrast to eutherians where

major development of the immune system occurs in a sterile intra-uterine environment, in marsupials this development occurs in an environment laden with antigenic challenge and potentially pathogenic micro-organisms (Yadav et al., 1972a; Charlick et al., 1981; Old and Deane, 1998). Thus marsupials afford not only the opportunity to study development but are also a unique system to examine the strategies used to protect an immunologically incompetent young mammal.

2. The Organisation and Function of Adult Marsupial Immune System

2.1. Organs, Tissues and Cells

Adult marsupials possess an array of primary and secondary lymphoid tissue similar to their eutherian counterparts (Basden et al., 1996; Basden et al., 1997). There are, however, two reported areas which warrant special comment – the thymus and the lymph nodes. Some marsupials, the Polyprotodonts, possess only a thoracic thymus, whilst most Diprotodonts, except for the Vombatidae, possess both a cervical and thoracic thymus (Old and Deane, 2000). The biological rationale for these differences is still a matter of conjecture, the most widely accepted being that it is related to different diets and potential exposure to antigens via oral exposure (Yadav, 1973; Ashman and Papadimitriou, 1975a). Marsupials all possess a distributed system of lymph nodes, although the literature raises some ambiguities about the actual number and distribution in the gut associated tissue. For example early studies of different species of American opossums claimed that both the white-eared opossum (*Didelphis azare*) and common opossum (*Didelphis marsupialis*) lacked significant numbers of lymph nodes in the small and large intestine (Azzali and Di Dio, 1965). In contrast, other studies documented 27 mesenteric lymph nodes in the pouchless opossum (*Marmosa mitis*), 44 in white-eared opossum (Coutinho et al., 1990) and 45 in Virginian opossum (*Didelphis virginiana*) (Bryant and Shifrine, 1974). In both

the koala and the tammar wallaby there have also been reports of low numbers of lymph nodes in the mucosal immune tissues of adults (Hemsley, 1996; Basden et al., 1997).

Within the lymphoid tissue beds and in blood similar cellular components have been observed as those reported for more well studied eutherian mammals, although limited availability of reagents such as antibodies to lymphoid cell markers has defined the extent of these studies (Canfield and Hemsley, 2000). Blood cell types, morphology and counts have been documented for a number of wild and captive populations of marsupials (Hogarth, 1974; Presidente, 1978; Hawkey et al., 1982) and a number of studies have documented changes in peripheral blood cell populations as a function of seasonality, age, gender and in health and disease (Giacometti et al., 1972; Presidente, 1978; Barnett et al., 1979; Baker and Gemmell, 1999; McKenzie et al., 2002; Young and Deane, 2006).

Histological and, in particular, immunocytochemical studies with antibodies to the lymphoid cell surface markers CD3, CD5 and CD79b have been able to document the presence and distribution of mature T and B cells in peripheral blood and the tissue beds of the spleen, lymph nodes, thymus and mucosal associated lymphoid tissues.

Immunocytochemical techniques have been successfully used to detect the presence of T and B cells in the tammar wallaby (Hemsley et al., 1995), the eastern grey kangaroo (*Macropus giganteus*) (Old and Deane, 2001), white-eared opossum (Coutinho et al., 1994), the koala (Canfield et al., 1996), the brushtail possum (Baker et al., 1999b), the ring tail possum (*Pseudocheirus peregrinus*) (Hemsley et al., 1995), the Brazilian white-belly opossum (*Didelphis albiventris*) (Coutinho et al., 1995), the northern brown bandicoot (Old and Deane, 2002), the long-footed potoroo (*Potorous longipes*), the long nosed potoroo (*Potorous tridactylus*) and the rufous hare-wallaby (*Lagorchestes hirsutus*) (Young and

Deane, 2003). All these studies support a strong commonality of structure of the immune system of marsupials with that found in eutherian mammals.

2.2. The Molecules of Immune Function

2.1. Molecules of Recognition – Immunoglobulins, the MHC and CD (surface) markers

Marsupial immunoglobulins have been extensively studied. Early work used protein purification and antibodies to identify IgM and IgG in a range of marsupial species including in the tammar wallaby and the grey kangaroo (Deane and Cooper, 1984), the brushtail possum (Ramadass and Moriarty, 1982), the Virginian opossum (Rowlands and Dudley, 1968), the quokka (*Setonix brachyurus*) (Bell et al., 1974a) and the koala (Wilkinson et al., 1991). Similar approaches were also used to document IgA in the milk of the tammar (Deane and Cooper, 1984), the brushtail possum (Ramadass and Moriarty, 1982) and the quokka (Bell et al., 1974b).

The characterisation of marsupial immunoglobulins (Ig) genes has provided a clear indication of the repertoire of Igs available in marsupial systems and their similarity with those present with their eutherian counterpart. With the exception of IgD, the isotypes IgM, IgG, IgE and IgA have all been identified in marsupials (Miller and Belov, 2000). The constant region of the heavy chains C μ and C α were first reported from opossums (Miller et al., 1999) and subsequently other constant regions of C ϵ and C γ , including C μ and C α , have been isolated from the brushtail possum (Belov et al., 1998; Belov et al., 1999b; Belov et al., 1999a). Initial techniques of immunoelectrophoresis were used to detect the presence of two light chains, Ig κ and Ig λ , in quokka (Bell et al., 1974a) and these were subsequently identified in the opossum (Lucero et al., 1998; Miller et al., 1999).

A similar progression in understanding is seen in documenting the important recognition molecules of the Major Histocompatibility Complex (MHC). Here early work in this area relied on functional studies and reported a possible lack of diversity in MHC Class II, in particular (McKenzie and Cooper, 1994). These claims led to long held beliefs that marsupials are evolutionarily less advanced (Wilkinson et al., 1992a; Jurd, 1994). However recent application of the tools of molecular biology and bioinformatics analysis of the opossum genome have quite clearly shown that marsupials have essentially the same repertoire of these genes and presumably the capacity to recognise and respond as eutherian mammals. Recent analysis of the opossum genome has documented the organisation of the MHC class I, II and III genes in opossums. This work has shown that although the marsupials possess MHC Class I, II and III, there are some differences in the organisation of these genes. Specifically Class I and II genes are interspersed and the framework region contains only framework genes, whilst in eutherians this region includes Class I genes. The organisation of these elements is similar to that found in birds and amphibians (Belov et al., 2006)

As indicated previously, a limited number of cell surface molecules involved in recognition and regulation have been identified using molecular protocols (Canfield and Hemsley, 2000). To date, the major immunohistochemical studies have relied on a limited collection of commercially available cross reactive antibodies to identify CD3, 5 and 79b (Hemsley et al., 1995). In addition, the epsilon chain of CD3 has been isolated in the tammar wallaby (Old et al., 2001) and most recently CD4 has been extensively characterised (Duncan et al., 2007). This is an area of research that requires much more extensive work but this will be helped significantly by the increasing sequence data available from the opossum genome sequencing project (http://www.ensembl.org/Monodelphis_domestica/index.html).

2.2.2. Regulatory Molecules – the Cytokines

Cytokines are a group of small secreted glycoproteins which play a major role in host immune response, inflammation and haematopoiesis (Gooding, 1992; Schwarzmeier, 1996). To date, eleven cytokines, and one receptor, have been identified in marsupials, some using extensive laboratory based protocols (Harrison and Wedlock, 2000), others most recently involving a bioinformatics analysis of the opossum genome (Belov et al., 2006). These cytokines include tumour necrosis factor alpha (TNF- α) (Harrison et al., 1999; Wedlock et al., 1999a), lymphotoxin (LT) - α and - β (Harrison et al., 1999; Harrison and Deane, 1999), Interleukin (IL-1 β) (Wedlock et al., 1999b), IL-1R2 (Harrison and Wedlock, 2000), IL-5 (Hawken et al., 1999), IL-10 (Wedlock et al., 1998), leukaemia inhibitory factor (LIF) (Cui and Selwood, 2000), three type 1 Interferons (Harrison et al., 2004) and a possible Interferon γ (Higgins et al., 2004). Apart from these studies focussed on identification, little has been done to investigate the circumstances leading to expression and functioning of these molecules in marsupial systems.

2.3. Functioning of the Adult Immune System

Studies on the function of the marsupial immune system have been patchy. A number of whole animal immunisation studies undertaken in the 1960's and 70's, demonstrated the capacity of the adult immune system to generate B-cell responses, albeit somewhat delayed and at low antibody titres. Specifically low secondary and anamnestic responses were observed in the grey short-tailed opossum (La Via et al., 1963; Rowlands et al., 1964), quokka (Stanley et al., 1972) and the koala (Wilkinson et al., 1992a) in response to exposure to *Salmonella typhi* flagella and bacteriophages. *In vitro* studies indicate of capacity for cellular immune response, have reported abnormally low levels of activity. Proliferative responses of lymphocytes to mitogens and to culture with allogenic

lymphocytes have been undertaken in a number of different marsupial species, including the quokka (Ashman et al., 1972), grey short-tailed opossum (Infante et al., 1991) and the koala (Wilkinson et al., 1992b) under a variety of culture conditions. The koala demonstrated delayed peripheral mononuclear cell (PMC) responses to a range of mitogens (Wilkinson et al., 1991). However, the production of a growth factor similar in behaviour to interleukin-2 (IL-2) suggested comparability with eutherian species with respect to T-cell response (Wilkinson et al., 1992a). Although PMC cells did not proliferate when exposed to a range of specific antigens, when 2-4- Dinitrofluorobenzene (DNFB) was applied to skin of the animal, delayed type hypersensitivity responses were observed (Wilkinson et al., 1992b). These studies led the authors to conclude that koalas had sluggish immune responses. Reflective of this historical perception of the unusually low functioning of the marsupial immune system was a review by Jurd (1994) entitled “Not proper mammals”. Recent studies however have sought to address the basis of these perceptions. An exploration of different culture protocols of tammar wallaby leucocytes would suggest that these reports may primarily be due to problems in defining the appropriate culture conditions for marsupial cells (Young and Deane, 2005).

3. The Development of the Marsupial Immune System

Histological studies of the development of lymphoid tissues and attainment of immune competence has been described in a number of marsupials, in varying levels of detail. These studies include the quokka (Yadav et al., 1972b; Ashman and Papadimitriou, 1975b), the Virginian opossum (Block, 1964; Cutts and Krause, 1980), the tammar wallaby (Basden et al., 1996; Basden et al., 1997) and the stripe-faced dunnart (Old et al., 2003a; Old et al., 2004) and to a limited extent in the grey short-tailed opossum (*Monodelphis domestica*) (Hubbard et al., 1991). In all these studies the pathways of development are essentially the

same as that observed in eutherian mammals, with the major variations being not in the sequence of events but in the actual time postpartum that they occur (Old and Deane, 2000).

In eutherian mammals, the yolk sac is the original haematopoietic organ. During embryonic development this shifts to the liver and spleen and, towards the end of gestation, to bone marrow (Roitt, 1991). In marsupials a similar developmental pattern is seen but differs in that at the time of birth the liver is the major haematopoietic organ with a shift to the bone marrow occurs about one to two months post partum. This has been consistently reported for the tammar wallaby (Basden et al., 1996) the quokka (Yadav, 1972; Ashman and Papadimitriou, 1975a), the opossum (*Didelphis Virginiana*) (Block, 1964) and stripe-faced dunnart (Old et al., 2004).

The appearance of different leukocyte population in the blood is a useful indicator of the early pathway of development in marsupial. In the neonatal tammar wallabies and other marsupials the blood consists primarily of nucleated, immature erythrocytes but also contains mature and immature neutrophils, platelets, and larger cells resembling stem cells (Basden et al., 1996). By day 2, small numbers of eosinophils become apparent, but interestingly neutrophils remain the dominant white cell type. Lymphocytes are first observed in tammar wallaby blood between day 5 to 6, in the quokka between days 3 to 4, in the stripe-faced dunnart by day 31 (Godfrey, 1969) and in the opossum between days 6 to 7 (Yadav et al., 1972c; Basden et al., 1996). The number of agranulocytes exceeds the number of granulocytes with an adult leukocyte profile achieved at about 60 days postpartum in the opossum, quokka and tammar wallaby (Block, 1964; Yadav et al., 1972b; Cutts and Krause, 1980; Basden et al., 1996).

Histological studies have documented the development of primary and secondary lymphoid tissue in the tammar (Basden et al., 1996; Basden et al., 1997), stripe-faced dunnart (Old et al., 2004) and the quokka (Yadav et al., 1972c; Ashman and Papadimitriou, 1975a).

Immunohistochemical techniques have been able to identify the appearance of mature T- and B-cells absent in the liver, bone marrow, thymus, spleen, gut associated and bronchial-associated lymphoid tissue during the early life of the young tammar wallaby (Old and Deane, 2003). The lymph nodes of the tammar, the quokka and the Virginian opossum first contain lymphocytes between days 4 and 7 after birth (Block, 1964), with differentiation into cortex and medulla in the Virginian opossum at days 10-12, day 14 in the quokka, day 12 in the stripe-faced dunnart, and day 30 in the tammar wallaby (Basden et al., 1997; Old and Deane, 2000). Specific T- and B-cell populations were detected using cross reactive antibodies to CD3 and HLA-DR and CD79 a and b in the spleen of white-eared opossum (Coutinho et al., 1994), the koala (Canfield et al., 1996), the brushtail possum (Baker et al., 1999a) and the tammar (Old and Deane, 2003), the appearance of such mature cells signalling the capacity to mount an active immunological response.

In a similar manner, the pathways of development of the spleen have been documented. Myeloid tissue was first observed at day 4 in the Virginian opossum (Block, 1964), at days 5-7 in the quokka (Basden et al., 1997), day 11 in the stripe-faced dunnart (Old et al., 2003b) and day 12 in the tammar (Basden et al., 1997) with distinct differentiation and appearance of T- and B-cells seen at 48 days in the white eared opossum (Coutinho et al., 1994) and brushtail possum (Baker et al., 1999a) and at day 50 in the tammar and complete differentiation into adult-like tissue by day 60 (Basden et al., 1997).

The last tissues to achieve adult-like structure and appearance for all marsupials studied have been the mucosal associated lymphoid tissues of the gut (GALT) and lungs (BALT). Although these studies have been limited, the development of the GALT has been documented in a number of metatherians with the appearance of lymphocytes in the gut by day 21 in the tammar wallaby (Tyndale-Biscoe and Renfree, 1987; Basden et al., 1997), day 31 in stripe-faced dunnart (Godfrey, 1969) and day 1 in the brushtail possum (Lyne et al., 1959). Similarly there are few reports on the development of BALT. In the stripe-faced dunnart and the tammar wallaby only single dispersed cells have been observed in these tissues in older pouch young (Old et al., 2003a).

3.1. Development of Functional Capacity

At the time of birth the young animal has no functional lymphoid tissue and capacity to mount an immunological response parallels the development of the tissues. Non specific inflammatory responses are the first step in capacity to respond to external threat. In the grey short tailed opossum an inflammatory response to skin damage is not observed until 6 days post partum and in the quokka until 8 days of age (Rowlands, 1970). Similarly, Block (1960) reported that the Virginian opossum was unable to mount an inflammatory response to wounds when less than 6 days old presumably due to lack of mature haematopoietic tissue (Block, 1960).

Studies of responses to immunisation have demonstrated the capacity of the immune system to produce antibodies to more diverse antigens as the animal ages (La Via et al., 1963; Rowlands et al., 1964; Rowlands et al., 1972; Yadav et al., 1972b; Ashman and Papadimitriou, 1975a). The production of specific antibodies to an injected antigen appears to coincide with the appearance of lymphocytes in the thymus, spleen and lymph nodes

(Rowlands et al., 1972; Yadav, 1972; Yadav et al., 1972c; Yadav, 1974; Coleman et al., 1991).

A number of transplantation experiments have been undertaken in young marsupials to document the development of cell mediated immunity, including in the quokka, the opossum and the tammar wallaby (LaPlante et al., 1966; Yadav, 1974; Walker and Tyndale-Biscoe, 1978; Rodger et al., 1985; Stone, 1997). These have largely been limited to studies on the impact of thymectomy on capacity to respond. In the Virginian opossum rejection of maternal skin transplants was not observed till 12 days post-partum, whilst in the quokka the rejection of transplanted thymus was not seen till 30 days post-partum (Ashman et al., 1975). Thymectomy in the Virginian opossum at 10-12 days post-partum resulted in reduction of lymphocytes in the tissues and peripheral circulation (Miller et al., 1965). Similarly, removal of cervical thymus in the quokka before day 5 (Ashman and Papadimitriou, 1975b) or day 10 (Yadav, 1974) lead to a decrease in the peripheral lymphocytes, but had little impact on the immune function. However, cervical thymectomy in the quokka delayed the onset of humoral response, but had no effect on graft rejection (Stanley et al., 1972).

4. Protection of the neonatal marsupial

It is clear from the previous description of the pathways of development of the young marsupial that there is an extensive post natal period during which the young animal needs to be immunologically protected. A number of possible maternal protective strategies have either been investigated or hypothesized. These broadly include (i) prenatal and postnatal transmission of protective molecules and cells, particularly via mammary gland secretions and (ii) mechanical and chemical preparation of the pouch for the neonate. What has been

less studied is the role that the young animal itself may actively play in this protection, particularly the role and development of innate immunity.

4.1. Prenatal and Postnatal protection

In eutherians passive immunological protection is afforded through either or both transplacental and/or postnatal transfer of immunoglobulins (Cooper, 1976; Turner, 1994). In marsupials reports of prenatal transfer of these protective molecules vary. Early studies failed to identify immunoglobulins in the newborn quokka and the American opossum respectively (Yadav and Eadie, 1973; Hindes and Mizell, 1976). In the tammar wallaby, however, IgG was reported in the serum of the prenatal tammar wallaby two days prior to birth and immediately after birth and prior to suckling (Deane et al., 1990) suggesting transfer of immunoglobulins from the mother to the foetus via the yolk-sac placenta. In contrast, transfer of passive immunity via milk immunoglobulins has been observed in a range of marsupial species. In the tammar wallaby, immunoglobulins and transferrin have been detected in milk over the complete period of lactation. During the period 0-90 days when the young animal is permanently attached to the teat, the mean IgG level in juveniles did not rise above the titre of immunoglobulins in the milk. However after teat release, the immunoglobulin levels rose dramatically, suggesting an active response by the young animal to its first oral encounter with micro-organisms (Deane and Cooper, 1984). Maternal immunoglobulins passed through milk have been shown to protect the neonate from infectious agents with the young of the common opossum protected from *Trypanosoma cruzi* infection through transfer of Igs in mother's milk (Jansen et al., 1994).

Milk is also a source of maternal leucocytes. Neutrophils are the most abundant cell observed in the early stages of lactation of the tammar wallaby, whilst macrophages were

the dominant leukocyte in late lactation (Young et al., 1997). Premature removal of the pouch young leads to an increase of macrophages, presumably involved in involution of the mammary gland. Plasma cells are also found in milk of tammar wallaby (Young et al., 1997) and koalas (Young and Deane, 2001). Newborn quokkas that have suckled have been shown to have leucocytes in their stomachs (Cockson and McNiece, 1980) and this has been postulated to protect the gut lining during early pouch life. The function of these cell populations however is largely unknown and *in vitro* studies have indicated that these cells have poor response to mitogens, phagocytic activity and limited anti-bacterial activity (Turner, 1994). This raises questions as to their viability and role in the GIT of young animal.

4.2. The Pouch

In macropods, pouch licking prior to birth of the young is frequently observed as a significant change in the appearance of the skin of pouch – from dry and dirty to clean and moist (Jackson, 2003). Licking leads to the deposition of saliva, a clear, slightly acidic mucoserous exocrine secretion. In humans saliva is known to contain a wide variety of proteins with immunological function, includes lysozyme, lactoferrin, peroxidase and secretory IgA (Kuroishia et al., 2007). It is reasonable to suggest that both mechanical action of the tongue and the presence of such compounds may serve an important role in removing potentially harmful micro-organisms from the pouch prior to the birth of the young.

It has long been argued that the skin of maternal pouch secretes antimicrobial compounds, which regulate microflora (Yadav, 1971). In eutherians, skin produces lactic acid and free fatty acids that decrease skin pH and inhibit the growth of micro-organisms (Roitt, 1991).

Sebaceous glands present in the skin produce sebum, consisting primarily of lipids, which act as a water repellent and surfactant for eccrine secretions (Porter, 2001). Skin also provides protection against mechanical injury (Haffner, 1998). Histological studies of the pouch skin of brushtail possums treated chemically to enter oestrus, clearly show sebaceous and eccrine glands, although no significant changes were observed as the animals came close to anticipated time of birth (Old et al., 2005).

In vertebrates and non-vertebrates, antimicrobial peptides are secreted by the epithelium or its equivalent and act as a first line of defense (Schonwetter et al., 1995). In eutherian mammals antimicrobial peptides, specifically the defensins and cathelicidins, play a significant role in the protection of epithelial surfaces (Schonwetter et al., 1995). Yadav (1971) first suggested the production of antimicrobial compounds based on his observations of the decline in micro-organism in the maternal pouch and the survival of the young under such potentially adverse conditions. However, attempts to isolate these compounds in marsupial pouch secretions have met with limited success. Whilst pouch epithelial secretions collected from the koala showed demonstrable antimicrobial activity, none of the peptide sequences derived from digested proteins in the secretions could be matched to any of the known proteins present in the translated database of mammals (Bobek and Deane, 2002). Subsequent studies on the pouch secretions of the tammar wallaby identified a peptide, eugenin, however, it failed to demonstrate any significant antimicrobial activity (Baudinette et al., 2005). Most recently, and during the course of this study, opossum genome analysis has provided some sequence data for cathelicidin and defensin and this could aid their complete isolation and sequencing by molecular means (Belov et al., 2007). A similar analysis of expressed sequence tags (ESTs) using thymus cDNA library from the newborn bandicoot (*Isodon macrourus*) has led to the identification of cathelicidin, whose

production may protect the immuno-compromised pouch young till they develop adaptive immune system (Baker et al., 2007).

4.3. Protective Strategies of the Neonate

It is reasonable that the young animal itself may have its own strategies of innate immune protection. Given the immaturity of the lymphoid tissue at the time of birth, it is extremely likely that innate immunity would play a large part in protection in the early stages of post-natal life. Innate immunity refers to the first-line of host defense that prevents infection from exposure to micro-organisms and there is a wide array of strategies used by mammals in pathogen recognition, signalling pathways and mechanisms of innate immunity. One potential fertile avenue of investigation is based on the observation of high levels of neutrophils in the blood of neonates (Basden et al., 1996) and macrophages in the quokka lung at one day post partum (Cockson and McNiece, 1980). In eutherian systems these cells produces antimicrobial compounds to kill invading pathogens. In addition, the epithelial tissues of the young animal, including the possible secretion of antimicrobial proteins, may play an important role by acting as a physical barrier to pathogens.

5. The Research Question

It has long been argued that the protection afforded to the young marsupial was in part due to antimicrobial secretions from the skin of the maternal pouch. This hypothesis is based on cultivation studies of the pouch microflora which showed a decline in number and diversity of resident micro-organisms (Yadav, 1971; Charlick et al., 1981; Old and Deane, 1998). However histological studies of the brushtail possum pouch showed no overt changes in secretory structures when the animal was chemically stimulated to go in oestrus (Old et al., 2005). No similar studies have been conducted on animals entering oestrus in a routine

physiological manner. To add to this picture, proteomic analysis of secretions for the koala pouch, which had demonstrated antimicrobial activity, gave *de novo* sequence data that had no match to any known proteins in the public access database at that time (Bobek and Deane, 2002); whilst a comprehensive study of low molecular weight compounds of the tammar pouch failed to isolate any compound with antimicrobial activity (Baudinette et al., 2005).

This project used a proteomics approach to investigate this particularly enigmatic area of marsupial immune function. In the first instance this focus was on neutrophils and their repertoire of proteins. In eutherian mammals, neutrophils contain a range of antimicrobials in their granules. Two of these groups of proteins – cathelicidins and defensins are also secreted by epithelial tissues. Thus, their successful isolation from neutrophils and sequencing would provide a foundation for identifying these compounds in epithelial secretions such as from the pouch skin.

5.1. Neutrophils

Neutrophils are blood cells capable of engulfing and destroying the invading microorganism (Roos and Winterbourn, 2002). They were discovered by Elie Metchnikoff in the 1880s, when he inserted a rose thorn into starfish larva and recognised the importance of phagocytosis as a defence mechanism for multicellular organisms (Metchnikoff, 1968). In eutherian mammals, neutrophils are non-proliferative cells generated from pluripotent haematopoietic stem cells in the bone marrow. These cells are capable of engulfing and destroying bacteria due to the wide repertoire of antimicrobial peptides and proteins present within their granules. As such, neutrophils and their granular proteins are a first line of defense against invading pathogens (Mollinedo et al., 1999).

The granules of neutrophils are classified as azurophilic (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules, lysosomes and secretory vesicles (Segal, 2005) and each contains a different functional repertoire of compounds.

Azurophilic granules, because of their mucopolysaccharide content, stain with azure dye A (Spicer and Hardin, 1969). However, when classified functionally, these granules are called peroxide-positive due to their myeloperoxidase (MPO) content (Bainton and Farquhar, 1966; Bainton et al., 1966). They contain neutral proteinases, including cathepsin G, elastase, proteinase 3, bactericidal permeability increasing protein (BPI), defensins (Bainton et al., 1966; Welsh and Spitznagel, 1971; Bretz and Baggiolini, 1974) and lysozyme (Baggiolini et al., 1969).

The second type of granule is the specific or secondary granule. These are peroxidase-negative granules and play a significant role in initiating inflammatory responses (Gallin, 1984). These granules store metalloprotease and antibiotic peptides in latent form (Gullberg et al., 1999) which are activated by the proteases released from azurophilic granules during degranulation (Gullberg et al., 1999). Specific granules contain gelatinase-associated lipocalin and lactoferrin (Bullen and Armstrong, 1979), lysozyme (Baggiolini et al., 1969) as well as a number of membrane proteins, such as flavocytochrome b_{558} of the NADPH oxidase (Segal and Jones, 1979).

The last granule type is gelatinase or tertiary granules. These granules are also peroxidase-negative (like specific granules) but have a high content of gelatinase (Gilbert et al., 1993; Kjeldsen et al., 1994). Neutrophils also contain lysosomes, themselves the source of acid hydrolases. The content of lysosomes is released into the phagocytic vacuoles after azurophilic granules release their contents (Segal et al., 1980).

5.2. Antimicrobial Proteins/Peptides

Antimicrobial proteins (AMPs) are evolutionarily ancient compounds widely distributed across the plant and animal kingdom and play a fundamental role in protection of self (Zasloff, 2002). Eutherian mammals produce a diverse range of AMPs, primarily secreted in large quantities from neutrophils and monocytes/macrophages, skin and mucosal surfaces of the gastrointestinal tract, genitourinary tract and trachea (Zanetti, 2004).

Since the discovery of the first antimicrobial peptide, cecropin (Steiner et al., 1981), over 600 antimicrobial proteins have been identified, with more than half isolated from insects (Andreu and Rivas, 1998; Bulet et al., 1999; Boix and Nogues, 2007). Due to the difference in physicochemical properties, these peptides have been grouped on the basis of structure and amino acid composition as follows:

(i) Peptides with cysteine residues: This group, consists of 3-4kDa cysteine-rich peptides collectively known as defensins, have been isolated from mollusc, acari, arachnids, insects and mammals. Defensins, classified as α - and β - defensins in vertebrates, are characterised by an anti-parallel β sheet structure sheet core stabilized by three disulfide bonds (Zasloff, 2002). In contrast, θ -defensins exist as a cyclic molecule (Lehrer and Ganz, 2002).

Recently, it was found that opossum genome contained one gene for α - and twelve for β - defensins (Belov et al., 2007).

(ii) Amphipathic α -helical peptides: This group consists of highly heterogeneous peptides, rich in specific amino acids exhibiting antibacterial and antifungal activity mainly consisting of precursor-derived peptides. The major example of this group is cathelicidins. These compounds consist of an N-terminal signal peptide, a highly conserved middle

domain known as cathelin, with a less conserved C-terminal antimicrobial domain cleaved by proteases present in the neutrophil granules. So far around 30 cathelicidins have been isolated from various mammalian species, however, only one has been identified from humans (Durra et al., 2006). Recent mining of the opossum genome has identified 12 possible cathelicidin gene sequences (Belov et al., 2007).

(iii) Peptides, rich in proline or glycine: This is a distinctive class of antibacterial and antifungal cationic peptides mostly isolated from insects and active against Gram-negative bacteria (Bulet et al., 1999; Otvos, 2000). Histidine rich peptides active against fungal pathogens have been isolated from mammals (Pollock et al., 1984), particularly from saliva of humans and primates (Tsai and Bobek, 1998).

Recently, a new group of anionic peptides exhibiting antimicrobial activity have been isolated prominently from mammals, these include neuropeptide derived molecules, aspartic-acid-rich molecules, aromatic dipeptides and oxygen binding proteins (Marshall and Arenas, 2003).

6. The Tools of Proteomics

The work described in this thesis has taken a proteomic approach to investigate aspects of innate immune protection. Such an approach is constrained by the lack of significant public access database of marsupial proteins and so the work describes in this thesis reflects strategies used to accommodate this deficit.

6.1. Overview of Proteomic Approach

All the proteins expressed by the genome of an organism at any one time is called the proteome and its study is collectively called proteomics. Proteomics can provide insights into changes of protein expression under physiological, developmental or disease conditions, protein function, characterisation of post-translational modifications and protein interactions (Williams and Hochstrasser, 1997). Proteomic studies are increasing as instrumentation becomes more user friendly and as the number of organisms whose genome has been sequenced increases. The latter is a major limitation in more widespread use of the technologies and has been of particular importance in this project.

Proteins are more problematic to study compared to either RNA or DNA. Due to their diversity and complexity in biological systems, no one technology can be singled out as a benchmark, rather a combination of strategies has to be employed to unravel different proteins and their functionality. An overview of the possible pathways for proteomic analysis is shown in Fig 1. As can be seen, in this Figure the essential tools of proteomic analysis can be grouped into (i) Gel Electrophoresis, primarily two-dimensional electrophoresis (2-DE PAGE) - a step which allows for the separation and visualization of proteins in a mixture, (ii) mass spectrometry (MS), where digested proteins (peptides) are separated on the basis of charge and mass and (iii) identification by analysis of their mass and spectral profile to obtain sequence data. Each step in the selection and use of these tools in this project are described in more detail below.

6.2. Two-Dimensional Polyacrylamide Gel Electrophoresis (2DE-PAGE)

Sample preparation is the most important step in obtaining reproducible 2DE PAGE gels and subsequent reliable data. There are a number of different methods for isolating

proteins from cells or tissues including, sonication, mechanically driven rapid pressure changes and homogenization. In addition, chemical processes using urea-based solutions containing detergents, reducing agents and protease cocktail inhibitors can be used. However, no single sample preparation or isolation protocol can be used for all samples, due to the difference in the physico-chemical properties of proteins and their source material.

Two dimensional polyacrylamide gel electrophoresis (2DE PAGE) is a two step technique most commonly used for separation and visualization of complex mixture of proteins. It is any extremely useful approach to use when one needs to understand the nature of the source protein (MW and pI) that is being sequenced. This method firstly separates proteins based on charge (pI) by isoelectric focusing (IEF) in the first dimension, and secondly according to molecular weight employing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimensions (O'Farrel, 1975). The protocol for 2DE was first developed by Gorg et al., (1988) and since then, considerable improvements have taken place with the introduction of immobilized pH gradient (IPG) for IEF (Bjellqvist et al., 1982). After equilibrating the IPG strip in the first dimension, the second dimension step is carried out on SDS-PAGE by applying an electric field. The coupling of these two methods increases resolution of proteins with reports of upto 10 000 proteins on a single gel with routine resolution of 2000 proteins (Lopez, 2007). The resolving power of 2DE PAGE gels can be further improved by the use of narrower pH range. The 2DE PAGE technique has its limitations. Spot selection for mass spectrometry is labor intensive; some proteins such as membrane proteins, may have poor solubility in the sample solubilisation solutions; very basic and acidic proteins may poorly focus; and low abundance proteins may be masked by those present at higher concentrations (Lopez,

2000). Nevertheless, 2DE PAGE is still a powerful tool in proteomic analysis (Rabilloud, 2002).

6.3. Detection and Digestion of Proteins

After the electrophoresis is complete, proteins are detected using a variety of staining methods of varying sensitivity. Stains need to be sensitive enough to stain low abundant proteins, should have wide linearity and dynamic range and need to be compatible with mass spectrometry. Although in practice there is no one staining protocol, most proteomic studies use a combination of stains such as Coomassie Brilliant Blue R-250 (CBB), Coomassie Brilliant Blue G-250 (colloidal Coomassie), silver and fluorescent stains. CBB stain has a dynamic range of 10-200 ng, whilst silver has a linear range of 0.3-10ng (Switzer et al., 1979). Higher sensitivity can be achieved with fluorescent dyes, such as Sypro Ruby, Sypro Orange, Deep Purple, and Flamingo (Patton, 2002). Once proteins are located on the gel they can be excised and subjected to enzymatic digestion by proteases. The most commonly used protease is trypsin, which cleaves proteins exclusively at the C-terminal end of lysine or arginine residues, provided the next amino acid is not proline. This creates peptides that can then be subjected to mass spectrometric analysis. By undertaking 2DE as a first step, this allows the peptides to be sourced to their proteins of origin and acts a validity check on the identification generated through MS analysis.

6.4. Mass Spectrometry Techniques

6.4.1. Overview of the Common Ionisation Techniques used in different Mass Spectrometers for Protein Identification

The two most commonly used ionization techniques used in mass spectrometers for protein analysis are (i) Matrix Assisted Laser Desorption Ionisation time of flight (MALDI-TOF)

and (ii) electrospray ionization (ESI) QTOF. In MALDI instrumentation a singly charged ion are created using a laser to excite the crystalline matrix containing analyte molecules and converts these into gas phase ions. In contrast, ESI generates multiply-charged ions are created by applying a potential difference between a capillary and the inlet of a mass spectrometer thus causing charged droplets to be released at the tip of the capillary. Solvent evaporates from the droplets and gas phase charged ions are formed. ESI is more sensitive to contaminants, such as salt, detergents and buffers than MALDI, but has found the widest application in proteomics as it measures the mass to charge ratio of an ion by the time it takes to progress in the field-free time of flight tube (QTOF instruments). Yet another method used to carry out large scale proteins analysis using electrospray ionisation is (iii) multi-dimensional chromatography (LC/LC-MS/MS). In this method, direct analysis of proteins mixtures circumvents the problems associated with SDS-PAGE, such as protein insolubility, high or low molecular weights, diffuse migration, comigration with contaminants, poor binding of proteins and limited recovery of peptides (Link et al., 1999).

6.4.2. Peptide Mass Fingerprinting (PMF) using MALDI-TOF Mass Spectrometer and its Limitations

Protein identification by peptide mass fingerprinting (PMF) was one of the first method by which proteins were identified using mass spectrometry (MS) (Henzel et al., 1993). This is relatively simple to operate, and the fastest method for identifying proteins recovered from 2-DE PAGE. After gel electrophoresis the protein spots are excised, washed and digested with trypsin to generate peptides which are eluted in small volumes and subjected to MS. In this method the proteins are identified by matching experimental peptide masses, determined with high accuracy, with the theoretical peptide masses obtained from an *in*

silco digestion of all proteins present in a database taking into consideration the specificity of the protease (Jensen et al., 1999). This approach is called peptide mass fingerprinting (PMF) for which several algorithms are available. As every protein results in a unique set of peptides masses after cleavage with trypsin, only a few peptide masses are required for confident protein identification. However, a major limitation to PMF identification is that the protein or close homologue must be present in the database for identification. In cases of unsequenced organisms, such as the tammar wallaby further MS analysis has to be done, involving the use of tandem mass spectrometry (MS/MS).

6.4.3. Tandem Mass Spectrometry using MALDI-TOF/TOF and its Limitations

Identification of proteins can be carried out by tandem mass spectrometry (MS/MS) using TOF/TOF analyzer. In this method the peptides are isolated and fragmented by post source decay (Spengler et al., 1991) or collision induced dissociation with inert gas (Papayannopoulos, 1995) which can then be interpreted and/or correlated to database entries. MALDI-TOF/TOF instruments use high energy collision leading to side chain cleavages and give rise to fragment ions denoted as d, v and w, which are useful means to differentiate isobaric amino acids (Ashcroft and Derrick, 1991). However the generation of singly charged peptides by desorption method leads to preferential cleavage of the peptide backbone (Wattenberg et al., 2002). One of the disadvantages is that due to preferential cleavage the y-ion series may not be detected leading to loss of sequence information (Wattenberg et al., 2002). This kind of fragmentation may not be a problem for protein identification as this change in fragmentation pattern can be accommodated in the search algorithm. However, the missing fragment ions can lead to ambiguous identification of proteins by *de novo* sequencing.

6.4.4. Tandem Mass Spectrometry using ESI-MS and its Limitation

Alternatively, tandem mass spectrometry can be carried out for protein identification using hybrid mass analyzers, such as a combination with quadrupole-time of flight (Q-TOF). The benefit of this instrumentation is that it can be interfaced with chromatographic separations like C18 and electrospray which allows complex peptide mixtures from a mixture to be successfully analysed. Low flow on-line or reverse-phase (RP) microcapillary liquid chromatography (LC) ESI MS/MS (Hunt et al., 1986) or nano-ESI (Wilm and Mann, 1996) greatly increases sensitivity. This liquid separation technique offers advantages, such as higher efficiencies, high speed separation, and analysis of small volumes samples (Alexander et al., 1998). The high performance liquid chromatographic system allows not only removal of the contaminants, but also purifies and concentrates the peptides which it elutes into the mass spectrometer for further analysis.

In this protocol this mass spectrometer first scans the full mass range to measure the masses of peptides (e.g. m/z 350-1750) eluting from the RP-nano LC. A specific peptide is selected by the software based on its mass-to-charge ratio and passed into a collision chamber to interact with collision gas (nitrogen or argon) leading to fragment ions that are detected in the second scan. This fragmentation occurs in a predictable manner between the amino acids bonds, resulting in the loss of amino acids between two adjacent peaks in the mass spectrum helping in interpretation of the spectra (MS/MS). The interpretation of the MS/MS spectra can be automated for the comparison of the experimental spectra with the theoretical spectra of the protein database using robust software, such as MASCOT (Perkins et al., 1999) or SEQUEST (Eng et al., 1994).

This technique is more sensitive to salts, buffer and hence a more stringent method needs to be used to clean up the peptide samples for MS/MS analysis through the high pressure liquid chromatography (HPLC). Even though LC-MS/MS is more laborious and time consuming than MALDI-TOF, it is a more reliable way to obtain a positive identification as it generates multiply charged peptide ions which readily fragment predominantly into intense y- and b- ions generating high quality and informative tandem mass spectra (Aebersold and Goodlett, 2001).

6.4.5. *De novo* Peptide Sequencing

If the aforementioned techniques fail to provide any positive protein identifications, *de novo* sequencing followed by BLAST searching provides an alternative identification strategy (Altschul et al., 1997; Taylor and Johnson, 1997). In this the amino acid sequence is obtained by analysing the mass difference between two adjacent y- and b-ion series in the fragmentation spectra of the precursor ion (Wilm and Mann, 1994). However, manual evaluation of the MS/MS spectrum is time consuming and is dependent on the quality of the spectrum, but it may be the only choice when dealing with organisms without a translated genomic database. Alternatively, to interpret tandem mass spectra of peptides, specialized software can be used to create amino acid sequence (Taylor and Johnson, 1997). This software uses different computational principles to produce a list of peptide sequences from each spectrum that are ranked in order of statistical confidence. Some of the common database search engines used to produce sequences are modified FASTA-based software (Taylor and Johnson, 2001) and MS BLAST accessible over the internet (Shevchenko et al., 2001).

6.4.6. Mascot Search Engine used for Protein Identification and their Statistical Validation

In our experimental approach, protein identification was carried out using Mascot, a search engine developed from the MOWSE computer program (Pappin et al., 1993; Pappin et al., 1996). The fragmentation spectra of the different proteins were searched against mammalian protein sequences present in the non-redundant NCBI database. To carry out the searches, Mascot relies on probability-based scoring for interpreting peptide mass fingerprint, sequence query and MS/MS ion search (Perkins et al., 1999). The basic principle of Mascot is to calculate the probability that the match between the experiment data and the database sequence is a random event. Hence the match with the lowest probability is reported as the best match (Perkins et al., 1999). However, the significance of the reported match as the best match depends on the size of the database, as the number of identifications goes up as the number of spectra searches increases (Moore et al., 2002). Nonetheless, a widely used significance threshold is the probability of the observed event occurring by chance is less than one in twenty ($p < 0.05$) (Perkins et al., 1999).

In experimental models with a translated genome, one individual peptide match is considered significant for a conclusive identification of a precursor protein (Link et al., 1999; Washburn et al., 2001). However, this peptide to identify the protein uniquely, it must be sufficiently long that is unlikely to exist in other unrelated proteins. Additionally, for a peptide of such length, many isobaric peptides may not be present in the database, and hence the identification of protein by the peptide cannot be considered valid (Moore et al., 2002). Moreover, proteins identified by a single peptide match exhibit higher false positive rates (Elias et al., 2005). The general support for this practice and the lack of translated

genome for Tammar wallaby, led us to base our approach to protein identification on the multiplicity of different peptide matches (Haynes et al., 1998; Kratcmarova et al., 2005).

The statistical validity of the probabilistic score calculated by Mascot can be tested by submitting the same search to different search engine for comparison of the results (Perkins et al., 1999). Alternatively, the validation can also be done by searching the identified sequence against a reverse (Elias et al., 2004; Peng et al., 2003) or randomized (Rice et al., 2000) target-decoy database containing all mammalian proteins sequences. These decoy-databases can be created by reversing the target protein sequences using the programme db-reverse.pl (Moore et al., 2002) or random protein sequences using the shuffleseq program (Rice et al., 2000). The general amino acid composition, sequence length, and the sequence homologies as the original database are preserved in both the reverse or random target-decoy database (Rice et al., 2000; Moore et al., 2002). Alternatively, the decoy database can also be created by stochastic method, such as Markov chain modeling (Haas, 2006). Once the decoy database is created, the MS/MS spectra are searched to provide an estimate of the false-positive rate of peptide identification, indicating the confidence of the protein identified from the identified peptides.

6.4.7. Multi-Dimensional Protein Identification Technology (MUDPIT)

This is a technology that holds great promise for large scale proteomic analysis. In this technique there is addition of a strong cation exchange resin in line with the reverse phase chromatography column. It does not involve separation through a 2DE PAGE gel and the digested peptides are directly eluted onto the column at low pH facilitating binding to the cation exchange column. Salt steps are used to elute peptides onto the reverse phase column using C 18 RP elution and analysis by MS. The peptides elute in an incremental manner as

the concentration of the salt increases. This process is easier to automate and certain classes of proteins can be analysed, such as very acidic, very basic and membrane proteins, which cannot be easily detected by 2DE PAGE. However, changes in the protein expression detected on 2DE PAGE cannot be detected using MUDPIT nor the specific pI and molecular weight of the source protein. In species with little or no translated genome data it is difficult to confidently identify proteins as the same peptide sequence may be present in multiple different proteins leading to ambiguity in discriminating proteins that share extensive homology e.g. belonging to “superfamilies” or are isoforms arising from alternatively spliced genes. Thus, the eluted peptides cannot be easily assigned to one source protein.

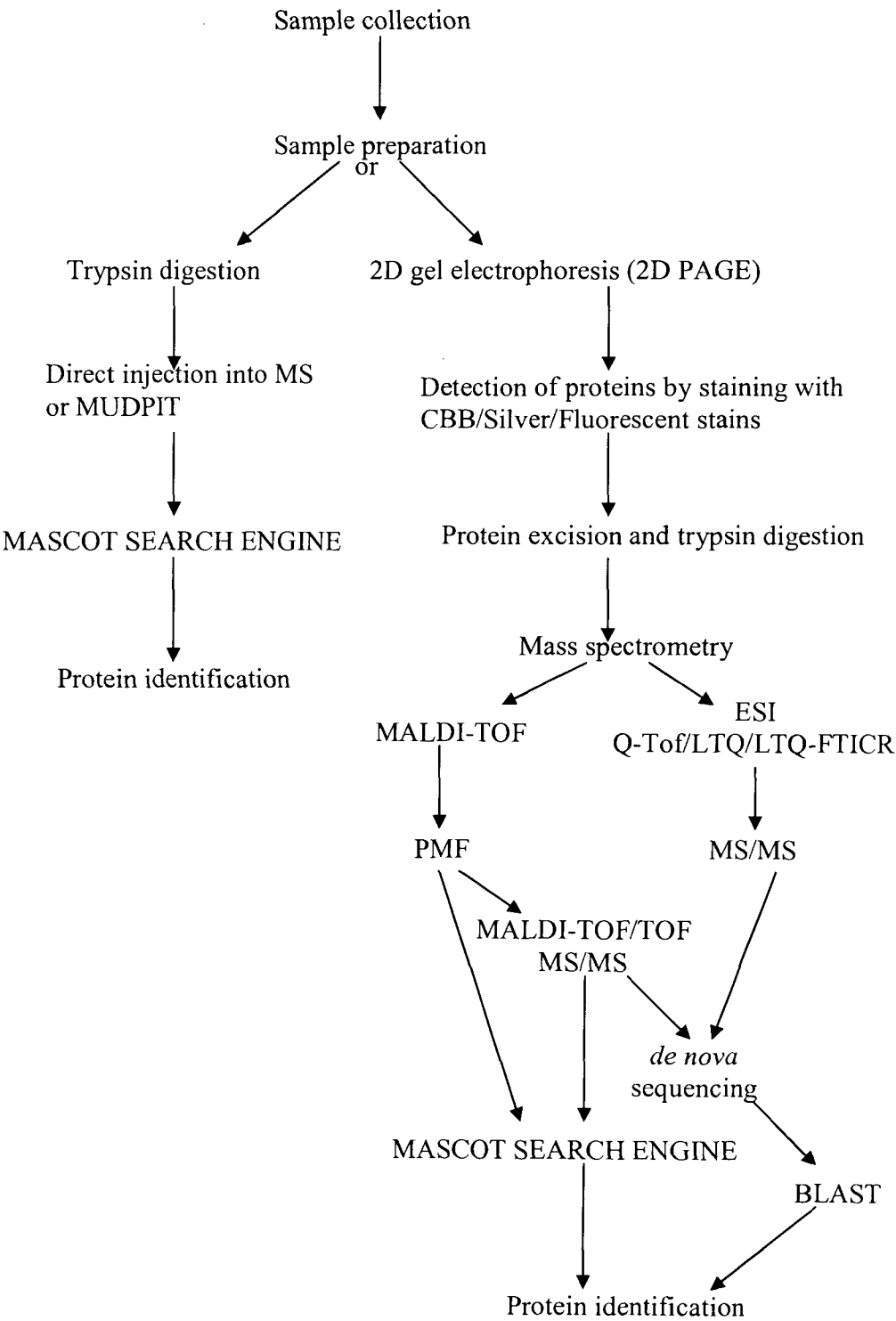
6.5. Cross Species Protein Identification in the Tammar Wallaby (*Macropus eugenii*)

The lack of a fully sequenced and translated genomic database for marsupials limits the capacity to use a number of the MS protocols described above. However, key features of primary structure and composition, of essential proteins are often conserved across species and this facilitates the identification of the proteins in unsequenced species by allowing cross species comparisons. This cross species matching can be done by comparing the amino acid composition and molecular mass (Cordwell et al., 1995; Wilkins and Williams, 1997). However, to use PMF for cross species matching, peptides need to have more than 80% identity (Wilkins and Williams, 1997).

In the work reported in this study a combination of strategies was employed to maximise the likelihood of positive protein identification in the tammar wallaby samples. Initially 2DE was used to separate proteins as this provided fundamental information on pI and molecular weight. It was seen to be of particular value in documenting changing patterns of

protein secretion from skin, and even such proteins could not be confidently identified. The constraint of limited identity of proteins across species boundaries was addressed by the use of tandem mass spectrometer analysis of peptides using ESI. This approach allows removal of contaminants and better ion spectra for both automated and manual spectra.

Figure1. Possible pathways for proteomic analysis



7. The Positioning of the Papers

The papers presented in this thesis are positioned to address the research question as follows:

Paper 1

Proteomic analysis of neutrophil proteins of the tammar wallaby (*Macropus eugenii*).

Comparative Biochemistry and Physiology, Part D: (2006) 1, 283- 291.

Kiran S. Ambatipudi, Julie M. Old, Michael Guilhaus, Mark Raftery, Lyn Hinds and Elizabeth M. Deane

This paper documents the protocols used to isolate neutrophils from the tammar wallaby and is the first description of the proteins of neutrophils of any marsupial species. The paper required a significant amount of protocol optimization to acquire sufficient neutrophil proteins for the proteomic analysis. It is focused on adult neutrophils primarily because of the availability of more reasonable amounts of blood compared to that available from young animals and because neutrophils are known sources of antimicrobials in eutherian mammals. Given the lack of a database for marsupials this approach offered the possibility of identification and acquisition of sequence data of marsupial antimicrobials.

Paper 2

In search of neutrophil granule proteins of the tammar wallaby (*Macropus eugenii*).

Molecular Immunology: (2008) 45, 690-700.

Kiran S. Ambatipudi and Elizabeth M. Deane.

Whilst paper 1 provided limited identification of neutrophil proteins, the techniques used failed to isolate the desired granule proteins. This paper describes the use of a range of compounds known to stimulate granule exocytosis in eutherian mammals, Phorbol myristate acetate, Ionomycin and calcium as well as protocols of differential centrifugation and lysis. A number of granule proteins were thus isolated and identified by mass spectrometry.

Paper 3

A proteomic approach to analysis of antimicrobial activity in marsupial pouch secretions. *Developmental and Comparative Immunology*: (2008) 32, 108-120.

Kiran S. Ambatipudi, Janice Joss, Mark Raftery and Elizabeth M. Deane

This paper documents the antimicrobial activity of maternal pouch skin secretions and uses proteomic techniques in an attempt to identify the actual compounds responsible for this activity. This paper firstly describes measurements of antimicrobial activity at various stages of the reproductive cycle followed by investigation of the protein components responsible for its antimicrobial activity.

Paper 4

A comparative proteomic analysis of skin secretions of the tammar wallaby (*Macropus eugenii*) and the wombat (*Vombatus ursinus*).

Comparative Biochemistry and Physiology, Part D: (2007) 2, 322-331.

Kiran S. Ambatipudi, Janice Joss and Elizabeth M. Deane.

This paper is complementary to paper 3 and is a more detailed proteomic analysis of how the secretions of the female tammar wallaby change over the life span of the animal- reproductively immature, mature and aged. By way of comparing with non-pouch skin it also examines the secretions from a mature female to document the differences between pouch skin and other ones to identify any unique aspects of pouch skin secretions.

References

- Aebersold, R. and Goodlett, D.R. (2001) Mass spectrometry in proteomics. Chemical Review 101, 269-295.
- Alexander, J.N., Schultz, G.A. and Poli, J.B. (1998) Development of a nano-electrospray mass spectrometry source for nanoscale liquid chromatography and sheathless capillary electrophoresis. Rapid Communication in Mass Spectrometry 12, 1187-1191.
- Altschul, S., Madden, T., Schafer, A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids 25, 3389-3402.
- Andreu, D. and Rivas, L. (1998) Animal antimicrobial peptides: an overview. Biopolymers 47, 415-433.
- Ashcroft, A.E. and Derrick, P.J. (1991) Four sector tandem mass spectrometry of peptides. CRC Press Inc, Boca Raton, Florida.
- Ashman, R., Keast, D., Stanley, N.F. and Waring, H. (1972) The in vitro response to phytohaemagglutinin (PHA) of leucocytes from intact and thymectomised quokkas. Australian Journal of Experimental Biology and Medical Science 50, 337-345.
- Ashman, R. and Papadimitriou, J. (1975a) Development of the lymphoid tissue in a marsupial, *S. brachyurus*. Acta Anatomy. 91, 594-611.
- Ashman, R. and Papadimitriou, J. (1975b) The effect of thymectomy on the lymphoid tissues of the quokka (*Setonix brachyurus*). Australian Journal of Wildlife Diseases. 53, 129-136.
- Ashman, R., Waring, H. and Stanley, N.F. (1975) Adult mortality after neonatal thymectomy in a marsupial, *Setonix brachyurus* (quokka). Proceedings of the Society for Experimental Biology and Medicine 144, 819-821.

- Azzali, G. and Di Dio, L.J.A. (1965) The lymphatic system of *Didelphis azarae* and *Didelphys marsupialis*. American Journal of Anatomy 116, 449-470.
- Baggiolini, M., Hirsch, J.G. and de Duve, C. (1969) Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. Journal of Cell Biology 40, 529-541.
- Bainton, D.F. and Farquhar, M. (1966) Origin of granules in polymorphonuclear leukocytes. Journal of Cell Biology 28, 277.
- Bainton, D.F., Ulliyot, J.L. and Farquhar, M.G. (1966) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Journal of Experimental Medicine 143, 907-934.
- Baker, M.L., Gemmell, E. and Gemmell, R.T. (1999a) Ontogeny of the brushtail possum, *Trichosurus vulpecula*. The Anatomical Record 256, 354-365.
- Baker, M.L., Gemmell, E. and Gemmell, R.T. (1999b) Ontogeny of the immune system of the brushtail possum, *Trichosurus vulpecula*. The Anatomical Record. 256, 354-365.
- Baker, M.L. and Gemmell, R.T. (1999) Physiological changes in the brushtail possum (*Trichosurus vulpecula*) following relocation from Armidale to Brisbane, Australia. Journal of Experimental Zoology 284, 42-49.
- Baker, M.L., Indiviglio, S., Nyberg, A.M., Rosenberg, G.H., Lindblad-Toh, K., Miller, R.D. and Papenfuss, A.T. (2007) Analysis of a set of Australian northern brown bandicoot expressed sequence tags with comparison to the genome sequence of the South American grey short tailed opossum. BMC Genomics 8, 1-10.
- Barnett, J.L., How, R.A. and Humphreys, W.F. (1979) Blood parameters in natural populations of *Trichosurus* species (Marsupialia: Phalangeridae I) Age, sex and

- seasonal variation in *T. caninum* and *T. vulpeula*. Australian Journal of Zoology 27, 913-926.
- Basden, K., Cooper, D.W. and Deane, E.M. (1996) Development of the blood-forming tissues of the tammar wallaby *Macropus eugenii*. Reproduction, Fertility and Development 8, 989-994.
- Basden, K., Cooper, D.W. and Deane, E.M. (1997) Development of the lymphoid tissues of the tammar wallaby *Macropus eugenii*. Reproduction, Fertility and Development 9, 243-254.
- Baudinette, R.V., Bootheung, P., Musgrave, I.G., Wabnitz, P.A., Maselli, V.M., Skinner, J., Alewood, P.F., Brinkworth, C.S. and Bowie, J. (2005) An immunomodulator used to protect young in the pouch of the tammar wallaby, *Macropus eugenii*. FEBS Journal 272, 433-443.
- Bell, R.G., Lynch, N.R. and Turner, K.J. (1974a) Immunoglobulins of the marsupial *Setonix brachyurus* (Quokka). Characterisation of three major serum classes, IgG2, IgG1 and IgM. Immunology 27.
- Bell, R.G., Stephens, C.J. and Turner, J.K. (1974b) Marsupial immunoglobulins: An immunoglobulin molecule resembling eutherian IgA in serum and secretions of *Setonix brachyurus* (Quokka). Journal of Immunology 113, 371-377.
- Belov, K., Deakin, J.E., Papenfuss, A.T., Baker, M.L., Melman, S.D., Siddle, H.V., Gouin, N., Goode, D.L., Sargeant, T.J., Robinson, M.D., Wakefield, M.J., Mahony, S., Cross, J.G.R., Benos, P.V., Samollow, P.B., Speed, T.P., Graves, J.A.M. and Miller, R.D. (2006) Reconstructing an ancestral mammalian immune supercomplex from a marsupial major histocompatibility complex. PLoS Bioolgy 4, 1-12.
- Belov, K., Harrison, G.A. and Cooper, D.W. (1998) Molecular cloning of the cDNA encoding the constant region of immunoglobulin A heavy chain (C alpha) from a

- marsupial: *Trichosurus vulpecula* (common brushtail possum). *Immunology Letters* 60, 165-170.
- Belov, K., Harrison, G.A., Miller, R.D. and Cooper, D.W. (1999a) Isolation and sequence of a cDNA coding for the heavy chain constant region of IgG from the Australian brushtail possum, *Trichosurus vulpecula*. *Molecular Immunology*. 36, 535-541.
- Belov, K., Harrison, G.A., Miller, R.D. and Cooper, D.W. (1999b) Molecular cloning of the brushtail possum (*Trichosurus vulpecula*) immunoglobulin E heavy chain constant region. *Molecular Immunology* 36, 1255-1261.
- Belov, K., Sanderson, C.E., Deakin, J.E., Wong, E.S.W., Assange, D., McColl, K.A., Gout, A., Bono, B.d., Barrow, A.D., Speed, T.P., Trowsdale, J. and Papenfuss, A.T. (2007) Characterization of the opossum immune genome provides insights into the evolution of the mammalian immune system. *Genome Research* In Press.
- Bjellqvist, B., Ek, K., Righetti, P., Gianazza, E., Gorg, A., Westermeier, R. and Postel, W. (1982) Isoelectric focussing in immobilized pH gradient: Principle, methodology and some applications. *Journal of Biochemical and Biophysical Methods* 6, 317-339.
- Block, M. (1960) Wound healing in the new-born opossum (*Didelphis virginiana*). *Nature* (London) 187, 340-341.
- Block, M. (1964) The blood forming tissues of the newborn opossum (*Didelphys virginiana*) Normal development through about the one hundredth day of life. *Ergebnisse der Anatomie und Entwicklungsgeschichte* 37, 1-237.
- Bobek, G. and Deane, E.M. (2002) Possible antimicrobial compounds from the pouch of the koala, *Phascolarctos cinereus*. *Letters in Peptide Science* 8, 133-137.

- Boix, E. and Nogues, M.V. (2007) Mammalian antimicrobial proteins and peptides: overview on the RNase A superfamily members involved in innate host defence. *Molecular BioSystems* 3, 317-335.
- Bretz, U. and Baggiolini, M. (1974) Biochemical and morphological characterisation of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. *Journal of Cell Biology* 63, 251.
- Bryant, B.J. and Shifrine, M. (1974) The immunohaematopoietic and lymphatic systems of *Marmosa mitis*: A developmental survey. *Journal of the Reticuloendothelial Society*. 16, 105-113.
- Bulet, P., Hetru, C., Dimarcq, J. and Hoffmann, D. (1999) Antimicrobial peptides in insects: structure and function. *Developmental and Comparative Immunology* 23, 329-375.
- Bullen, J. and Armstrong, J. (1979) The role of lactoferrin in the bactericidal function of polymorphonuclear leucocytes. *Immunology* 36, 781-791.
- Canfield, P., Hemsley, S. and Connolly, J. (1996) Histological and immunological study of the developing and involuting superficial cervical thymus in the koala (*Phascolarctos cinereus*). *Journal of Anatomy*. 189, 159-169.
- Canfield, P.J. and Hemsley, S. (2000) The roles of histology and immunohistology in the investigation of marsupial disease and normal lymphoid tissue. *Developmental and Comparative Immunology* 24, 455-471.
- Charlick, J., Manassis, C., Stanley, N., Waring, H. and Cockson, A. (1981) Quantitative alterations of the aerobic bacterial flora of the pouch of *Setonix brachyurus* (quokka), during oestrus, anoestrus, pregnancy and lactating anoestrus (pouch young). *Australian Journal of Experimental Biology and Medical Science* 59, 743-751.

- Cockson, A. and McNiece, R. (1980) Survival in the pouch: the role of macrophages and maternal milk cells. *Comparative Biochemistry and Physiology* 66, 221-225.
- Coleman, R.M., Lombard, M.F. and Sicard, R.E. (1991) *Fundamental Immunology*. Wm. C. Brown Publishers, London.
- Cooper, E.L. (1976) *Comparative Immunology*. Prentice-Hall, Incorporated, Edgewood Cliffs.
- Cordwell, S.J., Wilkins, M.R., Cerpa-Poljak, A., Gooley, A.A., Duncan, M. and Williams, K.L. (1995) Cross-species identification of proteins separated by two dimensional electrophoresis using matrix-assisted laser desorption time of flight mass spectrometry and amino acid composition. *Electrophoresis* 16, 438-443.
- Coutinho, H., Nogueira, J., King, G., Coutinho, V., Robalinho, T., Amorim, A., Carvalcanti, V., Robins, R. and Sewell, H. (1994) Immunocytochemical study of the ontogeny of Peyer's patches in the Brazilian marsupial *Didelphis albiventris*. *Journal of Anatomy* 185, 347-354.
- Coutinho, H.B., Sewell, H.F., Tighe, P., King, G., Nogueira, J.C., Robalinho, T.I., Coutinho, V.B. and Cavalcant, V.M.S. (1995) Immunocytochemical study of the ontogeny of the marsupial *Didelphis albiventris* immune system. *Journal of Anatomy* 187, 37-46.
- Coutinho, V.B., Coutinho, H.B., Robalinho, T.I., Silva, E.S.O., Sewell, H.F. and McKinnon, A.D. (1990) Histological and ultrastructural studies of the marsupial *Didelphis albiventris* Peyer's patches. *Memorias Institute Oswaldo Cruz*. 85, 435-443.
- Cui, S. and Selwood, L. (2000) cDNA cloning, characterization, expression and recombinant protein production of leukemia inhibitory factor(LIF) from the marsupial, the brushtail possum (*Trichosurus vulpecula*). *Gene* 243, 167-178.

- Cutts, H. and Krause, W.J. (1980) Leucocytes in the peripheral blood of the developing opossum. *Journal of Anatomy*. 130, 113-120.
- Deane, E.M. and Cooper, D.W. (1984) Immunology of pouch young marsupials. I. Levels of immunoglobulin transferrin and albumin in the blood and milk of euros and wallaroos (Hill kangaroos: *Macropus robustus*, Marsupialia). *Developmental and Comparative Immunology* 8, 863-876.
- Deane, E.M., Cooper, D.W. and Renfree, M.B. (1990) Immunoglobulin G levels in fetal and newborn tammar wallabies (*Macropus eugenii*). *Reproduction, Fertility and Development* 2, 369-375.
- Duncan, L., Nair, S.V. and Deane, E. (2007) Molecular characterisation and expression of CD4 in two distantly related marsupials: The gray short-tailed opossum (*Monodelphis domestica*) and tammar wallaby (*Macropus eugenii*). *Molecular Immunology* 44, 3641-3652.
- Durra, U.H.N., Sudheendraa, U.S. and Ramamoorthy, A. (2006) LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochimica et Biophysica Acta- Biomembranes* 1758, 1408-1425.
- Elias, J. E., Gibbons, F. D., King, O. D., Roth, F.P., and Gygi, S. P. (2004) Intensity-based protein identification by machine learning from a library of tandem mass spectra. *Nature Biotechnology* 22, 214 –219
- Elias, E. J., Haas, W., Faherty, K. B and Gygi, P. S. (2005) Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. *Nature Methods* 2, 667-675.
- Eng, J.K., McCormack, A.L. and Yates, J.R. (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequence in a protein database. *Journal of American Society for Mass Spectrometry* 5, 976-989.

- Gallin, J.I. (1984) Neutrophil specific granules. A fuse that ignites the inflammation response. *Clinical Research* 32, 320.
- Giacometti, L., Berntzen, A.K. and Bliss, M.L. (1972) Hematologic parameters of the opossum (*Didelphis virginiana*). *Comparative and Biochemical Physiology* 43A, 287-292.
- Gilbert, C.S., Parmley, R.T., Rice, W.G. and Kinkade, J.M.J. (1993) Heterogeneity of peroxidase-positive granules in normal human and chediak- Higashi neutrophils. *Journal of Histochemistry and Cytochemistry* 41, 837.
- Godfrey, G.K. (1969) Reproduction in a laboratory colony of the marsupial mouse *Sminthopsis larapinta* (Marsupialia: Dasyuridae). *Australian Journal of Zoology* 17, 637-654.
- Gooding, L.R. (1992) Virus proteins that counteract host immune defence. *Cell* 71, 5-7.
- Gullberg, U., Bengtsson, N., Bulow, E., Garwicz, D., Lindmark, A. and Olsson, I. (1999) Processing and targeting of granule proteins in human neutrophils. *Journal of Immunological Methods* 232, 201-210.
- Haffner, M. (1998) The size of sebaceous glands in relation to the size of hair follicles on the heads of some small mammals (Insectivora, Chiroptera, Rodentia). *Annals of Anatomy* 180, 165-171.
- Harrison, G.A., Broughton, M.J., Young, L.J., Cooper, D.W. and Deane, E.M. (1999) Conservation of 3' untranslated region elements in tammar wallaby (*Macropus eugenii*) TNF-alpha mRNA. *Immunogenetics* 49, 464-467.
- Harrison, G.A. and Deane, E.M. (1999) cDNA sequence of the lymphotoxin beta chain from a marsupial, *Macropus eugenii* (Tammar wallaby). *Journal of Interferon and Cytokine Research* 19, 1099-1102.

- Harrison, G.A., McNicol, K.A. and Deane, E.M. (2004) Interferon α/β genes from a marsupial, *Macropus eugenii*. *Developmental and Comparative Immunology* 28, 927-940.
- Harrison, G.A. and Wedlock, D.N. (2000) Marsupial cytokines. Structure, function and evolution. *Developmental and Comparative Immunology* 24, 473-484.
- Haas, W., Wilgelm, Faherty, K. B., Gerber, S. A., Elias, Joshua, E., Beausoleil, S. A., Bakalarski, C. E., Li, X., Villén, J. and Gygi, S. P. (2006) Optimization and Use of Peptide Mass Measurement Accuracy in Shotgun Proteomics. *Molecular and Cellular Proteomics* 5:1326-1337.
- Hawken, R.J., Maccarone, P., Toder, R., Marshall Graves, J.A. and Maddox, J.F. (1999) Isolation and characterisation of marsupial IL5 genes. *Immunogenetics* 49, 942-948.
- Hawkey, C.M., Ashton, D.G., Hart, M.G., Cindery, R.N. and Jones, D.M. (1982) Haematological findings in red-necked wallabies (*Protenmodon rufogrisea*) with necrobacillosis. *Research in Veterinary Science* 33, 290-294.
- Hayne, P., Fripp, N. and Aebersold, R. (1998) Identification of gel-separated proteins by liquid chromatography-electrospray tandem mass spectrometry: Comparison of methods and their limitations. *Electrophoresis* 19, 939-945.
- Hemsley, S.W., Canfield, P.J. and Husband, A.J. (1995) Immunohistological staining of lymphoid tissue in four Australian marsupial species using species cross-reactive antibodies. *Immunology and Cell Biology* 73, 321-325.
- Hemsley, S.W., Canfield, P. J. and Husband, A. J. (1996) Histological and immunohistological investigation of alimentary tract lymphoid tissue in the koala (*Phascolarctos cinereus*), brushtail possum (*Trichosurus vulpecula*) and ringtail possum (*Pseudocheirus peregrinus*). *Journal of Anatomy*. 188, 279-288.

- Henzel, W.J., Billeci, T.M., Stults, J.T., Wong, S.C., Grimely, C. and Watanabe, C. (1993) Identifying proteins from 2-dimensional gels by molecular mass searching of peptide-fragments in protein-sequence database. *Proceedings of the National Academy of Science* 90, 5011-5015.
- Higgins, D.P., Hemsley, S. and Canfield, P.J. (2004) Assessment of anti-bovine IL4 and IFN antibodies to label IL4 and IFN gamma in lymphocytes of the koala and brushtail possum *Veterinary Immunology and Immunopathology* 101, 153-160
- Hindes, R.D. and Mizell, M. (1976) The origin of immunoglobulins in opossum "embryos". *Developmental Biology* 53, 49-61.
- Hogarth, K.A. (1974) Preliminary investigation of the immune system of the tammar wallaby (*Macropus eugenii* Demarest). In. Australian National University., Canberra.
- Hubbard, G., Sapphire, D., Hackleman, S., Silva, M., Vandeberg, J. and Stone, W. (1991) Ontogeny of the thymus gland of a marsupial (*Monodelphis domestica*). *Laboratory Animal Science*. 41, 227-232.
- Hunt, D.F., III, J.R.Y., Shabanowitz, J., Winston, S. and Hauer, C.R. (1986) Protein sequencing by tandem mass spectrometry. *Proceedings of the National Academy of Science* 83, 6233-6237.
- Infante, A.J., Samples, N.K., Croix, D.A., Redding, T.S., VandeBerg, J.L. and Stone, W.H. (1991) Cellular immune response of a marsupial, *Monodelphis domestica*. *Developmental and Comparative Immunology*. 15, 189-199.
- Jackson, S.M. (2003) Australian Mammals: Biology and captive management. CSIRO, Collingwood.
- Jansen, A.M., Madeirea, F.B. and Deane, M.P. (1994) Trypanosoma cruzi infection in the opossum *Didelphis marsupialis*: absence of neonatal transmission and protection by

maternal antibodies in experimental infections. *Memorial Institute of Oswaldo Cruz* 89, 41-45.

Jensen, O.N., Wilm, M., Shevchenko, A. and Mann, M. (1999) Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. I: Link A, red. *2-D Proteome Analysis Protocols. Methods in Molecular Biology* 112, 513-530.

Jurd, R.D. (1994) "Not proper mammals": Immunity in monotremes and marsupials. *Comparative Immunology, Microbiology and Infectious Disease* 17, 41-52.

Kjeldsen, L., Sengelov, H., Lollike, K., Nielsen, M.H. and Borregaard, N. (1994) Isolation and characterization of gelatinase granules from human neutrophils. *Blood* 83, 1640-1649.

Kratchmarova, K. F., Blagoev, B., Haack-Sorensen, M., Kassem, M. and Mann, M. (2005) Mechanism of divergent growth factor effects in mesenchymal stem cells differentiation. *Science* 308, 1472-1477.

Kuroishia, T., Tanakaa, Y., Sakaia, A., Sugawarab, Y., Kominea, K.-I. and Sugawara, S. (2007) Human parotid saliva contains soluble toll-like receptor (TLR) 2 and modulates TLR2-mediated interleukin-8 production by monocytic cells. *Molecular Immunology* 44, 1969-1976.

La Via, M.F., Rowlands, D.T. and Block, M. (1963) Antibody formation in embryos. *Science* 140, 1219-1220.

LaPlante, E.S., Burrell, R., Watne, A.L. and Zimmerman, B. (1966) Permanent survival of skin allografts applied to the pouch young of the opossum. In: *Surgical Forum, Vol. XVII. American College of Surgeons, San Francisco, California*, p. 200-201.

Lehrer, R.I. and Ganz, T. (2002) Cathelicidins: a family of endogenous antimicrobial peptides. *Current Opinion in Hematology* 9, 18-22.

- Link, A.J., Eng, J., Schieltz, D., Carmack, E., Mize, G.J., Morris, D.R., Garvik, B.M. and Yates, J.R. (1999) Direct analysis of protein complexes using mass spectrometry. *Nature Biotechnology* 17, 676-682.
- Lopez, J.L. (2007) Two-dimensional electrophoresis in proteome expression analysis. *Journal of Chromatography B* 849, 190-202.
- Lopez, M.F. (2000) Better approaches to finding the needle in a haystack: Optimizing proteome analysis through automation. *Electrophoresis* 21, 1082-1093.
- Lucero, J.E., Rosenberg, G.H. and Miller, R.D. (1998) Marsupial light chains: Complexity and conservation of lamda in the opossum *Monodelphis domestica*. *Journal of Immunology* 161, 6724-6732.
- Lyne, A.G., Pilton, P.E. and Sharman, G.B. (1959) Oestrus cycle, gestation period and parturition in the marsupial *Trichosurus vulpecula*. *Nature (London)* 183, 622-623.
- Marshall, S.H. and Arenas, G. (2003) Antimicrobial peptides: A natural alternative to chemical antibiotics and a potential for applied biotechnology. *Electronic Journal of Biotechnology* 15, 271-284.
- McKenzie, L.M. and Cooper, D.W. (1994) Low MHC Class II variability in a marsupial. *Reproduction, Fertility and Development* 6, 721-726.
- McKenzie, S., Deane, E.M. and Burnett, L. (2002) Haematology and serum biochemistry of the tammar wallaby, *Macropus eugenii*. *Comparative Clinical Pathology* 11, 229-237.
- Metchnikoff, E. (1968) *Immunity in Infective Diseases*. Johnson Reprint Corp, New York, NY.
- Miller, J.F.A.P., Block, M., Rowlands, D.T. and Kind, P. (1965) Effect of thymectomy on hematopoietic organs of the opossum "embryo". *Proceedings of the Society for Experimental Biology and Medicine* 118, 916-921.

- Miller, R.D. and Belov, K. (2000) Immunoglobulin genetics of marsupials. *Developmental and Comparative Immunology* 24, 485-490.
- Miller, R.D., Bergemann, E.R. and Rosenberg, G.H. (1999) Marsupial light chains: Igk with four V families in the opossum *Monodelphis domestica*. *Immunogenetics* 50, 329-335.
- Mollinedo, F., Borregaard, N. and Boxer, L.A. (1999) Novel trends in neutrophil structure, function and development. *Immunology Today* 20, 535-537.
- Musser, A.M. (2003) Review of the monotreme fossil record and comparison of palaeontological and molecular data. *Comparative Biochemical and Physiology Part A* 136, 927-942.
- O'Farrel, F.H. (1975) High resolution two-dimensional electrophoresis of proteins. *Journal Biological Chemistry* 250, 4007-4021.
- Old, J.M. and Deane, E.M. (1998) The effect of oestrus and the presence of pouch young on aerobic bacteria isolated from the pouch of the tammar wallaby, *Macropus eugeii*. *Comparative Immunology, Microbiology and Infectious Diseases* 21, 237-245.
- Old, J.M. and Deane, E.M. (2000) Development of the immune system and immunological protection in marsupial pouch young. *Developmental and Comparative Immunology* 24, 445-454.
- Old, J.M. and Deane, E.M. (2001) Histology and immunohistochemistry of the gut-associated lymphoid tissue of the Eastern Grey Kangaroo, *Macropus giganteus*. *Journal of Anatomy* 199, 657-662.
- Old, J.M. and Deane, E.M. (2002) The gut-associated lymphoid tissues of the Northern brown bandicoot (*Isodon macrourus*). *Developmental and Comparative Immunology* 26, 841-848.

- Old, J.M. and Deane, E.M. (2003) The detection of mature T- and B-cells during development of the lymphoid tissues of the tammar wallaby (*Macropus eugenii*). *Journal of Anatomy* 203, 123-131.
- Old, J.M., Deane, E.M. and Harrison, G.A. (2001) Molecular characterisation of the tammar wallaby (*Macropus eugenii*) CD3 epsilon chain cDNA. *Molecular Immunology* 38, 359-365.
- Old, J.M., Irving, M. and Deane, E.M. (2005) Histology of the pouch epithelium and the mammary gland during chemically induced oestrus in the brushtail possum (*Trichosurus vulpecula*). *Journal of Anatomy* 207, 97-102.
- Old, J.M., Selwood, L. and Deane, E.M. (2003a) Development of the lymphoid tissues of the stripe-faced dunnart (*Sminthopsis macroura*). *Cells Tissues Organs* 175, 192-201.
- Old, J.M., Selwood, L. and Deane, E.M. (2003b) A histological investigation of the lymphoid and immunohaematopoietic tissues of the adult stripe-faced dunnart (*Sminthopsis macroura*). *Cells Tissues Organs* 173, 115-121.
- Old, J.M., Selwood, L. and Deane, E.M. (2004) A developmental investigation of the liver, bone marrow and spleen of the stripe-faced dunnart (*Sminthopsis macroura*). *Developmental and Comparative Immunology* 28, 347-355.
- Otvos, L. (2000) Antibacterial peptides isolated from insects. *Journal of Peptide Sciences* 6, 497-511.
- Papayannopoulos, I.A. (1995) The interpretation of collision-induced dissociation tandem mass spectra of peptides. *Mass Spectrometry Reviews* 14, 49-73.
- Pappin, D. J., Hojrup, P. and Bleasby, A. J. (1993) Rapid identification of proteins by peptide-mass fingerprinting. *Current Biology* 3, 327-332.

- Pappin, D. J., Rahman, D., Hansen, H.F., Bartlet-Jones, M., Jeffery, W., Bleasby, A. J., in: Burlingame, A. L. and Carr, S. A. (Eds) (1996) Chemistry in mass spectrometry and peptide mass database: Evolution of methods for the rapid identification and mapping of cellular proteins, Humana, Totowa, NJ, pp. 135-150.
- Patton, W.F. (2002) Detection technologies in proteome analysis. *Journal of Chromatography B* 771, 3-31.
- Perkins, D.N., Pappin, D.J.C., Creasy, D.M. and Cottrell, J.S. (1999) Probability-based identification by searching sequence database using mass spectrometry data. *Electrophoresis* 20, 3551-3567.
- Pollock, J.J., Denpitiya, L., Mackay, B.J. and Iacono, V.J. (1984) Fungistatic and fungicidal activity of human parotid salivary histidine-rich polypeptide on *Candida albicans*. *Infection and Immunity* 40, 702-707.
- Porter, A.M.W. (2001) Why do we have apocrine and sebaceous glands? *Journal of the Royal Society of Medicine* 94, 236-237.
- Presidente, P. (1978) Diseases seen in free-ranging marsupials and those held in captivity. *Post Graduate Comm Vet Sci* 36, 457-471.
- Rabilloud, T. (2002) Two-dimensional gel electrophoresis in proteomics: Old, old fashioned, but it still climbs up the mountains. *Proteomics* 2, 3-10.
- Ramadass, P. and Moriarty, K.M. (1982) Immunoglobulins of the Australian brushtail possum, *Trichosurus vulpecula*. *Developmental and Comparative Immunology* 1975, 549-556.
- Rice, P., Longden, I., Bleasby, A., 2000. EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet.* 16, 276-277.

- Rodger, J.C., Fletcher, T.P. and Tyndale-Biscoe, C.H. (1985) Active anti-paternal immunization does not affect the success of marsupial pregnancy. *Journal of Reproductive Immunology* 8, 249-256.
- Roitt, I. (1991) *Essential Immunology*. Blackwell Scientific Publications, London.
- Roos, D. and Winterbourn, C.C. (2002) Lethal Weapons. *Science* 296, 669-670.
- Rowlands, D.T., La via, M.F. and Block, M.H. (1964) The blood forming tissues and blood of the newborn opossum (*Didelphys virginiana*) II. Ontogenesis of antibody formation to flagella of *Salmonella typhi*. *Journal of Immunology* 93, 157-164.
- Rowlands, R.T. (1970) The immune response of adult opossums (*Didelphys virginiana*) to the bacteriophage f2. *Immunology* 18, 149-155.
- Rowlands, R.T., D., B. and Lin, H. (1972) The early immune response and immunoglobulins of opossum embryos. *Journal of Immunology*. 108, 941-946.
- Rowlands, R.T. and Dudley, M.A. (1968) The isolation of immunoglobulins of the adult opossum (*Didelphys virginiana*). *Journal of Immunology* 100, 736-743.
- Schonwetter, B.S., Stolzenberg, E.D. and Zasloff, M.A. (1995) Epithelial antibiotics induced at sites of inflammation. *Science* 267, 1645-1648.
- Schwarzmeier, J.D. (1996) The role of cytokine in haematopoiesis. *European Journal of Haematology* 60(Suppl), 69-74.
- Segal, A., Dorling, J. and Coade, S. (1980) Kinetic fusion of the cytoplasmic granules of the cytoplasmic granules with phagocytic vacuole in human polymorphonuclear leukocytes. Biochemical and morphological studies. *Journal of Cell Biology* 85, 42-59.
- Segal, A. and Jones, O. (1979) The subcellular distribution and some properties of the cytochrome b component of the microbicidal oxidase system of human neutrophils. *Biochemistry Journal* 182, 181-188.

- Segal, A.W. (2005) How neutrophils kill microbes. *Annual Review of Immunology* 23, 197-223.
- Shevchenko, A., Sunyaev, S., Loboda, A., Shevchenko, A., Bork, P., Ens, W. and Standing, K.G. (2001) Charting the proteomics of organisms with unsequenced genomes by MALDI-Quadrupole time-of-flight mass spectrometry and BLAST homology searching. *Analytical Chemistry* 73, 1917-1926.
- Spengler, B., Kirsch, D. and Kaufmann, R. (1991) Metastable Decay of Peptides and Proteins in Matrix-assisted Laser-desorption Mass Spectrometry. *Rapid Communication in Mass Spectrometry* 5, 198-202.
- Spicer, S.S. and Hardin, J.H. (1969) Ultrastructure, cytochemistry, and function of neutrophil leukocyte granules. A review. *Laboratory Investigation* 20, 488-497.
- Stanley, N.F., Yadav, M., Waring, H. and Eadie, M. (1972) The effect of thymectomy on response to various antigens of a marsupial *Setonix brachyurus* (Quokka). *Australian Journal of Experimental Biology and Medical Science* 50, 689-702.
- Steiner, H., Hultmark, D., Engstrom, A., Bennich, H. and Boman, H.G. (1981) Sequence and sepcificity of two antimicrobial proteins involved in insect immunity. *Nature* 292, 246-248.
- Stone, W.H., Manis, G. S., Hoffman, E. S., Saphire, D. G., Hubbard, G. B. and Vanderberg, J. L. (1997) Fate of allogeneic skin transplantations in a marsupial (*Monodelphis domestica*). *Laboratory Animal Science* 47, 283-287.
- Switzer, R.C., 3rd, R.C.S., Merril, C.R. and Shifrin, S. (1979) A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Analytical Biochemistry* 98, 231-237.

- Taylor, J.A. and Johnson, R.S. (1997) Sequence database searches via *de novo* peptide sequencing by tandem mass spectrometry. *Rapid Communication in Mass Spectrometry* 11, 1067-1075.
- Taylor, J.A. and Johnson, R.S. (2001) Implementation and uses of automated *de novo* peptide sequencing by tandem mass spectrometry. *Analytical Chemistry* 73, 2594-2604.
- Tsai, H. and Bobek, L.A. (1998) Human Salivary histatins: promising anti-fungal therapeutic agents. *Critical Reviews in Oral Biology and Medicine* 9, 480-497.
- Turner, R.J. (1994) *Immunology: A comparative approach*. J Wiley, Chichester ; New York.
- Tyndale-Biscoe, C.H. and Renfree, M. (1987) *Reproductive physiology of marsupials*. Cambridge University Press, Cambridge.
- Tyndale-Biscoe, H. (1973). In: *Life of Marsupials*. Edward Arnold (Publishers) Ltd, London, p. 1-16.
- Walker, K.Z. and Tyndale-Biscoe, C.H. (1978) Immunological aspects of gestation in the tammar wallaby, *Macropus eugenii*. *Australian Journal of Biological Sciences* 31, 173-182.
- Washburn, M. P., Wolters, D. and Yates, J. R., 3rd. (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nature Biotechnology* 19, 242-247.
- Wattenberg, A., Organ, A.J., Schneider, K., Tyldesley, R., Bordoli, R. and Bateman, R.H. (2002) Sequence dependent fragmentation of peptides generated by MALDI Quadrupole time-of-flight (MALDI Q-TOF) mass spectrometry and its implications for protein identification. *Journal of American Society for Mass Spectrometry* 13, 772-783.

- Wedlock, D.N., Aldwell, F.E. and Buddle, B.M. (1998) Nucleotide sequence of a marsupial interleukin-10 cDNA from the Australian brushtail possum (*Trichosurus vulpecula*). DNA sequence 964, 239-241.
- Wedlock, D.N., Goh, L.P., McCarthy, A.R., Midwinter, R.G., Parlane, N.A. and Buddle, B.M. (1999a) Physiological effects and adjuvanticity of recombinant brushtail possum TNF- α . Immunology and Cell Biology 77, 28-33.
- Wedlock, D.N., Goh, L.P., Parlane, N.A. and Buddle, B.M. (1999b) Molecular cloning and physiological effects of brushtail possum interleukin-1 β . Veterinary Immunology and Immunopathology 67, 359-372.
- Welsh, I.R.H. and Spitznagel, J.K. (1971) Distribution of lysosomal enzymes, cationic proteins, and bactericidal substances in subcellular fractions of human polymorphonuclear leukocytes. Infection and Immunity 4, 97.
- Wilkins, M.R. and Williams, K.L. (1997) Cross-species protein identification using amino acid composition, peptide mass fingerprinting, isoelectric point and molecular mass: a theoretical evaluation. Journal of Theoretical Biology 186, 7-15.
- Wilkinson, R., Allanson, M., Kolega, V., Lawrence, D. and Neville, S. (1991) Purification and initial characterisation of koala immunoglobulins. Veterinary Immunology & Immunopathology 29, 189-195.
- Wilkinson, R., Kotlarski, I. and Barton, M. (1992a) Koala lymphoid cells: analysis of antigen-specific responses. Veterinary Immunology & Immunopathology 33, 237-247.
- Wilkinson, R., Kotlarski, I., Barton, M. and Phillips, P. (1992b) Isolation of koala lymphoid cells and their in vitro responses to mitogens. Veterinary Immunology & Immunopathology 31, 21-33.

- Williams, K. and Hochstrasser, D. (1997) Proteome research: New frontiers in functional genomics. In: K.W. M Wilkins, R Appel, D Hochstrasser (Ed). Springer Press, Berlin, p. 1-12.
- Wilm, M. and Mann, M. (1994) Error tolerant identification of peptides in sequence databases by peptide sequence tags. *Analytical Chemistry* 66, 4390-4399.
- Wilm, M. and Mann, M. (1996) Analytical properties of the nanoelectrospray Ion source. *Analytical Chemistry* 68, 1-8.
- Yadav, M. (1971) The transmissions of antibodies across the gut of pouch young marsupials. *Immunology* 21, 839-851.
- Yadav, M. (1972) Characteristics of blood in the pouch young of a marsupial, *Setonix brachyurus*. *Australian Journal of Zoology* 20, 249-263.
- Yadav, M. (1973) The presence of the cervical and thoracic thymus lobes in marsupials. *Australian Journal of Experimental Biology and Medical Science* 21, 285-301.
- Yadav, M. and Eadie, M. (1973) Passage of maternal immunoglobulins to the pouch young of a marsupial, *Setonix brachyurus*. *Australian Journal of Zoology* 21, 171-181.
- Yadav, M., Stanley, N.F. and Waring, H. (1972a) The microbial flora of the gut of the pouch-young and the pouch of a marsupial, *Setonix brachyurus*. *Journal of General Microbiology* 70, 437-442.
- Yadav, M., Stanley, N.F. and Waring, H. (1972b) The thymus glands of a marsupial, *Setonix brachyurus* (quokka), and their role in immune responses - Effect of thymectomy on somatic growth and blood leucocytes. *Australian Journal of Experimental Biology and Medical Science*. 50, 357-364.
- Yadav, M., Stanley, N.F. and Waring, H. (1972c) The thymus glands of a marsupial, *Setonix brachyurus* (quokka), and their role in immune responses. *Structure and*

- growth of the thymus glands. Australian Journal of Experimental Biology and Medical Science 50, 347-356.
- Yadav, M., Waring, H. and Stanley, N. (1974) Effect of thymectomy on skin allograft survival in a macropodid marsupial, *Setonix brachyurus*. Transplantation 17, 30-36.
- Young, L., Basden, K., Cooper, D. and Deane, E. (1997) Cellular components of the milk of the tammar wallaby (*Macropus eugenii*). Australian Journal of Zoology. 45, 423-433.
- Young, L.J. and Deane, E.M. (2001) Cellular composition of the late milk of the koala (*Phascolarctos cinereus*). Australian Journal of Zoology 49, 195-202.
- Young, L.J. and Deane, E.M. (2003) Immunocytochemical identification of CD5 positive peripheral blood lymphocytes in four marsupial species. Comparative Clinical Pathology 12, 26-32.
- Young, L.J. and Deane, E.M. (2005) Culture and characterisation of peripheral blood monocytes and monocyte-derived adherent cells of the tammar wallaby, *Macropus eugenii*. Immunology Letters 96, 253-259.
- Young, L.J. and Deane, E.M. (2006) A longitudinal study of changes in blood leukocyte numbers in the tammar wallaby, *Macropus eugenii*. Comparative Clinical Pathology 15, 63-69.
- Zanetti, M. (2004) Cathelicidins, multifunctional peptides of the innate immunity. Journal of Leukocyte Biology 75, 39-48.
- Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. Nature 415, 389-395.

Chapter 2

Proteomic analysis of neutrophil proteins in the tammar wallaby (*Macropus eugenii*).

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Proteomic analysis of the neutrophil proteins of the tammar wallaby (*Macropus eugenii*)

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Abstract

A proteomic analysis of neutrophils from the tammar wallaby, *Macropus eugenii*, has been performed. Neutrophils were isolated from peripheral blood using density gradient centrifugation with Histopaque-1077, followed by treatment with ammonium chloride to lyse residual erythrocytes. Two-dimensional gel electrophoresis (2-DE) of lysed neutrophils was undertaken followed by in-gel trypsin digest and nanoliquid chromatography coupled tandem mass spectrometry (LC-MS) analysis and database searches. Seventy-seven proteins were isolated, 53 of which could be identified with high confidence as primarily of cytosolic origin. Protein identifications were only possible by matching identical peptide sequences within the NCBI mammalian database with the Mascot search program. Sequence identities were only deemed acceptable if more than three peptides were identified, the precursor/protein ion tolerances were less than ± 0.25 Da and the total Mowse scores were greater than 100. The validity of this approach was tested using a scrambled database where no single identified peptide showed Mowse scores greater than 55. This is the first report of the neutrophil proteins of any marsupial and represents a first step in examining the identity of proteins involved in innate defence in this marsupial.

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1. Introduction

Polymorphonuclear neutrophils are short-lived phagocytic cells that play a pivotal role in innate defence in eutherian mammals. Along with the other major phagocytic cell type, the macrophage, these cells are of ancient evolutionary origin, arising from the wandering phagocytic amoebocytes of invertebrates (Roitt et al., 2001). In eutherian mammals, neutrophils kill invading microorganisms by two mechanisms. The first method consists of an oxidative burst mediated via NADPH oxidase, which results in a cascade of reactive species including superoxide anions, subsequent production of hydrogen peroxide, release of myeloperoxidase (MPO) and the generation of

hypochlorous acid, all of which result in oxidative damage to microorganisms (Avram et al., 2004). The second mode of action depends on the controlled release of a repertoire of gene-coded antimicrobial polypeptides contained within neutrophil granules. These peptides kill by interaction with the phospholipid bilayers of the cell membranes of the ingested microorganisms, resulting in membrane permeabilisation, loss of ions and metabolites, and blockade of various essential cellular functions (Blondelle et al., 1999; Hancock and Chapple, 1999).

The advent of proteomic techniques has facilitated the study of protein expression in a range of cell types. However, to date, neutrophil proteins have only been documented in humans (Boussac and Garin, 2000; Fessler et al., 2002; Lominadze et al., 2005), the rat (Piubelli et al., 2002) and most recently in the cow (Lippolis and Reinhardt, 2005). Of these the most comprehensive analysis has been undertaken on human neutrophils with over 500 proteins identified from subcellular organelles (Lominadze et al., 2005). In the latter species, this extensive

Abbreviations: MPO, myeloperoxidase; HSP, heat shock protein; fMLP, N-formyl-methionyl-leucyl-phenylalanine.

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identification was facilitated by the existence of a substantial translated genomic database.

The tammar wallaby, *Macropus eugenii*, has been positioned as a model marsupial and a significant body of fundamental biological research has been undertaken. This research has primarily focused on unique features of reproduction and development, including development of the immune system (Old and Deane, 2000). This animal has been recently targeted as one of two marsupial species for whole genome sequencing (Wakefield and Marshall-Graves, 2003). However, to date, there is little publicly available sequence data making identification of proteins difficult. This problem of high confidence identification is compounded by the fact that there is limited comparability of protein sequences across species barriers (Verrillis et al., 2000).

In this paper, we present first report of the neutrophil proteins of any marsupial. To overcome the lack of database and achieve high confidence identification of these proteins from the tammar wallaby, we have used nanoliquid chromatography coupled tandem mass spectrometry analysis followed by database searching using Mascot search engine coupled with validity testing using a scrambled database.

2. Materials and methods

2.1. Animals and sample collection

Animals used in this study were adult male and female tammar wallabies maintained in a captive breeding colony at the Commonwealth Scientific and Industrial Research Organisation, Division of Sustainable Ecosystems, Canberra, ACT Australia. 10 to 15 ml of blood was collected from the caudal vein of 40 healthy adult animals into tubes coated with EDTA and to which gentamycin was added to a final concentration of 100 µg/ml to inhibit microbial growth. Blood collected from each animal was later pooled for neutrophil isolation. The collection of blood samples was approved by the CSIRO Sustainable Ecosystems Animal Ethics Committee (Approval No. 03/04-12).

2.2. Isolation of neutrophils

Neutrophils were isolated by density gradient centrifugation over endotoxin-free Histopaque-1077 (Sigma Diagnostic, St. Louis, MO, USA), according to the manufacturers instructions. Briefly, 20 ml of blood, diluted 1:1 with 1× PBS as layered over 20 ml of density gradient medium in a 50 ml sterile, polypropylene centrifuge tube and centrifuged at 645×g at 18 °C for 15–20 min. The granulocyte layer was retrieved and resuspended to a final volume of 50 ml at 37 °C with NH₄Cl (8.26% ammonium chloride, 1% potassium bicarbonate, 0.037% EDTA) as erythrocyte lysis solution for 10 min. The lysis was stopped by adding an equal volume of 2× PBS to restore the tonicity for the granulocytes. The cells in the suspension were disrupted by probe sonication with a Branson 450 probe for 60 s at 120 W and lysis was monitored by microscopic observation of granulocyte smears stained with Diff-Quik (Lab Aids, Narrabeen, NSW, Australia). The nuclei

and unbroken cells were removed by centrifugation at 420×g at 4 °C for 15 min and the supernatant stored at –80 °C until required for proteomic analysis.

2.3. Protein extraction

Proteins were precipitated from the supernatant and purified from the pellet using the Ready Prep™ 2-D Clean up kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The resultant material was then dissolved prior to subsequent analysis in 200 µl of 2-D sample solubilising solution. This solution consisted of 7 M urea, 2 M thiourea, 65 mM DTT, 2% CHAPS, 1% carrier ampholytes (3–10), 40 mM Tris and 0.002% bromophenol.

2.4. Two-dimensional gel electrophoresis (2-DE)

Two-DE was performed using 11 cm pH 3–10 linear IPG strips (Bio-Rad) in the first dimension. IEF was performed using the Multiphore II system (Amersham Pharmacia Biotech, Uppsala, Sweden) for 35 kV h at 20 °C. The IPG strips were then equilibrated for 20 min at room temperature by gentle rocking in 6 M urea, 2% SDS 20% glycerol, 1× Tris–HCl (pH 8.8), 5 mM tributylphosphine, 2.5% acrylamide and then embedded in 0.5% agarose on top of precast 4–20% gradient Tris–HCl criterion gels (Bio-Rad, Hercules, CA, USA). Electrophoresis was carried out using a cathode running buffer consisting of 192 mM glycine, 15 mM Tris, 0.1% SDS at pH 8.3. Gels were initially run at 5 mA/gel for 30 min, then at 200 V, until the bromophenol blue buffer front had completely run off the bottom of the gel. The gels were fixed in 10% methanol and 7% acetic acid followed by staining overnight with Sypro Ruby (Bio-Rad). Gels were destained in 10% methanol and 7% acetic acid for 1 h, and images were captured with the Molecular Imager Fx (Bio-Rad). Prior to excision of protein and to enhance visualisation, gels were further stained with 0.1% w/v colloidal Coomassie Brilliant Blue G-250 overnight and destained with 5% acetic acid for 1 h. The images were then scanned and captured with a Kodak Digital camera (Eastman Kodak, Rochester, NY, USA).

2.5. Mass spectrometry

Proteins were excised, washed three times in 50% v/v acetonitrile and 25 mM NH₄HCO₃, pH 7.8 at 37 °C for 10 min, dried at room temperature in a Speedy-Vac (Savant, Farmingdale, NY, USA) and finally covered with 8 µl of sequencing grade trypsin (15ng/µl) and digested overnight at 37 °C. Products were recovered from the gel by sequential extractions with 10% acetonitrile and 1% v/v formic acid. The combined extract was dried and peptides dissolved in 20 µl of 0.1% formic acid (Shevchenko et al., 1996). 5 µl of digests were concentrated and desalted using a micro C18 precolumn (500 µm×2 mm, Michrom Bioresources, Auburn, CA, USA) with H₂O/CH₃CN (98:2, 0.1% formic acid) at 20 µl/min. After a 4 min wash, the precolumn was automatically switched (Valco 10 port valve, Houston, TX, USA) into line with a fritless nanocolumn (Gatlin et al., 1998). Digested peptides were

Table 1
Identification of proteins from the tannur wallaby neutrophils using 3–10 pI range 2-D gel

Spot no.	Accession no.	Exp. and theor. pI	Exp. and theor. mol.	No. of peptide matches	Score and % coverage	Name
<i>Cytoskeletal proteins</i>						
85	gi 17511847	5.3, 5.31	~50,000, 41,766	30	956, 55	ACTG1 protein Type I hair keratin ^a Type II hair keratin ^a
34	gi 60389477	~5.5, 5.30	~49,000, 41,775	18	815, 61	Beta-actin
25	gi 4507877	~6.5, 5.89	~116,591, 116,649	13	627, 14	Vinculin
35	gi 49868	~5.2, 5.30	~100,000, 39,161	10	475, 34	Beta-actin
68	gi 338695	6.2, 4.75	50,000, 49,727	14	475, 24	Beta-tubulin
89	gi 17511847	5.3, 5.31	~51,000, 41,766	14	471, 32	ACTG1 protein
72	gi 49868	~5.0, 5.78	~50,000, 39,161	12	431, 28	Beta-actin
9	gi 264748	4.8, 4.80	~20,000, 19,683	7	356, 47	Myosin regulatory light chain
57	gi 60389477	5.4, 5.30	~45,000, 41,775	10	356, 27	Beta-actin
86	gi 49868	~5.2, 5.78	~50,000, 39,161	7	354, 24	Beta-actin
62	gi 33416530	5.9, 5.43	~36,000, 35,907	7	354, 16	Annexin Iv
8	gi 56703	~4.7, 4.78	~19,000, 19,826	10	351, 41	Myosin light chain
19	gi 71604	4.6, 4.65	38,000, 28,374	7	336, 22	Tropomyosin I
	gi 530049	4.8, 4.8	28,000, 26,279	6	300, 25	14-3-3 protein
29	gi 1703316	~7.1, 6.28	~38,000, ~38,000	5	296, 16	Annexin-I
59	gi 49868	5.2, 5.78	~49,000, 39,161	9	281, 23	Beta-actin
	gi 51477696	5.86, 5.9	~41,000, 45,154	5	205, 10	Parvin beta isoform
				5		Keratin 1 ^a
73	gi 49868	~6.1, 5.78	~52,000, 39,161	6	257, 17	Beta-actin
46	gi 49868	~5.2, 5.29	~75,000, 39,161	5	253, 16	Beta-actin
64	gi 62664759	~6.0, 5.44	~40,000, 27,700	5	212, 15	Prohibitin
	gi 2724046	5.6, 5.65	40,000, 35,814	5	161, 13	Beta-actin
20	gi 7441397	~4.3, 4.72	38,000, 28,703	7	196, 16	Tropomyosin
74	gi 61553085	~6.3, 6.37	~70,000, 38,967	6	192, 12	Annexin I
63	gi 1345668	~5.3, 5.47	~45,000, 31,216	6	184, 15	F-actin capping protein beta subunit
				5		Keratin
41	gi 47522618	~5.8, 5.62	~116,591, 116,803	4	146, 4	Vinculin
7	gi 136429	4.9, 5.76	15,000, 15,975	3	132, 22	Coactosin-like protein
18	gi 2897818	~5.3, 5.33	~32,000, 22,389	3	119, 16	Huntingtin interacting protein
<i>GTP-ases</i>						
80	gi 2463536	~5.9, ~5.8	~28,000, ~24,294	10	382, 36	GTP-binding protein
	gi 420272	5.98, 5.85	28,000, 23,850	4	161, 22	GTP-binding protein
61	gi 98453	5.4, 5.47	~38,000, 37,369	12	227, 11	G protein beta 1 subunit
				5		Keratin 1 ^a
				8		Cytokeratin 9 ^a
78	gi 595280	~6.3, 5.37	~24,000, 20,916	5	209, 19	Rap 1b
				9		Keratin, type II ^a Cytoskeletal 1 ^a
<i>Redox proteins</i>						
33	gi 65987	~8.1, 6.90	~40,000, 35,686	4	200, 13	Glyceraldehyde-3-phosphate dehydrogenase
81	gi 1717797	~6.2, 4.70	~24,000, 14,158	5	182, 27	Peroxioredoxin ^a (thioredoxin peroxidase 1)
6	gi 55971	4.6, 6.08	~16,000, 16,119	4	159, 15	Cytochrome c oxidase polypeptide
<i>Heat shock proteins</i>						
70	gi 56385	6.0, 5.43	71,000, 70,884	13	544, 18	Heat shock protein-70
82	gi 15010550	5.4, 4.73	~100,000, 92,413	8	315, 11	Heat shock protein-90
75	gi 396270	5.4, 5.89	71,000, 70,043	4	121, 6	Heat shock protein-70
	gi 3789970	5.4, 5.89	71,000, 43,865	4	100, 6	Fibrinogen A-alpha-chain
<i>Enzymes</i>						
60	gi 17369886	~5.7, 5.71	~37,000, 36,548	8	348, 22	Lactate dehydrogenase ^a
				9		Keratin 1 ^a
				9		Cytokeratin 9 ^a
56	gi 253706	4.8, 4.73	28,000, 27,696	8	291, 22	14-3-3 protein zeta-chain
55	gi 253706	4.8, 4.73	28,000, 27,696	9	289, 17	14-3-3 protein zeta-chain

(continued on next page)

Table 1 (continued)

Spot no.	Accession no.	Exp. and theor. pI	Exp. and theor. mol.	No. of peptide matches	Score and % coverage	Name
66	gi 29792061	6.5, 7.01	~55,000, 47,139	5	256, 11	EN01 protein
<i>Enzymes</i>						
88	gi 202549	3.9, 4.87	~60,000, 54,033	8	254, 15	Iodothyronine 5' monodeiodinase
				5		Keratin, type II ^a
						Cytoskeletal ^a
69	gi 1083063	~6.0, 6.51	~75,000, 54,939	6	210, 8	Protein disulphide isomerase
67	gi 693933	6.3, 7.01	~55,000, 47,079	5	185, 8	Enolase 1 variant
	gi 1703	6.3, 5.30	~55,000, 41,729	2	78, 5	Gamma non-muscle actin
14	gi 30582585	~5.6, 6.13	~17,000, 17,127	4	174, 28	Ubiquitin-conjugating enzyme
<i>Secretory vesicles</i>						
58	gi 162648	4.9, 5.82	48,000, 69,248	8	373, 8	Albumin
	gi 809190	4.9, 4.9	48,000, 35,783	6	297, 17	Annexin V
				7		Keratin 1
	gi 46250414	4.9, 5.01	48,000, 26,680	6	181, 15	EF hand domain containing 2
44	gi 3401945	~6.3, 6.21	72,000, 57,338	3	171, 6	Alkaline phosphatase
	gi 758306	~6.35, 9.3	72,000, 85,644	3	99, 2	Gelsolin
<i>Channels and transporters</i>						
32	gi 102	9.89, 9.21	~100,000, 59,683	7	367, 14	H ⁽⁺⁾ -transporting ATPase synthase
65	gi 4588526	~5.0, 5.02	~38,000, 26,907	3	143, 9	Nuclear chloride channel
				3		Keratin
<i>Other proteins</i>						
84	gi 54400730	~5.5, 6.01	~50,000, 57,422	6	214, 9	Similar to chaperone containing TCP-1 beta subunit
	gi 2136796	~5.5, 6.25	50,000, 50,947	4	145, 7	Actin binding protein p57
83	gi 10946928	6.2, 5.89	~50,000, 49,168	3	116, 4	Heterogeneous nuclear ribonuclear protein
<i>Contaminants</i>						
90	gi 122633	~7.9, ~7.9	~15,000, ~15,000	14	424, 56	Hemoglobin beta-chain
	gi 122429	7.23, 7.30	16,005, 15,210	10	331, 47	Haemoglobin alpha-chain
48	gi 122429	~7.3, ~7.9	~15,000, 15,210	12	401, 46	Hemoglobin alpha-chain
	gi 122633	7.36, 7.23	16,005, 15,000	9	293, 44	Hemoglobin beta-chain
52	gi 122633	~7.9, 7.23	~15,000, 16,005	4	209, 28	Hemoglobin beta-chain
				7		Cytokeratin 9 ^a
				5		Keratin 1 ^a
	gi 12963469	~7.9, 6.19	~15,000, 28,919	4	209, 22	Phosphoglycerate mutase 1
				3		Epidermal cytokeratin 2 ^a
47	gi 122633	~7.2, ~7.7	~15,000, 16,005	4	203, 28	Hemoglobin beta-chain
	gi 50979054	7.23, 7.77	~15,000, 40,388	3	122, 8	Vasodilator-stimulated phosphoprotein
49	gi 122633	~7.5, 7.23	~15,000, 16,005	3	136, 23	Hemoglobin beta-chain
30	gi 122633	~7.9, 7.23	~15,000, 16,005	3	133, 22	Hemoglobin beta-chain

All the peptides were ranked first except 81 and 60 with one peptide ranked second.
Tamarin wallaby proteins were separated by 2-D gel electrophoresis as described in Section 2.4. Proteins were identified by nano-LC-mass spectrometry as described in Section 2.5. Theoretical pI and M_r were obtained from NCBI nr database.
^a Indicates contaminant.

separated by nano-LC using a Cap-LC autosampler system (Waters, Milford, MA, USA). Peptides were eluted using a linear gradient of H₂O/CH₃CN (98:2, 0.1% formic acid) to H₂O/CH₃CN (50:50, 0.1% formic acid) at ~200 nl/min over 30 min. The precolumn was connected via a fused silica capillary (10 cm, 25 μm) to a low volume tee (Upchurch Scientific) where 2600 V was applied and the column tip positioned ~1 cm from the Z-spray inlet of a QTOF Ultima hybrid tandem mass spectrometer (Micromass, Manchester, UK). Positive ions were generated by electrospray and the QTOF operated in data dependent acquisition mode. A TOF MS

survey scan was acquired (m/z 350–1700, 1 s) and the two largest multiple charged ions (counts >20) were sequentially selected by Q1 for MS–MS analysis. Argon was used as collision gas and an optimum collision energy chosen, based on charge state and mass. Tandem mass spectra were accumulated for up to 6 s (m/z 50–2000).

2.6. Database searching

Peak lists were generated by MassLynx (version 4 SP1, Micromass) using the Mass Measure program to automatically

baseline subtract, smooth and centroid the data before submission to the database search program Mascot (version 2.1, Matrix Science, London, UK) with proteins identified by correlation of mass spectra to entries in the NCBI database (Sept 2005). Mascot MS/MS ion search criteria were as follows: taxonomy—mammalia, trypsin digestion allowing up to one miscleavage, variable modification—oxidation of methionine, cysteine as carboxyamidomethylation or propionamide, peptide tolerance of 0.25 Da, and MS/MS tolerance of 0.2 Da. The “ion score cutoff” was manually set to 20 thereby eliminating the lowest quality matches. A probability based Mowse score >48 indicated identity ($p < 0.05$). All protein sequences within the NCBI database were shuffled, without changing amino acid compositions or taxonomic information, using the shuffleseq program within the EMBOSS package (version 2.10.0-Win-0.8) (Rice et al., 2000) and Mascot searches repeated as with the standard database.

3. Results and discussion

3.1. Isolation of tammar wallaby neutrophils

The difference in the level of neutrophils in peripheral blood of the tammar wallaby compared to humans and other eutherian mammals (McKenzie et al., 2002) makes it difficult to successfully apply methods previously reported for these species in the isolation of neutrophils in marsupials. In cows, neutrophils can be separated from contaminating erythrocytes by hypotonic lysis with ice-cold water (Gennaro et al., 1983). However, in this study, we found that this hypotonic lysis procedure did not effectively remove contaminating erythrocytes resulting in significant levels of haemoglobin contamination in the gels. Moreover, this protocol resulted in loss of neutrophils due to the increased number of washes required to achieve erythrocyte lysis. We found that the use of ammonium

chloride was a compromise lysis protocol that prevented significant loss of neutrophils and minimised haemoglobin contamination, but still allowed separation and identification of neutrophil proteins. The retention of haemoglobin is apparent in the identity of proteins obtained (Table 1).

3.2. Protein isolation and sample preparation for 2-DE

Initially, Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) was used for the isolation of neutrophil proteins. However, the numbers of protein bands subsequently visible on a 4–20% Tris–glycine gradient gel (Invitrogen, Carlsbad, CA, USA) were low. This was possibly due to the use of SDS as the surfactant. Enhanced solubility of proteins and increased detection of protein bands was achieved using the Ready Prep™ 2D-Clean up kit and Sample Solubilising Solution. Pre-treatment of the sample prior to IEF was necessary to remove particulate matter and salts, lipids and carbohydrates. The pre-treatment of the sample using the Ready Prep™ 2D-Clean up kit also minimised the streaking effect observed when samples are not pre-treated.

3.3. Protein identification

2-DE resulted in the separation of over 100 proteins from tammar wallaby neutrophils (Fig. 1). To increase the visibility of the proteins for excision, the gel was stained firstly with Sypro Ruby (Fig. 1) and subsequently restained with Coomassie Brilliant Blue. The majority of proteins were visible in the range of pI 4–7 and molecular weight 50 to 250 kDa. Fewer proteins were visible at low molecular weight between 10 and 25 kDa. Due to the large number of proteins observed across a broad pI and molecular weight, the most intense proteins were excised, subjected to in situ tryptic digestion and analysed using LC-MS/MS.

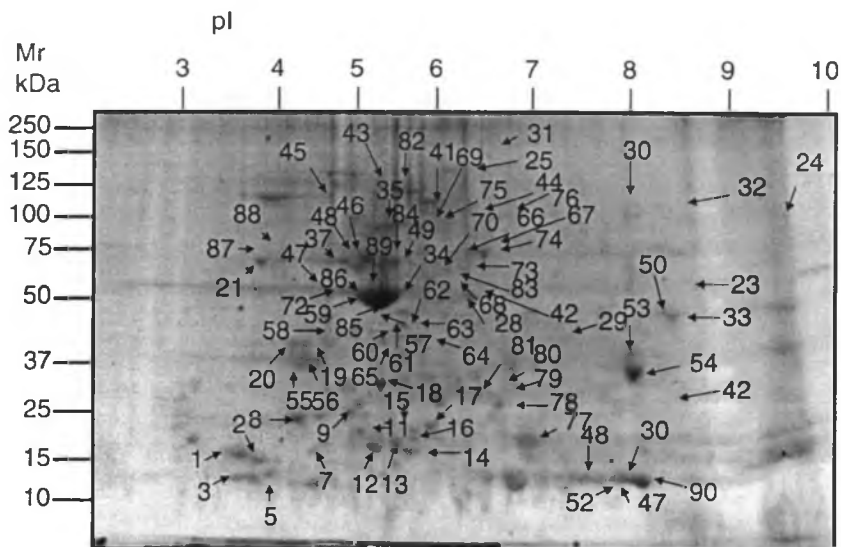


Fig. 1. Polymorphonuclear leukocyte proteins of tammar wallaby separated on 11 cm IPG strip pH 3–10 2-D gel stained with Sypro Ruby. Spot numbers refer to identification in Tables 1 and 2.

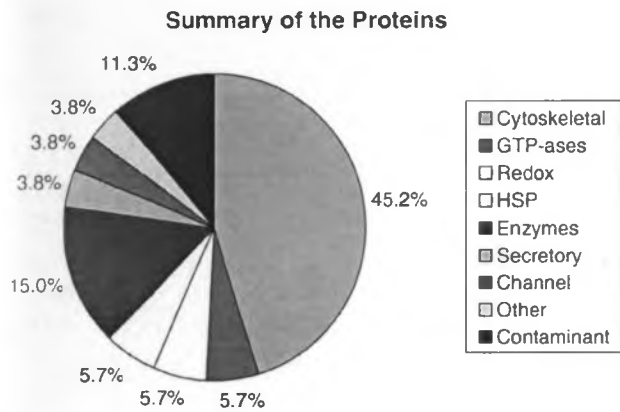


Fig. 2. Summary of proteins identified. 53 unique proteins were identified with high confidence, most were associated with fundamental metabolic pathways or with cytoskeletal function.

Of the 77 proteins analysed, 53 unique proteins were identified with very high confidence searching the NCBI mammalian database and are shown in Table 1 and Fig. 2. A minimum number of three peptide matches (Mowse scores > 20) and mass accuracy of 0.25 Da, in conjunction with M_r and pI search parameters, was used to attain a high confidence identification. Recently, database searches using reversed sequences as decoy databases were shown to allow an estimation of the likely error rate for large amounts of tandem mass spectra from two-dimensional liquid chromatography tandem MS (Moore et al., 2002; Peng et al., 2003). An NCBI database containing shuffled amino acid sequences was generated and the Mascot searches repeated with identical criteria. No peptides with Mowse scores > 55 were found and

only three matches had two different peptides identified (Fig. 3). This indicates that false positive protein identifications would be unlikely using the search parameters described here, despite the absence of any tammar wallaby protein sequence database.

The majority of the proteins identified were either cytoskeletal proteins or those involved in fundamental metabolic pathways. Table 1 lists both the deduced identity of these proteins plus their theoretical and experimental M_r . Table 2 lists more tentative identifications of proteins due to the lower number of peptide matches, but these still have reasonably high Mowse scores.

The proteins identified were common to proteins identified in neutrophils from other species with the exception of a few haemoglobin and keratin contaminants listed in Tables 1 and 2. Serine protease inhibitor identified in Table 2 may be from the protease cocktail inhibitor added to the proteins to prevent proteolytic degradation.

As a tammar wallaby protein database was not available, a search strategy based on identification of conserved peptide sequences was performed across species boundaries for the identification of all proteins. The experimental pI and molecular weight of most of the identified proteins was consistent with the theoretical values observed in eutherians, suggesting that the proteins identified are well conserved throughout evolution. However, the 2-D gel had many cytoskeletal proteins which were present in high abundance. Actin appears to have focussed poorly (Fig. 1). Moreover, actin (protein no. 35 and 46) and $H^{(+)}$ -transporting ATPase synthase (protein no. 32) have consistent pI but have a different molecular weight which may be due to a difference in the protein sequence in tammar wallaby. Alternatively, the molecules may have polymerised to form oligomers and hence increasing their molecular weight

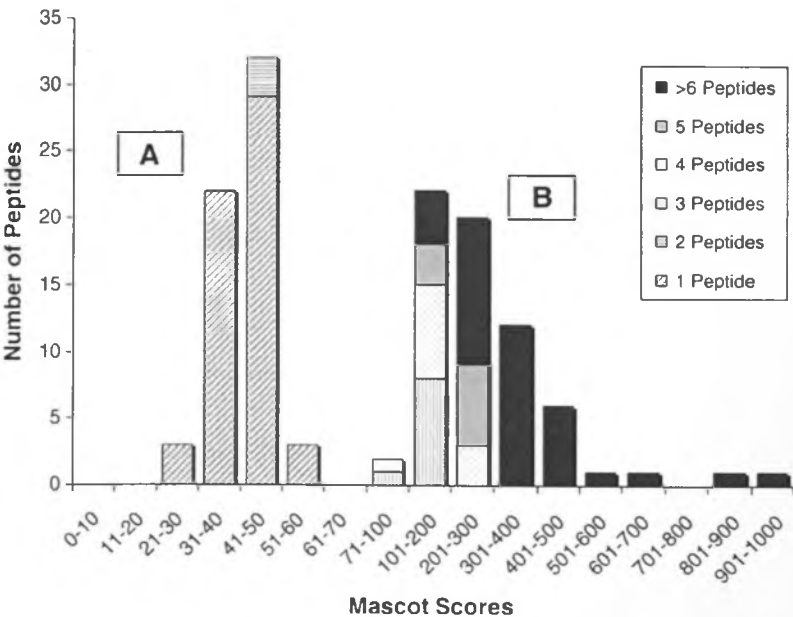


Fig. 3. Mascot scores for all proteins searched using NCBI mammal and a shuffled database. The Mascot scores 0–60 shown as A indicate low scoring matches obtained from searching the shuffled database. The Mascot scores 71–1000 shown as B indicate a minimum criteria of three high scoring peptides eliminates the possibility of a false positive identification.

Table 2
Tentative identification of proteins from tammar wallaby neutrophils using 3–10 pI range 2-D gel

Spot no.	Accession no.	No. of peptide matches	Score and % coverage	Identification	Sequence
13	gi 19697925	2	148, 15	Glia maturation factor	R. LVQTAELTK. V R. TTDDLTEAWLQEK. L
43	gi 122429	2	117, 23	Hemoglobin alpha-chain	FLAAVSTVLSTK IADALGQAVEHIDDLPGTLSK
28	gi 22347581	2	111, 4	Serine protease inhibitor	K. IPELLAK K. LVLVNAIYEK
79	gi 2463536	2	101, 9	GTP binding protein	K. VVLIGDSGVGK. S K. STIGVEFATR. S
24	gi 122633	2	100, 15	Hemoglobin beta-chain	VAIEQTGGEALGR VAIEQTGGEALGR
16	gi 13385866	1	54, 7	Actin related protein 2/3 complex	K. ALAAGGVGSIVR. V
54	gi 42415681	2	84, 15	Hemoglobin beta-chain	K. VLVAFGDAIK. N K. VAIEQTGGEALGR
42	gi 15593267	2	78, 15	Fibrinogen gamma-chain	IDGSLDFK EGFGHLSPGTGTFEFLGNEK
53	gi 122633	2	70, 13	Hemoglobin beta-chain	K. VLVAFGDAIK. N R. LLIVYPWTSR. F
5	gi 817960	2	62, 36	Histone H4	K. VFLENVIR. D K. TVTAMDVVYALK. R+ oxidation (M)

The tammar wallaby neutrophils separated by 2-D electrophoresis and identified by nano-LC-mass spectrometry. The table lists the proteins identified with low confidence as indicated by few peptide matches despite high score.

observed on gels by a factor of approx. 2 (Fig. 1). Interestingly, some other proteins were identified in spots separated by differences in pI (e.g. protein no. 6). These likely correspond to some type of post-translational modification such as phosphorylation, which increases the observed pI and occurs readily after activation (Berkow and Dodson, 1990). However, when searches were carried out for phosphorylation, no phosphopeptides were found and the differences could be due to the high abundance of the protein leading to poor focussing on the gel.

Approximately 19% of the proteins identified were associated with metabolic pathways. Such proteins are essential for the normal functioning of the cell and would be expected to be abundant. Glyceraldehyde-3-phosphate (Schmitz et al., 2003), glucose-6-phosphate and enolase (Piubelli et al., 2002) are key enzymes involved in the glycolytic pathway, while cytochrome oxidase is pivotal in electron transfer reactions across the inner mitochondrial membrane (Rahman et al., 2000). Thioredoxin peroxidase plays a significant role as an anti-oxidant and regulates the apoptotic pathway (Zhang et al., 1997). It is also produced in response to microorganisms and external stimuli, such as phorbol esters (Das and White, 2002). Protein disulphide isomerase which may be involved in the formation of disulphide bonds (McLaughlin and Bulleid, 1998). Enzymes mediators in the form of 14-3-3 protein have been found to be involved in the actin disassembly by the increase in the level of cytosolic Ca^{2+} and activation of protein kinase C (Burgoyne and Morgan, 2003). Albumin is present in secretory vesicles of neutrophils with alkaline phosphatase expressed on its membrane (Tapper et al., 2002). These markers are exocytosed when stimulated with fMLP (*N*-formylmethionyl-leucyl-phenylalanine) and ionomycin (Sengelov et al., 1993). The cells isolated here were not deliberately stimulated but activation may have occurred during isolation.

A variety of heat shock proteins (HSP) were also found. 90 kDa and 70 kDa HSPs are expressed in response to stress and

are highly conserved across different species (Villar, 2000). These HSP are involved in protecting against cellular damage when exposed to abrupt, sudden heat or stressful conditions (Villar, 2000). The production of HSP may have been up-regulated as the cells were exposed to repeated freezing and thawing during the various steps of neutrophil isolation.

A range of GTPases were also identified (Table 1). These proteins are involved in mediating extracellular signals to regulate cellular activity (Fenteany and Glogauer, 2004). These GTPases include Rab-like proteins, which are the largest of the Ras superfamily and are localised to specific intracellular compartments (Wennerberg et al., 2005). It has been suggested that this protein may be involved in the steps leading to respiratory burst and translocation of protein complexes by fusion of cellular components with the plasma membrane (Laura et al., 1998).

The other major groupings of identified protein were cytoskeletal proteins. These proteins play a fundamental role in neutrophil function. Mammalian actin occurs as three isoelectric variants— α -, β - and γ -actin (Provost et al., 2001). The different isoforms of actin undergo rapid reorganisation and, along with myosin and tropomyosin, play a significant role in migration and phagocytosis of microorganisms (Lippolis and Reinhardt, 2005). All these processes are regulated by various actin-binding proteins (Nunoi et al., 1999). Binding proteins attach to the actin filaments through electrostatic interaction involving basic and acidic amino acids (Vandekerckhove, 1990). A variety of binding proteins have been identified which regulate actin dynamics (Table 1). Coactosin-like protein is an actin-binding protein isolated from actin–myosin complex that binds to F-actin and opposes the activity of capping proteins (Provost et al., 2001). Intermediate filaments like vimentin have been shown to be secreted by macrophages in response to pro-inflammatory signals for killing bacteria and generation of reactive oxygen species (Mor-Vaknin et al., 2003). It is reasonable to consider that vimentin could play a similar role

in neutrophils (Fenteany and Glogauer, 2004). Tubulin, also detected in this study, is another cytoskeletal protein which regulates functions like cell polarization and migration (Fenteany and Glogauer, 2004). Similarly, vinculin is a part of the integrin family of receptor proteins that are found in a focal adhesion complex at the inner side of the plasma membrane and are required for changes in cell shape and movement (Ezzell et al., 1997). This focal adhesion complex consists of other proteins like α -actinins, paxillin and talin (Burridge et al., 1998), which are required for the regulation of actin organisation (Luna and Hitt, 1992) and mechanical signal transfer (Wang et al., 1993). Vasodilator-stimulated proteins are localized to the actin and act as ligands for the focal-adhesion-associated proteins like zyxin and vinculin and actin-binding profiling (Lawrence and Pryzwansky, 2001). Vasodilator-stimulated proteins play an important role in controlling cytoskeletal organisation by binding to F-actin and profiling, a protein that forms complexes with G-actin and regulate actin dynamics (Lawrence and Pryzwansky, 2001). Unlike vinculin associated with the process of exocytosis, Huntingtin interacting protein was identified as cytosolic proteins acting as a connecting link between the actin cytoskeleton and endocytic machinery (Engqvist-Goldstein et al., 1999). Annexin is a phospholipid and Ca^{2+} binding protein-like annexins are expressed in animal and plant kingdom, which participate in a variety of functions such as in phagocytosis (Gerke and Moss, 2001).

In addition to those proteins which could be identified with a high degree of confidence based on the minimum number of three peptide matches, a number of proteins could not be identified with similar confidence. These are shown in Table 2. These proteins had limited matches resulting in low scores and may represent proteins that probably have limited sequence identity between proteins in the current NCBI database. Another group of proteins (14) did not have any confident identification after Mascot searches. These were further divided into two groups of samples, one set (nine proteins) contained only a limited number (<10) of poor fragmentation spectra and we would not expect protein identification from these. These proteins were weakly stained and of low abundance. The other set (five proteins) contained a number (~50) of good quality fragmentation spectra and these probably represent proteins that have little identity with proteins contained within the current NCBI database (data not shown). Another search strategy where sequence tags are derived from de novo sequencing and BLAST searching may identify these proteins (Altschul et al., 1997; Taylor and Johnson, 1997). However, initial attempts at obtaining amino acid sequences de novo from the unidentified peptides were unsuccessful.

Proteomic analysis requires the matching of peptide peaks, either by peptide mass fingerprinting or low energy collision induced tandem mass spectrometry (CID MSMS), with those of known proteins within databases (Yates et al., 1995). In this study, the lack of a tammar wallaby database was a major constraint in the confident identification of proteins by using peptide mass fingerprinting as has been acknowledged by other researchers (Cordwell and Humphery-Smith, 1997). Proteins may be confidently identified by CID MSMS when a single

peptide fragmentation spectra results in a high Mascot score, and/or when more than 1 peptide is identified the confidence of assignment is increased. In this study, we used a series of techniques to optimise the chance of identification. Firstly, the technique of electrospray ionisation mass spectrometry generates multiple charged peptide ions that readily fragment forming predominately intense y- and b-type ions. Peptides were also separated by online nano-LC, which allows removal of hydrophilic contaminants like salts and improves the data-dependent detection efficiency by separating individual peptides in time. Finally, we implemented the Mascot search engine where identification was based on a probability score (Perkins et al., 1999). In the Mascot searches, the data obtained from the experimental fragmentation pattern for all the peptides are matched to the theoretical fragments, starting with the most intense peak. We believe this approach was important in delivering successful identification of 81% of the protein excised from the 2-DE gel. Some spots contained more than one protein, either because the proteins were not resolved by 2-DE or the proteins were contaminated with keratin during digestion or sample preparation. Our approach also allowed confident identification of the multiple proteins within these samples as has been previously shown, because each protein identified passed our strict search criteria (Lim et al., 2003).

4. Conclusion

This is the first proteomic analysis of the neutrophils of any marsupial. Although hampered by the lack of published proteomic information, identification and characterisation was achieved using 2-DE and LC-MS with cross-species matching of the peptide data. The problems associated with protein identification under these circumstances have previously been discussed (Lippolis and Reinhardt, 2005), but our strategy of applying strict criteria allowed confident identifications to be made. Most of the proteins identified were associated with fundamental metabolic pathways or with cytoskeletal function and appeared to be common to other cell types. Some proteins could not be identified with any degree of confidence. It may be that some neutrophil proteins of the tammar wallaby differ considerably from proteins in other sequenced organisms, preventing identification using the strategy outlined here. However, this seems unlikely given the high percentage of proteins, which could be identified. It may also be that the relative abundance of proteins present in tammar wallaby neutrophils differs from other organisms. In this study, we only sought reliable identifications from relatively abundant proteins. This study represents the first step in identification of proteins associated with immune function in marsupials, particularly those contained within the granules of neutrophils.

References

- Altschul, S., Madden, T., Schafer, A., Zhang, J., Zhang, Z., Miller, W., Lipman, D., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- Avram, D., Romijn, E.P., Pap, E.H.W., Heck, A.J.R., Wirtz, K.W.A., 2004. Identification of proteins in activated human neutrophils susceptible to

- tyrosyl radical attach. A proteomic study using a tyrosylating fluorophore. *Proteomics* 4, 2397–2407.
- Berkow, R.L., Dodson, R.W., 1990. Tyrosine-specific protein phosphorylation during activation of human neutrophils. *Blood* 75, 2445–2452.
- Blondelle, S.E., Lohner, K., Aguilar, M., 1999. Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: determination and biological specificity. *Biochim. Biophys. Acta* 1462, 89–108.
- Boussac, M., Garin, J., 2000. Calcium-dependent secretion in human neutrophils: a proteomics approach. *Electrophoresis* 21, 665–672.
- Burgoyne, R.D., Morgan, A., 2003. Secretory granule exocytosis. *Physiol. Rev.* 83, 581–632.
- Burridge, K., Fath, K., Kelly, T., Nuckolls, G., Turner, C., 1998. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4, 487–525.
- Cordwell, S.J., Humphrey-Smith, I., 1997. Evaluation of algorithms used for cross-species proteome characterisation. *Electrophoresis* 18, 1410–1417.
- Das, K.C., White, C.W., 2002. Redox system of the cell: possible links and implications. *Proc. Natl. Acad. Sci.* 99, 9617–9618.
- Engqvist-Goldstein, A.E.Y., Kessels, M.M., Chopra, V.S., Hayden, M.R., 1999. An actin-binding protein of the sla/Huntingtin interacting protein 1 family is a novel component of clathrin-coated pits and vesicles. *J. Cell Biol.* 147, 1503–1518.
- Ezzell, R.M., Goldmann, W.H., Wang, N., Parasharma, N., Ingber, D.E., 1997. Vinculin promotes cell spreading by mechanically coupling integrins to the cytoskeleton. *Exp. Cell Res.* 231, 14–26.
- Fenteany, G., Glogauer, M., 2004. Cytoskeletal remodelling in leukocytes function. *Curr. Opin. Hematol.* 11, 15–24.
- Fessler, M.B., Malcolm, K.C., Duncan, M.W., Worthen, G.S., 2002. A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase. *J. Biol. Chem.* 277, 31291–31302.
- Gatlin, C.L., Kleemann, G.R., Hays, L.G., Link, A.J., Yates, J.R., 1998. Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. *Anal. Biochem.* 263, 93–101.
- Gennaro, R., Dolzani, L., Romeo, D., 1983. Potency of bactericidal proteins purified from the large granules of bovine neutrophils. *Infect. Immun.* 40, 684–690.
- Gerke, V., Moss, S.E., 2001. Annexins: from structure to function. *Physiol. Rev.* 82, 331–371.
- Hancock, R.E., Chapple, D.S., 1999. Peptide antibiotic. *Antimicrob. Agents Chemother.* 43, 1317–1323.
- Laura, M.R., Coffey, P., Zwartkruis, F., Franke, B., Segal, A.W., Koenderman, L., Bos, J.J., 1998. Activation of the small GTPase Rap1 in human neutrophils. *Blood* 6, 2133–2140.
- Lawrence, D.W., Pryzwansky, K.B., 2001. The vasodilator-stimulated phosphorylation is regulated by cyclic GMP-dependent protein kinase during neutrophil spreading. *J. Immunol.* 166, 5550–5556.
- Lim, H., Eng, J., Yates, J.R.L., Tollaksen, S.L., Giometti, C.S., Holden, J.F., Adams, M.W.W., Reich, C.J., Olsen, G.J., Hays, L.G., 2003. Identification of 2D-gel proteins: a comparison of MALDI/TOF peptide mass mapping to μ LC-ESI tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* 14, 957–970.
- Lippolis, J.D., Reinhardt, T.A., 2005. Proteomics survey of bovine neutrophil. *Vet. Immunol. Immunopathol.* 103, 53–65.
- Lominadze, G., Powell, D.W., Leunman, G.C., Link, A.J., Ward, R.A., McLeish, K.R., 2005. Proteomic analysis of human neutrophil granules. *Mol. Cell. Proteomics* 4, 1503–1521.
- Luna, E., Hitt, A.L., 1992. Cytoskeleton–plasma membrane interactions. *Science* 258, 955–963.
- McKenzie, S., Deane, E.M., Burnett, L., 2002. Haematology and serum biochemistry of the tammar wallaby, *Macropus eugenii*. *Comp. Clin. Pathol.* 11, 229–237.
- McLaughlin, S.H., Bulleid, N.J., 1998. Thiol-independent interaction of protein disulphide isomerase with type X collagen during intra-cellular folding and assembly. *Biochem. J.* 331, 793–800.
- Moore, R.E., Young, M.K., Terry, D.L.J., 2002. An algorithm for evaluating SEQUEST database search results. *J. Am. Soc. Spectrom.* 13, 378–386.
- Mor-Vaknin, N., Punturieri, A., Sitwala, K., Markovitz, D.M., 2003. Vimentin is secreted by macrophages. *Nat. Cell Biol.* 5, 59–63.
- Nunoi, H., Yamazaki, T., Tsuchiya, H., Kato, S., Malechi, H.L., Matsuda, I., Kanegaski, S., 1999. A heterozygous mutation of beta-actin associated with neutrophil dysfunction and recurrent infection. *Proc. Natl. Acad. Sci. U. S. A.* 96, 8693–8698.
- Old, J.M., Deane, E.M., 2000. Development of the immune system and immunological protection in marsupial pouch young. *Dev. Comp. Immunol.* 24, 445–454.
- Peng, J., Elias, J.E., Thoreen, C.C., Licklider, L.J., Gygi, S.P., 2003. Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC–MS/MS) for large-scale protein analysis: the yeast proteome. *J. Proteome Res.* 2, 43–50.
- Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S., 1999. Probability-based protein identification by searching sequence database using mass spectrometry data. *Electrophoresis* 20, 3551–3567.
- Piubelli, C., Galvani, M., Hamdan, M., Domenici, E., Righetti, P.G., 2002. Proteome analysis of rat polymorphonuclear leukocytes: a two-dimensional electrophoresis/mass spectrometry approach. *Electrophoresis* 23, 298–310.
- Provost, P., Doucet, J., Stock, A., Gerisch, G., Samuelsson, B., Radmark, O., 2001. Coactosin-like, a human F-actin-binding protein: critical role of lysine-75. *Biochem. J.* 359, 255–263.
- Rahman, S., Lake, B.D., Taanman, J.-W., Hanna, M.G., Mark, C.J., Schapira, A. H.V., Leonard, J.V., 2000. Cytochrome oxidase immunohistochemistry: clues for genetic mechanism. *Brain* 123, 591–600.
- Rice, P., Longden, I., Bleasby, A., 2000. EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet.* 16, 276–277.
- Roitt, I., Brostoff, J., Male, D., 2001. Immunology, 6th edition. Mosby, Edinburgh.
- Schmitz, H.-D., Dutine, C., Bereiter-Hahn, J., 2003. Exportin 1-independent nuclear export of GAPDH. *Cell Biol. Int.* 27, 511–517.
- Sengelov, H., Kjeldsen, L., Borregaard, N., 1993. Control of exocytosis in early neutrophil activation. *J. Immunol.* 150, 1535–1543.
- Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* 68, 850–858.
- Tapper, H., Furuya, W., Grinstein, S., 2002. Localized exocytosis of primary (lysosomal) granules during phagocytosis: role of Ca^{+2} -dependent tyrosine phosphorylation and microtubules. *J. Immunol.* 168, 5287–5296.
- Taylor, J.A., Johnson, R.S., 1997. Sequence database searches via de novo peptide sequencing by tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 11, 1067–1075.
- Vandekerckhove, J., 1990. Actin-binding proteins. *Curr. Opin. Cell Biol.* 2, 41–50.
- Verrillis, N.M., Harry, J.H., Walsh, B.J., Hains, P.G., Robinson, E.S., 2000. Cross-matching marsupial proteins with eutherian mammal database: Proteome analysis of cells from UV-induced skin tumours of an opossum (*Monodelphis domestica*). *Electrophoresis* 21, 3810–3822.
- Villar, J., 2000. Heat shock protein gene expression and survival in critical illness. *Critical Care* 4, 2–5.
- Wakefield, M.J., Marshall-Graves, J.A., 2003. The kangaroo genome. *EMBO J.* 4, 143–147.
- Wang, N., Butler, J.P., Ingber, D.E., 1993. Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 260, 1124–1127.
- Wennerberg, K., Rossman, K.L., Der, C.J., 2005. The Ras superfamily at a glance. *J. Cell. Sci.* 118, 843–846.
- Yates, J.R., Eng, J.K., McCannack, A.L., Schieltz, D., 1995. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal. Chem.* 67, 1426–1436.
- Zhang, P., Liu, B., Kang, S.W., Seo, M.S., Rhee, S.G., Obeid, L.M., 1997. Thioredoxin peroxidase is a novel inhibitor of apoptosis with a mechanism distinct from that of Bcl-2. *J. Biol. Chem.* 272, 30615–30618.

Chapter 3

In search of neutrophil granule proteins of the tammar wallaby (*Macropus eugenii*).

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In search of neutrophil granule proteins of the tammar wallaby (*Macropus eugenii*)

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Abstract

Two approaches have been used to isolate and identify proteins of the granules of neutrophils of the tammar wallaby, *Macropus eugenii*. Stimulation with PMA, Ionomycin and calcium resulted in exocytosis of neutrophil granules as demonstrated with electron microscopy. However proteomic analysis using two dimensional gel electrophoresis, in-gel trypsin digestion followed by nano liquid chromatography coupled tandem mass spectrometry (LC–MS/MS) failed to identify any anticipated granule proteins in the reaction supernatants. Subsequent use of differential centrifugation and lysis followed by the application of the same proteomic analysis approach resulted in the isolation and confident identification of 39 proteins, many of which are known to be present in the granules of neutrophils of eutherian mammals or play a role in degranulation. These proteins notably consisted of the known antimicrobials, myeloperoxidase (MPO), serine proteinase, dermcidin, lysozyme and alkaline phosphatase. A number of important known antimicrobials, however, were not detected and these include defensins and cathelicidins. This is the first report of the neutrophil granule proteins of any marsupial and complements previous reports on the cytosolic proteins.

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Keywords: Marsupials; Neutrophils; Electrospray ionisation-tandem mass spectrometry; Two-dimensional gel electrophoresis

1. Introduction

Polymorphonuclear neutrophils are a major component of innate defence in the mammalian immune system. They are phagocytic cells, engulfing and killing invading micro-organisms by two main mechanisms (Mollinedo et al., 1999). The first of these is an oxygen dependent process, mediated by NADPH oxidase and involving a series of reactive molecules – hydrogen peroxide, myeloperoxidase (MPO), and hyperchlorous acid (HOCl) – all of which cause oxidative damage to the target micro-organisms (Avram et al., 2004). The second of these mechanisms is oxygen independent and involves the release of a range of proteases, antimicrobial proteins and peptides and other enzymes from neutrophil granules. These components act to destroy the integrity of the cell membrane of the target micro-organisms resulting in disruption of cellular activities and cell

death. In eutherian mammals these granules are classified as azurophilic (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules, lysosomes and secretory vesicles and each contains a different functional repertoire of compounds (reviewed by Segal, 2005).

Azurophilic granules, because of their mucopolysaccharide content stain with azure dye A (Spicer, 1969) and contain myeloperoxidase (Bainton and Farquhar, 1966), bactericidal permeability increasing protein, neutral proteinases, elastase, defensins (Bainton and Farquhar, 1966; Welsh and Spitznagel, 1971; Bretz and Baggiolini, 1974), and lysozyme (Baggiolini, 1969). Specific or secondary granules store metalloprotease and antibiotic peptides in latent form which are themselves activated by the proteases released from azurophilic granules during degranulation (Gullberg et al., 1999). Specific granules also contain lactoferrin, gelatinase-associated lipocalin (Bullen and Armstrong, 1979) and lysozyme (Baggiolini et al., 1969). Finally gelatinase or tertiary granules are characterised by a high content of gelatinase (Gilbert et al., 1993; Kjeldsen et al., 1994) whose release is essential for migration of neutrophils through basement membranes (Delclaux et al., 1996). Neutrophils also

Abbreviations: MPO, myeloperoxidase; ALP, alkaline phosphatase; PMA, Phorbol myristate acetate

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contain lysosomes a source of acid hydrolases which is released into the phagocytic vacuoles after azurophilic granules release their contents (Segal et al., 1980).

Neutrophil proteins, including granule proteins, have been documented in a range of eutherian mammals, including humans (Boussac and Garin, 2000; Fessler et al., 2002; Lominadze et al., 2005); rats (Piubelli et al., 2002) and cows (Lippolis and Reinhardt, 2005). Recently we reported the high confidence identification of fifty three cytosolic proteins from the neutrophils of the tammar wallaby (*Macropus eugenii*), a model metatherian mammal (marsupial) native to Australia (Ambatipudi et al., 2006). This study highlighted both the difficulty in isolating the granule proteins of marsupial neutrophils and the inherent difficulties in high confidence identification of proteins from organisms which lack a translated genomic database.

In this study we have used two approaches to isolate and identify neutrophil granule proteins from the tammar wallaby. The first approach consisted of stimulation with Phorbol myristate acetate (PMA), Ionomycin and calcium, compounds, whose actions in stimulating neutrophil degranulation in eutherian mammals are well documented (Goldstein et al., 1975; Sengelov et al., 1993). In these species stimulation of neutrophils with PMA causes degranulation of specific granules, releasing lysozyme (Goldstein et al., 1975) and lactoferrin (Gaudry et al., 1997), whilst stimulation with Ionomycin coupled with an increased intracellular calcium concentration leads to exocytosis of all granule subsets releasing myeloperoxidase, serine proteinase, lysozyme, defensins, cathelicidins, lactoferrin and gelatinase (Sengelov et al., 1993). As this first approach failed to yield the desired granule proteins, a second approach, consisting of a stepwise process of centrifugation and lysis was used to improve isolation of neutrophil granule proteins. This approach has been used successfully in the rabbit (Zarembek et al., 2002), cow (Gennaro et al., 1983), rat (Eisenhauer et al., 1989) and monkey (Rausch and Canonica, 1975).

The proteins obtained through each process were visualised using two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and identified following a proteomic analysis approach consisting of in-gel trypsin digestion followed by nano liquid chromatography coupled tandem mass spectrometry (LC-MS/MS). Finally, to identify the protein components we used database searches with cut-off scores set to eliminate low quality matches. Although identification was hampered, as indicated previously, by a lack of significant translated genomics database for marsupials we did identify a number of proteins of granule origin and known antimicrobial capacity.

2. Materials and methods

2.1. Animals and sample collection

Adult tammar wallabies used in this study were maintained in a captive breeding colony at Macquarie University, Sydney, Australia. For each experimental protocol 10 mL of blood was collected from the caudal vein of 14 healthy adult animals into tubes coated with ethylene diamine tetra acetic acid (EDTA)

and was pooled for neutrophil isolation. The amount of blood which could be collected from any one animal at any one time was limited by animal ethics considerations to 10 mL per month and was approved by the Macquarie University Animal Ethics Committee (Approval No. 2004/007).

2.2. Isolation of neutrophils

Isolation of neutrophils was carried out as previously described (Ambatipudi et al., 2006). In brief, 10 mL of blood, diluted 1:1 with 1X PBS as layered over 10 mL of Histopaque-1077 (Sigma Diagnostic, St. Louis, MO, USA) density gradient medium in a 50 mL sterile, polypropylene centrifuge tube and centrifuged at $645 \times g$ at 18°C for 20 min. The granulocyte layer was removed and resuspended to a final volume of 50 mL at 37°C with NH_4Cl (8.26% ammonium chloride, 1% potassium bicarbonate, 0.037% EDTA) as erythrocyte lysis solution for 15 min. The lysis was stopped by addition of an equal volume of $2 \times \text{PBS}$ to restore the tonicity for the granulocytes. Trypan blue staining revealed 2×10^6 cells/mL neutrophils were isolated with $>98\%$ viability.

Isolated neutrophils were subjected to two sets of protocols in order to obtain neutrophil granule proteins (i) treatment with Phorbol myristate acetate (PMA), Ionomycin and calcium to elicit degranulation and (ii) differential centrifugation and hypotonic lysis.

2.3. Treatment with Phorbol myristate acetate (PMA) and Ionomycin

Purified neutrophils were treated with Phorbol myristate acetate (PMA), Ionomycin and calcium at a range of different concentrations identified in the literature and summarised in Table 1 as follows:

(i) Phorbol myristate acetate (PMA)

PMA was used to stimulate protein release from specific granules. To achieve this, isolated neutrophils were resuspended in phosphate buffer saline (PBS) at a concentration of 1×10^6 cells/mL and stimulated with PMA at a range of concentrations and under conditions shown in Table 1. The initial concentrations selected were based on successful published protocols and included 0.1 ng/mL (Biggar, 1978), 20 ng/mL (Wang-Iverson et al., 1978), 50 ng/mL (Gaudry et al., 1997) and 1 $\mu\text{g/mL}$ (Lollike et al., 2002). Subsequently, as these methods did not appear to result in measurable protein release a higher range of concentrations was used, including 10 ng/mL, 100 ng/mL and 10 $\mu\text{g/mL}$ (Table 1). After all treatments, cells were centrifuged at $1000 \times g$ for 15 min at 4°C and the supernatant containing the secreted proteins were stored at -80°C for subsequent proteomic analysis.

(ii) Ionomycin with calcium

Neutrophils were treated with Ionomycin and calcium to stimulate exocytosis of all granule types (Sengelov et al., 1993). Isolated neutrophils were resuspended in PBS at a concentration of 1×10^6 cells/mL with calcium at a con-

Table 1
Summary and source of conditions used to stimulate neutrophils in this study

Stimulant	Concentration	Time (min)	Reference
PMA	0.1 ng/mL	30	Biggar (1978)
PMA	50 ng/mL	30	Gaudry et al. (1997)
PMA	20 ng/mL	30	Wang-Iverson et al. (1978)
PMA	1 µg/mL	60	Lollike et al. (2002)
Ionomycin with calcium	1 µM + 1.3 mM	15	Boussac and Garin (2000)
PMA + Ionomycin with calcium	20 ng/mL + 2 µM + 1 mM	15	Chatila et al. (1989)

All incubations were performed at 37 °C. Each stimulation experiment was performed in triplicate.

centration of 1.3 mM. Degranulation was initiated by the addition of Ionomycin to a final concentration of 1 µM (Boussac and Garin, 2000) and incubation at 37 °C for 15 min. The cells were then centrifuged at 1000 × g for 15 min at 4 °C and the supernatant containing the secreted proteins were stored at –80 °C for further analysis.

(iii) PMA with Ionomycin and calcium

To maximise degranulation of all the different granule types, neutrophils were stimulated with PMA, Ionomycin and calcium (Chatila et al., 1989). Neutrophils at a concentration of 1 × 10⁶ cells/mL were resuspended in PBS with calcium at a concentration of 1 mM. Exocytosis was induced by the addition of 20 ng/mL of PMA and 2 µM Ionomycin and incubated with at 37 °C for 15 min. The cells were centrifuged at 1000 × g for 15 min at 4 °C and the supernatant containing the secreted proteins were stored at –80 °C for subsequent proteomic analysis.

(iv) Electron microscopy of stimulated cells

To verify whether such treatments caused degranulation in marsupials, isolated neutrophils were also stimulated with 10 ng/mL of PMA in 0.1% dimethyl sulfoxide and incubated at 37 °C for 1 min. Pellets of treated and control cells were fixed in 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4 for 4 h. The pellets were washed in this buffer and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Pellets were again washed in buffer and *en bloc* stained with 2% aqueous uranyl acetate for 20 min, dehydrated in a graded series of ethanol, infiltrated with LR White resin and embedded in gelatin capsules. The resin was polymerised at 60 °C for 24 h. Semi-thin (1 µm) and ultrathin sections (~70 nm) were cut using a Reichert Ultracut-S ultramicrotome. Semi-thin sections were stained with methylene blue and ultra-thin sections were stained with 2% aqueous uranyl acetate and Reynold's lead citrate, mounted on pioloform coated copper grids and viewed in a Philips CM10 transmission electron microscope using 100 kV.

2.4. Isolation of granules

Neutrophils were disrupted by probe sonication with a Branson 450 probe for 30 s at 120 W and lysis was verified by microscopic observation of smears stained with Diff-Quik (Lab Aids, Narrabeen, NSW, Australia). The nuclei and unbroken cells were removed by centrifugation at 1000 × g at 4 °C for

15 min and the supernatant containing cytosolic proteins and granules was then centrifuged at 20,000 × g at 4 °C for 1 h to sediment the granules. The supernatant was discarded and the granule pellet resuspended in 10% acetic acid and subjected to sonication with a Branson 450 probe for 30 s at 120 W. The suspension was then centrifuged at 20,000 × g at 4 °C for 1 h resulting in separation of a pellet containing unbroken granules and supernatant containing granule proteins. Both fractions were stored at –80 °C until further analysis.

2.5. Protein extraction

Proteins were precipitated from the supernatant using 90% TCA and the resultant material was then dissolved in 200 µl of 2-D Sample Solubilising solution. Similarly the pellet was resuspended and proteins extracted in 200 µl of 2-D Sample Solubilising solution. This solution consisted of 7 M urea, 2 M thiourea, 65 mM DTT, 2% CHAPS, 1% carrier ampholytes (3–10), 40 mM Tris and 0.002% bromophenol. An aliquot of the protein in PBS was used to determine the protein concentration using FluoroProfile (Sigma Diagnostics, St. Louis, MO, USA) according to the manufacturers' instructions.

2.6. Two-dimensional gel electrophoresis (2DE)

For the PMA, Ionomycin and calcium and PMA plus Ionomycin and calcium stimulated cells, 150 µg of protein was loaded onto the 2DE gel using 11 cm pH 6–11 linear IPG strips (Bio-Rad, Hercules, CA, USA). For the pellet and supernatant fractions approximately 130 µg of protein was loaded onto a 11 cm pH 3–10 linear IPG strips (Bio-Rad, Hercules, CA, USA). IEF was performed using the Ettan IPGphor 3 (Amersham Pharmacia Biotech, Uppsala, Sweden) for 100 kVh at 20 °C. The IPG strips were then equilibrated for 20 min at room temperature by gentle rocking in 6 M urea, 2% SDS 20% glycerol, 1 × Tris–HCl (pH 8.8), 5 mM tributylphosphine, 2.5% acrylamide and then embedded in 0.5% agarose on top of precast 4–20% gradient Tris–HCl Criterion gels (Bio-Rad, Hercules, CA, USA). Electrophoresis was carried out using a cathode running buffer consisting of 192 mM glycine, 15 mM Tris, 0.1% SDS at pH 8.3. Gels were initially run at 5 mA/gel for 30 min, then at 200 V, until the bromophenol blue buffer front had completely run off the bottom of the gel. The gels were fixed in 10% methanol and 7% acetic acid followed by staining overnight with Sypro Ruby (Bio-Rad, Hercules, CA, USA). Gels were destained in 10%

methanol and 7% acetic acid for 1 h, and images were captured with the Typhoon 9410 scanner (GE Healthcare, Bio-Sciences Inc., Quebec, Canada).

2.7. Trypsin digestion and mass spectrometry

Proteins were excised automatically from all gels using ExQuest spot cutter (Bio-Rad, Hercules, CA, USA), washed three times in 50% (v/v) acetonitrile and 25 mM NH_4HCO_3 , pH 7.8 at 37 °C for 10 min, dried at room temperature in a Speedy-Vac (Savant, Farmingdale, NY, USA), covered with 8 μL of sequencing grade trypsin (15 ng/ μL) and digested overnight at 37 °C. Products were recovered from the gel by sequential extractions with 10% acetonitrile and 1% (v/v) formic acid. Samples (5 μL) were concentrated and desalted onto a micro C18 precolumn (500 $\mu\text{m} \times 2 \text{ mm}$, Michrom Bioresources, Auburn, CA) with $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ (98:2, 0.05% HFBA) at 20 $\mu\text{L}/\text{min}$. After a 4 min wash the pre-column was switched (Valco 10 port valve, Dionex, Houston, TX, USA) into line with a fritless nano column (Gatlin et al., 1998). Digested peptides were separated by nano-LC using an Ultimate 3000 HPLC and autosampler system (Dionex, Amsterdam, Netherlands) and were eluted using a linear gradient of $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ (98:2, 0.1 % formic acid) to $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ (50:50, 0.1 % formic acid) at $\sim 300 \text{ nL}/\text{min}$ over 30 min. High voltage (1800 V) was applied to the low volume tee (Upchurch Scientific) and the column tip positioned $\sim 0.5 \text{ cm}$ from the heated capillary of a LTQ FT Ultra mass spectrometer (Thermo Electron, Bremen, Germany). Positive ions were generated by electrospray and the LTQ FT Ultra operated in data dependent acquisition mode.

A survey scan (m/z 350–1750) was acquired in the FTICR cell (resolution = 100,000 at m/z 400), with an initial accumulation target value of 1,000,000 ions in the linear ion trap. Up to the nine most abundant ions (>2000 counts) with charge states of +2 or +3 were sequentially isolated and fragmented within the linear ion trap using collision induced dissociation with an activation $q=0.25$ and activation time of 30 ms at a target value of 30,000 ions. The m/z ratios selected for MS/MS were dynamically excluded for 60 s.

2.8. Database searching

Peak lists for spectra obtained from the LTQ-FT Ultra were generated using Mascot Daemon/extract.msn (Matrix Science, London, England, Thermo) using the default parameters, and submitted to the database search program Mascot (version 2.1, Matrix Science). Proteins were identified by correlation of mass spectra to entries in the NCBI_{nr} database (May 2007) with ion search criteria as follows: taxonomy – Mammalia, trypsin digestion allowing up to one miscleavage, variable modification – oxidation of methionine, cysteine as carboxyamidomethylation or propionamide, precursor tolerance 8 ppm and product ion tolerances $\pm 0.6 \text{ Da}$. The “ion score cutoff” was manually set to 20 thereby eliminating the lowest quality matches. A probability based Mowse score >32 indicated identity ($p < 0.05$).

3. Results and discussion

3.1. Neutrophils and their response to PMA and Ionomycin

PMA and Ionomycin have frequently been used in eutherian mammals to stimulate neutrophil exocytosis (Biggar, 1978; Chatila et al., 1989; Gaudry et al., 1997; Lollike et al., 2002). PMA, in particular, is known to stimulate release of the contents of specific granules in humans (Goldstein et al., 1975; Wang-Iverson et al., 1978; Wright and Gallin, 1979; Gaudry et al., 1997), mice (Abdel-Latif et al., 2005), rats (Biggar, 1978) and cows (Gennaro et al., 1983). Similarly, Ionomycin with calcium has been reported to cause degranulation of all the neutrophil granules in humans (Boussac and Garin, 2000) and cows (Gennaro et al., 1983). In this study we used a range of different PMA, Ionomycin and calcium concentrations and combinations (Table 1) to stimulate marsupial neutrophils to degranulate. To verify whether such treatments actually resulted in degranulation we undertook EM of treated cells. Fig. 1 shows a representative electron micrograph of neutrophils treated with PMA. Granules can be seen scattered throughout the cell, some in contact with the plasma membrane in the process of exocytosis (Fig. 1). This visual analysis suggests that chemical stimulation results in granule exocytosis, moreover, from the morphology of the granules, it appears that the neutrophils of marsupials, like humans (Lollike et al., 2002), may also undergo compound exocytosis.

3.2. Two-dimensional gel electrophoresis (2DE) and protein identification

EMs of PMA stimulated cells (Fig. 1) suggested that such treatments (Table 1) resulted in release of proteins from

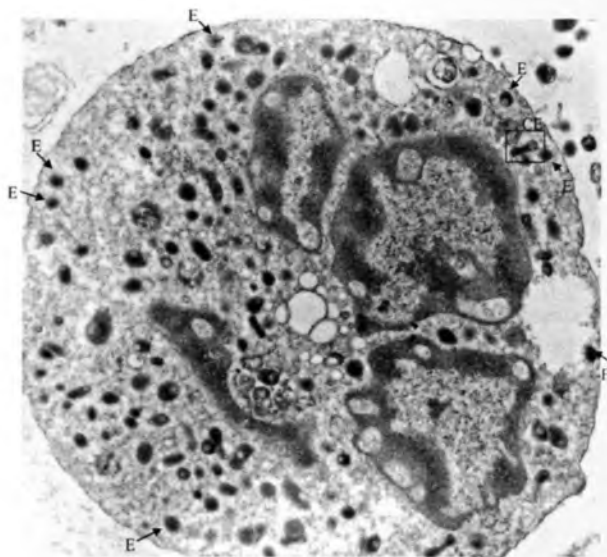


Fig. 1. Electron micrograph of the tammar wallaby (*Macropus eugenii*) neutrophils showing exocytosis. Neutrophils were incubated for 1 min with 10 ng/ml of Phorbol myristate acetate (PMA) for 1 min at 37 °C and then were fixed and processed for EM. A number of granules are visible in the cell with exocytosis (E) and compound exocytosis (CE and delineated with a square) apparent. Scale bar = 1 μM .

Table 2
Summary of data on antimicrobial proteins from the neutrophil granules of eutherian mammals

Antimicrobial protein	Species	Accession no.	pI	Molecular weight
Lysozyme	Human	P61626	9.38	16537.02
	Bovine	Q06285	6.8	16278.60
	Mouse	P08905	9.11	16688.98
	Pig	P12069	9.06	14667.78
	Sheep	P17607	6.4	16195.49
Myeloperoxidase	Human	P05164	9.19	83868.57
	Mouse	P11247	9.6	81070.52
Dermcidin	Human	P81605	6.09	11283.86
	Rat	Q71DI1	6.09	11283.86
Serine Proteinase	Human	Q9Y5Q5	4.87	116565.47
	Mouse	O08797	5.27	42259.42
	Bovine	O02739	5.44	42560.66
	Rat	Q8K3K4	6.11	45386.62
Lactoferrin	Human	P02788	8.5	78181.95
	Mouse	P08071	8.9	77865.81
	Bovine	P24627	8.69	78056.36
	Horse	O77811	8.30	75991.23
Cathelicidin	Human	P49913	9.48	19301.38
	Bovine	P22226	7.58	17600.07
	Mouse	Q0VB78	8.9	19581.54
	Horse	O62841	4.98	18152.79
Beta-Defensins	Human	P60022	8.96	7419.71
	Bovine	P46159	8.98	4278.07
	Mouse	P56386	9.14	7749.17
	Sheep	O19038	11.49	7243.76
BMAP	Bovine	P54228	9.39	17851.74
Lipocalin (NGAL)	Human	P80188	9.02	22588.07
	Mouse	P11672	8.96	22875
	Rat	P30152	8.41	22558.69
	Pig	P53715	5	19352.34
	Horse	Q28388	9.87	20804.59
Bactericidal/permeability increasing protein	Human	Q8N4FO	8.82	49172.5
	Mouse	Q8C1E1	6.03	49521.11
Gelatinase	Human	P08253	5.26	73882.3
	Bovine	Q9GLE5	5.42	73776.85
	Mouse	P33434	5.31	74102.41

neutrophils by degranulation. However, 2DE of reaction supernatants from cells treated with PMA, Ionomycin plus calcium and PMA plus Ionomycin and calcium (data not shown) failed to provide any indication of the release of the anticipated granule proteins. Table 2 presents a summary of the known pIs and MW of granule proteins from eutherian mammals sourced from www.expasy.org. Fig. 2 shows a representative 6–11 pI 2DE gel of PMA treated neutrophils, whilst Fig. 3 shows a representative 6–11 pI 2DE of marsupial neutrophils stimulated with Ionomycin with calcium. Twenty-two protein spots were visualised from the PMA stimulated cells but none were apparent in the basic region of the gel (circled in Fig. 2) where it was anticipated that most of the granule proteins would be located (see Table 2). Fig. 3 shows that around thirty protein spots were detected when cells were treated with Ionomycin and calcium. A larger number of proteins spread across a wide pI range would be expected as this treatment is known to cause

degranulation of azurophilic, specific and gelatinase granules in eutherian mammals (Gennaro et al., 1983; Boussac and Garin, 2000) (Fig. 3). A total of 52 spots were excised from both gels, digested and confidently identified by mass spectrometry. This analysis yielded no known granule protein matches. Haemoglobin, carried over during neutrophil isolation because of its relatively high concentration, was identified in spots 1, 2 and 3 (Fig. 2) and spots 1 and 2 in Fig. 3. Cytosolic proteins such as actin were identified from spot 16 (Fig. 2) and spots 24 and 26 (Fig. 3) while annexin was identified spot 17 (Fig. 2) and spot 25 (Fig. 3). Enzymes of the metabolic pathway such as glyceraldehyde-3-phosphatase dehydrogenase were identified from spots 8, 9 and 18 (Fig. 2) and spot 10 (Fig. 3). Similarly periredoxin was identified from spots 12 and 13 (Fig. 2) and spot 22 (Fig. 3). All other proteins (data not shown) had been identified previously in marsupial neutrophils (Ambatipudi et al., 2006).

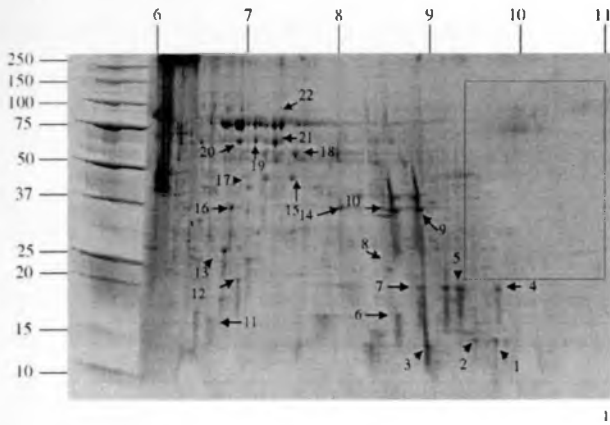


Fig. 2. 2DE PAGE (pI 6–11) of proteins released from neutrophils when stimulated with 50 ng/mL of Phorbol myristate acetate (PMA). Spots excised for MS analysis indicated with numbers 1–22. The black square on the gel indicates the likely region for the presence of granular proteins given the data in Table 2.

There is no obvious reason why the stimulation protocols, successfully used in eutherian mammals, appeared to cause granule exocytosis when viewed with EM (Fig. 1) but failed to provide proteomic evidence of the desired granule proteins (Figs. 2 and 3). It is possible that the target proteins were present at concentrations below the level of detection of the fluorescent stain (sensitivity down to 1 ng). This in turn could be due to incomplete exocytosis under the stimulation conditions used or the low numbers of neutrophils present in marsupial blood compared with eutherian mammals (Ambatipudi et al., 2006) coupled with an associated low level of protein released on stimulation.

Given the apparent lack of success using chemical stimulation protocols a second approach was used to isolate granule proteins that involved a stepwise process of centrifugation and lysis. The approach has successfully been used to isolate the total granule fraction in a range of eutherian mammal species, including rabbit (Zarembek et al., 2002), bovine (Gennaro et al., 1983), rat (Eisenhauer et al., 1989) and monkey (Rausch and Canonica, 1975). Fig. 4 shows a representative 3–10 pI 2DE

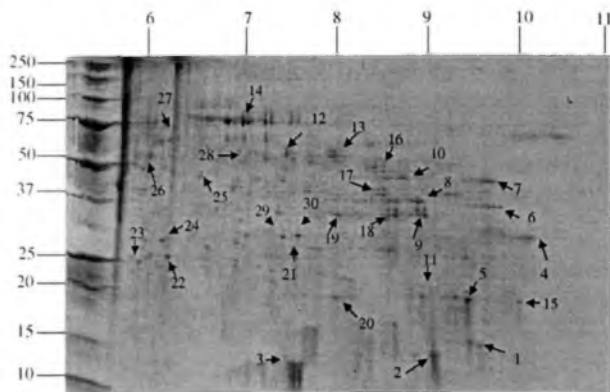


Fig. 3. 2DE PAGE (pI 6–11) of proteins released from neutrophils when stimulated with 1 μM of Ionomycin and 1.3 mM of calcium. Spots excised for MS analysis indicated with numbers from 1 to 30.

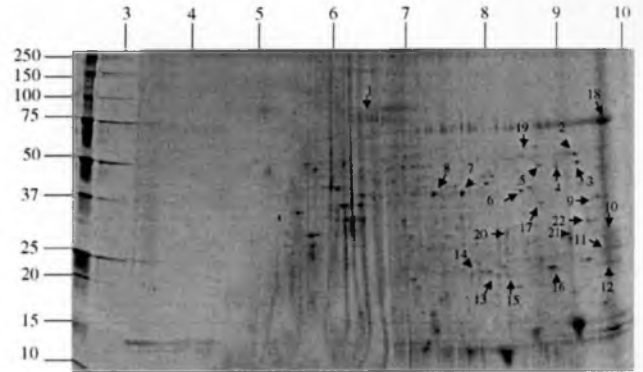


Fig. 4. 2DE PAGE (pI 3–10) of proteins extracted from the granule pellet prepared as described in Section 2.4. Spots excised for MS analysis are indicated by numbers from 1 to 22 and data presented in Table 3.

of the granule pellet isolated from tammar wallaby neutrophils, whilst Fig. 5 shows a representative 3–10 pI 2DE of the supernatant fraction. Over 20 protein spots were visualised in the gel of the supernatant fraction, with approximately equal numbers of proteins seen in both the 4–7 pI range at approximately 35–100 kDa and at the basic end of the gel at approximately 15–20 kDa (Fig. 5). Over 20 proteins were also detected in the granule pellet fraction (Fig. 4). The majority of the proteins were visible towards the basic end of the gel in the 20–70 kDa molecular weight range with a small number of proteins visible at pI 4–7. The pIs and MW of the proteins evident in these gels compared favourably with those anticipated from eutherian data (Table 2) and suggested that this approach to isolation of granule proteins proved more successful than the approach using PMA, Ionomycin and calcium.

Table 3 presents confident protein identifications based on high MOWSE scores and number of peptide matches for spots shown in Figs. 4 and 5 along with observed and expected pI and MW values. In some cases, there is a significant difference between observed and expected pIs and MWs. This may be due to a difference in amino acid content reflective of species diversity, polymerisation to form oligomers with associated changes in molecular weight and post-translational

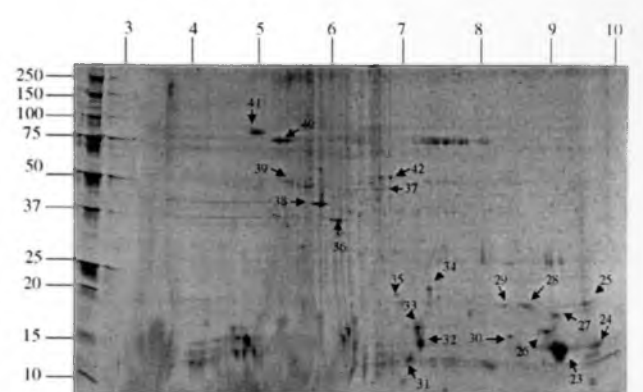


Fig. 5. 2DE PAGE (pI 3–10) of proteins from the supernatant prepared as described in Section 2.4. Spots excised for MS analysis are indicated by numbers from 23 to 42 and data presented in Table 3.

Table 3
Confident identification of proteins from the neutrophil granules of the tammar wallaby using 3–10 pI range 2DE and LC–MS/MS

Spot no.	Name	Function	Experimental pI (approx.)	Theoretical pI	Experimental molecular weight (approx.)	Theoretical molecular weight	Mowse score and % coverage	No. of peptides	Accession no.
1	Alkaline phosphatase	Biomarker for degranulation	6.5	6.2	75000	57338	438, 21	17	gi 3401945
	Fab-lysozyme antibody	Antibody		5.2		14304	155, 21	3	gi 22916
	Actin	Phagocytosis and structure		5.2		41786	154, 13	3	gi 28336
2	ATP synthase	Proton gradient	9.2	9.2	55000	59716	904, 31	28	gi 6680748
	Annexin	Exocytosis		7.3		59077	106, 6	4	gi 7304885
3	Fibrinogen	Precursor to fibrin	9.1	8.2	50000	27037	149, 20	5	gi 15593264
	ATP synthase	Proton gradient				55000	137, 7	3	gi 114511
	Intestinal alkaline phosphatase	Biomarker for degranulation				36142	64, 2	1	gi 3510659
4	Serine proteinase	Antimicrobial	8.9	5.8	50000	42860	116, 10	3	gi 27413904
	Serin peptidase	Plasminogen activator		5.9					gi 109122404
5	Fibrinogen	Precursor to fibrin	8.8	8.2 6	50000	27037	166, 23	7	gi 15593264
	Dermcidin	Antimicrobial				11277	101, 20	3	gi 16751921
6	Phosphoglycerate kinase	Glycolysis	8.5	7.9	37000	38000	284, 17	7	gi 5593264
	Fibrinogen	Precursor to fibrin		8.2		27037	160, 20	6	gi 15593264
	Dermcidin	Antimicrobial		6.1		11277	104, 20	3	gi 16751921
7	Fibrinogen	Precursor to fibrin	7.8	6.3	40000	35078	141	6	gi 73977986
8	Myosin	Phagocytosis and structure	7.3	5.6	37000	26217	395, 5	9	gi 73921193
	Fibrinogen	Precursor to fibrin		6.7		54718	313, 5	16	gi 33859809
9	Actin	Phagocytosis and structure	9.6	5.2	38000	41786	245, 18	7	gi 28336
	Fibrinogen	Precursor to fibrin		8.3		54861	86, 14	4	gi 182430
	Dermcidin	Antimicrobial		6.1		11277	72, 3	3	gi 16751921
10	Tubulin	Microtubule	9.8	4.8	32000	49710	221, 11	5	gi 49481
	Non-histone protein	Nuclear		5.4		24968	185, 26	6	gi 478813
	Beta-actin	Motor activity		5.2		41786	162, 13	6	gi 28336
	Integrin-linked kinase	Enzyme		8.3		51386	122, 8	5	gi 4758606
	Myeloperoxidase	Antimicrobial		9.4		53130	112, 5	3	gi 494395
	Galectin-3	Anti-apoptotic		8.6		27412	101, 9	3	gi 52851
11	G3PD	Glycolysis	9.7	6.2	26000	35813	100, 6	3	gi 56188
	Neutrophilic cytosolic proteins	Oxidative burst				59448	71, 5	3	gi 6754798
12	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	9.7	8.3	22000	35000	189, 6	4	gi 31645
13	Rap1b	Protein	8	5.65	20000	20812	375, 42	9	gi 7661678
	Cdc42	Chemotaxis		6.92		21473	355, 56	14	gi 2624582
	β-Haemoglobin	Oxygen transport		7.23		16005	180, 23	3	gi 122633
14	Ras-related protein	Protein	7.8	7.5	22000	21415	235, 41	9	gi 45006381
	Ubiquitin	Control of polymerisation		6.6		24353	85, 5	3	gi 114676157
	Flavin reductase	Electron transport		7.1		22065	84, 9	4	gi 73948324
15	Rap1a	Regulate Ras	8.2	4.6	20000	19011	106, 15	3	gi 1942609
	Haemoglobin	Oxygen transport		7.1		16122	298, 8	8	gi 62901558
16	Myosin	Phagocytosis and structure	9	5.4	22000	26217	284, 3	7	gi 73921193
	Elongation factor	Protein synthesis		6.3		50029	221, 10	8	gi 109459860
	Ubiquitin	Proteolysis		7.2		16005	134, 22	3	gi 122633
	Cytochrome c- reductase	Oxidative burst		5.8		90327	123, 3	3	gi 24485
	Dermcidin	Antimicrobial		9.1		29434	101, 10	4	gi 57036700
	Haemoglobin	Oxygen transport		6.1		11277	85, 20	2	gi 16751921

Table 3 (Continued)

Spot no.	Name	Function	Experimental pI (approx.)	Theoretical pI	Experimental molecular weight (approx.)	Theoretical molecular weight	Mowse score and % coverage	No. of peptides	Accession no.
17	ATP synthase	Proton gradient	8.8	9.16	37000	59714	709, 25	15	gi 4757810
	Actin	Phagocytosis and Structure		5.78		39161	530, 38	17	gi 49868
	ATP synthase	Proton gradient		5.39		56861	384, 16	8	gi 179279
	Myosin	Phagocytosis and structure		5.23		144996	344, 5	9	gi 189036
	Filamin-1	Structural		5.01		53109	157, 11	7	gi 13897886
	Aldehyde dehydrogenase	Enzyme		7.55		56673	114, 5	3	gi 118502
	Coronin	Cell locomotion		6.05		50943	102, 6	4	gi 4895037
	Dermcidin	Antimicrobial		6.08		11277	96, 20	4	gi 16751921
18	Myeloperoxidase	Antimicrobial	9.5	9.6	75000	81019	275, 6	9	gi 6754732
	Thyroid peroxidase	Enzyme		8.1		100398	89, 2	4	gi 50978626
19 ^a	Lysozyme	Antimicrobial	8.5	9.6	50000	15928	56	2	gi 88180
20	Actin	Phagocytosis and structural	8.2	6.9	30000	34311	133, 13	6	gi 5031599
	Annexin	Exocytosis		7.5		53984	104, 4	3	gi 113969
21 ^a	Actin	Phagocytosis and structure	9.1	5.2	30000	41786	79, 3	2	gi 28336
22 ^a	Myosin	Phagocytosis and structure	9.3	5.5	32000	26217	155, 3	2	gi 73921193
23	Fibrinogen	Precursor to fibrin	9.2	8.2	15000	27037	149, 20	5	gi 5593264
	ATP synthase	Proton gradient		9.5		38852	137, 7	3	gi 11451
24	Histone	Nuclear	9.8	10.4	15000	13766	324, 52	11	gi 223096
	Myosin	Exocytosis		5.2		144996	141, 2	4	gi 189036
	Thioredoxin peroxidase	House-keeping		8.9		11976	80, 12	4	gi 6166493
	Histone	Nuclear		11		14164	72, 20	3	gi 32111
25	Cyclophilin	Calcium signaling ligand	9.8	8.2	18000	32952	108, 27	4	gi 118090
26	Histone	Nuclear	9	10.3	16000	11260	142, 24	6	gi 32097
	Flotillin-1	Vascular		7.08		47326	102, 9	3	gi 5031699
27	Histone	Nuclear	9	11	18000	14164	82, 21	3	gi 32111
28	Histone	Nuclear	8.8	11.4	19000	11360	188, 42	6	gi 4504301
	α -Haemoglobin	Oxygen transport		7.36		15210	129, 24	6	gi 122429
	Myosin	Phagocytosis and structure		5.54		22621	105, 1	3	gi 73921193
	β -Haemoglobin	Oxygen transport		7.23		16005	88, 15	3	gi 122633
29	Histone	Nuclear	8.2	10.9	19000	11260	112, 33	4	gi 32097
30	Histone	Nuclear	8.2	10.2	16000	13899	215, 41	9	gi 31979
31	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	7	8.2	13000	24662	253, 12	9	gi 50978862
	Histone	Nuclear		10.9		13899	190, 33	11	gi 68435381
32	Histone	Nuclear	7.2	10.2	15000	13899	113, 41	13	gi 31979
33	Histone	Nuclear	7.2	11.4	18000	11360	119, 33	4	gi 4504301
	T Plastin	Actin binding		5.73		63825	106, 4	3	gi 190028
	β -Haemoglobin	Oxygen transport		6.23		16005	87, 17	3	gi 122633
34	Histone	Nuclear	7.2	10.8	20000	23437	111, 12	4	gi 68362800
	Integrin-linked kinase	Morphogenesis		8.3		51386	101, 8	4	gi 4758606
35	Rap 1B	Protein	7	5.6	18000	20000	375, 42	9	gi 7661678
	Cdc42	Chemotaxis		6.9		21473	355, 56	14	gi 2624582
36	Actin	Phagocytosis and structure	6.1	5.29	36000	41724	914, 57	39	gi 71620
	Filamin-A	Actin binding		5.9		41643	218, 10	5	gi 53791221

Table 3 (Continued)

Spot no.	Name	Function	Experimental pI (approx.)	Theoretical pI	Experimental molecular weight (approx.)	Theoretical molecular weight	Mowse score and % coverage	No. of peptides	Accession no.
37	Myosin	Phagocytosis and structure	6.7	10.9	49000	11260	207, 42	7	gi 189036
	Actin	Phagocytosis and structure		5.78		39161	506, 35	20	gi 49868
	Coronin-1A	Motility		7.1		41327	320, 17	15	gi 73958506
	Glucone-6-phospahte dehydrogenase	Glycolysis		6.16		59277	183, 9	9	gi 2494652
	Histone	Nuclear		11.36		11360	128, 29	3	gi 4504301
38	Myosin	Phagocytosis and structure	6	5.5	39000	26392	993, 10	27	gi 12667788
	Actin	Phagocytosis and structure		5.78		39161	806, 20	28	gi 49868
	Atpb5	Transcription		5.24		56632	713, 34	23	gi 23272966
	Histone	Nuclear		10.87		11260	214, 50	7	gi 32097
	MHC antigen -1	Cell adhesion		5.28		37882	154, 14	7	gi 83375992
	β -Haemoglobin	Oxygen transport		7.23		16005	95, 17	4	gi 122633
	Histone	Nuclear		11.04		14164	90, 25	4	gi 32111
	Histone	Nuclear		7.57		46757	88, 6	3	gi 32111
	Tubulin	Phagocytosis and Structure		4.95		49761	87, 6	3	gi 32015
	ATP synthase	Proton gradient		5.2		59714	709	15	gi 4757810
39	β -Actin	Structural	9.2	5.3	50000	39161	530	17	gi 49868
	ATP Synthase	Proton gradient		5.2		57919	384	8	gi 28940
	Non-muscle myosin	Structural		4.8		144996	344	9	gi 189036
	Filamin-1	Structural		5.1		280586	157	7	gi 13897886

Bold font highlight the antimicrobials.

^a Proteins identified with two peptides.

modifications such as phosphorylation or myristalation observed in eutherian sequences (www.expasy.org).

Of the 42 spots excised from gels in Figs. 4 and 5 (Table 3), five proteins were identified as antimicrobial proteins found in the neutrophil granules of eutherian mammals but have not been described in marsupials. Of the remaining 37 spots, 34 spots were confidently identified as either enzymes or structural proteins as a part of the degranulation process, whereas three proteins could not be confidently identified due to poor fragmentation spectra (spots 40, 41 and 42). The identity and general roles of the proteins identified in Table 3 are discussed below.

3.2.1. Host defence granule proteins

Two proteins (spots 10 and 18) were confidently identified as myeloperoxidase (MPO) based on a high MOWSE score and number of peptide matches but with slight differences in the expected compared to observed pI and MW (Table 3). Myeloperoxidase is a major constituent of azurophilic granules, stored as a tetramer consisting of two glycosylated alpha chains (MW 59–64 kDa) and two unglycosylated beta chains (MW 14 kDa), with a molecular weight of 150 kDa and theoretical pI of 9.2 (Nauseef, 1988). MPO is a hemoprotein that plays a critical role in the conversion of hydrogen peroxide to hypochlorous acid, a potent micro-biocidal and cytotoxic agent and an important defence mechanism of leukocytes (reviewed by Mayer-Scholl et al., 2004). Another antimicrobial protein confidently identified

with high MOWSE and three peptide matches was serine proteinase, a known component of azurophilic granules of eutherian mammals (spot 4, Fig. 4, Table 3). Serine proteinase plays a critical role in many physiological processes, such as digestion of phagocytosed micro-organisms (Rao et al., 1996). It also enables neutrophils to migrate from the circulatory system through the basement membrane to the site of inflammation (Rao et al., 1996).

Two proteins known to be present in specific granules of eutherian neutrophils were also identified. Dermcidin was confidently identified in protein spots 5, 6, 9 and 17 (Fig. 4). This low molecular weight antimicrobial protein is constitutively expressed in sweat glands and may play an important role in the regulation of human skin flora (Schitteck et al., 2001). It has recently been isolated from specific granules of human neutrophils (Lominadze et al., 2005). Once again there was considerable variation in the expected pI and MW, with the observed pI/s of proteins in spots 5, 6, 9 and 17 more basic than observed in the two eutherian dermcidin proteins which are reported at pI 6.09 (Table 2). This may be due to a difference in the protein sequence in tammar wallaby, whilst increased molecular weight may reflect polymerisation to form oligomers. Another protein identified as arising from specific granules was lysozyme (spot 19) although this was a low confidence identification as indicated by two peptide matches despite a reasonably high MOWSE score (Table 3). Alkaline phosphatase (ALP) was confidently identified in spot 1 (Fig. 4). ALP is a specific granule marker

localised to the cytoplasmic membrane in humans (Spitznagel et al., 1974) and rabbits (Smith et al., 1982). It is also found on the surface of the secretory vesicle (Tapper et al., 2002) and exocytosed when stimulated with fMLP (*N*-formylmethionyl-leucyl-phenylalanine) and Ionomycin (Sengelov et al., 1993). ALP activity is known to increase drastically in response to bacterial infection (McCall et al., 1969).

It is interesting to note that although a number of proteins known to be present in the specific granules of eutherian mammals were detected, two most important groups of antimicrobials namely the defensins and cathelicidins were not. It is possible that cathelicidins may have been present at concentrations below the level of detection of the system. Eutherian defensins have MW <10 kDa and it is likely that their marsupial counterparts would not be detected in the gel systems used in this study.

3.2.2. Structural proteins

Another group of proteins that were confidently identified were cytoskeletal proteins which play a fundamental role in neutrophil function. Structural proteins such as, actin, myosin were identified from spots 8, 17, 20, 21, 22, 24, 36, 37, 38 and 39 (Figs. 4 and 5). These compounds are known to function in the rapid reorganisation occurring in neutrophils during migration and phagocytosis of micro-organisms (Lippolis and Reinhardt, 2005) and neutrophil exocytosis (Rothwell et al., 1989; Borregaard and Cowland, 1997; Prywansky and Merricks, 1998; Faurschou and Borregaard, 2003). Annexin, identified in spot 20, is a phospholipid and Ca²⁺ binding protein localised in neutrophil cytoplasm, whose exact function is unknown. However, it has been reported to participate in degranulation (Boussac and Garin, 2000) and phagocytosis (reviewed by Gerke and Moss, 2001). In addition to proteins identified as part of the cell cytoskeleton, coronin was identified in protein spot 37 (Fig. 5). This protein is associated with cytoskeletal rearrangements and plays an important role in phagocytosis and formation of the phagocytic vacuole (Lippolis and Reinhardt, 2005).

3.2.3. Enzymes

A range of GTPases (spots 13, 14 and 15) (Fig. 4) and other enzymes (e.g. spots 2, 11, 12, 14, 24 and 31) (Figs. 4 and 5) have also been identified and are reported in Table 3. These GTPases include Rab like proteins, which are the largest of the Ras superfamily and are localised to specific intracellular compartments (Wennerberg et al., 2005). Monomeric GTPases of the Rab family and Cdc 42 have been reported to be associated with granule exocytosis in different cell types (Hong-Geller and Cerione, 2000; Deacona and Gelfanda, 2001; Zerial and McBride, 2001), mediating extracellular signals to regulate cellular activity (reviewed by Fenteany and Glogauer, 2004) and in steps leading to respiratory burst and translocation of protein complexes by fusion of cellular components with the plasma membrane (Laura et al., 1998). Glyceraldehyde-3-phosphate dehydrogenase (Schmitz et al., 2003) was identified from spots 11, 12 and 31, (Figs. 4 and 5 and Table 3) whilst thioredoxin peroxidase was identified in protein spot 24 (Fig. 5 and Table 3). This enzyme plays a significant role as an anti-oxidant and regulates the apoptotic pathway (Zhang et al., 1997).

In this study a number of proteins known to originate from other peripheral blood cells were identified. These include haemoglobin identified in protein spots 13, 15, 16, 28, 33 and 38 (Figs. 4 and 5, Table 3) an abundant protein difficult to eliminate in neutrophil preparations (Ambatipudi et al., 2006). Similarly, histone was identified in protein spots 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 37 and 38 (Figs. 4 and 5, Table 3). This probably reflects nuclear breakage and non-specific binding to granule membranes during preparation. Histones have been detected in murine macrophages (Hiempstra et al., 1993) and reported to co-localise with extruded DNA and granular enzymes in forming the extracellular traps of neutrophils (NET) (Brinkman et al., 2004). It maybe that DNA in such structures could also bind granule proteins and limit their detection by proteomic protocols used in this study. It is likely that histones play a similar NET role in marsupial neutrophils as they do in eutherian mammals.

We have, in this study, identified a number of proteins known to be present in the granules of neutrophils from eutherian mammals, although we note our failure to detect the major antimicrobials, defensins and cathelicidins. In a previous paper, we identified 54 proteins of cytosolic origin (Ambatipudi et al., 2006) and the data presented in this paper complements that work. We have also highlighted the difficulty faced in the isolation of neutrophils and their granules from a marsupial. One reason for this is the low levels of neutrophils present in marsupial blood compared to eutherian mammals and the problems encountered in isolation of the granules themselves. This low level of neutrophils and our failure to detect some of the major known antimicrobial granule proteins may have implications for the efficacy of the innate arm of immunological defence in this animal. To confirm this will require detailed functional studies. We have successfully shown, using EM, that compounds known to stimulate granule exocytosis on eutherian mammals also elicit such a response in marsupial cells, although the extent of this response may have limited the protein available for analysis. This paper thus adds to our growing knowledge of the components and operation of a model marsupial immune system.

References

- Abdel-Latif, D., Steward, M., Lacy, P., 2005. Neutrophil primary granule release and maximal superoxide generation depend on Rac2 in a common signalling pathway. *Can. J. Physiol. Pharmacol.* 83, 69–75.
- Ambatipudi, K., Old, J., Guilhaus, M., Raftery, M., Hinds, L., Deane, E., 2006. Proteomic analysis of the neutrophil proteins of the tammar wallaby (*Macropus eugenii*). *Comp. Biochem. Physiol. D* 4 (1), 283–291.
- Avram, D., Romijn, E.P., Pap, E.H.W., Heck, A.J.R., Karel, W.A., 2004. Identification of proteins in activated human neutrophils susceptible to tyrosyl radical attack. A proteomic study using a tyrosylating fluorophore. *Proteomics* 4, 2397–2407.
- Baggiolini, M., Hirsch, J.G., de Duve, C., 1969. Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. *J. Cell Biol.* 40, 529–541.
- Bainton, D.F., Farquhar, M., 1966. Origin of granules in polymorphonuclear leukocytes. *J. Cell Biol.* 28, 277.
- Biggar, W.D., 1978. Effect of Phorbol myristate acetate on cellular metabolism and lysozyme release from alveolar macrophages and polymorphonuclear leukocytes. *Infect. Immun.* 21, 669–671.
- Borregaard, N., Cowland, J.B., 1997. Granules of the human neutrophilic polymorphonuclear leukocytes. *Blood* 89, 3503–3521.

Boussac, M., Garin, J., 2000. Calcium-dependent secretion in human neutrophils: a proteomic approach. *Electrophoresis* 21, 665–672.

Bretz, U., Baggiolini, M., 1974. Biochemical and morphological characterisation of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. *J. Cell Biol.* 63, 251.

Brinkman, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria. *Science* 303, 1532–1535.

Bullen, J., Armstrong, J., 1979. The role of lactoferrin in the bactericidal function of polymorphonuclear leucocytes. *Immunology* 36, 781–791.

Chatila, T., Silverman, L., Miller, R., Geha, R., 1989. Mechanism of T cell activation by the calcium ionophore ionomycin. *J. Immunol.* 143, 1283–1289.

Deacon, S.W., Gelfand, V.I., 2001. Of yeast, mice, and men: Rab proteins and organelle transport. *J. Cell Biol.* 152, F21–F24.

Delclaux, C., Delacourt, C., d'Ortho, M.P., Boyer, V., Lafuma, C., Harf, A., 1996. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration in basement membrane. *Am. J. Resp. Cell Mol. Biol.* 14, 288–295.

Eisenhauer, P.B., Harwig, S.S.L., Szklarek, D., Ganz, T., Selsted, M.E., Lehrer, R.I., 1989. Purification and antimicrobial properties of three defensins from rat neutrophils. *Infect. Immun.* 57, 2021–2027.

Faurschou, M., Borregaard, N., 2003. Neutrophil granule and secretory vesicles in inflammation. *Microb. Infect.* 5, 1317–1327.

Fenteany, G., Glogauer, M., 2004. Cytoskeletal remodelling in leukocytes function. *Curr. Opin. Hematol.* 11, 15–24.

Fessler, M.B., Malcolm, K.C., Duncan, M.W., Worthen, G.S., 2002. A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase. *J. Biol. Chem.* 277, 31291–31302.

Gatlin, C.L., Kleemann, G.R., Hays, L.G., Link, A.J., Yates, J.R., 1998. Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. *Anal. Biochem.* 263, 93–101.

Gaudry, M., Bregerie, O., Andrieu, V., Benna, J.E., Pocard, M.-A., Hakim, J., 1997. Intracellular pool of vascular endothelial growth factor in human neutrophils. *Blood* 90, 4153–4161.

Gennaro, R., Dolzani, L., Romeo, D., 1983. Potency of bactericidal proteins purified from the large granules of bovine neutrophils. *Infect. Immun.* 40, 684–690.

Gerke, V., Moss, S.E., 2001. Annexins: from structure to function. *Physiol. Rev.* 82, 331–371.

Gilbert, C.S., Parmley, R.T., Rice, W.G., Kinkade, J.M.J., 1993. Heterogeneity of peroxidase-positive granules in normal human and chediak–Higashi neutrophils. *J. Histochem. Cytochem.* 41, 837–849.

Goldstein, I.M., Hoffstein, S.T., Weissmann, G., 1975. Mechanism of lysosomal enzyme release from human polymorphonuclear leukocytes. *J. Cell Biol.* 66, 647–652.

Gullberg, U., Bengtsson, N., Bulow, E., Garwicz, D., Lindmark, A., Olsson, I., 1999. Processing and targeting of granule proteins in human neutrophils. *J. Immunol. Methods* 232, 201–210.

Hiempstra, P.S., Eisenhauer, P.B., Harwig, S.S., Barselaar, M.T.V.D., Furth, R., Lehrer, R.I., 1993. Antimicrobial proteins of murine macrophages. *Infect. Immun.* 61, 3038–3046.

Hong-Geller, E., Cerione, R.A., 2000. Cdc42 and Rac stimulate exocytosis of secretory granules by activating the IP (3)/calcium pathway in RBL-2H3 mast cells. *J. Cell Biol.* 148, 481–494.

Kjeldsen, L., Sengelov, H., Lollike, K., Nielsen, M.H., Borregaard, N., 1994. Isolation and characterization of gelatinase granules from human neutrophils. *Blood* 83, 1640–1649.

Laura, M.R., Coffey, P., Zwartkruis, F., Franke, B., Segal, A.W., Koenderman, L., Bos, J.J., 1998. Activation of the small GTPase Rap1 in human neutrophils. *Blood* 6, 2133–2140.

Lippolis, J.D., Reinhardt, T.A., 2005. Proteomics survey of bovine neutrophil. *Vet. Immunol. Immunopathol.* 103, 53–65.

Lominadze, G., Powell, D.W., Leurman, G.C., Link, A.J., Ward, R.A., McLeish, K.R., 2005. Proteomic analysis of human neutrophil granules. *Mol. Cell. Proteomics* 4, 1503–1521.

Lollike, K., Lindau, M., Calafat, J., Borregaard, N., 2002. Compound exocytosis of granules in human neutrophils. *J. Leuk. Biol.* 71, 973–980.

Mayer-Scholl, A., Averhoff, P., Zychlinsky, A., 2004. How do neutrophils and pathogens interact? *Curr. Opin. Microbiol.* 7, 62–66.

McCall, C.E., Katayama, I., Cotran, R.S., Finland, M., 1969. Lysosomal and ultrastructural changes in human “toxic” neutrophils during bacterial infection. *J. Exp. Med.* 129, 267–293.

Mollinedo, F., Borregaard, N., Boxer, L.A., 1999. Novel trends in neutrophil structure, function and development. *Immunol. Today* 20, 535–537.

Nauseef, W.M., 1988. Biosynthesis and processing of myeloperoxidase—a marker for myeloid cell differentiation. *Eur. J. Biochem.* 40, 97–110.

Piubelli, C., Galvani, M., Hamdan, M., Domenici, E., Righetti, P.G., 2002. Proteome analysis of rat polymorphonuclear leukocytes: a two-dimensional electrophoresis/mass spectrometry approach. *Electrophoresis* 23, 298–310.

Prywansky, K.B., Merricks, E., 1998. Chemotactic peptide-induced changes of intermediate filament organization in neutrophils during granule secretion: role of cyclic guanosine monophosphate. *Mol. Biol. Cell* 9, 2933–2947.

Rao, N.V., Rao, G.V., Marshall, B.C., Hoidal, J.R., 1996. Biosynthesis and processing of proteinase 3 in U937 cells. *J. Biol. Chem.* 271, 2972–2978.

Rausch, P.G., Canonica, P.G., 1975. Characterization of monkey peripheral neutrophil granules during infection. *Infect. Immun.* 12, 687–693.

Rothwell, S.W., Nath, J., Wright, D.G., 1989. Interactions of cytoplasmic granules with microtubules in human neutrophils. *J. Cell Biol.* 108, 2313–2326.

Schitteck, B., Hipfel, R., Sauer, B., Bauer, J., Kalbacher, H., Stevanoic, S., Schirle, M., Schroeder, K., Blin, N., Meier, F., Rassner, G., Garbe, C., 2001. Dermicidin: a novel human antibiotic peptide secreted by sweat glands. *Nat. Immunol.* 2, 1133–1137.

Schmitz, H.-D., Dutine, C., Bereiter-Hahn, J., 2003. Exportin 1-independent nuclear export of GAPDH. *Cell Biol. Int.* 27, 511–517.

Segal, A., Dorling, J., Coade, S., 1980. Kinetic fusion of the cytoplasmic granules of the cytoplasmic granules with phagocytic vacuole in human polymorphonuclear leukocytes. *Biochemical and morphological studies. J. Cell Biol.* 85, 42–59.

Segal, A.W., 2005. How neutrophils kill microbes. *Annu. Rev. Immunol.* 23, 197–223.

Sengelov, H., Kjeldsen, L., Borregaard, N., 1993. Control of exocytosis in early neutrophil activation. *J. Immunol.* 150, 1535–1543.

Smith, G.P., Macgregor, R.R., Peters, T.J., 1982. Localization of leucine aminopeptidase and vitamin B12-binding protein in rabbit peripheral blood polymorphonuclear leukocytes. *Biochim. Biophys. Acta* 719, 532–538.

Spitznagel, J.K., Dalldorf, F.H., Leffell, M.S., Folds, J.D., Welsh, I.R.H., Cooney, M.H., Martin, L.E., 1974. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. *Lab. Invest.* 30, 774–785.

Tapper, H., Furuya, W., Grinstein, S., 2002. Localized exocytosis of primary (lysosomal) granules during phagocytosis: role of Ca²⁺-dependent tyrosine phosphorylation and microtubules. *J. Immunol.* 168, 5287–5296.

Wang-Iverson, P., Pryzwansky, K.B., Spitznagel, J.K., Cooney, M.H., 1978. Bactericidal capacity of Phorbol myristate acetate-treated human polymorphonuclear leukocytes. *Infect. Immun.* 22, 945–955.

Welsh, I.R.H., Spitznagel, J.K., 1971. Distribution of lysosomal enzymes, cationic proteins, and bactericidal substances in subcellular fractions of human polymorphonuclear leukocytes. *Infect. Immun.* 4, 97–102.

Wennerberg, K., Rossman, K., Der, C., 2005. The Ras superfamily at a glance. *J. Cell Sci.* 118, 843–846.

Wright, D.G., Gallin, J.I., 1979. Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. *J. Immunol.* 123, 285–294.

Zarembek, K.A., Katz, S.S., Tack, B.F., Doukhan, L., Weiss, J., Elsbach, P., 2002. Host defense function of proteolytically processed and parent (unprocessed) cathelicidins of rabbit granulocytes. *Infect. Immun.* 70, 569–576.

Zerial, M., McBride, H., 2001. Rab proteins as membrane organizers. *Nat. Rev.* 2, 107–119.

Zhang, P., Liu, B., Kang, S.W., Seo, M.S., Rhees, S.G., Obeid, L.M., 1997. Thioredoxin peroxidase is a novel inhibitor of apoptosis with a mechanism distinct from that of Bcl-2. *J. Biol. Chem.* 272, 30615–30618.

Chapter 4

A proteomic approach to analysis of antimicrobial activity in marsupial pouch secretions.

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A proteomic approach to analysis of antimicrobial activity in marsupial pouch secretions

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Abstract

We have documented the antimicrobial activity of pouch secretions of the tammar wallaby, *Macropus eugenii*, over the period leading up to birth and after birth of the young animal. This activity was greatest against the Gram-negative *Escherichia coli* and highest at the time of birth. Fractionation of the pouch secretions showed that activity at different times over the reproductive periods was associated with different molecular-weight (MW) components, with compounds in the range up to 50 kDa active immediately prior to and at the time of birth. Proteomic analysis using 1 and 2DE PAGE and LC–MS/MS identified the major components of the pouch secretions at these times, at a range of pI's and MWs. The majority of high-confidence identifications, at a wide range of pI's and MW, were β -lactoglobulin, a known component of marsupial milk. We subsequently conducted a proteomic analysis of mammary gland secretions and digest products from the gut of the young animal, using 2DE PAGE and MALDI MS/MS, to confirm its source and compare it with the observed MW and pI's of β -lactoglobulin. Although we did not directly identify an effector molecule responsible for antimicrobial activity, these results lead us to propose that β -lactoglobulin plays a role in the protection of the young marsupial, a role previously thought to be primarily due to specific secretions from the epithelial surface of the pouch. © 2007 Elsevier Ltd. All rights reserved.

1. Introduction

Marsupials are born after a short gestation period ranging from 13 days in the bandicoot (*Isodon macrourus*) [1] to 35 days in the koala (*Phascolarctos cinereus*) [2]. At birth, the

young animal has well-developed limbs that enable it to crawl unaided into the maternal pouch (the marsupium), but other major body systems, including respiratory, digestive, urinary and immune systems, are not well developed [3,4]. Subsequent development and maturation occur in the pouch in an environment known to harbour a variety of micro-organisms [5–7]. Prior to birth and during this period of development in the pouch, immunological protection of the pouch young is presumed to rely on a combination of maternal strategies including maternal licking of the pouch

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providing mechanical and chemical cleansing, prenatal transfer of maternal immunoglobulins across the yolk sac placenta and transfer of cellular and soluble components of milk including immunoglobulins and lactoferrin [8].

Yadav [9] initially proposed that the epithelial tissues of the maternal pouch may secrete antimicrobial compounds that regulate bacterial numbers and provide protection to the young marsupial. To date, there have been limited investigations of this hypothesis. Examination of the changing microflora of the pouch over the period leading up to birth of the young and in the immediate postnatal periods supported this proposition in the quokka (*Setonix bachyurus*) [5,10] and in the tammar wallaby (*Macropus eugenii*) [7]. In the koala, however, the presence of culturable microorganisms in the maternal pouch was linked to the subsequent death of the pouch young [6]. There have been two reported investigations of marsupial pouch secretions. A study of pouch secretions from the koala [11], documented antimicrobial activity against Gram-negative *Escherichia coli* but not Gram-positive *Staphylococcus aureus* and identified a number of peptides using one-dimensional polyacrylamide gel electrophoresis (1DE PAGE), Edman sequencing and MALDI mass spectrometry (MS). None of these peptides matched known protein sequences in public access databases at that time. Baudinette et al. [12] in a study of the pouch secretions of the tammar wallaby failed to identify any significant antimicrobial activity, but did isolate a peptide they subsequently called eugenin, the synthetic form of which caused smooth muscle to contract and splenocytes to proliferate. They postulated that this peptide had the capacity to stimulate immune cells and regulate bacterial flora in the pouch. Recently, we examined the histological appearance of epithelial tissues of the pouch of the brushtail possum, *Trichosurus vulpecula*, over the period of induced oestrus and failed to detect any histological changes reflective of increased secretory activity from this location [13].

In this study, pouch secretions were collected from female tammar wallabies across several breeding cycles, with a view to investigating antimicrobial activity against standard culture types *E. coli* and *S. aureus* and to apply recently developed proteomic techniques to identify possible antimicrobial proteins. Pouch secretions with demonstrable antimicrobial activity were analysed using both 1- and 2-DE PAGE, in-gel trypsin digestion, followed by nano-liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). Database searches with cut-off scores set to eliminate low-quality matches were then conducted to identify the protein components of these secretions. Subsequently, we also undertook proteomic analysis of early mammary gland secretions and digested milk products from the gut of the resident pouch young to investigate the possible origin of the proteins identified in pouch washes. This approach has allowed us to construct a picture of the possible roles of maternal secretions in the protection of the young animal.

2. Materials and methods

2.1. Animals

Pouch and mammary gland secretions were collected from female tammar wallabies maintained in a captive breeding

colony at the Fauna Park, Macquarie University, Australia. The protocols used in this study were approved by the Animal Ethics Committee of Macquarie University (Approval No. 2004/007 and Approval No. 2003/026).

2.2. Sample collection and preparation

Pouch secretions, as wash samples, were collected from 15 reproductively mature females across the breeding season from February to mid-July over a 2-year period. The samples collected covered the period immediately prior to oestrus, oestrus and post-oestrus. In the tammar wallaby, these periods reflect the reproductive stages immediately prior to and covering the birth of the young animal.

Secretions were collected by a gentle lavage of the pouch with 1 ml of sterile water. The wash was placed in a sterile collection tube, and, as protein concentrations in these samples was presumed to be low, the sample was lyophilised and resuspended in 100 µl of sterile phosphate-buffered saline (PBS) to provide concentrated samples for antimicrobial and proteomic analysis. Subsequently, samples from each of the three periods were pooled to obtain sufficient protein for the analysis, and the protein concentration was determined in triplicate using FluoroProfile (Sigma Diagnostics, St. Louis, MO, USA) according to the manufacturers' instructions. One hundred microlitres of reconstituted pooled pouch washes were fractionated on the basis of molecular weight (MW) using Centricon spin columns (Amicon, Bedford, MA, USA) according to the manufacturers' directions. The MW cut-off range for these columns was: > 50, 30–50, 3–30 and < 3 kDa. The fractions and whole reconstituted washes were stored at –80 °C until required for antimicrobial and proteomic analyses.

The mammary gland secretion samples were collected by gentle massage of the mammary gland of mature female tammar wallabies in the 24 h immediately prior to the anticipated birth of the pouch young. The sample was then centrifuged at 2900 rpm for 10 min at 4 °C to separate the lipid and whey fractions. The whey fraction was retained frozen at –80 °C until analysis.

The stomach and intestine samples, containing milk digest products, were collected post-mortem from a 12-day-old female pouch young. The contents of the stomach and intestines were collected into sterile tubes and retained frozen at –80 °C until analysis.

2.3. Assessment of antimicrobial activity

2.3.1. Bacterial strains and growth conditions

The bacterial test strains used in this study were *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. To prepare the assay culture, *E. coli* and *S. aureus* were grown in Luria broth overnight at 37 °C. After this period, 10 ml of fresh Luria broth was inoculated with the test organism and cultured to mid-logarithmic phase for 2 h at 37 °C with gentle agitation. A standard curve of cell numbers versus absorbance at 600 nm was determined for each culture, and for antimicrobial assays the cell concentration was adjusted to 1.1×10^5 CFU/ml by dilution with culture media.

2.3.2. Antibacterial activity of whole and fractionated pouch washes

Antibacterial activity was assessed using a modification of the method of Midorikawa [14]. In brief, 180 µl of reconstituted wash and fractionated samples were incubated with 1.1×10^5 CFU/ml of test organism for 4 h at 37 °C with constant agitation. A test using 180 µl PBS instead of wash was used as a control, and all assays were performed in duplicate. A positive control was also undertaken using stock solution of the antimicrobial Cecropin with minimum inhibitory concentration of 12 µg/ml (Sigma-Aldrich, St. Louis, MO, USA).

After incubation, 20 µl of the resultant culture at dilutions of 10^{-3} , 10^{-4} and 10^{-5} was plated onto nutrient agar plates and incubated at 37 °C for 18 h. Antibacterial activity was determined by comparing colony numbers after exposure to pouch washes compared with the colony numbers on control plates. Bacterial survival was expressed as a percentage of the control. For reconstituted wash samples, results were standardised to allow comparisons on the basis of the protein concentration in the test samples.

2.4. Protein precipitation and concentration

For proteomic analysis, proteins in the wash samples, mammary gland secretions and stomach and intestine samples were precipitated using 90% TCA and the resultant pellet was then re-suspended in 200 µl of 2-D sample-solubilising solution. This solution consisted of 7 M urea, 2 M thiourea, 65 mM DTT, 2% CHAPS, 1% carrier ampholytes (3–10), 40 mM Tris and 0.002% bromophenol. Mammary gland secretions and stomach and intestine samples were then reduced and alkylated with 5 mM tributyl phosphine and 10 mM acrylamide for 1 h in the dark.

2.5. One-dimensional gel electrophoresis

To overcome anticipated detection and sequencing problems associated with the low protein concentration in the pouch washes, 1-DE on a 4–20% Tris–glycine SDS-PAGE gradient gel (Invitrogen, Carlsbad, CA, USA) was employed, in the first instance, to separate the major protein components of reconstituted pouch washes. Electrophoresis was carried out using a cathode running buffer of 192 mM glycine, 15 mM Tris and 0.1% SDS, pH 8.3. Samples were diluted two-fold in $2 \times$ loading buffer (50 mM Tris–HCl pH 6.8, 100 mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue and 10% (v/v) glycerol) and boiled for a minimum of 5 min before loading onto the gel. The gels were run at 125 V, until the bromophenol blue front had completely run off the bottom of the gel. The gels were fixed in 10% methanol and 7% acetic acid, followed by staining overnight with Sypro Ruby (Molecular Probes, Eugene, CA, USA). Gels were destained in 10% methanol and 7% acetic acid for 1 h, and images were captured with the Typhoon 9410 scanner (GE Healthcare, Bio-Sciences Inc., Quebec, Canada).

Prior to excision of proteins and to enhance visualisation, gels were restained with 0.1% w/v colloidal Coomassie Brilliant Blue G-250 overnight and destained with 5% acetic acid for 1 h. The images were then scanned and captured

with a Kodak Digital camera (Eastman Kodak, Rochester, NY, USA).

For the separation and visualisation of lower MW proteins 10–20% Tris–Tricine SDS-PAGE gradient gel electrophoresis (Invitrogen, Carlsbad, CA, USA) was performed according to the methods of Schagger and von Jagow [15]. The cathode running buffer consisted of 0.1 M Tris–HCl, 0.1 M Tricine, 0.1% SDS, pH 8.25. Running, staining and visualisation conditions were the same as described above for Tris–glycine gels.

2.6. Two-dimensional gel electrophoresis

In order to have sufficient quantity of protein for visualisation and identification, pouch wash samples from mature reproductively active females in all three periods of pre-oestrus, oestrus and post-oestrus were pooled for analysis. 2-DE was performed over two pI ranges, using 11 cm pH 4–7 and 7–10 linear IPG strips (Bio-Rad Hercules, CA, USA) in the first dimension. Isoelectric focusing (IEF) was performed using the Multiphore II system (Amersham Pharmacia Biotech, Uppsala, Sweden) for 100 kVh at 20 °C. The IPG strips were then equilibrated for 20 min at room temperature by gentle rocking in 6 M urea, 2% SDS, 20% glycerol, $1 \times$ Tris–HCl (pH 8.8), 5 mM tributylphosphine and 2.5% acrylamide and then embedded in 0.5% agarose on top of precast 4–20% gradient Tris–HCl Criterion gels (Bio-Rad, Hercules, CA, USA). Electrophoresis was carried out using a cathode running buffer consisting of 192 mM glycine, 15 mM Tris and 0.1% SDS at pH 8.3. Gels were initially run at 5 mA/gel for 30 min, then at 200 V, until the bromophenol blue buffer front had completely run off the bottom of the gel. The gels were fixed in 10% methanol and 7% acetic acid, followed by staining overnight with Sypro Ruby (Molecular Probes, Eugene, CA, USA). Gels were destained in 10% methanol and 7% acetic acid for 1 h. Protocols for the visualisation and staining for protein excision were as described above.

For mammary gland secretions, stomach and intestine samples, 200 µg of protein was loaded onto 11 cm pI 3–10 IPG strips (Bio-Rad, Hercules, CA, USA). The strips were focused for 40 or 70 kVh depending on the IPG strip length, with the Multiphore II system (Amersham Pharmacia Biotech, Uppsala, Sweden), and proteins were subsequently separated using precast 4–20% gradient Tris–HCl Criterion gels (Bio-Rad, Hercules, CA, USA) for the 11 cm strips or a manually made 8–18% polyacrylamide SDS gel for the 17 cm strips. Gels were double stained with Sypro Ruby (Molecular Probes, Eugene, Oregon, USA) and then with Coomassie Blue G250 (Bio-Rad, Hercules, CA, USA).

2.7. Mass spectrometry

2.7.1. Pouch wash secretions: mass spectrometry using LC-MS/MS

Proteins were excised from 1D SDS-PAGE and 2D gels for identification by MS. The gel plug was extracted three times in 50% v/v acetonitrile and 25 mM NH_4HCO_3 , pH 7.8, at 37 °C for 10 min, dried at room temperature in a Speedy-Vac (Savant, Farmingdale, NY, USA), covered with 8 µl of sequencing grade trypsin (15 ng/µl) and digested overnight at 37 °C. Products were recovered from the gel by sequential

extractions with 10% acetonitrile and 1% v/v formic acid. The combined extract was dried and peptides were dissolved in 20 µl of 0.1% formic acid [16]. Five microliters of digests were concentrated and desalted using a micro-C18 precolumn (500 µm × 2 mm, Michrom Bioresources, Auburn, CA, USA) with water:acetonitrile (98:2, 0.1% formic acid) at 20 µl/min. After a 4 min wash, the pre-column was automatically switched (Valco 10 port valve, Houston, TX, USA) into line with a fritless nano-column [17]. Digested peptides were separated by nano-LC using a Cap-LC autosampler system (Waters, Milford, MA, USA). Peptides were eluted using a linear gradient of water:acetonitrile (98:2, 0.1% formic acid) to water:acetonitrile (50:50, 0.1% formic acid) at ~200 nl/min over 30 min. The pre-column was connected via a fused silica capillary (10 cm, 25 µm) to a low-volume tee (Upchurch Scientific, Oak Ridge, WA, USA) where 2600 V was applied and the column tip positioned ~1 cm from the Z-spray inlet of a QTOF Ultima hybrid tandem mass spectrometer (Micromass, Manchester, UK). Positive ions were generated by electrospray and the QTOF operated in a data-dependent acquisition mode. A TOF MS survey scan was acquired (m/z 350–1700, 1 s) and the two largest multiple charged ions (counts > 25) were sequentially selected by Q1 for MS–MS analysis. Argon was used as the collision gas and an optimum collision energy was chosen, based on charge state and mass. Tandem mass spectra were accumulated for up to 6 s (m/z 50–2000).

2.7.2. Milk and milk digests: mass spectrometry using MALDI MS/MS

Excised gel pieces were washed three times with 50% acetonitrile in 25 mM NH_4HCO_3 and evaporated to dryness using a Speed Vac concentrator. The gel plug was rehydrated with 10 µl of 0.2 µg sequencing grade trypsin (Promega, Madison, WI, USA) in 25 mM NH_4HCO_3 and digested overnight at 37 °C. Peptide products were recovered from the gel by sonication in 10 µl 0.1% TFA. Ten microlitres of extract was concentrated and desalted using C18 Zip tips (Millipore, Bedford, MA, USA). Peptides were deposited onto the ABI 4700 MALDI target plate using 5 mg/ml α cyano 4 hydroxy cinnamic acid (Sigma Aldrich, St. Louis, MO, USA) in 70% acetonitrile and 0.1% TFA (Applied Biosystems, Warrington, UK). Peptide mass fingerprint (PMF) and collision-induced dissociation (CID) were performed using ABI 4700 TOF TOF.

2.8. Protein identification and database searching

For pouch wash secretions, protein identification was carried out by matching the peptide peaks with known proteins present within the public access databases [18]. Peptide peak lists were generated by MassLynx (version 4 SP1, Micromass) using the Mass Measure program to automatically baseline subtract, smooth and centroid the data before submission to the database search program Mascot (version 2.1, Matrix Science, London, UK). Proteins were identified by the correlation of mass spectra to entries in the NCBI_{nr} database (June 2006). Mascot MS/MS ion search criteria were as follows: taxonomy—Mammalia, trypsin digestion allowing up to one miscleavage, semi-trypsin digestion for peptides formed as cleaved products, variable modification—oxidation of methionine, cysteine as carbox-

amidomethylation or propionamide, peptide tolerance of 0.25 Da and MS/MS tolerance of 0.2 Da. The “ion score cut-off” was manually set to 20, thereby eliminating the lowest-quality matches. A probability-based Mowse score > 42 indicated identity ($p < 0.05$).

For mammary gland secretions and milk digest products, peptide peak lists were similarly matched against known proteins present in the NCBI_{nr} database, using automated MASCOT Deamon software (version 2.1.0, Matrix Science, London, UK). Mascot searches criteria were set as follows: taxonomy—Mammalia, trypsin digestion allowing up to one miscleavage, variable modification—oxidation of methionine, cysteine as propionamide, peptide tolerance of 0.2 Da and MS/MS tolerance of 0.2 Da. A probability-based Mowse score using $-10 \log(P) > 65$ indicated identity ($p < 0.05$).

3. Results

3.1. The pouch

Fig. 1 shows the appearance of the tammar pouch at the three periods at which wash samples were collected from reproductively mature females. Fig. 1A shows a pre-oestrus pouch in which dried secretions are clearly visible. Fig. 1B clearly shows the clean moist pouch that is observed at oestrus and at the birth of the young animal. A newborn tammar wallaby is clearly seen attached to the mammary gland teat. Fig. 1C shows a post-oestrus pouch from an animal with an older pouch young, dried material is now once again apparent.

3.2. Estimation of protein concentration

Attempts to obtain accurate readings of the protein concentration in each of the concentrated pooled pouch wash secretions were problematic and may have been due to the presence of interfering compounds in the concentrated wash. Protein concentrations obtained by the triplicate analysis of the pooled sample for each reproductive stage were pre-oestrus 190 ± 81 µg/ml, oestrus 439 ± 70 µg/ml and post-oestrus 228 ± 46 µg/ml, indicating that the protein concentration of the pooled oestrus sample was about twice that observed in pre- and post-oestrus samples.

3.3. Antibacterial activity

Antibacterial activity of pooled pouch wash secretions from each of the three periods and of fractionated samples were investigated against stock cultures of *E. coli* and *S. aureus*. Whole concentrated pouch wash secretions had no activity against the Gram-positive *S. aureus* (Fig. 2a). The positive control, the antibacterial, Cecropin, showed complete inhibition of *S. aureus* colony growth (data not shown). Fig. 2a shows % survival (\pm standard error) of *E. coli* after exposure to the reconstituted pouch washes from the three different stages of the reproductive cycle. These assays were conducted in triplicate. Results have been calculated with respect to a standard amount of protein of 3 µg, this being the lowest protein concentration in the test samples. Highest antimicrobial activity against *E. coli* was observed in

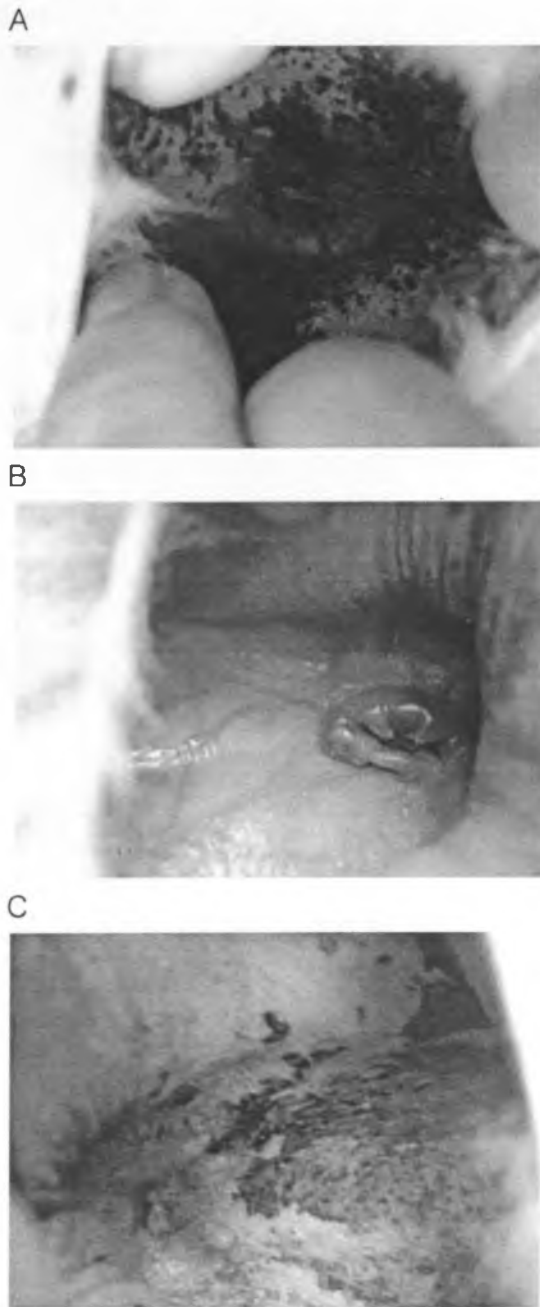


Fig. 1 Macroscopic appearance of the pouch of the tammar wallaby at different stages in the reproductive cycle. (A) Prior to the birth of the pouch young, dried secretions are apparent. (B) The pouch at oestrus, with a newborn pouch young attached to the teat. The pouch is characteristically clean and moist. (C) Post-oestrus dried, secretions are once again apparent.

the oestrus sample, with an average of 15% bacterial survival compared to 65% for pre-oestrus. The post-oestrus sample had little inhibitory effect on bacterial growth.

Pouch wash secretions fractionated according to MW were assessed for antibacterial activity against *E. coli*. The activity of all fractions for all three reproductive stages is shown in Fig. 2b. Greatest inhibition of growth is seen in MW

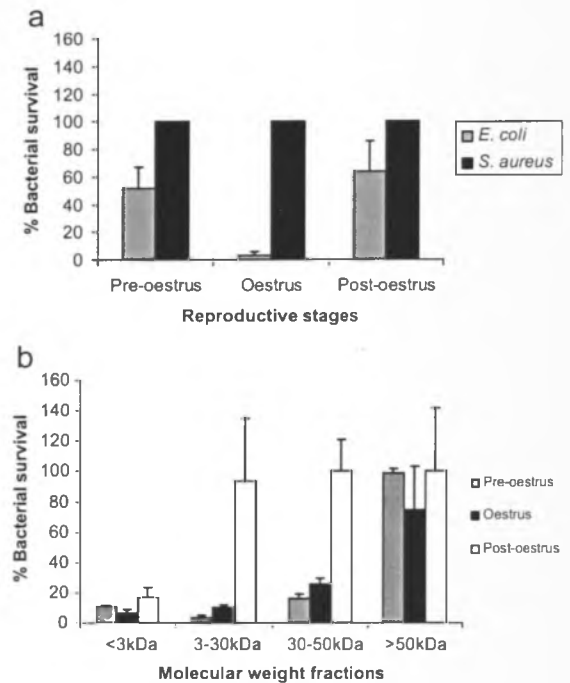


Fig. 2 (a) Antimicrobial activity against *E. coli* and *S. aureus* of whole concentrated pouch wash secretions collected at different stages of the reproductive cycle. Data show a mean % survival with standard error bars ($n = 3$) expressed per 3 μ g of protein. (b) Antimicrobial activity against *E. coli* of fractionated pouch wash secretions collected at different stages of the reproductive cycle. Data show a mean % survival with standard error bars ($n = 3$).

fractions less than 50 kDa, particularly in the pre-oestrus and oestrus samples. For the post-oestrus sample, significant inhibition was observed only in the fraction < 3 kDa.

3.4. Separation and identification of proteins in pouch secretions

Given the limited amount of sample available for the analysis 1D gel electrophoresis, followed by LC-MS/MS, was initially undertaken to separate and identify proteins, particularly in the MW range up to 50 kDa.

Two 1D gel systems were used to cover the desired MW range. A 4–20% Tris–glycine gradient gel (Fig. 3) shows the protein components of the unfractionated pouch washes collected at the three different reproductive stages. The protein bands covered a wide range of molecular weights, from 14 to 85 kDa and it can be seen that a number of these bands appear to be common to all reproductive stages. In order to detect low-MW proteins, a 10–20% Tris–Tricine gel system was used. No proteins were detected below the 3 kDa MW marker (data not shown). This may reflect the low concentration of such proteins and the limits of detection of the gel staining system used. To address this, 5 μ l of this sample was analysed by direct application to nanoLCMS. Few multiple charged ions were observed and the fragmentation spectra were weak. No high-scoring identifications were obtained. Although intense singly charged ions were

detected, these were attributed to low-MW contaminants that may have also suppressed the ionisation of any other low-MW peptides in the sample.

Twelve protein bands from the 4–20% Tris–glycine gradient gel (Fig. 3) were excised, subjected to in-gel trypsin digestion and analysed using LC–MS/MS. Peptide identification was obtained by matching fragmentation spectra against the mammalian NCBI nr database (Table 1). Seven of these bands (bands 2, 6, 7, 8 and 10–12) were confidently identified as β -lactoglobulin. Some peptides gave less confident identifications on the basis of low number of peptide matches and low MOWSE score (bands 1, 3, 4 and 9), and some bands had more than one confident protein identification, suggesting that more than one

protein was contained within a single band (spots 3, 6–8, 10 and 11). It can also be seen that for a number of β -lactoglobulin bands the experimental MW is significantly different from the theoretical MW.

The apparent ubiquitous presence of β -lactoglobulin at a range of MWs and in all samples from all three reproductive stages (Fig. 3) and the observation that other proteins were identified in the same bands suggested that it was possible that low-abundance proteins were masked by β -lactoglobulin, present at higher concentrations. Such proteins would potentially be exposed by 2DE separation.

Fig. 4a and b shows 2DE analysis of pooled whole-pouch wash secretions. The majority of protein spots were observed in the 4–7 pI gel across a wide range of molecular weights between 10 and 80 kDa (Fig. 4a), whilst less than 10 proteins were observed in the 7–10 pI gel at low MW <15 kDa (Fig. 4b).

A total of 37 protein spots were excised from these 2DE gels, subjected to in-gel trypsin digestion and analysed using LC–MS/MS. To overcome the lack of a substantial tammar wallaby protein database, a search across the species barrier was carried out. This allows proteins to be identified on the basis of conserved peptide sequences. Table 2 presents the results of this analysis. Only five proteins (spots 2–4, 24 and 30) could be identified with a high degree of confidence based on the minimum number of three peptide matches and high Mowse scores and these identifications were β -lactoglobulin. Their observed MW ranged from around 15 kDa (spot 30) to 40 kDa (spot 3). Less confident identifications, deemed so because of the low number of peptide matches or low Mowse score, also matched to β -lactoglobulin (spots 8, 17, 21, 23, 27 and 34), whilst the observed MWs ranged from 13 to 60 kDa. One spot gave a tentative peptide match to dermcidin (spot 2), although the observed MW and the theoretical MW are so significantly different as to question the validity of the match. Other less confident matches were to serotonin (spot 1) and

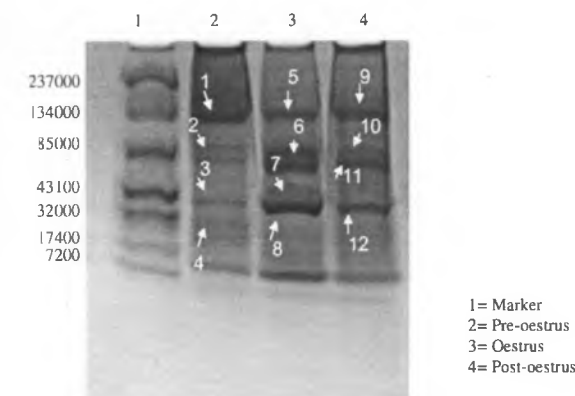


Fig. 3 4–20% Tris–glycine gradient gel of whole concentrated pooled pouch wash secretions collected at different stages of the reproductive cycle, showing proteins predominantly present in the MW range 14–85 kDa. Lane 1, MW marker; Lane 2, pre-oestrus sample; Lane 3, oestrus; lane 4, post-oestrus. Bands subjected to MS analysis numbered and data presented in Table 1.

Table 1 Identification of proteins from tammar wallaby pouch secretions separated using 1D SDS-PAGE gel

Band no.	Name	Exp. mol. wt (approx.)	Theor. mol. wt.	Mowse score	No. of peptides	Accession no.
1	Interferon, β 1	120,000	21,215	42	1	gi 9506801
2	β -Lactoglobulin	85,000	20,220	86	3	gi 86574
3	Carbonic anhydrase	40,000	37,563	92	2	gi 30466252
	β -Lactoglobulin		20,220	45	1	gi 3122325
4	β -Lactoglobulin	20,000	20,220	62	1	gi 1146
5	Keratin	120,000	108,313	70	2	gi 55956899
6	β -Lactoglobulin	70,000	20,220	133	3	gi 86574
	Transferrin		68,507	90	2	
7	β -Lactoglobulin	40,000	20,220	136	4	gi 3122325
	α -Casein		28,123	58	2	gi 115649
8	β -Lactoglobulin	32,000	20,220	131	4	gi 3122325
	β -Casein		35,882	64	2	gi 115662
9	β -Lactoglobulin	120,000	20,220	73	1	gi 1146
10	β -Lactoglobulin	85,000	20,220	179	8	gi 3122325
	Transferrin		91,555	163	3	gi 5051646
11	β -Lactoglobulin	60,000	20,220	178	7	gi 3122325
	Interferon		21,215	62	1	gi 3122325
12	β -Lactoglobulin	32,000	20,220	108	4	gi 3122325

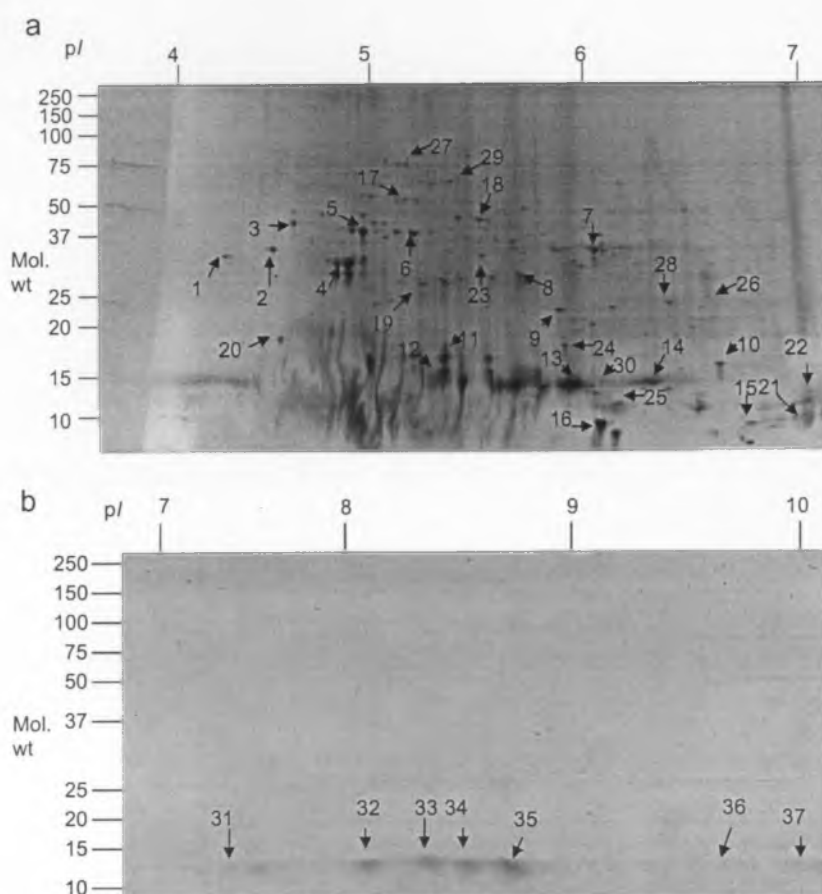


Fig. 4 2DE PAGE of pooled pouch wash secretions from female tammar wallabies. Spots subjected to MS analysis numbered and data presented in Table 2. (a) electrophoresis over pI range 4–7. (b) electrophoresis over pI range 7–10.

apolipoprotein J (spot 5). Only one protein could be identified on the 7–10 pI gel and it was identified with high confidence as β -lactoglobulin (spot 34). A number of spots could not be matched primarily because the protein concentration was too low to generate good fragmentation spectra (spots 10, 12, 13, 18, 20, 22, 25, 26, 28, 29, 31–33 and 35–37).

The consistent detection and confident identification of β -lactoglobulin in the 2D gel system at a range of pI's and MWs raised questions as to the source of this compound. Most obviously, it could have come directly from mammary gland secretions prior to the attachment of the young animal to the teat, at pre-oestrus, or it could have arisen as a digest product from the gut of the young animal, at post-oestrus. To examine this possibility, we undertook comparative proteomic analysis of both mammary gland secretions collected just before the birth of the young, representative of the cusp of pre-oestrus/oestrus, and gut samples from a young animal, representative of post-oestrus.

3.5. Separation and identification of proteins of mammary gland secretions and milk digests

Fig. 5 shows 2DE of the mammary gland secretions collected prior to birth (Fig. 5a), and milk digest from a 12-day-old animal—the stomach (Fig. 5b) and duodenum (Fig. 5c). For

the mammary gland secretion, most proteins were observed in the 4–7 pI gel across a wide range of MWs from 15 to 150 kDa (Fig. 5a). The gel is dominated by two abundant proteins in the 50–100 kDa regions (spots 11 and 12), the similar MWs and varying pI's suggestive of significant post-translational modification of these proteins. The gel analysis of the milk digested in the stomach and duodenum (Fig. 5b and c) shows abundant proteins spread across a MW range of 10–150 kDa, with the majority in the 4–7 pI range (Fig. 5c). The pattern of distribution of protein spots across these gels does not have any strong resemblance to that seen in the pouch wash secretion gels (Fig. 4a and b).

A total of 64 protein spots from mammary gland secretions and milk digest gels were excised, subjected to in-gel trypsin digestion and analysed using MALDI mass spectrometry: 12 protein spots from the mammary gland secretion 4–7 pI gel (Fig. 5a), 22 protein spots from the 3–10 pI gel of milk digest from the stomach (Fig. 5b) and 30 protein spots from the 3–10 pI gel of milk digest from the duodenum (Fig. 5c). Peptide identification was obtained by matching the fragmentation spectra obtained from MALDI PMF and MS/MS against the mammalian NCBI database.

Ten out of the 12 proteins from the mammary gland secretion gel (Fig. 5a) were confidently identified as having high Mowse scores and a high number of peptide matches (Table 3). Only two spots were identified with high

Table 2 Identification of proteins from tammar wallaby pouch secretions separated using 4–7 pI range 2D gel

Spot no.	Name	Exp. pI (approx.)	Theor. pI	Exp. mol. wt. (approx)	Theor. mol. wt.	Mowse score	No. of peptides	Accession no.
1	Serotonin	4.2	9.13	36,000	46,107	39	1	gi 62655081
2	β -Lactoglobulin	4.7	5.82	36,000	20,220	106	4	gi 3122325
	Periaxin		5.82		20,220	40	1	gi 2143901
	Dermcidin ^a		6.09		11,283	75	2	gi 16751921
3	β -Lactoglobulin	5	5.82	40,000	20,220	312	10	gi 3122325
	Folate transporter 1		10.64		64,868	50	1	gi 74001578
	α -Lactoglobulin		4.65		13,776	48	1	gi 126003
4	β -Lactoglobulin	5	5.82	36,000	20,220	217	8	gi 3122325
	α -Lactoglobulin		4.65		13,776	69	2	gi 126003
5	Apolipoprotein J ^a	5	5.98	37,000	41,213	71	1	gi 178855
6	Diacylglycerol kinase κ	5	5.36	37,000	141,829	32	1	gi 57753888
7	Heat shock protein	6	4.87	36,000	14,962	31	1	gi 82897995
8	β -Lactoglobulin	5.8	5.82	27,000	20,220	75	2	gi 3122325
	Apoferitin		5.2		22,161	48	1	gi 55639021
9	60s Ribosomal protein	5.8	10.26	24,000	47,581	28	1	gi 62657863
11	Occludin	5.3	10.01	18,000	29,404	26	1	gi 46520134
14	Glucokinase	6.3	5.77	15,000	54,089	32	1	gi 44888570
15	CG6004-PB	6.8	3.84	10,000	157,141	29	1	gi 82959506
16	Ectodysplasin A	6.1	5.23	9000	24,784	31	1	gi 62655081
17	β -Lactoglobulin	5.2	5.82	60,000	20,220	47	1	gi 3122325
19	Occludin	5.2	10.01	26,000	29,404	26	1	gi 46520134
21	β -Lactoglobulin	6.9	5.82	13,000	20,220	109	2	gi 125916
23	β -Lactoglobulin	5.5	5.82	36,000	20,220	54	2	gi 125916
24	β -Lactoglobulin	5.6	5.82	19,000	20,220	182	5	gi 125916
27	β -Lactoglobulin	5.3	5.82	75,000	20,220	47	2	gi 125916
30	β -Lactoglobulin	6	5.82	15,000	20,220	90	3	gi 125916
34 ^b	β -Lactoglobulin	8.5	5.82	14,000	20,220	47	2	gi 125916

^aProteins identified when semi-trypsin was specified as the enzyme in the MASCOT searches.

^bProtein identified from the mature female tammar wallaby pouch secretions using 7–10 pI range 2D gel.

confidence as β -lactoglobulin (spots 7 and 8), with an experimental MW close to 15 kDa compared to the theoretical one of 20 kDa. A number of other intense spots observed at a range of MW and pI's were identified as β -actin. The most abundant proteins (spots 11 and 12) did not match any known protein within the database.

Twelve spots out of 22 were confidently identified from the 2DE gel of milk digest in the stomach of the 12-day-old pouch young (Table 4). High Mowse scores and a minimum of three matched peptides gave high confidence in the protein identification. Only spot 8, with an observed MW of around 18 kDa and pI 5.8, was identified with high confidence as β -lactoglobulin.

Of the 30 protein spots excised from the 2DE gel of milk digested in the duodenum, 19 proteins were identified with high confidence (Table 5) using similar criteria as noted previously. Of these, only spot 7 was confidently identified as β -lactoglobulin, with an observed MW of around 14 kDa and pI around 6.2. A number of other proteins were identified with high confidence to enzymes (spots 1, 4, 6, 8, 9 and 11–18).

4. Discussion

As can be seen in Fig. 1, there are obvious macroscopic changes in the appearance of the pouch over the period prior to birth and when the young animal is in residence. These differences, particularly as the pouch becomes clean and moist prior to birth, have prompted repeated statements that the pouch produces secretions that regulate the microflora and reduce the threat to the immunologically incompetent neonate. Our observation of antimicrobial activity of pouch secretions against a Gram-negative bacterial species are consistent with previous reports of inhibitory activity observed in pouch secretions in the koala [11] and with our previous observation that the Gram-negative bacteria of the pouch do decrease over the period of birth and oestrus in the tammar wallaby [7]. However, they are at odds with the reported results of Baudinette et al. [12] for the tammar wallaby. In this latter study, samples were collected as swabs from 2 days before the birth of the young and up to 4 weeks after the birth, a similar time frame to that used in our study. As indicated, they observed no antimicrobial activity in their lyophilised water:methanol

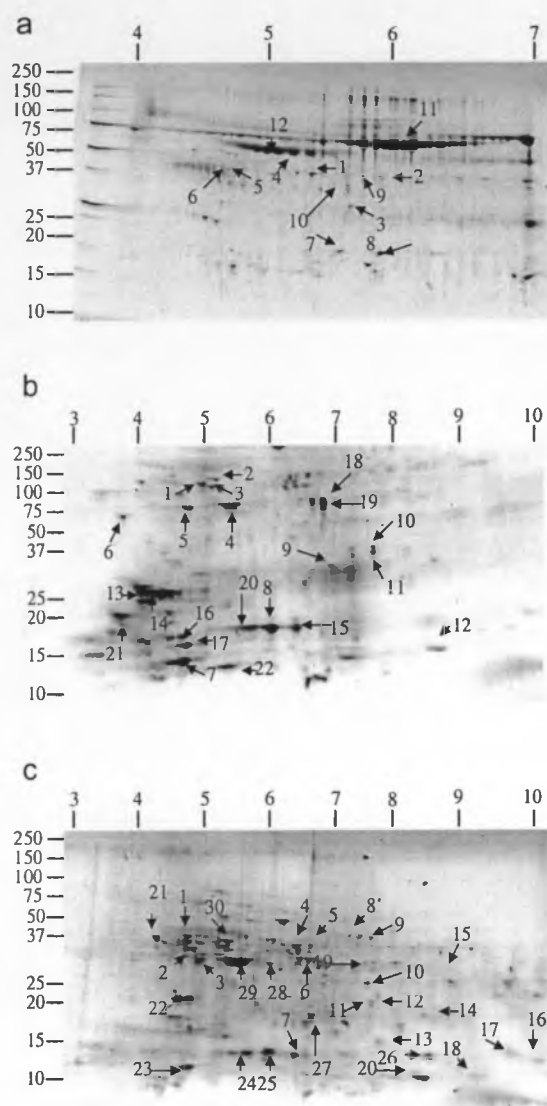


Fig. 5 2DE PAGE of mammary gland secretions and milk digest from a 12-day-old pouch young. Spots subjected to MS analysis numbered and data presented in Tables 3–5, respectively. (a) Sample collected from mammary gland immediately prior to birth, *p*l 4–7. (b) Sample collected from the stomach of pouch young, *p*l 3–10. (c) Sample collected from the duodenum of pouch young, *p*l 3–10.

extracts and the active immunomodulator, eugenin, was only isolated from swabs collected from pouches in the postnatal period up to 2 weeks after birth [12]. This is the period equivalent to post-oestrus in our study.

In our study, the nature of the biological system meant that little protein was available for the analysis from any one pouch wash collection. To address this, we collected samples over a 2-year period and concentrated them by lyophilisation prior to analysis. We specifically used a protein measurement protocol designed for low-concentration, low-volume samples. Even so, attempts to obtain accurate measurements of protein in the samples were not reliable, although we have included the indicative data and

we have used this to allow a direct comparison of antimicrobial activity of reconstituted pouch wash samples based on protein concentration. It may be that lyophilisation and the consequent increase in salt or other compounds in the sample have affected these assays.

Fractionation of pouch wash samples and subsequent analysis of antimicrobial activity in these different fractions, even allowing for differing protein concentrations, suggested that MW compounds <50 kDa were present in samples collected at different stages during the reproductive period and had significant activity against Gram-negative bacteria, particularly during the pre-oestrus and oestrus periods (Fig. 2b).

We subsequently undertook proteomic analysis of the pouch wash secretion in order to identify compounds that could be responsible for this antimicrobial activity. This process itself was problematic due to the extremely small amounts of material, with low protein concentration, available for analysis. To address this, we initially undertook 1D PAGE analysis as this could be undertaken with a smaller amount of sample material. 1D PAGE showed a number of proteins in the MW range 15–85 kDa, but no proteins were detected in the gel system used to provide separation over the small MW range, in particular <3 kDa. The latter observation may be due to the amount of protein present in the wash falling below the level of detection of the system (1–2 µg/ml). In the Baudinette et al. study [12], HPLC was used on post-oestrus samples to successfully isolate compounds <2 kDa and it was in this fraction that they identified the immunomodulator, eugenin.

Proteomic analysis of these visible proteins on the 1D gel system in the MW range of interest (Fig. 3) primarily identified β -lactoglobulin with high confidence, although other important antimicrobial proteins such as transferrin were co-identified with high confidence (spot 6—Table 1) [19]. As it was possible that β -lactoglobulin was present in such high concentrations relative to other proteins that it masked their presence, we subsequently undertook 2DE analysis. In order to increase the likelihood of detection and subsequent identification of these proteins, we firstly used a staining protocol aimed at increasing the visibility of proteins down to 1 ng. Secondly, as a comprehensive tammar wallaby protein database was not available, we used a combination of MS techniques to increase the chances of identification. These entailed electrospray ionisation mass spectrometry coupled to a chromatographic nano-LC column that allows online separation of peptides and removal of hydrophilic contaminants, such as salt, and improves the data-dependent acquisition. Finally, we used the Mascot search engine where identification was based on the probability of conserved peptide sequences across species boundaries [20]. Using this approach we confidently identified a number of proteins (Table 2). Although the majority of these confident identifications were β -lactoglobulin, other proteins with known antimicrobial activity were also identified. Dermcidin, an antimicrobial secreted by epithelial cells and sweat glands [21], was co-identified at spot 2 (Table 2). Although identified with low confidence, having a significantly different experimental versus observed MW and only two peptide matches, it is possible that dermcidin is present in pouch secretions and contributes to the observed antimicrobial activity. Its co-identification in spot 2 with

Table 3 Identification of proteins from mammary gland secretion pre-oestrus separated using 4–7pI 2D gel

Spot no.	Name	Exp. pI (approx.)	Theor. pI	Exp. mol. wt. (approx.)	Theor. mol. wt.	Mowse score	No. of peptides	Accession no.
1	β -Actin	5.3	5.78	37,000	39,161	159	16	gi 49868
2	Actin	5.8	5.3	37,000	41,775	178	18	gi 60389477
3	β -Actin	5.8	6.03	28,000	41,638	142	16	gi 55741585
4	Vimentin	5.5	5.06	50,000	53,615	102	13	gi 57114172
5	Vimentin	4.8	5.08	37,000	52,840	112	14	gi 73948972
6	Vimentin	4.8	5.08	37,000	52,840	102	12	gi 73948972
7	β -Lactoglobulin	5.6	5.82	15,000	20,220	86	11	gi 125916
8	β -Lactoglobulin	5.8	5.82	15,000	20,220	71	10	gi 125916
9	β -Actin	5.8	6.03	37,000	41,638	142	16	gi 55741585
10	β -Actin	5.7	6.03	35,000	41,638	122	15	gi 55741585

Table 4 Identification of proteins from milk digest from the stomach of pouch young separated using 3–10 pI range 2D gel

Spot no.	Name	Expt. pI (approx.)	Theor. pI	Expt. mol. wt. (approx.)	Theor. mol. wt.	Mowse score	No. of peptides	Accession no.
1	α -Tubulin	5	4.75	100,000	49,596	204	12	gi 7838279
2	Tubulin-podophyllotoxin	5	4.91	100,000	51,091	105	7	gi 47169127
3	Mitochondrial ATP synthase	5	5.13	100,000	45,390	247	10	gi 89574037
4	β -Actin	5.5	5.56	77,000	41,661	199	8	gi 14250401
5	Keratin 19	4.8	4.86	77,000	44,018	115	6	gi 886904
6	Albumin	4	5.82	74,000	71,815	79	5	gi 162648
7	α -Lactalbumin	4.8	4.73	14,000	16,344	124	3	gi 295498
8	β -Lactoglobulin	5.8	5.83	18,000	20,220	130	6	gi 125916
9	Albumin	6.2	5.82	36,000	71,815	143	6	gi 162648
10	Casein α S1	7.1	4.85	37,000	24,587	149	5	gi 225632
11	Casein α S1	7.1	4.85	36,000	24,587	118	5	gi 225632
12	α -Globin	8	7.22	14,000	15,414	152	7	gi 42517335

β -lactoglobulin also raises the possibility that even in the 2DE gel system some low-concentration proteins may have been masked by this relatively abundant compound.

The large number of spots confidently identified as β -lactoglobulin at a range of MW and pI's raised the possibility that the peptides may have been derived from proteins with significant shared sequence homology to β -lactoglobulin. We thus mapped peptide sequences obtained from the MS analysis of the 1D and 2D experiments to the whole sequence of tammar wallaby β -lactoglobulin available on ExPASy (www.expasy.org) (Fig. 6). The identified peptides have a wide coverage, indicating that the proteins we have identified are truly β -lactoglobulin.

β -Lactoglobulin is a significant component of milk of a number of animals, including marsupials [22]. It is a member of the lipocalin family of proteins, a diverse family characterised by a common protein fold of β -sheet that acts as a ligand-binding site and is associated with transport functions [23]. In marsupials, three milk proteins have been identified as belonging to the lipocalins— β -lactoglobulin, late lactation protein and a novel protein, trichosurin, isolated from the brushtail possum [24]. In this study, we identified β -lactoglobulin in both pre-oestrus mammary gland secretion and in the stomach and duodenum digests.

The observed MWs and pI's were close to those expected, with MW around 20 kDa and pI close to 5.9. These observations, and their appearance in the gels, are consistent with those reported for milk whey of the brushtail possum [24], where two forms of β -lactoglobulin were detected as two protein spots using 2DE. In contrast, in our pouch wash gels and in the LC-MS/MS data, β -lactoglobulin was confidently identified over a wide range of MWs, from around 15 to 60 kDa, and at a range of pI's, from around 5 to 7. These observations suggest that β -lactoglobulin in the pouch environment may polymerise and that the molecule itself maybe glycosylated. Under physiological conditions, bovine β -lactoglobulin exists as a dimer and polymerises to an octamer at low temperature and pH [25]. It is possible that this transition could occur in the maternal pouch as the pH of the skin of the pouch young varies from neutral (pH~6.5) at birth to slightly acidic during the first few weeks post birth. Moreover, the pH of the microenvironment of the pouch could be influenced by epithelial secretion of lactic and fatty acids known to favour the growth of commensal microflora. The ScanProsite tool (www.expasy.org) was used to scan the β -lactoglobulin sequence for any potential glycosylation sites. The consensus sequence NISD with a high probability of *N*-glycosylation was identified at residues

Table 5 Identification of proteins from milk digest from the duodenum of pouch young separated using 3–10pI range 2D gel

Spot. no.	Name	Exp. pI (approx.)	Theor. pI	Exp. mol. wt. (approx.)	Theor. mol. wt.	Mowse score	No. of peptides	Accession no.
1	Prolyl 4-hydroxylase, β -polypeptide	4.7	4.77	37,000	57,600	223	8	gi 42415475
2	Keratin 10	4.8	5.4	36,000	10,7761	93	5	gi 94391959
3	Keratin 19	4.9	4.86	35,000	44,018	129	3	gi 886904
4	Aldehyde dehydrogenase	6.2	6.06	36,000	48,881	210	7	gi 16073616
5	Keratin 8	6.8	5.35	36,000	35,277	129	3	gi 437903
6	Ornithine aminotransferase	6.5	6.1	34,000	48,654	72	5	gi 73586966
7	β -Lactoglobulin	6.2	5.83	14,000	20,220	177	8	gi 125916
8	Catalase	7.2	6.7	37,000	57,031	154	8	gi 7245758
9	Catalase	7.7	6.88	37,000	60,424	127	5	gi 12082093
10	Annexin A2	7.6	7.55	27,000	39,015	104	6	gi 73586982
11	Carbonic anhydrase	7.6	6.4	22,000	29,013	229	8	gi 46016009
12	Carbonic anhydrase	7.6	6.4	23,000	29,013	167	8	gi 46016009
13	Carbonic anhydrase	7.7	6.4	17,000	29,013	154	8	gi 46016009
14	Carbonic anhydrase	8.2	6.4	19,000	29,013	149	6	gi 46016009
15	Glyceraldehyde-3-phosphate dehydrogenase	8.8	8.14	36,000	36,320	86	5	gi 46016019
16	Carbonic anhydrase	10	6.4	13,000	29,013	148	8	gi 46016009
17	Carbonic anhydrase	9.5	6.4	14,000	29,013	149	6	gi 46016009
18	Carbonic anhydrase	9.1	6.4	12,000	29,013	149	6	gi 46016009
19	α -Globin	7.3	7.22	34,000	15,414	109	4	gi 42517335

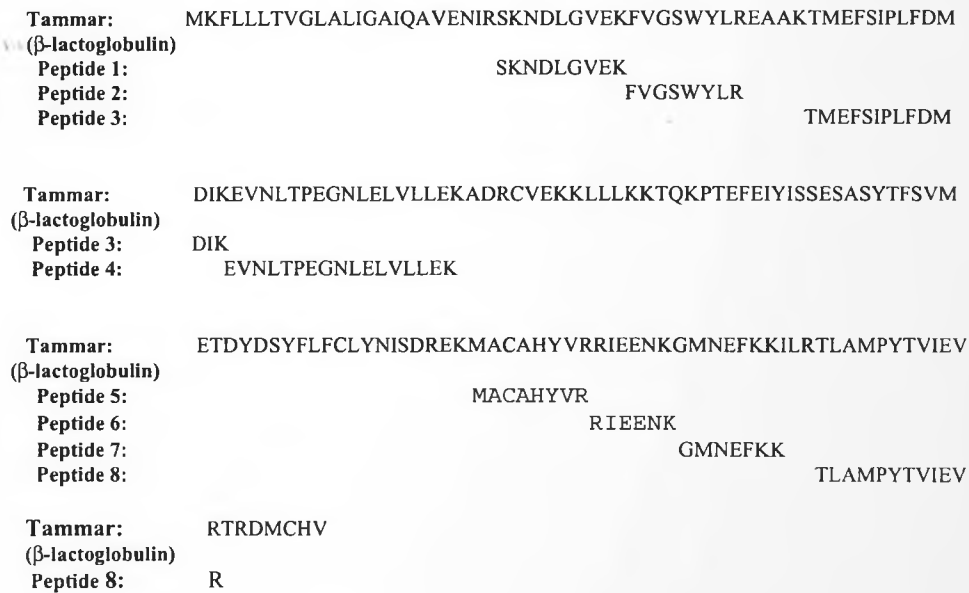


Fig. 6 Amino acid sequence of tammar wallaby β -lactoglobulin (www.expasy.org), accession no. Q29614LACB_MACEU. Individual peptides from both 1D and 2D gels and identified through MS analysis (Tables 1 and 2) have been mapped to various regions of the β -lactoglobulin sequence and validate the identification of the source protein spot as β -lactoglobulin.

125–128. Post-translational modifications, such as the addition of carbohydrate moieties to β -lactoglobulin, have been detected in other species [26].

Whilst polymerisation and glycosylation might explain the observed wide distribution of β -lactoglobulin on the gels, it does little to explain the observed antimicrobial activity. β -Lactoglobulin has been investigated, and found wanting, as

a modulator of intestinal development and activity in piglets [27], although some early studies have suggested that its secretion in animals that secrete colostrum (equivalent to the pre-oestrus mammary gland secretion in this study) is related to its role in the establishment of early immunity [28]. Pellegrini et al. [29] have, however, demonstrated that proteolytic digestion of bovine β -lactoglobulin produces four

peptides with anti-bacterial activity. In their study, these peptides had greatest activity against Gram-positive organisms, but slight modification of the amino acid sequence resulted in peptides with activity against Gram-negative organisms. It is possible that β -lactoglobulin released into the maternal pouch from the mammary gland, during pre-oestrus or from the gut of the neonate, post-oestrus, is cleaved by pouch or gut enzymes to produce bioactive peptides that were not detected in our study. β -Lactoglobulin has been shown to pass intact through the stomach of piglets [30] and the intestine of rabbits [31], and our experimental observations would suggest that this also occurs in marsupials.

5. Conclusion

We have demonstrated antimicrobial activity in pouch washes collected from mature female tammar wallabies in the period leading up to and at the time of birth of the pouch young. Proteomic analysis of these washes revealed the presence of a known antimicrobial, dermcidin, but suggests that β -lactoglobulin is a significant component of the pouch environment at crucial early stages of development of the young animal, although we have no evidence that the compound is responsible for antimicrobial activity. This β -lactoglobulin may have been sourced directly from the mammary gland or via the digestive tract of the young animal, but has undergone significant post-secretion modification. Other compounds present in concentrations below the limits of detection may be responsible for this antimicrobial activity. This project provides further insights into possible immune protective strategies operating in the marsupial pouch and suggests future research to focus on antimicrobial activity of peptides derived from β -lactoglobulin.

References

- [1] Gordon G, Hulbert AJ. Fauna of Australia, Mammalia. Canberra: Australian Government Publishing Service; 1989.
- [2] Tyndale-Biscoe CH, Renfree M. Reproductive physiology of marsupials. London: Cambridge University Press; 1987.
- [3] Lyne AG, Pilton PE, Sharman GB. Oestrus cycle, gestation period and parturition in the marsupial *Trichosurus vulpecula*. Nature (London) 1959;183:622–3.
- [4] Gemmell RT, Rose R. Organ development in some new born marsupial with particular reference to the rat-kangaroos. In: Grigg G, Jarman P, Hume I, editors. Kangaroos, wallabies and rat-kangaroos. Surrey Beatty and Sons; 1989. p. 349–54.
- [5] Yadav M, Stanley NF, Waring H. The microbial flora of the gut of the pouch-young and the pouch of a marsupial, *Setonix brachyurus*. J Gen Microbiol 1972;70:437–42.
- [6] Osawa R, Blanshard WH, O'Callaghan BG. Microflora of the pouch of the koala (*Phascolarctos cinereus*). J Wildlife Dis 1992;28:276–80.
- [7] Old JM, Deane EM. The effect of oestrus and the presence of pouch young on aerobic bacteria isolated from the pouch of the tammar wallaby, *Macropus eugenii*. Comp Immunol Microbiol Infect Dis 1998;21:237–45.
- [8] Deane EM, Cooper DW, Renfree MB. Immunoglobulin G levels in foetal and newborn tammar wallabies. Rep Fertl Dev 1990;2: 369–75.
- [9] Yadav M. The transmission of antibodies across the gut of the pouch young marsupials. Immunology 1971;21: 839–51.
- [10] Charllick J, Manassis C, Stanley N, Waring H, Cockson A. Quantitative alterations of the aerobic bacterial flora of the pouch of *Setonix brachyurus* (quokka), during oestrus, anoestrus, pregnancy and lactating anoestrus (pouch young). Aust J Exp Biol Med Sci 1981;59:743–51.
- [11] Bobek G, Deane EM. Possible antimicrobial compounds from the pouch of the koala, *Phascolarctos cinereus*. Lett Pept Sci 2002;8:133–7.
- [12] Baudinette RV, Boontheung P, Musgrave IF, Wabnitz PA, Maselli VM, Skinner J, et al. Eugenin: an immunomodulator used to protect young in the pouch of the tammar wallaby, *Macropus eugenii*. FEBS J 2005;272:433–42.
- [13] Old JM, Irving M, Deane EM. Histology of the pouch epithelium and the mammary glands during chemically induced oestrus in the brushtail possum (*Trichosurus vulpecula*). J Anat 2005;207: 97–102.
- [14] Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, et al. *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, β -defensins and CAP-18, expressed by human keratinocytes. Infect Immunol 2003;71: 3730–9.
- [15] Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987; 166:368–79.
- [16] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 1996;68:850–8.
- [17] Gatlin CL, Kleemann GR, Hays LG, Link AJ, Yates JR. Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. Anal Biochem 1998;263:93–101.
- [18] Yates JR, Eng JK, McCormack AL, Schieltz D. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. Anal Chem 1995;67: 1426–36.
- [19] Valenti P, Antonini G, Fanelli MRR, Orsi N, Antonini E. Antibacterial activity of matrix-bound ovotransferrin. Antimicrob Agents Chemother 1982;21:840–1.
- [20] Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence database using mass spectrometry data. Electrophoresis 1999;20: 3551–67.
- [21] Schitteck B, Hipfel R, Sauer B, Bauer J, Kalbacher H, Stevanovic S, et al. Dermcidin. A novel human antibiotic peptide secreted by sweat glands. Nat Immunol 2001;2: 1133–7.
- [22] Godovac-Zimmermann J, Shaw D. Beta-lactoglobulin identified in marsupial milk. The primary structure, binding site and possible function of beta lactoglobulin from eastern-grey kangaroo (*Macropus giganteus*). Biol Chem Hoppe Seyler 1987; 368:879–86.
- [23] Flower DR. The lipocalin family: structure and function. Biochem J 1996;318:1–14.
- [24] Plette CP, Hunter AK, Marshall CJ, Grigor MR. Phylogenetic analysis of three lipocalin-like proteins present in the milk of *Trichosurus vulpecula* (Phalangeridae, Marsupialia). J Mol Evol 1998;46:361–9.
- [25] Iametti S, Rasmussen P, Frokiaer H, Ferranti P, Addeo A, Bonomi F. Proteolysis of bovine β -lactoglobulin during thermal treatment in sub denaturing conditions highlights some structural features of the temperature-modified protein and yields fragments with low immunoreactivity. Eur J Biochem 2002;269:1362–72.

- [26] Kalidas C, Joshi L, Batt C. Characterisation of glycosylated variants of β -lactoglobulin expressed in *Pichia pastoris*. *Protein Eng* 2001;14:201–7.
- [27] Sutton Jr. LF, Alston-Mills B. β -Lactoglobulin as a potential modulator of intestinal activity and morphology in neonatal piglets. *Anat Rec A* 2006;288A:601–8.
- [28] Jenness R. Biosynthesis and composition of milk. *J Invest Dermatol* 1974;63:109–18.
- [29] Pellegrini A, Dettling C, Thomas U, Hunziker P. Isolation and characterisation of four bactericidal domains in the bovine β -lactoglobulin. *Biochim Biophys Acta* 2001;1526:131–40.
- [30] Lovegrove JA, Osman DL, Morgan JB, Hampton SM. Transfer of cow's milk beta-lactoglobulin to human serum after a milk load: a pilot study. *Gut* 1993;34:203–7.
- [31] Caillard I, Tome D. Modulation of beta-lactoglobulin transport in rabbit ileum. *Am J Physiol* 1994;266:G1053–9.

Chapter 5

A comparative proteomic analysis of skin secretions of the tammar wallaby (*Macropus eugenii*) and the wombat (*Vombatus ursinus*).

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A comparative proteomic analysis of skin secretions of the tammar wallaby (*Macropus eugenii*) and the wombat (*Vombatus ursinus*)

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Abstract

The secretome of the pouch skin of the model marsupial the tammar wallaby, *Macropus eugenii* has been investigated using techniques of two-dimensional gel electrophoresis, in-gel trypsin digestion followed by nanoliquid chromatography coupled tandem mass spectrometry (LC-MS/MS). Differences in the patterns of secreted proteins were observed in the female pouch at three stages of maturity — reproductively immature; reproductively mature and active and mature, postreproductively active. Skin from the underarm area of mature females had a markedly different secreted protein profile. The greatest diversity of proteins was seen in the mature reproductive pouch and from an opportunistic sample collected from the pouch another mature female marsupial, the common wombat, *Vombatus ursinus*. A total of 20 proteins were confidently identified from the pouch skin secretions of the tammar wallaby and wombats, whilst 20 proteins were tentatively identified. In all skin secretomes, globins were the most abundant proteins whilst the antimicrobial, dermcidin was detected in the wombat sample. Some proteins such as keratin and actin could be sourced to sloughed and degraded skin cells. A number of proteins were present at such low concentrations that confident identification was not possible. This was compounded by the lack of a comprehensive database of marsupial proteins which constrains the reliability of automated identification protocols.

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Keywords: Skin; Epithelium; Secretome; Marsupials; Electrospray ionisation-tandem mass spectrometry; Two-dimensional gel electrophoresis

1. Introduction

In all mammals the epithelial tissues, particularly skin, provide both a physical and chemical barrier to invasion by potential pathogens and against the stressors of the external environment (Huang et al., 2005). In marsupial mammals the female has a unique area of skin, the *marsupium* or pouch which forms the site of major development and maturation of the young (Tyndale-Biscoe, 1973) and as such this area of skin plays an additional role in protecting the neonate. Marsupials, in contrast to eutherian mammals, are born after a short gestation period with no functional lymphoid tissue and are known to rely on a number of maternal strategies for immunological protection whilst they develop in the maternal pouch (Old and

Deane, 2000). One strategy that has long been hypothesised but has yet to be proven definitively has been the possible secretion of antimicrobial compounds by the skin of the maternal pouch (Yadav et al., 1972; Bobek and Deane, 2002; Baudinette et al., 2005; Ambatipudi et al., in press).

The structure of the female marsupial pouch varies between different groups of marsupials. In macropods such as the tammar wallaby, *Macropus eugenii* (Order Diprotodontia; Family Macropodidae) and the common wombat, *Vombatus ursinus*, (Order Diprotodontia; Family Vombatidae) the pouch of a mature parous female is deep and moist whilst in others like the small carnivorous dasyurid, *Sminthopsis crassicaudata*, (Order Diprotodontia; Family Dasyuridae) the pouch is little more than folds of skin around a smooth area containing the mammary gland teats. However, despite these structural differences, in all marsupials the pouch plays a pivotal role in provision of a site for nurturing and protection of the young.

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In macropods like the tammar wallaby there are visible morphological changes to the pouch during the reproductive life time of the female. The skin of the pouch of an immature animal is clean and dry and the pouch is shallow. As the animal becomes reproductively mature the pouch deepens and as the animal enters the breeding season the pouch skin becomes clean, moist and translucent. In contrast, in the non-breeding season the pouch is still deep but the skin is dry and covered with a dry brown secretion (Tyndale-Biscoe, 1973; Ambatipudi et al., in press). These macroscopic changes in the pouch as the animal enters the reproductive stage of oestrus have been attributed to maternal licking and possible secretions from the pouch skin itself. However histological studies of the pouch skin of the possum as it entered an artificially induced oestrus failed to demonstrate any changes in the epithelial tissue beds reflective of increased secretory activity (Old et al., 2005).

In this study, we have utilised a proteomics approach to investigate possible changes in secretions onto the surface of the pouch skin at different stages in reproductive maturity of the tammar wallaby. We have also used an opportunistically collected sample from a reproductively mature common wombat for its potential to provide a comparison across two different groups of marsupials albeit with similar pouch structures. The majority of published proteomic studies of skin have used either extracted whole cells, usually fibroblasts, or skin biopsies (Huang et al., 2005) and these studies themselves have largely been restricted to humans and mice. In contrast in this study we have collected secretions from the skin surface, both from females at different stages of their reproductive life and from a control area of non-pouch skin. Samples collected from all animals were analysed using 2D PAGE (two-dimensional polyacrylamide gel electrophoresis), in-gel trypsin digestion followed by nanoliquid chromatography coupled tandem mass spectrometry (LC-MS/MS). To identify the protein components of these secretions we used database searches with cut-off scores set to eliminate low quality matches, although identification was hampered by a lack of significant translated genomic database for marsupials. This is the first study of the secretome of the skin of a marsupial.

2. Materials and methods

2.1. Animals

The female tammar wallabies (*M. eugenii*) used in the study were maintained in a captive breeding colony at Macquarie University, Australia. Samples were collected during the breeding season from February to mid July over a two year period. The protocols used in this study were approved by the Animal Ethics Committee of Macquarie University (Approval No. 2004/007 and Approval No. 2003/026). Pouch secretions were collected from a total of 3 immature females less than 2 years of age; 8 mature reproductively active animals, with and without pouch young, and 7 mature postreproductive active animals. Secretions were also collected from under the forelimbs of 15 mature females to act as a control. In addition, secretions were collected opportunistically from the pouch of

one mature female wombat (*V. ursinus*) with a pouch young held in Taronga Zoological Park, Sydney, Australia.

2.2. Sample collection and preparation

Secretions were collected by a gentle lavage of the pouch and skin area with 1 ml of sterile water using a sterile plastic pipette. Sterile gloves were worn to protect against human contamination of the site. The resultant wash was placed in a sterile collection tube and transported to the laboratory and frozen at -80°C until use. As protein concentration in the individual samples was presumed to be low, prior to analysis all samples were lyophilized, resuspended and pooled, according to maturity and reproductive stage, in 100 μL of sterile phosphate buffered saline (PBS) to provide concentrated samples for proteomic analysis.

2.3. Protein extraction

Proteins in the pooled reconstituted samples were precipitated using 90% TCA. The resultant pellet was then resuspended in 200 μL of 2-D sample solubilising solution. This solution consisted of 7 M urea, 2 M thiourea, 65 mM DTT, 2% CHAPS, 1% carrier ampholytes, 40 mM Tris and 0.002% bromophenol. An aliquot of the precipitate resuspended in PBS was used to determine the protein concentration using FluoroProfile (Sigma Diagnostics, St. Louis, MO, USA) according to the manufacturers' instructions.

2.4. Two-dimensional gel electrophoresis (2-DE)

Approximately forty, ninety and forty five micrograms of protein was loaded onto IPG strips for 2-DE from immature, reproductively mature and active and postreproductive mature animals respectively. Two-DE was performed over two pI ranges, using 11 cm pH 4–7 and 7–10 linear IPG strips (Bio-Rad, Hercules, CA, USA) in the first dimension. IEF was performed using the Multiphore II system (Amersham Pharmacia Biotech, Uppsala, Sweden) for 100 kVh at 20°C . The IPG strips were then equilibrated for 20 min at room temperature by gentle rocking in 6 M urea, 2% SDS 20% glycerol, 1X Tris–HCl (pH 8.8), 5 mM tributylphosphine, 2.5% acrylamide and then embedded in 0.5% agarose on top of precast 4–20% gradient Tris–HCl Criterion gels (Bio-Rad). Electrophoresis was carried out using a cathode running buffer consisting of 192 mM glycine, 15 mM Tris, 0.1% SDS at pH 8.3. Gels were initially run at 5 mA/gel for 30 min, then at 200 V, until the bromophenol blue buffer front had completely run off the bottom of the gel. The gels were fixed in 10% methanol and 7% acetic acid followed by staining overnight with Sypro Ruby (Molecular Probes, Eugene, OR, USA). Gels were destained in 10% methanol and 7% acetic acid for 1 h. Prior to excision of protein and to enhance visualisation, gels were further stained with 0.1% w/v colloidal Coomassie Brilliant Blue G-250 overnight and destained with 5% acetic acid for 1 h.

For control non-pouch skin samples 2DE was performed over two pI ranges, using 11 cm pH 4–7 and 7–10 linear IPG

strips (Bio-Rad) in the first dimension. IEF was performed using the Multiphore II system (Amersham Pharmacia Biotech, Uppsala, Sweden) for 100 kVh at 20 °C. Subsequently, proteins were separated using a precast 4–20% gradient Tris–HCl Criterion gels (Bio-Rad) for the 11 cm strips. Gels were double stained with Sypro Ruby (Molecular Probes) and then Coomassie Blue G250 (Bio-Rad).

2.5. Trypsin digestion and mass spectrometry of pouch and skin washes

Proteins were excised from 2D gels for identification by mass spectrometry. The gel plug was extracted three times in 50% v/v acetonitrile and 25 mM NH₄HCO₃, pH 7.8 at 37 °C for 10 min, dried at room temperature in a Speedy-Vac (Savant, Farmingdale, NY, USA), covered with 8 µL of sequencing grade trypsin (15 ng/µL) and digested overnight at 37 °C. Products were recovered from the gel by sequential extractions with 10% acetonitrile and 1% v/v formic acid. The combined extract was dried and peptides dissolved in 20 µL of 0.1% formic acid (Shevchenko et al., 1997). Five µL of digests were concentrated and desalted using a micro C18 precolumn (500 µm×2 mm, Michrom Bioresources, Auburn, CA, USA) with H₂O:CH₃CN (98:2, 0.1% formic acid) at 20 µL/min. After a 4 min wash the precolumn was automatically switched (Valco 10 port valve, Houston, TX, USA) into line with a fritless nanocolumn (Gatlin et al., 1998). Digested peptides were separated by nano-LC using a Cap-LC autosampler system (Waters, Milford MA, USA). Peptides were eluted using a linear gradient of H₂O:CH₃CN (98:2, 0.1% formic acid) to H₂O:CH₃CN (50:50, 0.1% formic acid) at ~200 nL/min over 30 min. The precolumn was connected via a fused silica capillary (10 cm, 25 micron) to a low volume tee (Upchurch Scientific, Oak Ridge, WA, USA) where 2600 V was applied and the column tip positioned ~1 cm from the Z-spray inlet of a QTOF Ultima hybrid tandem mass spectrometer (Micromass, Manchester, UK). Positive ions were generated by electrospray and the QTOF operated in data-dependent acquisition mode. A TOF MS survey scan was acquired (m/z 350–1700, 1 s) and the 2 largest multiple charged ions (counts >25) were sequentially selected by Q1 for MS-MS analysis. Argon was used as collision gas and an optimum collision energy chosen, based on charge state and mass. Tandem mass spectra were accumulated for up to 6 s (m/z 50–2000).

2.6. Protein identification and database searching

Protein identification was carried out by matching the peptide peaks with known proteins present within public access databases (Yates et al., 1995). Peptide peak lists were generated by MassLynx (version 4 SP1, Micromass) using the Mass Measure program to automatically baseline subtract, smooth and centroid the data before submission to the database search program Mascot (version 2.1, Matrix Science, London, UK). To overcome the lack of protein database of the tammar wallaby and wombat, a search across species barrier was undertaken by correlation of mass spectra to entries in the NCBI_{nr} database

(June 2006). Additionally, to increase our chances of further identification, a combination of techniques were used, such as electrospray ionisation mass spectrometry to generate multiply charged peptide ion which readily fragment, predominantly into y- and b-type ions. Chromatographic nano-LC column was used to remove hydrophilic contaminants, such as salts, improving data-dependent acquisition and Mascot search engine was used to identify the proteins based on probability.

Mascot MS/MS ion search criteria was as follows: taxonomy — Mammalia, trypsin digestion allowing up to one miscleavage, semi-trypsin digestion for peptides formed as cleaved products, variable modification — oxidation of methionine, cysteine as carboxyamidomethylation or propionamide, peptide tolerance of 0.25 Da, and MS/MS tolerance of 0.2 Da. The “Ion score cut-off” was manually set to 20 thereby eliminating the lowest quality matches. A probability based Mowse score >42 indicated identity (*p*<0.05).

3. Results

3.1. Two-dimensional gel electrophoresis (2-DE) of skin secretions of the tammar wallaby and protein identification

Representative 2DE gels of pouch skin secretions from female tammar wallabies at the three different stages of reproductive maturity are shown in Figs. 1, 2 and 3. Fig. 1a and b are 4–7 and 7–10 pI gels from immature animals respectively.

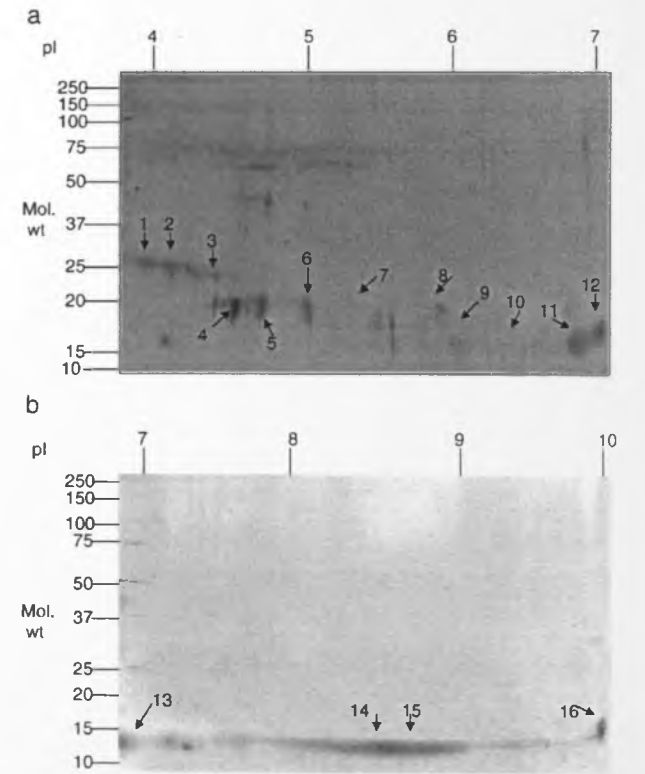


Fig. 1. 2DE PAGE of pouch secretions from immature female tammar wallabies (a) pI range 4–7 (b) pI range 7–10. Protein spots subjected to MS analysis are numbered and data presented in Table 1.

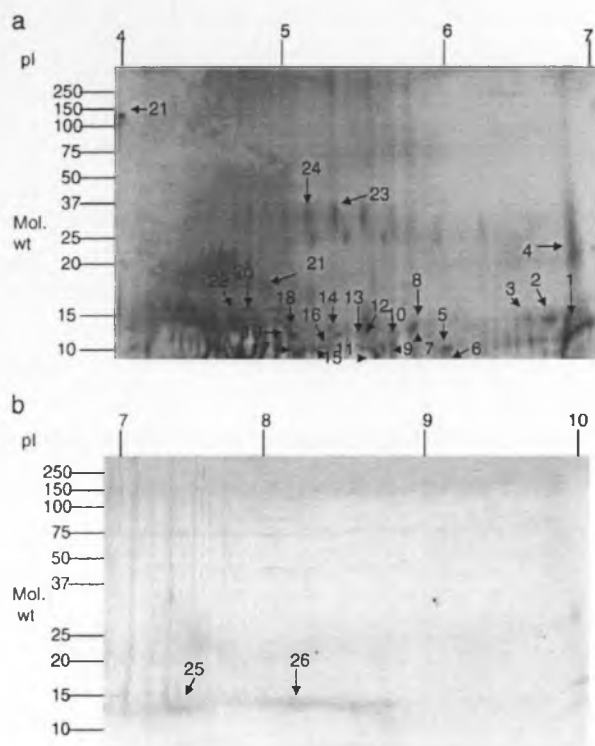


Fig. 2. 2DE PAGE of pouch secretions from reproductively active mature female tammar wallabies (a) pI range 4–7 (b) pI range 7–10. Protein spots subjected to MS analysis are numbered and data presented in Table 2.

Fig. 2a and b are 4–7 and 7–10 pI gels from mature reproductively active animals, whilst Fig. 3a and b are 4–7 and 7–10 pI gels from mature postreproductive animals. 2DE gels of control tammar skin secretions are shown in Fig. 4. Fig. 4a is 4–7 pI gel, whilst Fig. 4b covers pI 7–10.

Twelve protein spots were observed in the 4–7 pI 2DE of the pouch secretions collected from immature tammar wallabies (Fig. 1a) whilst four protein spots were observed in the 7–10 pI 2DE (Fig. 1b). In the 4–7 pI gel, the majority of the proteins (spots 4, 5, 6, 7, 8, 9, 10, 11, 12) were observed in the 10–20 kDa molecular mass range, whilst fewer proteins (spots 1, 2 and 3) were observed at approximately 25 kDa. The 4 proteins in the 7–10 pI gel (spots 13, 14, 15 and 16) were spread across the gel at approximately at 10–15 kDa molecular mass and poorly resolved. Of the 16 spots excised from gels from immature females only 2 proteins could be tentatively identified as mutant type hexokinase (Spot 4) and histone (Spot 5) (Table 1). The remaining proteins could not be identified, despite attempts to determine *de novo* sequence data. In some instances this was due to the low protein concentration and resultant poor spectra whilst for others the lack of sequence commonality with other species failed to provide a confident identification match for these proteins.

In contrast, 2DE of pouch secretions collected from reproductively active mature animals resulted in the separation of approximately 24 proteins in the 4–7 pI gel (Fig. 2a), with only two protein spots observed in the 7–10 pI gel at

approximately 14 kDa molecular mass (Fig. 2b). The latter gel, similar to that observed for immature animals, was also poorly resolved. In the 4–7 pI gel (Fig. 2a) the majority of proteins, except for spots 21, 23 and 24, were observed in the 10–20 kDa molecular mass range. A number of these proteins (spots 5, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19) migrated ahead of the bromophenol dye front, making it difficult to resolve other proteins on the gel without sustaining loss of these proteins. Some proteins (spots 23, 24) were poorly resolved, whilst others (spots 20, 21 and 22) were embedded within the dye front making it difficult to for visualisation for excision. Of the twenty six proteins excised from these gels (Fig. 2a and b) for analysis only fourteen proteins could be identified with high confidence (Table 2). Of the remaining proteins, 5 could be identified tentatively (Table 2), whilst 7 had poor fragmentation spectra preventing reliable identification. The majority of high confidence identifications were either proteins of the globin super family — α -globin (spots 1, 2, 4 and 5) and β -globin (spot 1) or proteins of the lipocalin family — α -lactalbumin (spots 15, 17 and 19) and β -lactoglobulin (spots 3, 7, 8, 14, 18, 21, 23) (Table 2).

In postreproductive animals, 7 protein spots were observed in the 4–7 pI gel (Fig. 3a), whilst once again the 7–10 pI gel failed to resolve satisfactorily (Fig. 3b) although two possible protein spots are evident (spots 8 and 9). In contrast to proteins

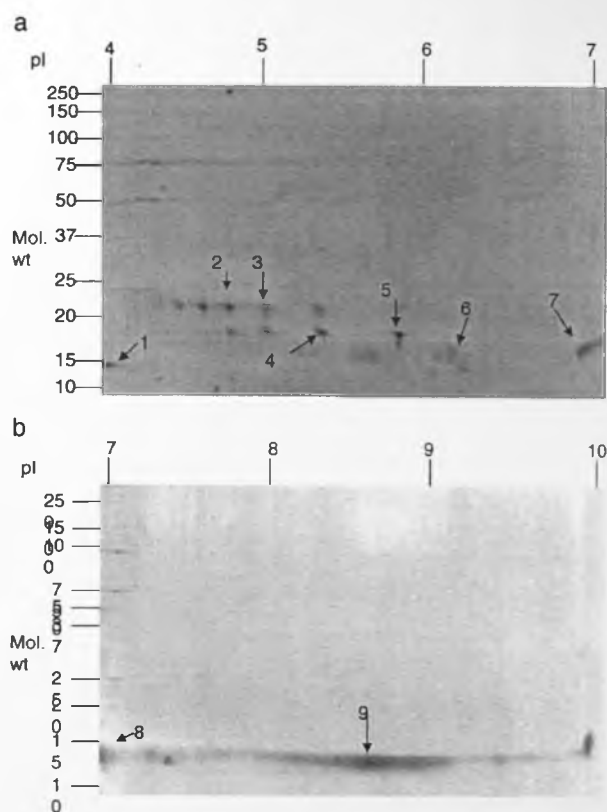


Fig. 3. 2DE PAGE of pouch secretions from postreproductive mature female tammar wallabies (a) pI range 4–7 (b) pI range 7–10. Protein spots subjected to MS analysis are numbered and data presented in Tables 3a and 3b.

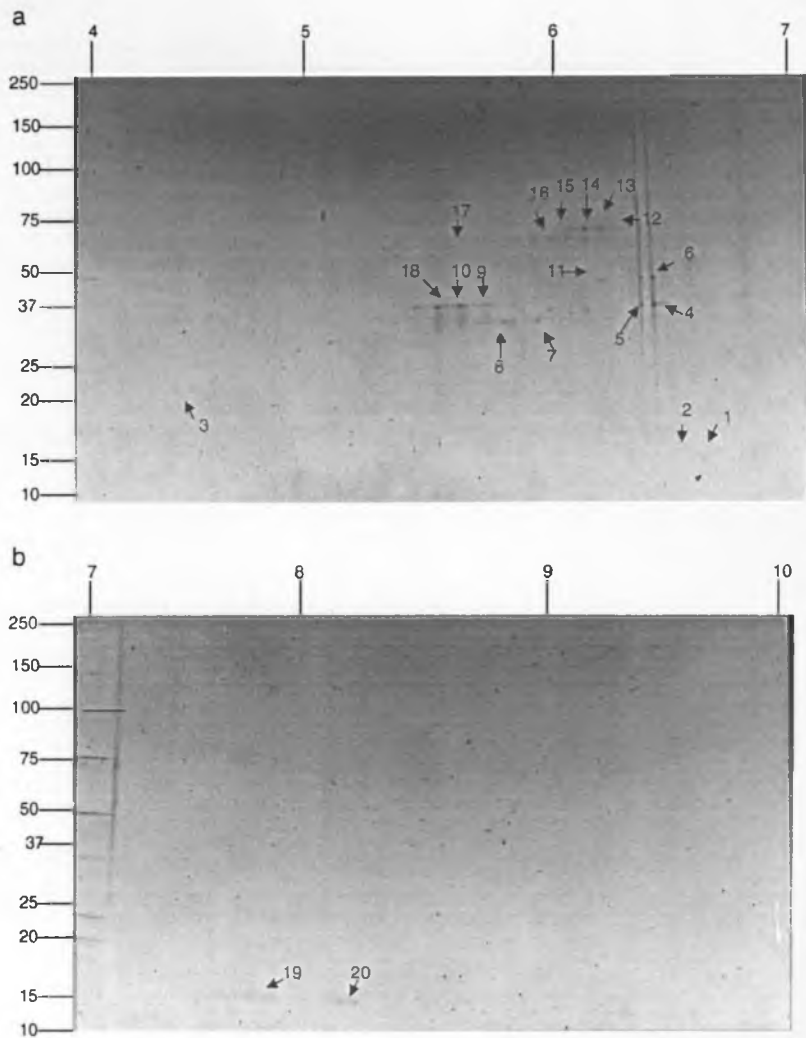


Fig. 4. 2DE PAGE of secretions from non-pouch skin of a mature tammar wallaby (a) pI range 4–7 (b) pI range 7–10. Protein spots subjected to MS analysis are numbered and data presented in Table 4.

from inactive and mature animals, the proteins in the 4–7pI gels in mature, postreproductive animals were well resolved with no dye front obstructing the visualisation of the proteins, particularly at the bottom of the gel. In the 4–7 pI gel (Fig. 3a) the majority of the proteins (spots 1, 2, 3, 4, 5, 6 and 7) were observed in the 15–25 kDa molecular mass range and at 10–15 kDa in the 7–10 pI range gel (Fig. 3b). Seven high intensity spots were excised from the 4–7 pI gel and three spots were identified with high confidence (Table 3a). Lysozyme was tentatively identified from spot 1 with 2 peptides and high Mowse scores (Table 3a). When a semi-trypsin digest match was carried out, lysozyme was confidently identified with high Mowse score and 5 peptides. This could be due to the possible cleavage product of the original protein or due to non-specific tryptic digestion. Multiple proteins were identified along with lysozyme in the same spot, such as alpha and beta-globin, along with tentative identification of dermcidin. In contrast, only one protein was confidently identified as α and β globin (spot 8) from the 7–10 pI gel (Table 3b).

Eighteen protein spots were observed in the 4–7 pI gel of non-pouch skin secretions (Fig. 4a), whilst only two protein spots were detected in the 7–10 pI gel (Fig. 4b). In the 4–7pI gel, nine proteins (spots 4, 5, 6, 7, 8, 9, 10 and 18) were observed in the molecular mass range 36 to 50 kDa whilst six proteins (spots 12, 13, 14, 15, 16 and 17) were observed between 70 and 90 kDa molecular mass. Of the three proteins

Table 1
Identification of proteins from pouch secretions of immature females using 4–7 pI 2DE gels

Spot no.	Name	Sequence	Mowse score and % coverage	No. of peptides	Accession no.
4	Hexokinase	R. ENRGLDNP. K	51, 1	1	gi 2689658
5	Histone	R. SSVCAFVMSGR. K	45, 1	2	gi 28174908

Table 2

Confident and tentative identification of proteins from the pouch secretions of mature reproductively active animals

Spot no.	Name	Theor pI	Expt. pI (app.)	Theor. mol. wt	Expt mol. wt (app.)	Mowse score and % coverage	No. of peptides	Accession no.
Confident identifications 4–7 pI								
1	α -globin	7.22	6.9	15,336	15,000	397, 61	11	gi 122429
	β -globin	7.09		16,132		140, 23	4	gi 122633
	α -globin	7.22		15,336		76, 17	3	gi 122272
	β -lactoglobulin	5.82		20,220		30, 1	1	gi 1146
	α -lactalbumin	4.73		15,722		57, 1	1	gi 126003
2	α -globin	7.22	6.8	15,336	16,000	184, 42	7	gi 122429
	β -globin	7.09		16,132		117, 15	2	gi 122633
	β -lactoglobulin	5.82		20,220		53, 9	2	gi 1146
3	β -lactoglobulin	5.82	6.7	20,220	16,000	97, 22	3	gi 1146
	α -globin	7.22		15,336		49, 14	1	gi 122429
	β -globin	7.09		16,132		30, 8	1	gi 122633
4	α -globin	7.22	6.9	15,336	25,000	147, 31	4	gi 122429
	β -globin	7.09		16,132		105, 15	2	gi 122633
	β -lactoglobulin	5.82		20,220		51, 9	1	gi 1146
	β -casein	6.48		35,881		38, 2	1	gi 115662
	fatty acid binding protein	7.74		34,301		30, 2	1	gi 16758094
5	α -globin	7.22	6	15,336	11,000	89, 25	3	gi 122429
	α -lactalbumin	4.73		15,722		49, 9	1	gi 126003
7	β -lactoglobulin	5.82	5.8	20,220	15,000	208, 30	8	gi 125916
	α -globin	7.22		15,336		55, 14	2	gi 122633
8	β -lactoglobulin	5.82	5.8	20,220	16,000	190, 30	8	gi 125916
14	β -lactoglobulin	5.82	5.4	20,220	15,000	165, 32	8	gi 125916
	α -s1-casein	5.73		28,122		89, 6	1	gi 115662
	Transthyretin	5.41		16,445		71, 13	2	gi 1174820
	α -lactalbumin	4.73		15,722		45, 1	1	gi 126003
15	α -lactalbumin	4.73	5.3	15,722	11,000	201, 27	4	gi 295498
17	α -lactalbumin	4.73	5.2	15,722	10,000	71, 9	3	gi 126003
	cysteine protease inhibitor	6.82		42,887		33, 1	1	gi 544087
	α -globin	7.22		15,336		29, 1	1	gi 122429
18	β -lactoglobulin	5.82	5.2	20,220	14,000	75, 6	4	gi 125916
	β -casein	6.48		35,881		33, 2	1	gi 115662
	Myomegalin	8.79		12,180		31, 2	1	gi 11693144
	α -lactalbumin	4.73	5.1	15,722	12,000	153, 52	4	gi 126003
	β -lactoglobulin	5.82		20,220		126, 27	3	gi 125916
21	β -lactoglobulin	5.82	4.8	20,220	18,000	124, 27	3	gi 125916
	α -lactalbumin	4.73		15,722		59, 4	1	gi 126003
	Thymopoietin	7.56		75,492		30, 1	1	gi 8856685
23	β -lactoglobulin	5.82	5.4	20,220	37,000	139, 32	5	gi 125916
	α -lactalbumin	4.73		15,722		40, 1	1	gi 126003
	Clusterin	4.68		48,125		35, 1	1	gi 27806907
Tentative identifications 4–7 pI								
Spot no.	Name	Sequence				Mowse score and % coverage	No. of peptides	Accession no.
9	β -lactoglobulin	K.FVGSWYLR.E				45, 12	2	gi 125916
11	Cystatin	K.TQVVAGTNYIYK.V				43, 20	2	gi 11311947
12	α -lactalbumin	R.KCQASQILK.E + Acrylamide (C)				64, 21	2	gi 126002
		K.CQASQILK.E + Acrylamide (C)						
	Cystatin	K. TQVVAGTNYIYK.V				49, 12	1	gi 1311047
16	α -s1-casein	K.YELDKYR.E.R				100, 9	2	gi 115649
		R.QVEYNFNEEDSSASR.E						
	α -lactalbumin	K.CQASQILK.E + Acrylamide (C)				73, 16	2	gi 126003
		K.ILQLPEGLGYWK.A						
	β -casein	K.VLPLLQHR.V				68, 5	2	gi 115662
		K.EILHNDER.E						
	α -globin	R.TFHSFPTTK.T				64, 14	2	gi 122429
		R. VLSAADKGHV.K						
24	β -lactoglobulin	R.TLAMPYTVIEVR.T + Oxidation (M), K.EVNLTPEGNLELVLEK.T				78, 22	2	gi 1146
	α -lactalbumin	K.ILQLPEGLGYWK.K				50, 9	1	gi 126003
	Clusterin	R.MKDQCEK.C + Acrylamide (C)				34, 1	1	gi 27806907

Table 3a
Confident and tentative identification of proteins from pouch secretions of postreproductive mature animals for 4–7 pI 2DE gels

Spot no.	Name	Theor pI	Expt. pI (approx.)	Theor. mol. wt	Expt mol. wt (approx)	Mowse score and % coverage	No. of peptides	Accession no.
Confident identifications 4–7 pI								
1	α-globin	7.36	4.2	15,210	15,000	232, 36	6	gi 122429
	Beta-globin	7.23		16,005		110, 15	2	gi 122633
	Lysozyme	9.13		16,700		70, 18	2	gi 47523838
	Alpha integrin	5.46		103,180		53, 1	2	gi 62460554
	Dermcidin	6.08		11,277		42, 1	1	gi 16751921
*1	Lysozyme	9.13	4.2	16,700	15,000	181, 5	5	gi 47523838
	α-globin	7.36		15,210		232, 36	6	gi 122429
	Beta-globin	7.23		16,005		110, 15	2	gi 122633
	Alpha integrin	5.46		103,180		53, 1	2	gi 62460554
	Dermcidin	6.08		11,277		42, 1	1	gi 16751921
2	Gamma non-muscle actin	5.3	5	41,729	250,000	152, 9	4	gi 1703
	Actin	5.3		39,161		111, 4	4	gi 49868
7	β-globin	7.23	7	15,906	18,000	71, 8	6	gi 229302

Tentative identifications 4–7 pI range 2DE gel

Spot no.	Name	Sequence	Mowse score and % coverage	No. of peptides	Accession no.
6	Nesprin	R.AMTGLIK.K	51, 10	1	gi 55627590

*Peptides identified by semi-trypsin digestion of proteins.

Table 3b
Confident and tentative identification of proteins from pouch secretions of postreproductive mature animals 7–10 pI 2DE gel

Spot no.	Name	Theor pI	Expt. pI (approx.)	Theor. mol. wt	Expt mol. wt (approx)	Mowse score and % coverage	No. of peptides	Accession no.
8	α-globin	7.36	7	15210	15000	140, 15	4	gi 122429
	β-globin	7.23		15210		110, 14	4	gi 1143317

Tentative identification

Spot no.	Name	Sequence	Mowse score and % coverage	No. of peptides	Accession no.
9	Dermcidin	K. ENAGEDPG. K	61, 10	1	gi 16751921

detected below 20 kDa, one protein (spot 3) was observed at the acidic end of the gel, whilst two proteins (spots 1 and 2) were observed at an approximate pI of 6.8. This contrasts strongly with protein patterns observed in gels of pouch skin secretions where most proteins were detected in the 4–7 pI gels and in the molecular mass range 10–37 kDa. The protein profile seen in the non-pouch skin secretions shows a train of proteins e.g. (spots 9, 10 and 18; Fig. 4a) indicative of possible protein isoforms. Of the 18 spots excised and analysed from the pI 4–7 gel (Fig. 4a), only three proteins (spots 6, 9 and 10) were confidently identified (Table 4), whilst neither of the 2 proteins excised from pI 7–10 could be confidently identified. Enolase was identified from spot 6, whereas actin was identified from spots 9 and 10. However, the remaining proteins could not be

identified with similar confidence and can be divided into two groups. Group one (spots 1, 7, 11 and 12) contained a number (~50) of good quality fragmentation spectra which were identified as keratin. The second set contained a limited number of spectra (~15) from which no protein identification was possible. Moreover, these protein spots were weakly stained, limiting the capacity for reliable identification.

3.2. Two-dimensional gel electrophoresis (2-DE) of the secretions of wombats and protein identification

Fig. 5a and b show 4–7pI 2DE and 7–10 pI 2DE gels respectively of secretions collected from a mature wombat. Similar to observations in the tammar wallaby samples, the

Table 4
Identification of proteins from the non-pouch skin secretions of the tammar wallaby, 4–7 pI 2DE gel

Spot no.	Name	Theor pI	Expt. pI (approx.)	Theor. mol. wt	Expt mol. wt (approx)	Mowse score and % coverage	No. of peptides	Accession no.
6	Enolase	6.16	6.3	47,086	48,000	82, 6	3	gi 6978809
9	Actin	5.22	5.7	47,086	37,000	151, 16	5	gi 28336
10	Actin	5.22	5.8	41,786	37,000	88, 12	3	gi 28336

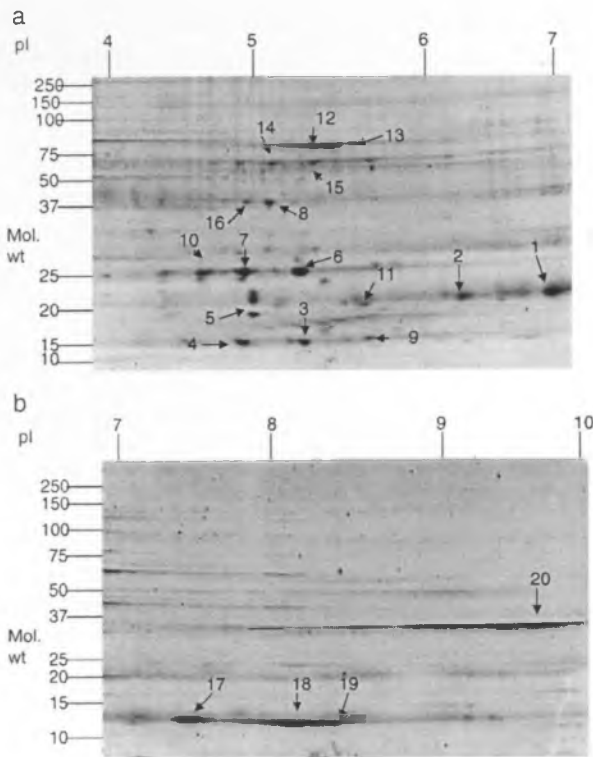


Fig. 5. 2DE PAGE of pouch secretions from a mature female wombat (a) pI range 4–7 (b) pI range 7–10. Protein spots subjected to MS analysis are numbered and data presented in Table 5.

majority of the proteins were detected in the molecular mass range of 10–37 kDa in the 4–7 pI gel, with three protein spots detected in the 7–10 pI range. Of these 10 proteins (spots 1, 2, 3,

4, 5, 6, 7, 9, 10 and 11) were observed in the 4–7 pI gel in the molecular mass range 15–25 kDa (Fig. 5a), with 6 proteins (spots 8, 12, 13, 14, 15 and 16) in the 37–75 kDa molecular mass range. Of the 20 protein spots detected in both gels (Fig. 5a and b) and excised for analysis, only two proteins could be identified with confidence (Table 5). These proteins (spots 7 and 9) from the 4–7 pI gel were identified as β -globin and dermcidin respectively (Table 5). A number of other proteins were also co-identified from spot 7, including hornerin and dermcidin. Although identified with high Mowse scores, of 133 and 101 respectively, these identifications cannot be deemed confident due to fewer peptides matches and, in the case of hornerin, the significant difference in theoretical versus experimental MW. Of the four proteins (spots 17, 18, 19 and 20) excised from the 7–10 pI gel only one (spot 19) was tentatively identified with one peptide match to prelamina with a Mowse score of 45.

4. Discussion

This study has identified a number of proteins secreted from the skin of female marsupials. Although the focus of the study has been the unique area of the marsupial female pouch, some proteins have been identified from another non-pouch skin area. Figs. 1, 2 and 3 clearly illustrates that the proteins secreted onto the surface of the pouch skin of female tammar wallabies changes over their reproductive life with the greatest diversity of proteins seen in reproductively active females (Fig. 2a and b). Furthermore comparison with proteins from non-pouch skin of reproductively active females (Fig. 4a and b) shows a clear difference, in the MW and pI of detected proteins although many of the proteins present could not be confidently identified

Table 5
Confident and tentative identification of proteins from pouch skin secretions of the wombat, 4–7 pI and 7–10 pI 2DE gels

Spot no.	Name	Theor pI	Expt. pI (approx.)	Theor. mol. wt	Expt.mol. wt (approx)	Mowse score and % coverage	No. of peptides	Accession no.
7	β -globin	7.23	4.9	16,132	25,000	136, 21	3	gi 122633
	Hornerin			282,366		133	2	gi 11341258
	Dermcidin			11,284		101	2	gi 16751921
9	Dermcidin	6.08	5.7	11,283.9	15,000	144	3	gi 16751921
Spot no.	Name	Mowse score and % coverage			No. of peptides		Sequence	Accession no.
Tentative identifications								
1	Prelamin	49, 1			1		R.NIQNVVLK.L	gi 74013283
3	Dermcidin	48, 10			1		K.ENAGEDPGLAR.Q	gi 16751921
	Profilin	37, 3			1		R.CPATVQSR.R	gi 73980614
5	Neuron navigator	62, 1			1		K.KEVSEL.R.S	gi 21654877
11	Phospholipase A2	47, 1			2		R.KTLTLTLR.T	gi 73969133
							K.TLTLTLR.T	
12	Profilin	37, 3			1		R.CPATVQSR.R	gi 73980614
13	Dermcidin	39, 10			1		K.ENAGEDPGLAR.Q	gi 16751921
14	Protease inhibitor	97, 5			2		K.LVLVNAIYFK.G	gi 13489087
	Dermcidin	50, 10			1		R.DIFISKIVHK.S	gi 16751921
	Poly Ig receptor	47, 2			1		K.ENAGEDPGLAR.Q	gi 514366
							R.ASVDSGSSEEQGGSSR.A	
16	GEF factor	31, 1			1		K.LADVLSPR.L	gi 72888183
Tentative identification from 7–10 pI 2DE gel								
*19	Prelamin	45, 1			1		R.NIQNVVLK.L	gi 74013283

*Peptides identified by semi-trypsin digestion of proteins.

or were there at too low a concentration to be reliably identified. Such differences between non-pouch and pouch skin and with reproductive status support a hypothesis that secretions from pouch skin are unique and influenced by the reproductive stage of the female and potentially play a role related to the well being of the young.

A variety of globins were detected in this study, at all stages in the tammar wallaby pouch and from the wombat pouch. Two of these, α - and β -globin, were common to the mature active and mature postreproductive pouch secretions. α - and β -globin are well documented oxygen transporters in adult mammals. Their genes are expressed in a wide range of tissues leading to speculation that globins may have functions apart from oxygen transportation (Wheeler et al., 2001; Bhaskaran et al., 2005), such functions may include antimicrobial activity under acidic conditions and low ionic strength (Hirsch, 1960).

The majority of proteins identified in the mature reproductively active pouch were confidently identified as β -lactoglobulin and α -lactalbumin (Table 2). These globular proteins have known biological activities including anticancer, antihypertensive, antimicrobial and opiodergic effects (Chatterton et al., 2006). These two proteins also account for 70–80% of the milk whey proteins (Chatterton et al., 2006) and, in an earlier investigation of antimicrobial activity in the maternal pouch, it was suggested that their abundance was most likely due to either mammary gland secretion or excretion from the digestive tract of a resident pouch young marsupial attached to the mammary teat (Ambatipudi et al., in press). In a previous study we reported the major proteins present in marsupial milk (Joss et al., 2007), this work clearly shows β -lactoglobulin is a major component of marsupial milk. However many other abundant proteins that were observed in milk were not observed in pouch wash samples. Future work should thus be focused on analysis of skin secretions from mature females, who are not actively reproducing, to clarify the possible source of this protein.

Hornerin, detected in this study in pouch secretions of wombats, has been reported to be expressed in developing and regenerating stratified epithelium of human and mouse skin as well as in a range of other tissues including tongue, oesophagus and forestomach. It is thought to play a role in the process of cornification (Makino et al., 2003; Takaishi et al., 2005). Dermcidin was detected with low confidence in the pouch secretion of postreproductive mature tammar wallabies and the wombat. This is a known antimicrobial expressed by human sweat glands (Schitteck et al., 2001). It is likely that this compound would play such a role in skin in marsupials.

Published proteomic studies of skin have largely used either cultured cells or skin biopsies coupled with extraction protocols that display the whole cell proteome. They have also largely focused on documenting the changing proteome under adverse conditions. Human keratinocytes and fibroblasts exposed *in vitro* to irritants such as UV, sodium lauryl sulphate and carbon nanoparticles (Huang et al., 2005; Fletcher and Basketter, 2006; Witzmann and Monteiro-Riviere, 2006) showed altered levels of a variety of proteins, including stress

proteins such as the heatshock proteins; cell signalling molecules such as calmodulin; serine proteases as well as vimentins, annexin, keratins and actins. *In vivo* studies using skin biopsies from affected humans and mice has also documented changed levels of membrane and adhesion proteins (Park et al., 2007), signal transduction proteins (Choi et al., 2005), vimentin, tropomyosin and galactin isoforms (Huang et al., 2005) and a range of enzymes involved in fundamental metabolic pathways (Vaudens et al., 2003; List et al., 2007). The sole report of proteins of marsupial skin is that of *in vitro* studies of UV induced tumours of the South American opossum, *Monodelphis domestica* (Verrills et al., 2000). This study, similar to those cited above, used total proteomic extraction protocols but it did highlight the difficulties, still relevant today, of identification of proteins from marsupial mammals due to the lack of a comprehensive database of sequenced proteins as is found for mice and humans. The proteins identified in the skin of the opossum consisted of tropomyosin and vimentin isoforms similar to those found in mouse and human malignant cells (Huang et al., 2005). They also confidently identified a number of components of the cytoskeleton including actin, keratin and myosin as well as cell signalling compounds such as calumenin and calmodulin – once again very similar to proteins documented in human and mouse skin (Verrills et al., 2000). In our study we focused on proteins released onto the skin surface – the skin secretome. It is probably not surprising that we did not detect some of these high MW high abundance proteins such as the keratins, although we did isolate actin. Modelling studies on the mouse secretome (Greenbaum et al., 2001; Grimmond et al., 2003) have suggested that the majority of secreted proteins would be of lower MW, between 50 and 99 amino acids in length (Grimmond et al., 2003) although this would vary between tissues. In this study a number of the proteins present on the skin surface have not been confidently identified but there is a clear difference in the skin “secretome” of the female pouch at different stages of reproductive life. Such changes could be linked to changes in hormonal differences a situation similar to that observed in humans at the onset of puberty, although previous studies have shown no overt changes in the epithelial secretory structures of the female pouch as the animal nears birth of the young (Old et al., 2005).

5. Conclusions

In this study we have illustrated the differences in proteins secreted onto the skin surface of the pouch of the tammar wallaby at different stages of reproductive maturity and activity. We have also compared the patterns of protein secretion with that observed in non-pouch skin from the same species and from the pouch of another marsupial, the wombat. Although a number of these proteins could not be confidently identified our observations support a hypothesis that changes in pouch skin secretions are related to reproductive activity and possibly play a role in protection of the young. These results also represent the first reports of the skin secretome of a marsupial mammal.

References

- Ambatipudi, K., Joss, J., Raftery, M., Deane, E., in press. A proteomic approach to analysis of antimicrobial activity in marsupial pouch secretions. *Dev. Comp. Immunol.*
- Baudinette, R.V., Bootheung, P., Musgrave, I.G., Wabnitz, P.A., Maselli, V.M., Skinner, J., Alewood, P.F., Brinkworth, C.S., Bowie, J., 2005. An immunomodulator used to protect young in the pouch of the tammar wallaby, *Macropus eugenii*. *FEBS J.* 272, 433–443.
- Bhaskaran, M., Chen, H., Chen, Z., Liu, L., 2005. Hemoglobin is expressed in alveolar epithelial type II cells. *Biochem. Biophys. Res. Commun.* 333, 1348–1352.
- Bobek, G., Deane, E.M., 2002. Possible antimicrobial compounds from the pouch of the koala, *Phascolarctos cinereus*. *Lett. Pept. Sci.* 8, 133–137.
- Chatterton, D.E.W., Smithers, G., Roupas, P., Brodkorb, A., 2006. Bioactivity of β -lactoglobulin and α -lactalbumin-technological implications for processing. *Int. Dairy J.* 16, 1229–1240.
- Choi, K.-C., Lee, S., Kwak, S.Y., Kim, M.-S., Choi, H.K., Kim, K.H., Chung, J.H., Park, S.H., 2005. Increased expression of 14-3-3 ϵ protein in intrinsically aged and photo aged human skin in vivo. *Mech. Ageing Dev.* 126, 629–636.
- Fletcher, S.T., Basketter, D.A., 2006. Proteomic analysis of the response of EpiDerm TM cultures to sodium lauryl sulphate. *Toxicol. In Vitro* 20, 975–985.
- Gatlin, C.L., Kleemann, G.R., Hays, L.G., Link, A.J., Yates, J.R., 1998. Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. *Anal. Biochem.* 263, 93–101.
- Greenbaum, D., Luscombe, N.M., Jansen, R., Qian, J., Gerstein, M., 2001. Interrelating different types of genomic data, from proteome to secretome: 'oming in on function. *Genome Res.* 11, 1463–1468.
- Grimmond, S.M., Miranda, K.C., Yuan, Z., Davis, M.J., Hume, D.A., Yagi, K., Tominaga, N., Bono, H., Hayashizaki, Y., Okazaki, Y., Teasdale, R.D., 2003. The mouse secretome: functional classification of the proteins secreted into the extracellular environment. *Genome Res.* 13, 1350–1359.
- Hirsch, J.G., 1960. Antimicrobial factors in tissues and phagocytic cells. *Microbiol. Mol. Biol. Rev.* 24, 133–140.
- Huang, C.-M., Elmets, C.A., Kampen, K.R., DeSilva, T.S., Barnes, S., Kim, H., Tang, D.-C.C., 2005. Prospective highlights of functional skin proteomics. *Mass Spectrom. Rev.* 24, 647–660.
- Joss, J., Molloy, M., Hinds, L., Deane, E.M., 2007. Proteomic analysis of early lactation milk of the tammar wallaby (*Macropus eugenii*). *Comp. Biochem. Physiol. D* 2, 150–164.
- List, E.O., Berryman, D.E., Palmer, A.J., Qiu, L., Sankaran, S., Kohn, D.T., Kelder, B., Okada, S., Kopchick, J.J., 2007. Analysis of mouse skin reveals proteins that are altered in a diet-induced diabetic state: a new method for detection of type 2 diabetes. *Proteomics* 7, 1140–1149.
- Makino, T., Takaishi, M., Toyoda, M., Morohashi, M., Huh, N.-h., 2003. Expression of homerin in stratified squamous epithelium in the mouse: a comparative analysis with profilaggrin. *J. Histochem. Cytochem.* 51, 485–492.
- Old, J.M., Deane, E.M., 2000. Development of the immune system and immunological protection in marsupial pouch young. *Dev. Comp. Immunol.* 24, 445–454.
- Old, J., Irving, M., Deane, E., 2005. Histology of the pouch epithelium and the mammary gland during chemically induced oestrus in the brushtail possum (*Trichosurus vulpecula*). *J. Anat.* 207, 97–102.
- Park, Y.-D., Lyou, Y.-J., Yang, J.-M., 2007. Two-dimensional electrophoresis analyses of atopic dermatitis and the chances to detect new candidate proteins by the variation in immobilized pH gradient strips. *J. Dermatol. Sci.* 47, 9–17.
- Schitteck, B., Hipfel, R., Sauer, B., Bauer, J., Kalbacher, H., Stevanoic, S., Schirle, M., Schroeder, K., Blin, N., Meier, F., 2001. Dermicidin: a novel human antibiotic peptide secreted by sweat glands. *Nat. Immunol.* 2, 1133–1137.
- Shevchenko, A., Chernushevich, I., Werner, E., Standing, K.G., Thomson, B., Wilm, M., Mann, M., 1997. Rapid 'de novo' peptide sequencing by a combination of nanoelectrospray, isotopic labelling and a quadrupole/time-of-flight mass spectrometer. *Rapid Commun. Mass Spectrom.* 11, 1015–1024.
- Takaishi, M., Makino, T., Morohashi, M., Huh, N., 2005. Identification of human homerin and its expression in regenerating and psoriatic skin. *J. Biol. Chem.* 280, 2696–4703.
- Tyndale-Biscoe, H., 1973. The developing marsupial. Edward Arnold Publishers Ltd, London, pp. 1–16.
- Vaudens, F., Crettaz, D., Scelatta, C., Servis, C., Quadroni, M., Bienvenut, W.-V., Schneider, P., Hohlfield, P., Applegate, A.L., Tissot, J.-D., 2003. Plasticity of protein expression during culture of fetal skin cells. *Electrophoresis* 24, 1281–1291.
- Verrills, N.M., Harry, J.H., Walsh, B.J., Hains, P.G., Robinson, E.S., 2000. Cross-matching marsupial proteins with eutherian mammal databases: proteome analysis of cells from UV-induced skin tumours of an opossum (*Monodelphis domestica*). *Electrophoresis* 21, 3810–3822.
- Wheeler, D., Hope, R., Cooper, S.J.B., Dolman, G., Webb, G.C., Bottema, C.D.K., Gooley, A.A., Goodman, M., Holland, R.A.B., 2001. An orphaned mammalian β -globin gene of ancient evolutionary origin. *PNAS* 98, 1101–1106.
- Witzmann, F.A., Monteiro-Riviere, N.A., 2006. Multi-walled carbon nanotubes exposure alters protein expression in human keratinocytes. *Nonomed. Nanotech. Biol. Med.* 2, 158–168.
- Yadav, M., Stanley, N.F., Waring, H., 1972. The microbial flora of the gut of the pouch-young and the pouch of a marsupial, *Setonix brachyurus*. *J. Gen. Microbiol.* 70, 437–442.
- Yates, J., Eng, J., McConnack, A., Schieltz, D., 1995. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal. Chem.* 67, 1426–1436.

Chapter 6

Conclusions

The primary objective of this study has been to extend our knowledge of the immune protective strategies used in marsupials, with a particular emphasis on how this may be enacted in protection of the pouch young. The vast body of literature available on eutherian mammals suggests that the skin and antimicrobial proteins/peptides produced by phagocytes are an important component in the host defence mechanism. Nevertheless, this aspect of host immune response remains a poorly understood area, primarily due to the complex multi-step process of host-pathogen interaction. This project aimed to extend our knowledge of these processes in marsupials.

The first step in this project was directed at the isolation and identification of antimicrobial proteins/peptides (AMP's) present within the neutrophil granules of the tammar wallaby (*Macropus eugenii*). This study used adult neutrophils, in the first instance, because of their greater availability. Neutrophils themselves were chosen because, in eutherian immune systems, these cells contain a great diversity of AMPs and given that no sequence information was available about AMPs at the commencement of this study using neutrophils offered the logical pathway to obtain the sequence of such molecules. The second phase of this study sought to identify any possible antimicrobial compounds secreted by the maternal pouch at the time of birth of the altricial pouch young. The first step was to determine if indeed there was such antimicrobial activity as reports in the literature were variable. Having confirmed activity against gram positive

micro-organisms the tools of proteomics could then be applied to potentially identify such compounds.

It was necessary to address a number of problems of working with marsupials and the unique pouch environment. The isolation of neutrophils, in the first instance, used reported protocols which had been developed from work on eutherian species. This proved problematic due to the relatively low numbers of these cells and the variation of neutrophil numbers with age and season. Neutrophils increase from around 1.41×10^6 cells/ml in a young animal to 1.64×10^6 cells/ml in an adult, whilst seasonal variations range from 1.64×10^6 cells/ml in summer to 2×10^6 cells/ml in autumn. Consequently, the initial task was to establish a protocol to maximise the isolation of neutrophils with minimal contamination with haemoglobin and minimal loss of target cells.

Secondly, the lack of translated genomic database for the tammar wallaby hindered confident identification of neutrophil proteins. To address this issue, a search strategy was developed for the identification of peptide sequences across species boundaries. Using this method we were not only successful in identifying the majority of abundant proteins, but also validated the identifications using a decoy database to allow an estimation of the likely error rate of the protein identification. The proteins identified in this phase of the study were common to other cell types, either part of the fundamental pathway or cytoskeleton of the cell. However, a few proteins could not be identified with any degree of confidence. Owing to poor quality spectra, some of the unidentified spots could not be matched with any known proteins in the database. In contrast, other

unidentified spots exhibited good quality spectra but failed to match with any known proteins within the database, suggesting these proteins may be unique to marsupials. Predominantly, however, the results indicated that the abundant proteins present in the neutrophils of the tammar wallaby do not differ from those observed other mammals.

Moving from the isolation of neutrophils themselves to working on granules and their proteins also proved problematic. Neutrophils were initially stimulated with Phorbol myristate acetate (PMA) and Ionomycin with calcium to cause degranulation, according to published protocols for eutherian systems. However, using this approach we had little success in isolating AMP's from the granules. The stimulation studies were repeated with a wide range of concentration of stimulants with little success. Further attempts to optimise AMPs yield using a combination of PMA and Ionomycin with calcium were tried with a view to producing a synergetic effect for degranulation, but no AMP's were identified on subsequent proteomic analysis. Finally a protocol consisting of centrifugation and lysis allowed some success in the isolation of granules and identification of some antimicrobial proteins, including lysozyme, serine proteinase and dermcidin, and enzymes such as myeloperoxidase and alkaline phosphatase. In addition, a number of proteins, such as GTPases of the Rab family and Cdc 42 associated with granule exocytosis were also successfully identified. Identification of these proteins revealed a large degree of sequence conserved across species, despite evolutionary divergence between mammalian lineages. This very likely reflects the fact that the proteins identified in this study perform a fundamental role in the immune system across all mammals.

The constraints of working with an unsequenced, untranslated database coupled to the limitations of protein sample concentration meant that only relatively abundant proteins were identified. Future work in this area will benefit not only from the current roll out of sequence from the Opossum Sequencing project but from improvements in proteomic technologies, some of which eg MUDPIT, started to appear during this project.

Nevertheless this study has been the first step in identification of proteins associated with immune function in marsupials, particularly those present within the granules of neutrophils.

The second major portion of this project focused on that unique feature of marsupials – the maternal pouch and what role it may play in immunological protection of the young animal. Whilst skin in all mammals plays an important role both as a physical and chemical barrier against infectious agents, in marsupials the skin of the pouch has long been presumed to play an additional role in active protection of the neonate. Such a perception has been supported by macroscopic observations of change in the pouch associated with birth of the young and by a limited number of studies of changes in the culturable pouch microflora.

In this study, pouch secretions of the mother were collected as a wash over a two year sampling period, with this collection coinciding with the period immediately prior to birth, at birth and post birth. The small amounts of proteins present in these washes, even over such an extensive sampling period, posed a severe constraint on the amount and type

of analysis that could be undertaken. In particular, this limitation affected capacity to identify proteins below the level of sensitivity of the gel stains and the MS protocols.

Nevertheless, it has been clearly demonstrated that the pouch wash collected across the three different reproductive stages exhibited inhibitory activity against *E. coli*, with highest activity at oestrus or the anticipated time of birth. This inhibitory activity was exhibited by compounds that ranged up to 50kDa and proteomic analysis subsequently identified all the major protein bands as belonging to β -lactoglobulin. It is extremely likely that the abundance of β -lactoglobulin masked the detection of low abundance proteins although; 2DE should have allowed such proteins to be detected.

The consistent and overwhelming detection of β -lactoglobulin raised questions about its origin. Logically it could be sourced to mammary gland secretions before the attachment of the pouch young to the teat but this would not be feasible once the young were attached. Alternatively then, it may have originated as a digestive product from the gut of the young animal. To investigate this proteomic analysis of the mammary gland secretions collected just before the birth of the young animal was carried out along with analysis of gut samples from a young animal. Both these sample sources clearly showed the presence of β -lactoglobulin. However two important and interesting observations emerged. Firstly there were many other high abundance proteins present in these samples that did not appear in the pouch wash samples and secondly the MW and pI values were not totally congruent between the washes and the other samples. These results clearly raise questions about the long held belief that the maternal pouch skin is the source of

antimicrobials and suggest that the mammary gland (and perhaps the gut of the neonate) play a more substantial role.

To further investigate the role of maternal pouch skin, wash samples were collected from females at three key stages of their reproductive lives - immature, mature and reproducing and post-reproductive, mature. An opportunistic sample from a mature female wombat (*Vombatus ursinus*), was also analysed at this time. There were clear differences in the nature and amount of the proteins identified over these three periods. In the immature animals no proteins could be confidently identified, this is probably due to low protein concentrations, falling below the limits of detection of the stain. Post reproductive maturity the pattern of proteins detected differed significantly from that observed in females actively reproductive. Of the proteins that were reliably identified some, such as dermcidin, may be the product sweat glands of the pouch skin and are similar to those produced by the skin of eutherians.

In some ways this project has raised more questions about the strategies used to protect the neonatal marsupial. Although a number of antimicrobial proteins of the neutrophil granules have been identified sampling limits and the limitations of the technology have, we suspect, prevented isolation and identification of many compounds. Nevertheless antimicrobial activity within the pouch at the time of birth has been proven and further experimentation is needed to clarify the nature and the source of such activity. In view of the significant advances made in the field of proteomics combined with improvements in available instrumentation, such as high resolution mass spectrometers, future efforts must

be directed at isolating other antimicrobial proteins present within the neutrophil granules, such as cathelicidin, bacterial permeability membrane and low molecular weight proteins, such as defensins. In addition, future research should also target the pouch young to isolate the wide repertoire of antimicrobial proteins present within their neutrophil granules and compare with those isolated from adults. Finally, skin secretions from the pouch young should be collected to isolate and identify any possible antimicrobial compounds secreted during early developmental phases of the young animal.

The results reported in this study of the marsupial model animal, the tammar wallaby, can serve as a paradigm for future investigations of other antimicrobial proteins from neutrophil granules and epithelia employed for the protection of altricial pouch young. These studies also provide tantalising details about the likely conservation of the antimicrobial responses in mammals, despite the evolutionary distance between the metatherian species studied here and eutherians. Future research directed at the investigation of specific antimicrobial activity of the identified proteins of neutrophils and skin could potentially form the basis of development new immunotherapies to fight against micro-organisms in immunocompromised hosts, including humans.