Appendix 1.

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RESEARCH ARTICLE

Proteome mapping of the *Trichoderma reesei* 20S proteasome

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Abstract The 26S proteasome, a multicatalytic protease comprising the catalytic 20S core particle and the 19S regulatory particle has a crucial role in cellular protein quality control. We have used a chromatography-based approach to purify and map the protein content of the 20S core particle from the industrially-exploited filamentous fungus *Trichoderma reesei*. There are no previous reports on the isolation or proteomic mapping of the proteasome from any filamentous fungus. From the reference map, 13 of the 14 20S proteasome subunits and many related proteins that co-purified with the 20S proteasome have been identified. These include 78 kDa glucose-regulated protein (BIP) and several chaperones including heat shock proteins involved in the unfolded protein response (UPR). Some proteasome interacting proteins (PIPs) were also identified on the proteome map and included 14-3-3-like protein, glyceraldehyde-3-phosphate dehydrogenase, transaldolase, actin, translation elongation factor, enolase, ATPase in the ER (CDC48), and eukaryotic initiation factor. We present here a master map for the 20S catalytic core to pave the way for future differential display studies addressing intracellular degradation of endogenous and foreign proteins in filamentous fungi.

Keywords *Trichoderma reesei* · Filamentous fungi · 20S proteasome · Proteomics · Protein secretion

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Introduction

The unicellular yeast *Saccharomyces cerevisiae* is an acknowledged model of a eukaryotic cell and most of the information concerning ER-mediated protein quality control in fungi comes from yeast studies. For example, the yeast proteasome has been characterised at the biochemical, structural and genetic level (Glickman et al. 1998; Cagney et al. 2001; Verma et al. 2000). While extracellular proteolysis has been studied in high protein secreting, multicellular filamentous fungi, very little is known about pathways for intracellular protein degradation and their regulation. This observation is somewhat unexpected considering the importance of filamentous fungi as biotechnologically-exploited production hosts for a variety of homologous and heterologous gene products (Nevalainen et al. 2005).

Trichoderma reesei can secrete up to 100 g l^{-1} of protein into the culture medium (Cherry and Fidantsef 2003). This activity implies a considerable stress on protein folding and secretion and thereby the cellular machinery for protein quality control. The two main

ER-mediated protein quality control mechanisms that sense the presence and participate in the clearing of faulty proteins are the unfolded protein response (UPR; Sims et al. 2005) and ER associated degradation (ERAD; Romisch 2005; Nishikawa et al. 2005). Accumulation of unfolded or incorrectly folded proteins triggers the UPR, which activates chaperone expression (BIP, protein disulfide isomerase and heat shock proteins) in an attempt to correctly fold proteins. If unsuccessful, the terminally misfolded proteins are retrotranslocated from the ER to the cytoplasm. Once retrotranslocated from the ER, misfolded proteins are tagged with ubiquitin (Ciechanover 1998) and degraded by the 26S proteasome.

The 26S proteasome is a 2.5 MDa multicatalytic protease that degrades proteins using an ATP-dependent mechanism (Babbit et al. 2005). The 26S proteasome consists of a 20S core particle (CP) and a 19S regulatory particle (RP). The 20S CP is a barrel shaped structure composed of four stacked rings, each containing seven subunits ($\alpha_7\beta_7\beta_7\alpha_7$). The two outer rings are composed of seven different α-subunits, which regulate the opening of the proteasome to misfolded proteins. The two inner rings are formed by seven different β-subunits and enclose a large central chamber containing the catalytic sites of the proteasome. The CP is characterised by three proteolytic activities: chymotrypsin-like (subunit β5), trypsin-like (subunit β2) and peptidylglutamyl peptide-hydrolysing activities (subunit β1; Heinemeyer et al. 1997; Wolf and Hilt 2004). The RP is composed of at least 18 different subunits in *S. cerevisiae* (Guerrero et al. 2006). The base of the RP is composed of six ATPases, which directly associate with the alpha subunit rings of the CP. The ATPases function to provide ATP for the ATP-dependent proteolysis carried out by the 26S proteasome. The base of the RP facilitates protein unfolding and translocation into the CP. The lid of the RP is composed of twelve subunits and is involved in recognition of ubiquitin tagged proteins, de-ubiquination and unfolding of the proteins in preparation for their entry into the 20S CP. Recently, 64 proteasome-interacting proteins (PIPs) have been found to associate with the 26S proteasome, providing evidence for a highly dynamic protein interacting network (Guerrero et al. 2006).

The aim of this work was to separate the 20S proteasome subunits from *T. reesei* using two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (2D SDS-PAGE). A translated protein database for identification of proteins by mass spectrometry (peptide mass fingerprinting; PMF) was developed from the recently completed genome sequence of *T. reesei* (http://www.jgi.doe.gov/sequencing/DOEprojseqplans.html) and comparisons were made between this species-specific protein database and databases containing all known fungal proteins. The work presented here will facilitate further studies addressing the function of the fungal proteasome and its involvement in the eradication of faulty foreign proteins from the secretory pathway.

Materials and methods

Fungal strain and cultivation conditions

Trichoderma reesei strain Rut-C30 (Eveleigh and Montenecourt 1979) was conidiated and maintained on potato dextrose agar (Difco Laboratories, Detroit, MI, USA). Conidia were collected using 5 ml of 0.9% sodium chloride (w/v), 0.01% Tween-80 (v/v) and 1×10^7 conidia were used to inoculate 500 ml minimum medium (MM; 110 mM potassium phosphate, 38 mM ammonium sulphate, 2.4 mM magnesium sulphate, 4.1 mM calcium chloride, 2.9 mM manganese sulphate, 7.2 mM iron sulphate, 0.35 mM zinc sulphate, 0.71 mM cobalt sulphate, pH 5.5) supplemented with 2% glycerol (v/v) in five 1 l conical flasks. Cultures were grown at 28°C on a shaker at 250 rpm for 48 h. The mycelia were collected and washed three times with 50 ml Milli-Q water by inverting and centrifugation at 4,000*g* for 10 min at 20°C. Washed mycelia were immediately processed for the proteasome purification.

Proteasome purification

After washing, the mycelia were resuspended in a threefold volume of mycelia in proteasome extraction buffer (PEB; 50 mM tris (pH 7.5), 5 mM $MgCl_2$, 10% glycerol, 5 mM ATP, 1 mM DTT, 1 mM PMSF) and 0.1% (v/v) fungal and yeast protease inhibitor cocktail. The mycelia were lysed by sonicating at 60% intensity for five cycles of 15 s on a Branson Sonifier 250 (Branson Ultrasonic, CT, USA). The samples were kept on ice throughout the sonication cycles. Cellular debris was pelleted by centrifuging at 4,000*g* for 30 min at 4°C followed by ultracentrifugation of the supernatant at 126,000*g* for 1 h at 4°C. Finally, the extract was passed through Miracloth to remove lipid material. Proteasome purification was performed three times to obtain the results presented here.

The cell extract from above was used as the starting sample for chromatographic purification steps, which were performed at 4°C to minimise dissociation of the 20S proteasome complex. A method similar to Legget et al. (2005) was used with minor modifications.

Briefly, the cell extract was applied onto a 100 ml DEAE-Affigel Blue resin (Biorad) column (affinity interactions) at 2 ml min^{-1}, washed with 100 ml PEB then 200 ml PEB, 50 mM NaCl at 4 ml min^{-1} before eluting with 200 ml PEB, 150 mM NaCl at 4 ml min^{-1}. Each 10 ml fraction collected (Pharmacia FLPC System) was assayed for chymotrypsin-like activity using the 20S Proteasome Assay Kit AK-740 (Biomol, USA) following the manufacturers' instructions. Fractions positive for chymotrypsin-like activity were pooled and further purified on a 50 ml Source Q resin ion exchange column (GE Healthcare, USA) at 2 ml min^{-1}. The sample was eluted using a linear salt gradient of 100 mM NaCl to 500 mM NaCl in PEB buffer at 4 ml min^{-1}. Fractions of 6 ml were collected and assayed for chymotrypsin-like activity. All fractions showing chymotrypsin-like activity that eluted at approximately 300 mM NaCl were pooled and concentrated to 1 ml on a 30 kDa centrifugal concentrator (Amicon® Ultra−15, Millipore, USA). The concentrated sample was separated on a 100 ml size exclusion column containing Superose 6 (Biorad) at 1 ml min^{-1} and eluted with PEB, 100 mM NaCl at 1 ml min^{-1}. Fractions of 2 ml were collected and assayed for chymotrypsin-like activity as described below. Pooled fractions positive for chymotrypsin-like activity were concentrated by 30 kDa centrifugal concentrator and stored at −80°C until used.

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Protein separation was performed as described Laemmli (1970) using 4–20% (w/v) Tris–glycine gels (iGels™, Life Therapeutics, Australia). Approximately 45 µg of the proteasome sample was loaded onto the gel and run at 130 V for 90 min, after which proteins were stained with Coomassie Blue (BioRad) for 16 h. Proteasome samples were resolved by SDS-PAGE and then electrotransferred to a PVDF membrane (Immobilon™, Millipore) with a wet transfer system (Bio-Rad) for immunoblotting. Detection of 20S subunit proteins was performed with a yeast anti-rabbit 20S antibody (BIOMOL PW9355). The primary antibody was visualised with an alkaline phosphatase-labeled goat anti-rabbit IgG immunoglobulin (Calbiochem®) and nitroblue tetrazolium and 5-bromo-4-chloro−3-indolylphosphate (Sigma).

Desalting the proteasome prior to 2D SDS PAGE

The concentrated samples (in duplicate) were desalted using a 2-D Clean up Kit following manufacturers' instructions (GE Healthcare, USA). Precipitated proteins were resuspended in 200 µl of sample solution (7 M urea, 2 M thiourea, 1% (w/v) C7Bz0, 40 mM tris, 5 mM tributylphosphine, 10 mM acrylamide, 1 mM PMSF and 0.1% (v/v) protease inhibitor cocktail) and incubated at room temperature for 90 min to allow complete reduction and alkylation of proteins (Herbert et al. 2003). The reactions were quenched with 10 mM dithiothreitol before insoluble material was removed by spinning at 16,000*g* for 10 min. The conductivity of each sample was tested and was acceptable if salt levels gave a reading of less than 300 µS cm^{-1}, otherwise further precipitation using the 2-D Clean up Kit was performed. Samples were prepared at RT to prevent urea from precipitating out of solution. The samples were used directly to rehydrate immobilized pH gradient (IPG) strips.

Isoelectric focusing and 2D SDS PAGE

The samples were used directly to passively rehydrate 3–10 and 4–7, 11 cm IPG strips (Amersham Pharmacia, Uppsala, Sweden) by applying 180 µl of each sample (equivalent to 230 µg of protein). IPG's were focused to a total of 80,000 Volt hours (Vh) using a three-step focusing program. The focusing program included a rapid ramp to 300 V for 4 h, a linear ramp to 10,000 V over 8 h, and a 10,000 V step until 80,000 Vh were reached. IPGs were equilibrated for 20 min in 6 M urea, 2 % (w/v) SDS, 50 mM Tris–HCl buffer, pH 8.8, 0.1% (w/v) bromophenol blue. The IPGs were then placed on top of Proteome Systems 6–15% Gelchips (Proteome Systems, Australia) and ran at 30 mA constant until the bromophenol blue dye reached the bottom of the gel. Gels were fixed in 10% (v/v) methanol, 7% (v/v) acetic acid solution for 30 min, then stained with Sypro Ruby solution (Molecular Probes) for 16 h. Gels were destained in the fixing solution before being scanned on a fluorescence scanner (Alpha Innotech Corporation, California). Gels were restained for 16 h with Coomassie Colloidal Blue G250 (17% (w/v) ammonium sulphate, 34% (v/v) methanol, 3.6% (v/v) orthophosphoric acid, 0.1% (w/v) Coomassie G-250) and destained with 1% (v/v) acetic acid for further analysis as required.

Mass spectrometry and identifications

Protein spots were excised using the Xcise™ apparatus (Shimadzu Biotech, Japan). Gel pieces were destained and dried. Trypsin was added to each gel piece and they were incubated at 37°C for 16 h for protein digestion. Each peptide solution was desalted

and concentrated using ZipTips™ from Millipore (USA) and spotted onto the target plate with 1.0 µl matrix solution (4 mg ml^{-1} alpha-cyano-4-hydroxy-cinnamic acid in 70% (v/v) acetonitrile, 1% (v/v) trifluoroacetic acid). Peptide mass fingerprints of tryptic peptides were generated by matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) using an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in the MS mode. A Nd:YAG laser (355 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range 750–3,500 Da. The instrument was then switched to MS/MS (TOF/TOF) mode where the eight strongest peptides from the MS scan were fragmented by collision-induced dissociation. A near point external calibration was applied to give a mass accuracy within 50 ppm. Mass spectrometry data was searched against proteins from all fungal species using Mascot Peptide Mass Fingerprint where a modified MOWSE scoring algorithm was used to rank results (http://www.matrixscience.com/help/scoring_help.html) (Pappin et al. 1993), or using the *T. reesei* database as described below.

Trichoderma reesei database development

The entire set of 8619 protein coding sequences as predicted by the DOE *T. reesei* genome sequencing project (http://www.jgi.doe.gov/sequencing/DOEprojseqplans.html), was downloaded as a single file in FASTA format. This file was converted to a searchable format using tools available in MASCOT (http://www.matrixscience.com). The resulting database was then searched using MS data by MASCOT, utilizing peptide mass fingerprinting as the method of protein identification. Briefly, amino acid sequences and monoisotopic masses were calculated for theoretical peptides arising from the in silico tryptic digestion of all translated gene sequences were searched by MASCOT. The peptides searched included potential sequence and mass changes arising from missed tryptic cleavage sites, oxidation of methionine residues, and the alkylation of cysteine residues with acrylamide. This database was queried with experimentally generated MALDI-TOF data using either an interactive web-accessible search tool, or an automated batch-mode version of the software. Results were ranked by the number of peptides matched and by percentage of total protein sequence covered. To be considered as a positive match one or more of the following criteria needed to be met: a minimum of four peptide matches and/or a MASCOT score >50 and/or 20% peptide coverage of the protein sequence.

Results

Purification of the 20S proteasome was performed by three-step chromatography. Chymotrypsin activity, indicating the presence of the 20S CP in eluted fractions, was monitored by a fluorometric assay using SUC-LLVY-AMC as a substrate. First, a fungal cell extract was fractionated on a DEAE-Affigel blue column. Active fractions were eluted at 270–312 ml with 100 mM NaCl. Pooled eluates were then loaded onto Resource Q column and the proteasome-containing fractions were eluted at a salt concentration of approximately 300–340 mM NaCl. These fractions were pooled and concentrated into 1 ml for gel filtration by Superose 6. Proteasomes were eluted in fractions 10–30 with 100 mM NaCl. Fractions 10–22 and 23–30, showing chymotrypsin activity, were pooled and purification of the 20S catalytic particle was confirmed by 1D SDS-PAGE and Western blotting using a yeast 20S antibody (Fig. 1).

Two-dimensional protein maps of the *T. reesei* proteasome were produced after chromatography-based purification and desalting by protein precipitation. The 2D gels produced, contained approximately 150 protein spots when displayed across either 3–10 or 4–7 pH ranges (Fig. 2). While the gel representing the 3–10 pH range of the proteasome protein extract was more compacted than the gel representing the 4–7 pH range, it provided a complete picture of the proteins present

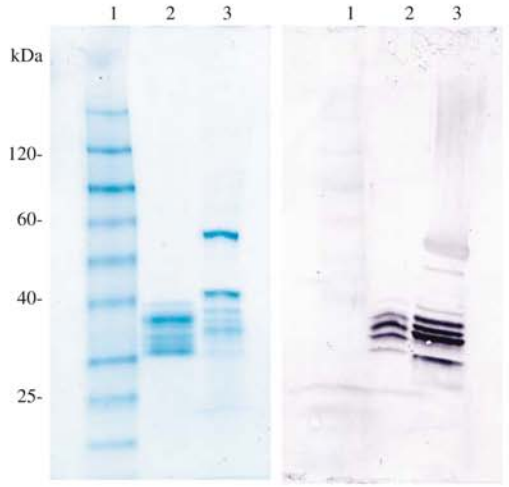

Fig. 1 **a** Coomassie-stained 1D SDS-PAGE of the purified *T. reesei* 20S proteasome. **b** Western blot using anti-rabbit yeast 20S antibody. *Lane 1* Prestained molecular weight marker (Invitrogen 10748-010), *lane 2* human 20S proteasome, lane 3: purified 20S proteasome from *T. reesei*

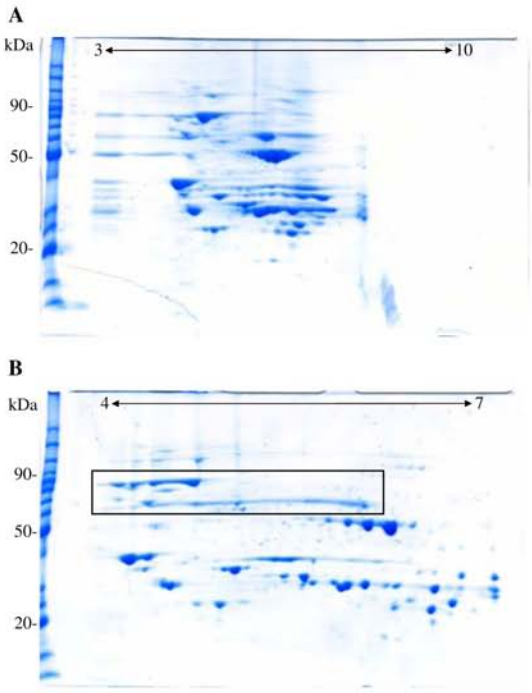

Fig. 2 Coomassie-stained 2D SDS-PAGE of the *T. reesei* 20S proteasome purified by the chromatographic method described. The gels were run on **a** 11 cm 3–10 IPG strips and **b** 11 cm 4–7 IPG strips in the first dimension and 6–15% SDS-PAGE in the second dimension. The *boxed region* on **b** represents an area of incomplete protein focusing

in this extract with minimal protein smearing. Protein smearing was more apparent across the 4–7 pH range of the proteasome extract as indicated by the boxed area in Fig. 2b. For reasons described above, the 2D gel containing proteins displayed across the 3–10 pH range was used for protein identification.

From the gel represented in Fig. 2a, 122 protein spots were excised and processed for mass spectrometry analysis and protein identification. Two approaches were taken to identify these proteins. The first was cross species identification (CSI) where peptide masses were compared to all known fungal proteins in the non-redundant protein database from the National Centre of Biotechnology Institute using Mascot (see Methods for further details). The second approach was to construct a custom protein database derived from the *T. reesei* sequencing project for peptide mass fingerprinting (PMF) for species-specific protein identification. Protein identification results from the CSI method are outlined in Table 1 and results from the *T. reesei* database are shown in Table 2.

Thirty-two unique proteins were identified using the CSI approach (Table 1) represented by 50 different protein spots including one spot, which was identified as a mixture of 2 different proteins (Fig. 3a). Most of these proteins were identified from closely related fungal species including *Gibberella zeae* and *Neurospora crassa*. Each protein identified was submitted to a homologous protein search using the BLASTP tool from the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) to determine if they contained a conserved protein domain. This examination was particularly important for the proteasome alpha and beta subunits, which were predominantly identified as hypothetical proteins or conserved hypothetical proteins. Table 1 provides further information on each identified protein, including the number of peptides matched, amino acid coverage and the presence of conserved protein domains. Where possible, proteins with known conserved domains have been labelled as proteasome alpha/beta subunits, proteins derived from the UPR response, chaperones including heat shock proteins (HSP) or known PIP.

Thirty-four unique proteins representing 81 different protein spots including the identification of 11 protein spots as a mixture of more than one protein (see Fig. 3b) were identified from the custom *T. reesei* protein database. Protein matches from the *T. reesei* PMF database are shown in Table 2 and generally contain a greater number of protein isoforms identified, greater numbers of peptide matches and higher levels of amino acid coverage when compared to PMF results in Table 1. The amino acid sequence corresponding to each gene reference number was used to conduct a BLASTP search to provide additional protein information on each result. This search provided information on the name of every protein identified and also highlighted conserved protein domains present for each of these proteins. Where possible, proteins with known conserved domains have been labelled as proteasome alpha/beta subunits, chaperones including heat shock proteins (HSP) or known PIP.

Discussion

We were able to identify all alpha proteasome subunits and six of the seven beta proteasome subunits of the *T. reesei* 20S proteasome, with the exception of Beta 7. The Beta 7 subunit has a predicted pI and mass of 7.7 and 29.1 kDa, suggesting that it is masked by the Alpha 7 subunit, which has a pI of 7.6 and a mass of 29.3 kDa. Other proteins that co-purified with the 20S proteasome feature some UPR proteins such as 78 kDa

Table 1 Proteins identified by cross species identification from the purified proteasome map of *Trichoderma reesei*

Protein name	Species	Accession no.	Number of spots identified	MW (Da)	pI	No. of peptides matched	Coverage (%)	Conserved domain present
Conserved hypothetical protein	*Gibberella zeae*	gil42545254	1	21,209	4.98	7	39	Proteasome beta
Hypothetical protein	*Yarrowia lipolytica*	gil50556048	1	23,592	5.55	11	43	Proteasome beta
Hypothetical protein	*G. zeae*	gil42553892	1	26,488	7.71	7	29	Proteasome beta
Hypothetical protein	*Neurospora crassa*	gil32423127	2	32,151	5.68	9–10	35	Proteasome beta
Hypothetical protein	*Magnaporthe grisea*	gil39977139	1	29,338	7.66	12	36	Proteasome alpha
Conserved hypothetical protein	*G. zeae*	gil42551634	3	27,980	5.89	11	39–60	Proteasome alpha
Conserved hypothetical protein	*G. zeae*	gil42544646	3	29,147	5.93	6–10	36–56	Proteasome beta
Hypothetical protein	*N. crassa*	gil32410633	1	27,917	6.12	14	70	Proteasome alpha
Hypothetical protein	*N. crassa*	gil32411629	2	29,584	6.92	8–13	36–37	Proteasome alpha
Conserved hypothetical protein	*G. zeae*	gil42552615	4	27,913	5.81	9–11	31–45	Proteasome alpha
Probable proteasome component C2	*N. crassa*	gil28949952	1	29,020	5.21	9	34	Proteasome alpha
Probable proteasome subunit alpha type 2	*G. zeae*	gil42549948	1	30,543	4.99	8	34	Proteasome alpha
Hypothetical protein	*N. crassa*	gil32408341	1	26,857	4.85	9	36	Proteasome alpha
Hypothetical protein	*G. zeae*	gil42544315	1	31,985	4.71	8	30	Proteasome alpha
14-3-3-like protein 2	*Paracoccidioides brasiliensis*	gil38569374	1	29,624	4.68	5	26	PIP
14-3-3-like protein	*Hypocrea jecorina*	gil12054274	1	29,579	4.79	14	42	PIP
14-3-3-like protein	*H. jecorina*	gil12054276	1	30,404	4.89	8	36	PIP
Glyceraldehyde 3-phosphate dehydrogenase	*Trichoderma koningii*	gil422228	1	35,952	6.28	6	18	PIP
Ketol acid reductoisomerase	*N. crassa*	gil18376395	1	44,596	8.52	10	28	N/A
Actin	*Emericella nidulans*	gil113277	1	41,622	5.64	12	40	PIP
Putative serine/threonine phosphatase 2C ptc2	*H. jecorina*	gil33087518	3	47,457	4.57	10–18	34–50	UPR
Eukaryotic initiation factor 4α	*N. crassa*	gil32414453	1	47,937	5.9	8	23	PIP
Hypothetical protein	*G. zeae*	gil42551306	1	53,193	5.5	16	28	N/A
Enolase	*G. zeae*	gil42545184	1	47,367	5.01	6	20	PIP
Enolase	*Penicillium chrysogenum*	gil34392443	2	47,207	5.14	5–9	14–21	PIP
Hypothetical protein F06655.1	*G. zeae*	gil42554748	5	53,916	5.37	7–10	18–22	M18 peptidase and LAP4 domain
78 kDa glucose-regulated protein	*G. zeae*	gil42553353	1	74,524	5.08	15	29	UPR/HSP70
78 kDa glucose-regulated protein	*N. crassa*	gil45645170	1	72,287	5.01	14	22	UPR/HSP70
Heat shock protein 70	*H. jecorina*	gil30961863	3	73,319	5.17	8–21	18–35	HSP70
Heat shock protein 60	*G. zeae*	gil42550894	1	61,430	5.57	12	20	HSP60
Hypothetical protein AN0858.2	*Aspergillus nidulans*	gil67517159	1	103,464	5.54	32	29	HSP98
Hypothetical protein	*N. crassa*	gil32402982	2	90,515	5.01	26–40	25–47	ATPase domain

PIP Proteasome interacting protein, *UPR* unfolded protein response, *N/A* no applicable proteasome related domain or function known

Table 2 Proteins identified by matching PMF data to the translated *Trichoderma reesei* cDNA database

Protein name	Gene reference number	Number of spots identified	MW (Da)	pI	No. of peptides matched	Coverage (%)	Conserved domain present
Proteasome Alpha-1 subunit	6837	7	31,645	4.82	7–13	33–49	Proteasome alpha
Proteasome Alpha-2 subunit	1126	3	32,165	5.7	7–11	23–44	Proteasome alpha
Proteasome Alpha-3 subunit	482	6	31,696	4.79	6–12	22–40	Proteasome alpha
Proteasome Alpha-4 subunit	6123	4	27,835	6.14	8–12	31–49	Proteasome alpha
Proteasome Alpha-5 subunit	2768	1	29,091	4.62	12	45	Proteasome alpha
Proteasome Alpha-6 subunit	8317	4	27,862	6.16	8–18	31–55	Proteasome alpha
Proteasome Alpha-7 subunit	8176	7	29,309	7.61	10–17	48–63	Proteasome alpha
Proteasome Beta-1 subunit	6377	2	29,171	6.88	7–13	27–51	Proteasome beta
Proteasome Beta-2 subunit	2077	4	31,001	5.16	6–11	27–38	Proteasome beta
Proteasome Beta-3 subunit	5333	3	26,542	5.1	4–11	24–39	Proteasome beta
Proteasome Beta-4 subunit	1749	1	31,939	8.62	11	31	Proteasome beta
Proteasome Beta-5 subunit	5857	2	32,232	6.09	10	34	Proteasome beta
Proteasome Beta-6 subunit	2563	4	27,607	5.35	5–11	24–43	Proteasome beta
14-3-3-like protein	5837	2	29,551	4.49	4–9	13–28	PIP
Glyceraldehyde-3-phosphate dehydrogenase	168	1	36,247	7.14	7	24	PIP
Alcohol dehydrogenase	3678	2	37,637	6.85	10–11	35–42	PIP
Ketol-acid reductoisomerase	4266	1	44,749	9.09	11	33	N/A
Deoxyhypusine synthase	105	1	39,159	5.29	7	21	N/A
Transaldolase	1855	2	35,584	5.16	6–16	21–48	PIP
Actin	2112	1	41,595	5.44	8	26	PIP
Translation elongation factor	5185	6	45,927	6.53	13–21	38–59	PIP
Enolase	5647	3	47,286	4.84	9–13	29–36	PIP
ATP citrate lyase	706	1	52,985	5.35	20	37	N/A
Aspartyl aminopeptidase	622	8	54,325	6.03	14–25	25–43	N/A
Histone acetyltransferase	2072	1	59,831	4.13	13	25	N/A
Histone binding protein	3622	1	55,843	4.12	19	43	N/A
Heat shock protein 70 kD	3072	2	73,697	4.88	9–15	17–25	Chaperone
Heat shock protein 70 kD	7312	2	66,584	5.03	6–22	17–35	Chaperone
Heat shock protein 60 kD	179	2	61,105	5.26	16–22	32–43	Chaperone
Phosphoglycerate mutase	8591	1	58,272	4.82	20	38	N/A
Transketolase	5330	1	75,108	6.21	22	31	N/A
SEC23-like protein	5122	1	85,729	6.24	17	19	Role in proteasomal sorting
Cobalamin-independent methionine synthase	702	2	85,621	6.48	21–25	31–34	N/A
Heat shock protein 98 kD	1905	1	91,807	5.34	35	32	Chaperone
ATPase in endoplasmic reticulum (CDC48)	3274	2	89,647	4.67	22–35	25–38	PIP
Hypothetical protein	1276	1	133,773	4.02	18	21	N/A

PIP Proteasome interacting protein, *N/A* no applicable proteasome related domain or function known

glucose regulated-protein (BIP; Sims et al. 2005), chaperones including HSP60, HSP70, and HSP98 and the negative UPR regulator, serine/threonine phosphatase (Welihinda et al. 1998). Some PIPs also co-purified with the 20S proteasome and include 14-3-3-like protein, glyceraldehyde-3-phosphate dehydrogenase, transaldolase, actin, translation elongation factor, enolase, ATPase in the ER (CDC48), and eukaryotic initiation factor 4a. These PIPs have been found to co-purify with the 26S proteasome under different affinity based purifications (Verma et al. 2000) and also by treatment of yeast cells with formaldehyde to retain the 26S proteasome and interacting proteins as complete entities (Guerrero et al. 2006). A similar strategy may be applied to map globally the 26S proteasome interaction network in filamentous fungi.

The chromatography purification used throughout this work also resulted in co-purification of other proteins that have not been described to associate with the proteasome (Tables 1, 2). These include ketol acid reductoisomerase, deoxyhypusine synthase, ATP citrate lyase, aspartyl aminopeptidase, histone acetyltransferase, histone binding protein, phosphoglycerate mutase, transketolase, cobalamin-independent methionine synthase and a hypothetical protein. It is unlikely that these proteins co-eluted from the column, as the final chromatography column is a size exclusion column and only very large proteins (approximately 700 kDa–1 MDa) will elute at similar positions to the 20S proteasome. Most of the proteins mentioned above have a mass of less than 100 kDa. If these proteins have no natural interactions with the 20S proteasome, it is

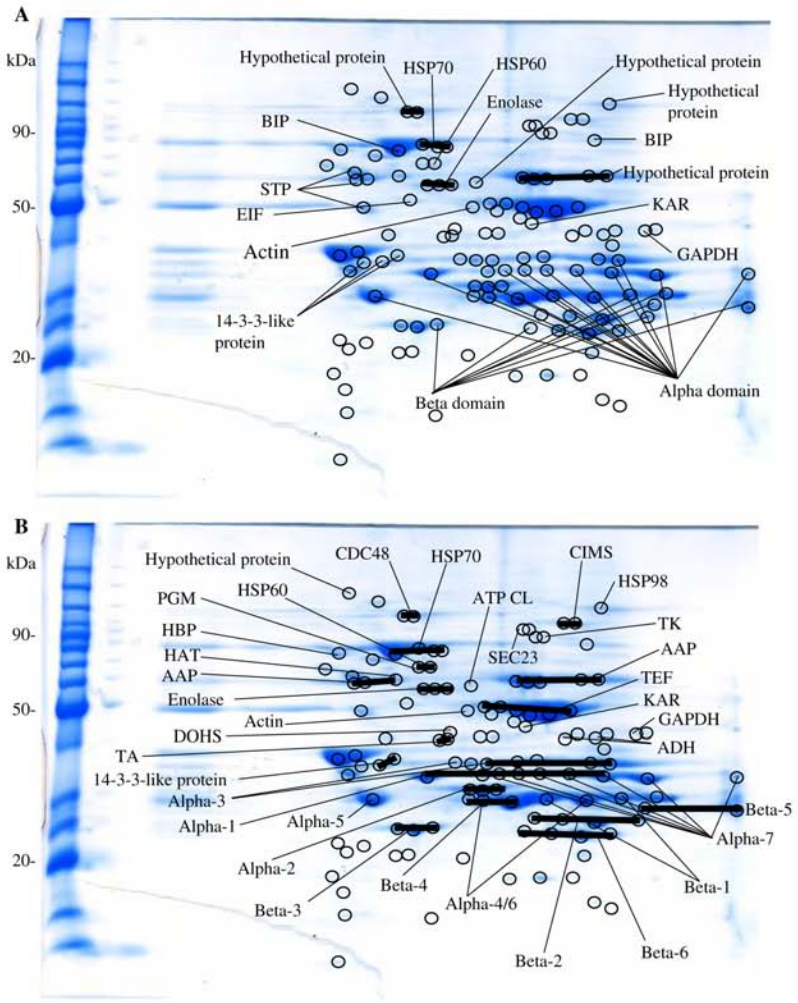

Fig. 3 Protein identifications of the *T. reesei* 20S proteasome by mass spectrometry. **a** Protein identification map produced by comparing PMF data to all fungal proteins on NCBI non-redundant protein database. **b** Protein identification map produced by comparing PMF data to in-house *T. reesei* protein database. Abbreviations used on protein maps include *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *KAR* ketol acid reductoisomerase, *STP* serine/threonine phosphatase, *EIF* eukaryotic initiation factor, *BIP* 78 kDa glucose-regulated protein, *HSP* heat shock protein, *ADH* alcohol dehydrogenase, *DOHS* deoxyhypusine synthase, *TA* transaldolase, *TEF* translation initiation factor, *ATP CL* ATP citrate lyase, *AAP* aspartly aminopeptidase, *HAT* histone acetyltransferase, *HBP* histone binding protein, *PGM* phosphoglycerate mutase, *Tk* transketolase, *CIMS* cobalamin-independent methionine synthase, *CDC* cell division control

likely that they have formed a complex or association with the proteasome during or shortly after cell lysis. Whatever the case, the proteins consistently co-purified with the proteasome across triplicate purifications and are likely to have some association to the 20S proteasome. Their possible true role as PIPs remains to be elucidated.

It may be surprising that components of the 19S regulatory subunit were not found in the 2D map of *T. reesei* shown in Fig. 3, even though 4–5 mM ATP was included throughout the purification procedure in an attempt to retain the 26S proteasome structure, as previously reported (Legget et al. 2005). It is possible that the 19S regulatory subunit dissociated from the 20S

Table 3 Protein identifications made by comparing cross species matching all known fungi proteins with *Trichoderma reesei* specific protein database

Protein database searched	Number of protein spots identified	Percentage of spots identified	Unique proteins identified
All fungal proteins on the NCBI nr protein database	50 (including 1 spot identified as a mixture)	41.0	32
Translated *T. reesei* cDNA sequences from genome release 1	81 (including 11 spots identified as mixtures)	66.4	34

proteasome during cell lysis, where a high-powered ultrasonic probe was used to break the hyphae. Ultrasonication was found to be preferred to homogenisation in a blender, as much higher levels of the proteasome were extracted and detected based on the 20S proteasome assay kit using chymotrypsin-like activity as an indicator. A bead mill has been used to break up yeast cells to retain the 26S proteasome complex (Legget et al. 2005). However, it was not attempted during this work. A regulatory particle protein, RPN5, from the 19S lid of the 26S proteasome was found on a 2D gel from a replicate proteasome purification along with an additional PIP eukaryotic translation initiation factor 3 and chaperone HSP90 (data not shown). These proteins were present on the 2D gel in regions of low resolution due to protein streaking and incomplete focussing and hence were not visible as individual protein spots in Fig. 2a. The presence of the single regulatory particle protein from a replicate proteasome extraction does not provide sufficient evidence that we have purified the 26S proteasome, as other regulatory particles and ATPases described from the yeast 19S lid were not observed. Most of the 19S regulatory particles have a predicted pI suitable for the 3–10 pH range (2D gel shown in Fig. 2a), indicating that the 19S RP subcomplex of proteins is missing from this preparation. We recognise the importance of having the entire 19S regulatory particle for further studies into protein degradation. Therefore, we will explore more gentle methods such as using the French Press for cell lysis. In addition, less powerful ultrasonication will be trialled. The addition of ATP should have kept the 19S regulatory particle associated to the 20S core particle. However, since purification of the proteasome was monitored by chymotrypsin-like activity, it may be possible that the 19S subunits were present in non-active fractions and therefore discarded.

The availability of a species-specific database for PMF searching has made a substantial difference in the number of protein identifications made from the 122 spots cut out and analysed from Fig. 2a. The findings have been summarised in Table 3 and show that a 41% identification frequency was achieved by CSI whereas a 66% identification frequency was achieved with a species-specific protein database for *T. reesei*. The increase in protein identification frequency using the *T. reesei* database is due to identification of a greater number of protein pI isoforms rather than a substantial increase in the different types of proteins identified. The limitations of CSI have been known for some time (Wilkins and Williams 1997; Lester and Hubbard 2002). However, it has been successfully used for protein mapping where species-specific databases do not exist (Grinyer et al. 2004a; Grinyer et al. 2004b). The availability of the species-specific protein database is highly advantageous for proteomics-based protein identification projects.

This is the first reported example of purification of the 20S proteasome from a filamentous fungus. The purified 20S proteasome from *T. reesei* was displayed by 2D SDS-PAGE and protein identifications were made by mass spectrometry. Thirteen of the 14 subunits were identified from the map of the 20S proteasome. Several UPR-related proteins were identified including a number of chaperones (HSP). Several PIPs found to interact with the yeast proteasome were identified, indicating that they co-purify with the fungal 20S proteasome. The proteins displayed and identified form a master map of the *T. reesei* 20S proteasome, which can be readily applied for future studies into the function of the fungal proteasome in cellular protein quality control of this high protein-yielding cell factory.

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Appendix 2.

Protein Expression and Purification 67 (2009) 156–163

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Rapid purification method for the 26S proteasome from the filamentous fungus *Trichoderma reesei*

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ABSTRACT

We have developed a fast and simple two column chromatographic method for the purification of the 26S proteasome from the filamentous fungus *Trichoderma reesei* that simplifies the overall procedure and reduces the purification time from 5 to 2.5 days. The combination of only the anionic exchange POROS® HQ column (Applied Biosystems) together with a size exclusion column has not been used previously for proteasome purification. The purified complex was analysed further by two-dimensional electrophoresis (2DE) and examined by transmission electron microscopy (TEM). A total of 102 spots separated by 2DE were identified by mass spectrometry using cross-species identification (CSI) or an in-house custom-made protein database derived from the *T. reesei* sequencing project. Fifty-one spots out of 102 represented unique proteins. Among them, 30 were from the 20S particle and eight were from the 19S particle. In addition, seven proteasome-interacting proteins as well as several non-proteasome related proteins were identified. Co-purification of the 19S regulatory particle was confirmed by TEM and Western blotting. The rapidity of the purification procedure and largely intact nature of the complex suggest that similar procedure may be applicable to the isolation and purification of the other protein complexes.

Introduction

The 26S proteasome is a 2.5 MDa proteinase complex found in the cytoplasm and nuclei of all animal, plant and fungal cells [1]. It is involved in a multitude of cellular processes including cell differentiation, signal transduction, cell-cycle control, regulation of metabolic activity and stress response. This organelle functions to remove and recycle misfolded proteins as well as intracellular regulatory proteins rapidly and selectively [2,3]. Eventually, 80–90% of total cellular proteins are degraded by the 26S proteasome [4]. Depending on protein breakdown rates, the proteasome can constitute up to 1% of the cellular protein content [5].

The 26S proteasome is composed of at least 32 different subunits arranged in two sub-complexes: the 20S proteolytic complex, called the core particle (CP)[1] and the 19S regulatory particle (RP). The cylindrical structure of the 20S proteasome is composed of four heptameric rings of α and β-subunits. The two outer rings consist of seven α-subunits while the two central rings are composed of seven β-subunits. The centre of the multicatalytic protease chamber contains three subunits which perform the proteolytic function. Subunit β1 has peptidyl-glutamyl peptide-hydrolysing activity, subunit β2 has trypsin-like activity and subunit β5 has chymotrypsin-like activity [6,7]. The exact number of 19S complex subunits in different organisms is unknown. In yeast, as well as mammals, 19 different subunits have been identified [8,9].

The 19S RP associates with the 20S proteasome via an ATP-dependent reaction to form an active 26S complex. The RP of the 26S proteasome contains six ATPases of the "AAA" family (RPT; for Regulatory Particle Triple-A ATPase subunit) and four non-ATP-

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[1] *Abbreviations used:* CP, core particle; RP, regulatory particle; WB, washing buffer; IEF, isoelectric focusing; IPG, immobilized pH gradient; TEM, transmission electron microscopy; UPR, unfolded protein response; PiPs, proteasome-interacting proteins; CDC, cell division control.

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ase subunits (RPN), which form the base complex at the interface between the 19S RP and 20S CP [10]. The ATPases play a role in substrate binding and unfolding and assist in the translocation of the unfolded substrate into the 20S proteasome cavity [11]. The lid of the 19S RP is composed of nine non-ATPase subunits and its major activity is proposed to be deubiquitination of proteins. Therefore, the lid is required for the recognition and degradation of ubiquitinated target proteins [10].

The unicellular yeast *Saccharomyces cerevisiae* is an acknowledged model of a eukaryotic cell and the majority of published purification methods for the 26S proteasome come from yeast studies [8,11,12]. 26S proteasomes have been isolated from higher plants (e.g. [13,14]), mammalian cells (e.g. [15,16]) and from yeast [8,17]. Isolation of the 20S proteasome particle from a filamentous fungus also has been reported [18]. While the earlier proteasome purification methods involved the application of glycerol gradients, PEG precipitation followed by multistep chromatography or His-tags combined to immunochromatography, more sophisticated high affinity chromatography systems can be applied to simplify this process. We present here a simple and rapid isolation and purification method for the entire and enzymatically active 26S proteasome from the industrially exploited filamentous fungus *Trichoderma reesei*. This method can be applied to the purification of other large protein complexes of interest.

Materials and methods

Strain and cultivation

T. reesei strain RutC-30 [19] was grown in five 2 L conical flasks (500 mL culture volume) in minimal medium [20] supplemented with 2% glycerol (v/v) at 28 °C for 48 h, with shaking at 250 rpm.

Isolation of the 26S proteasome

T. reesei mycelia were harvested by centrifugation at 4000*g* for 20 min, washed three times with distilled water and placed in two times the sample volume of buffer A (50 mM Tris, pH 7.5, containing 5 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF, 10% (v/v) glycerol, 0.1% (v/v), and fungal and yeast protease inhibitor cocktail [Sigma, P8215]). Complete cell lysis was achieved by twice passing the fungal mycelia through a French Press (French® Press Cell Press, Thermo Scientific, USA) at 15,000 psi. The extract was centrifuged at 4000*g* for 30 min and the supernatant spun at 100,000*g* for 1 h at 4 °C. The final supernatant was filtered through Miracloth (Calbiochem®) and a membrane filter (pore size 0.2 μm) to remove lipid material. The extract was concentrated to 6 mL using an Ultra free concentrator with molecular weight cut-off of 5 kDa (Amicon® Ultra-15, Millipore, USA). The protein concentration of the fungal extract was determined using a modified Bradford method [21] with bovine serum albumin as a standard. The fungal extract was stored at −80 °C until used.

The extract was applied onto a 1 mL POROS® HQ column (anion exchange, Applied Biosystems) at 5 mL min^{-1}, using an ÄKTA Explorer 10S FPLC system (GE Healthcare), washed with 25 mL buffer B (10 mM Tris–10 mM NaH_2PO_4, pH 7.5–1 mM ATP) at 5 mL min^{-1} before eluting using a linear salt gradient of 0–1 M NaCl in buffer B at 5 mL min^{-1}. One milliliter fractions were collected and assayed for chymotrypsin activity using a fluorometric assay (20S Proteasome Assay Kit AK-740, Biomol, USA) following the manufacturers' instructions. Fractions positive for chymotrypsin-like activity were pooled and concentrated as mentioned earlier. The concentrated sample was separated on a 150 mL size exclusion column containing Sephacryl S-500 HR (GE Healthcare Life Sciences, Uppsala, Sweden) at 0.5 mL min^{-1} with buffer C (1 M NaCl in buffer B). Fractions of 1 mL were collected and assayed for chymotrypsin-like activity as described above. Fractions containing high chymotrypsin-like activity (eluting approximately at 230–280 mM NaCl) were pooled and concentrated to 1 mL and stored at −80 °C until used. Protein concentration was determined as previously described.

1D SDS–PAGE and Western blotting

The purified 26S proteasome was separated on one-dimensional 12–20% (w/v) SDS–PAGE gel and protein bands were visualised with Coomassie Colloidal Blue 250G (CCB; 17% (w/v) ammonium sulphate, 34% (v/v) methanol, 3.6% (v/v) orthophosphoric acid, 0.1% (w/v) Coomassie G-250). For Western blotting, proteins were separated by SDS–PAGE as above and blotted onto PVDF (Immobilon-P, Millipore, USA) membranes at 30 V for 1 h. Following blocking with 3% (w/v) skim milk in the washing buffer (WB; PBS–0.5% Tween 20) for 1 h, the membranes were incubated for 1 h with primary antibody (rabbit polyclonal anti-20S and five anti-19S proteasome subunits Ig, Abcam Inc., USA; 1:2000 dilution). After washing the blot with WB three times, it was incubated for 1 h with secondary antibody (alkaline phosphatase conjugated goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA; 1:500 dilution). After three washes with WB, the secondary antibody was detected with alkaline phosphatase substrate (*FAST*™ BCIP/NBT; 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Sigma).

Isoelectric focusing and 2D SDS–PAGE

A buffer exchange was performed using a 5 kDa centrifugal concentrator to remove residual salt from chromatograph fractions prior to isoelectric focusing (IEF) and 2D electrophoresis. For each 2DE gel, a 300 μg portion of concentrated sample was purified using a 2D Clean up Kit following the manufacturer's instructions (GE Healthcare, USA). Precipitated proteins were resuspended in 200 μL of sample solution (7 M urea, 2 M thiourea, 1% (w/v) C7Bz0, 40 mM Tris, 5 mM tributylphosphine, 10 mM acrylamide, 1 mM PMSF, and 0.1% (v/v) protease inhibitor cocktail). The samples were used directly to passively rehydrate 4–7, 11 cm immobilized pH gradient (IPG) strips (Bio-Rad, CA, USA) by applying 200 μL of each sample. IPGs were focused to a total of 80,000 Volt hours (Vh) using a three-step focusing program. The focusing program included a rapid ramp to 300 V for 4 h, a linear ramp to 10,000 over 8 h, and a 10,000 V step until 80,000 Vh were reached. IPGs were equilibrated for 20 min in 6 M urea, 2% (w/v) SDS, 50 mM Tris–HCl buffer, pH 8.8, 0.1% (w/v) bromophenol blue for 20 min. The IPGs were then placed on top of Bio-Rad Criterion™ XT Precast Gel, 4–12% (Bio-Rad, CA, USA), and run at 30 mA constant until the bromophenol blue dye reached the bottom of the gel. Gels were fixed in 10% (v/v) methanol, 7% (v/v) acetic acid solution for 30 min, then stained with Coomassie colloidal blue G250 (17% (w/v) ammonium sulphate, 34% (v/v) methanol, 3.6% (v/v) orthophosphoric acid, 0.1% (w/v) Coomassie G-250).

Mass spectrometry and protein identifications

Protein spots were excised manually and gel pieces were destained and dried. Each gel spot was subjected to in-gel digestion with trypsin as described previously [18]. Mass fingerprints of tryptic peptides were generated by matrix assisted laser desorption/ionization-time of flight–mass spectrometry (MALDI-TOF–MS) using an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in the MS mode. A Nd:YAG laser (355 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range 750–3500 Da. The instrument was then switched to MS/MS (TOF/TOF) mode where the eight strongest peptides from the MS scan were fragmented by collision-induced

dissociation. A near-point external calibration was applied to give a mass accuracy within 50 ppm. Mass spectrometry data were searched against proteins from all fungal species using Mascot Peptide Mass Fingerprint where a modified MOWSE scoring algorithm was used to rank results (http://www.matrixscience.com/help/scoringhelp.html) [22], or using an in-house species-specific *T. reesei* database (described in Ref. [18]).

Transmission electron microscopy

The purified proteasome sample was diluted to a final protein concentration of 0.2 μg mL^{-1} with phosphate buffer (50 mM pH 7.5 containing 2 mM ATP) for transmission electron microscopy (TEM). Negative staining of samples was carried out Pioloform coated, 300 mesh copper grids (Proscitech, Australia) prior to sample application. The grids were floated on 20 μL drops of the sample solution for 1 min, and excess solution was removed by blotting. The grids were then floated onto drops of 2% (w/v) uranyl acetate solution for 2 min. After blotting of excess stain solution and the sample was dried and observed and photographed using a Philips CM10 TEM (Philips, Eindhoven) at an operating voltage of 100 kV. All negatives were scanned using a Microtek scanner and further processed using Image J imaging software (NIH).

Results and discussion

Purification of the fungal 26S proteasome

The 26S proteasome and associated proteins were purified from *T. reesei* cell lysates by anion exchange and size exclusion chroma-

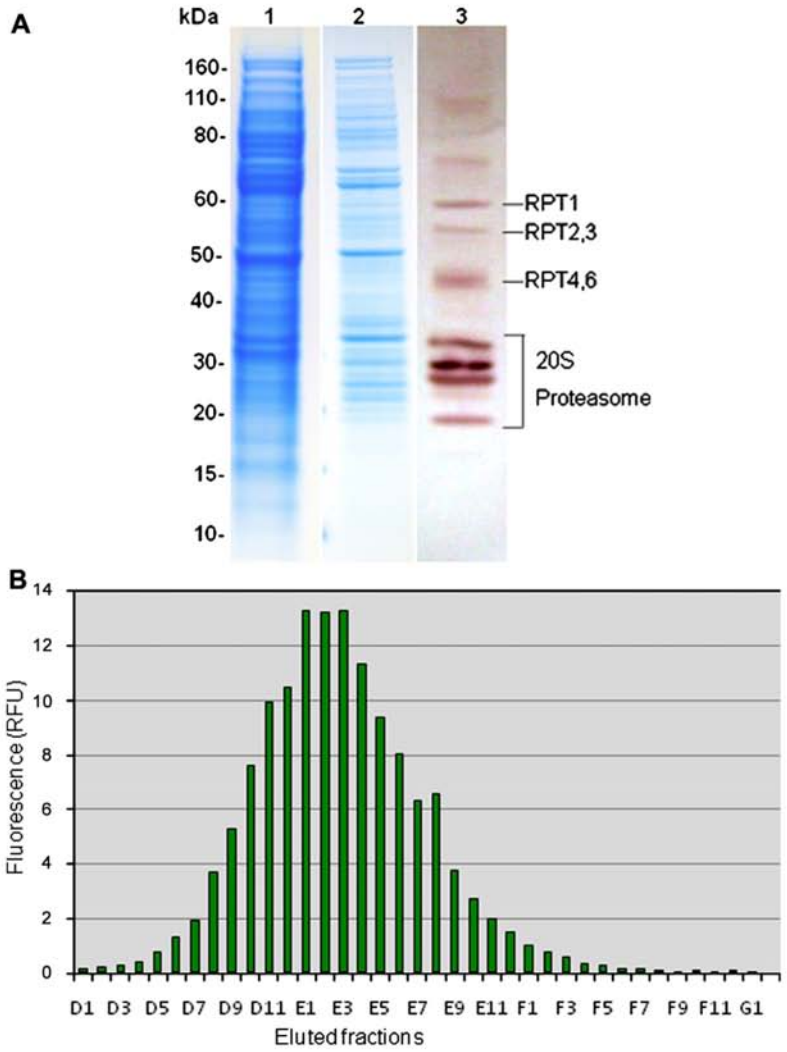

Fig. 1. (A) 1D SDS–PAGE gels and Western blot of the *T. reesei* 26S proteasome. Lane 1, eluted and pooled fractions, E1–E8, from POROS® HQ column. Lane 2, eluted and pooled fractions D1–G1 from the Sephacryl S-500 HR column. Lane 3, purified 26S proteasome detected by yeast polyclonal antibodies (anti-20S and five anti-19S proteasome antibodies). (B) Chymotrypsin-like activity of fractions D1–G1 eluted from the Sephacryl S-500 HR column.

tography. This process is less time consuming and simpler than the methods previously published for purification of the 26S proteasome complex [23–25]. Notably, the buffer used is also very simple containing only 10 mM Tris–10 mM NaH_2PO_4, pH 7.5–1 mM ATP. The time taken to prepare the crude fungal extract was similar but the two-step chromatography method reduced the purification time by 50% compared to conventional purification methods. The POROS® HQ column (anion exchange; Cat. No. 1-2332-26, Applied Biosystems) is packed with polystyrenedivinylbenzene particles which provides a dynamic protein-binding capacity (55 mg mL^{-1}, bovine serum albumin, pH 8.0, at 3600 cm h^{-1}) and performs bio-separations 10–100 times faster than conventional chromatography without loss of capacity or resolution (http://products.appliedbiosystems.com). Total protein concentration of purified 26S proteasome sample was 2.7 mg mL^{-1} which reflects the high protein-binding capacity of POROS® HQ column.

1D SDS–PAGE and Western blotting

Purification of the 26S proteasome was confirmed by 1D SDS–PAGE and Western blotting using a yeast 20S antibody and a mixture of six 19S subunit antibodies as shown in Fig. 1. This blot indicated the presence of RPT1, RPT2, RPT3, RPT4, and RPT8 subunits of 19S subunits and all 20S proteasome subunits. All antibodies used detected bands on the blot corresponding to proteins of the expected molecular weight.

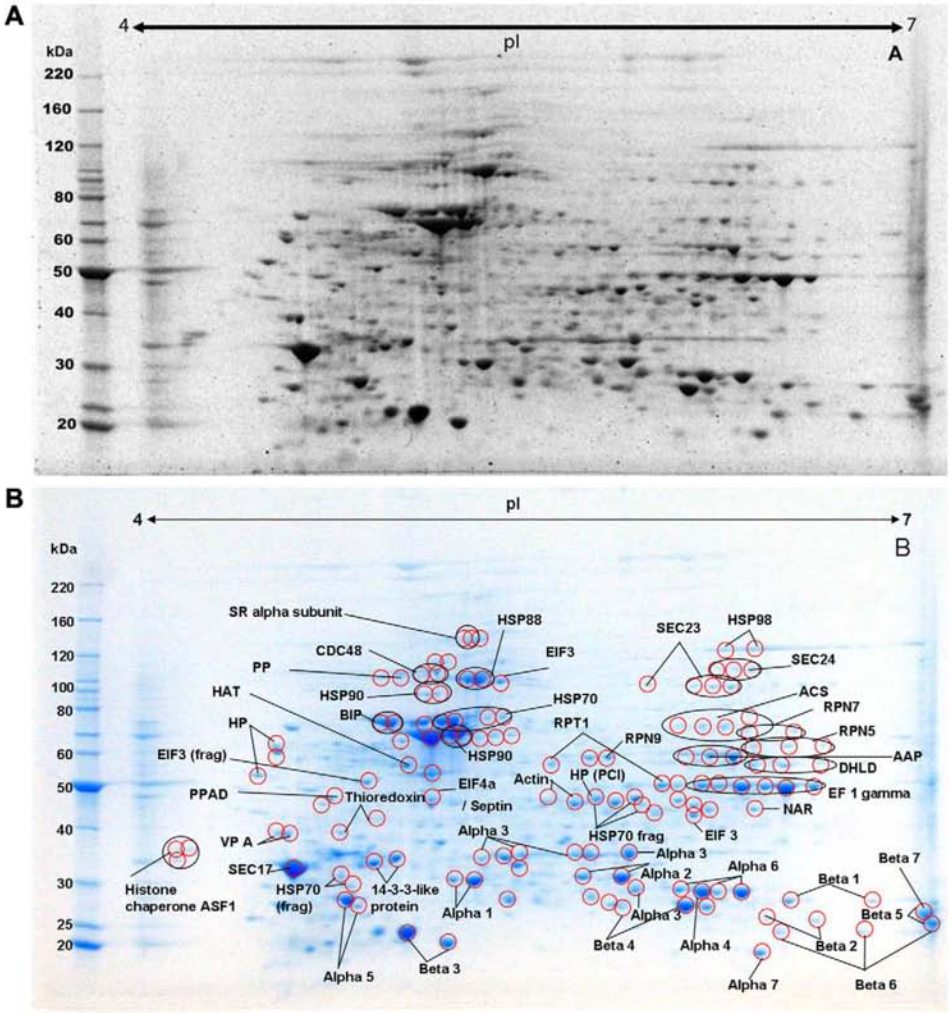

Fig. 2. The proteome map of purified 26S proteasome from *T. reesei* with protein identifications (in B). The 26S proteasome is displayed across an IPG (pH 4–7, 11 cm) in the first dimension and 4–12% SDS–PAGE in the second dimension, stained with stained with Sypro Ruby (A) and Coomassie colloidal blue (B). Protein identification made from MALDI and MS/MS analysis and PMF data compared to all fungal proteins on NCBI non-redundant protein database and in-house *T. reesei* protein database. Abbreviations used on protein maps include EIF, eukaryotic initiation factor; BIP 78 kDa glucose-regulated protein; HSP, heat shock protein; EF, elongation factor; HAT, histone acetyltransferase; CDC, cell division control; HP, hypothetical protein; PPAD, porhyromonas-type peptidyl-arginine deiminase; PP, predicted protein; SR, sulfide reductase; VP, vacuolar protease; ACS, acetyl coA synthetase; DHLD, dihydrolipolyl dehydrogenase; NAR, norsolorinic acid reductase; RPT, regulatory subunit, ATP-ase; RPN non-ATP-ase regulatory subunit. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

Table 1
Proteins identified from the proteasome preparation of *Trichoderma reesei* using an in-house *T. reesei* database and cross-species identification (CSI).

Protein name	Species	Accession No.	MW (Da)	p*I*	No. of peptides matched	Coverage (%)	MALDI or MS/MS	Relation to 26S proteasome
Proteasome α1 subunit	*Trichoderma reesei, Chaetomium globosum* CBS 148.51	6837, gi|116199241	31,645	4.82	16–19	48–57	MALDI and MS/MS	20S proteasome α1 subunit
Proteasome α2 subunit	*T. reesei, Gibberella zeae PH-1*	1126, gi|46117136	32,165	5.7	12–15	33–40	MALDI and MS/MS	20S proteasome α2 subunit
Proteasome α3 subunit	*T. reesei, Saccharomyces cerevisiae*	482, gi|3114275	31,696	4.49	5–10	14–34	MALDI and MS/MS	20S proteasome α3 subunit
Proteasome α4 subunit	*T. reesei, G. zeae PH-1*	6123, gi|46121975	27,835	6.14	4	14	MALDI and MS/MS	20S proteasome α4 subunit
Proteasome α5 subunit	*T. reesei, Coccidioides immitis RS*	2768, gi|119178760	29,090	4.62	6	32	MALDI and MS/MS	20S proteasome α5 subunit
Proteasome α6 subunit	*T. reesei, Botryotinia fuckeliana B05.10*	8317, gi|154296309	27,862	6.16	14–18	28–43	MALDI and MS/MS	20S proteasome α6 subunit
Proteasome α7 subunit	*T. reesei*	8176	29,309	7.61	4	18	MALDI	20S proteasome α7 subunit
Proteasome β1 subunit	*T. reesei, G. zeae PH-1*	6377, gi|46108556	29,171	6.88	9–11	28–33	MALDI and MS/MS	20S proteasome β1 subunit
Proteasome β2 subunit	*T. reesei, Encephalitozoon cuniculi GB-M1*	2077, gi|19173354	31,001	5.16	6–8	21–30	MALDI	20S proteasome β2 subunit
Proteasome β3 subunit	*T. reesei, G. zeae PH-1*	5333, gi|46108708	26,542	5.1	9	30	MALDI	20S proteasome β3 subunit
Proteasome β4 subunit	*T. reesei*	1749	31,939	8.62	4–5	16	MALDI	20S proteasome β4 subunit
Proteasome β5 subunit	*T. reesei*	5857	32,232	6.09	4–8	18–24	MALDI	20S proteasome β5 subunit
Proteasome β6 subunit	*T. reesei, Phaeosphaeria nodorum SN15*	2563, gi|111067693	27,606	5.35	6–10	24	MALDI and MS/MS	20S proteasome β6 subunit
Proteasome β7 subunit	*E. cuniculi GB-M1*	gi|85014261	24,751	6.65	9	36	MALDI	20S proteasome β7 subunit
HSP70 (fragment)	*Hypocrea jecorina, T. reesei*	gi|30961863, 3072	73,319 (fragment)	5.17	3–19	4–32	MALDI and MS/MS	Chaperone, UPR
Vesicular fusion protein sec17	*T. reesei*	194	33,455	4.98	8	27	MALDI	N/A
14-3-3-like protein	*H. jecorina*	gi|12054274	29579	4.79	12–22	46–75	MALDI and MS/MS	PiP
Vacuolar protease A	*T. reesei*	8529	42,404	4.5	4–9	11–15	MALDI and MS/MS	N/A
Thioredoxin	*T. reesei*	4055	35,053	4.4	7–9	25–38	MALDI	PiP
Norsolorinic acid reducatase	*T. reesei*	507	45,783	6.41	12	23	MALDI	N/A
Eukaryotic translation initiation factor 3	*T. reesei*	8266	37,674	6.01	15	35	MALDI	PiP
Hypothetical protein (with PCI domain)	*T. reesei*	5787	48,501	5.01	15	32	MALDI	N/A
Actin	*T. reesei, C. globosum CBS 148.51*	2112, gi|116198005	41,595	5.44	8–15	24–44	MALDI and MS/MS	PiP
Septin	*T. reesei*	2371	43,260	4.74	10	33	MALDI	N/A
Eukaryotic initiation factor 4α	*T. reesei, B. fuckeliana B05.10*	5842, gi|154324134	44,846	5.13	16	35	MALDI and MS/MS	PiP
Eukaryotic initiation factor 3 (fragment)	*T. reesei*	5363	65,026 (fragment)	5.00	20	28	MALDI	PiP
Porphyromonas-type peptidyl-arginine deiminase	*T. reesei*	3630	39,158	4.33	13	44	MALDI	N/A
Hypothetical protein	*T. reesei*	4413	52,066	4.07	7	13	MALDI	N/A
Hypothetical protein	*T. reesei*	3622	55,843	4.12	10	19	MALDI	N/A
Histone acetyltransferase type B subunit 2	*T. reesei*	2072	59,832	4.13	13	29	MALDI	Non-PiP
Hypothetical protein FG11597.1 (RPT1)	*G. zeae PH-1*	gi|46140165	50,864	4.93	7	21	MALDI	19S proteasome ATPase subunit
Probable 26S proteasome regulatory particle chain RPT1	*Neurospora crassa*	gi|11265294	48,972	6.35	10	20	MALDI	19S proteasome ATPase subunit
26S proteasome regulatory subunit RPN9	*S. cerevisiae*	RPN9_YEAST	45,754	5.51	7	20	MALDI	19S proteasome non-ATPase subunit
Elongation factor 1γ	*T. reesei*	5185	45,927	6.53	8–23	27–64	MALDI	PiP
Dihydrolipoyl dehydrogenase	*T. reesei, C. globosum CBS 148.51*	8362, gi|116179598	54,075	7.17	23–26	47–50	MALDI and MS/MS	N/A
Aspartyl aminopeptidase	*T. reesei*	622	54,326	6.03	14–19	26–32	MALDI	N/A
RPN5	*T. reesei, C. globosum CBS 148.51*	4412, gi|116193225	55,532	6.53	7–12	16–23	MALDI and MS/MS	19S proteasome non-ATPase subunit
Hypothetical protein SNAO_01062 (RPN7)	*P. nodorum SN15*	gi|111071437	52,390	6.93	8–10	22–24	MALDI	19S proteasome non-ATPase subunit
Acetyl coA synthetase	*T. reesei, G. zeae PH-1*	318, gi|46105404	81,201	6.82	7–20	12–25	MALDI and MS/MS	N/A
SEC23	*T. reesei, N. crassa OR74A*	5122, gi|157071286	85,729	6.24	5–14	5–18	MALDI and MS/MS	Non-PiP

Table 1 (*continued*)

Protein name	Species	Accession No.	MW (Da)	p*I*	No. of peptides matched	Coverage (%)	MALDI or MS/MS	Relation to 26S proteasome
SEC24	*T. reesei, G. zeae PH-1*	8518, gi|46138569	113,434	6.43	11–12	11–14	MALDI and MS/MS	Non-PiP
HSP98	*T. reesei*	1905	91,807	5.34	7–17	9–21	MALDI	Chaperone, UPR
HSP90	*T. reesei, N. crassa*	2288, gi|6979704	80,012	4.65	29–35	46–48	MALDI and MS/MS	Chaperone, UPR
BIP	*N. crassa OR74A*	gi|85080590	72,287	5.01	16–18	25–27	MALDI and MS/MS	Chaperone, UPR
HSP70	*T. reesei, H. jecorina*	3072, gi|30961863	73,697	4.88	10–30	20–48	MALDI and MS/MS	Chaperone, URP
Eukaryotic translation initiation factor 3	*T. reesei*	1032	85,232	4.83	32	45	MALDI	PiP
HSP88	*T. reesei, N. crassa*	7151, gi|14285475	77,637	4.78	26	35	MALDI and MS/MS	Chaperone, UPR
CDC48 (AAA family of ATPases)	*T. reesei, Magnaporthe grisea 70–15*	3274, gi|39940094	89,647	4.67	24	29	MALDI and MS/MS	Non-PiP
Predicted protein	*T. reesei*	3798	88,771	4.22	11	13	MALDI	N/A
Sulphite reductase α	*T. reesei*	8319	115,208	4.83	20	21	MALDI	N/A
Histone Chaperone ASF1 (anti-silence protein)	*T. reesei, G. zeae PH-1*	8602, gi|46105444	27,911	3.72	4–6	19–30	MALDI and MS/MS	Chaperone, N/A

PiP, proteasome interacting protein.
Non-PiP, known to have interaction with proteasome, but not yet classified as PiP.
UPR, unfolded protein response.
N/A, no applicable proteasome-related domain or function known.

2D SDS–PAGE and protein identification

The purified sample was analysed further by 2-DE over a 4–7 pH range and approximately 200 spots with molecular masses ranging from 20 to 150 kDa were detected with Sypro Ruby staining (Fig. 2A). The 172 most intense spots were excised for mass spectrometry. Overall, 102 spots (as shown in Fig. 2B), corresponding 51% of the total, were identified by MALDI and MS/MS. Fifty-one unique proteins were identified by matching MS data using a combination of two approaches. The first was cross-species identification (CSI) where peptide masses were compared to all known fungal proteins in the non-redundant protein database of the National Centre of Biotechnology Institute using Mascot (http://www.matrixscience.com). The second approach was a custom-made protein database derived from the *T. reesei* sequencing project for peptide mass fingerprinting (PMF) for species-specific protein identification [18]. Forty five protein identifications obtained from the *T. reesei* database and an additional six proteins were identified from CSI. The combined protein identification results are presented in Table 1, which also provides further information on each protein identified including the number of peptides matched, amino acid coverage and its relation to the proteasome.

We were able to identify all seven α and β 20S proteasome subunits by this rapid approach. Four 19S proteasome subunits were identified including RPN5, RPN7, and RPN9, which correspond to lid subunits; in addition, one base subunit, RPT1 was found [24,26]. In general, little is known about the assembly of 19S regulatory particle. The particle is formed from two distinct sub-complexes, the ATPase-containing base and the ubiquitin-recognising lid, which may assemble together at the time the 19S particle has recognised the substrate for degradation and an active 26S proteasome consisting of both the 20S and 19S particles is required. Because of the poorly understood dynamics, the 20S and 19S particles traditionally have been purified separately and assembled *in vivo* [27]. In this work, we have shown that both complexes can be purified by the simple and fast two-column method described.

Other proteins that co-purified with the 20S proteasome include some unfolded protein response (UPR) proteins such as chaperones (HSP70, HSP90, and HSP98) and proteasome-interacting proteins (PiPs) including 14-3-3 like protein, thioredoxin, actin, eukaryotic initiation factor 3 and 4α, elongation factor 1γ and CDC48. These PiPs have been found to co-purify with the 26S proteasome under different affinity-based purifications [12] and also by treatment of yeast cells with formaldehyde to retain the 26S proteasome complex and interacting proteins as complete entities [28].

SEC23, SEC24, septin cell division control (CDC), and histone acetyltransferase were co-purified with the proteasome and identified on the 2DE map. SEC23 and SEC24 are known to play a role in sorting proteins for proteasome degradation [29]. The septin CDC protein is known to associate with E3 ubiquitin ligases in humans suffering from Parkinson's disease [30]. The histone acetyltransferase complex (SAGA) is recruited by the 19S RP to target promoters during gene activation [31] supporting the contention that the 26S proteasome may have further roles beyond proteolytic degradation.

Our research also resulted in the co-purification of nine other proteins that have not been described as associating with the proteasome previously (Table 1). These proteins were co-purified across duplicate proteasome purifications and are likely to have some association with the 26S proteasome. However, their role as PiPs needs to be confirmed by further studies using quantitative analysis of tandem affinity purified *in vivo* cross-linked protein complexes (QTAX) [28].

The 19S regulatory particles in *S. cerevisiae* have a predicted p*I* range of 4.32–5.92, except Rpn6 (p*I* 9.09) [8]. Therefore, a 4–7 pH range should have been suitable to display the 19S RP on the 2D gel (shown in Fig. 2B). The Western blot in Fig. 1 shows the presence of five 19S sub-complex proteins that were extracted and purified. These were the RPT1 (ATPase) of 19S regulatory subunit and three RPNs (non-ATPases) were present also as a spot in 2D gel. The absence of some of 19S RP subunits, as detected by Western blotting, on the 2D map could be explained by the fact that the 19S complex itself is very stable and difficult to dissociate by the methods used for 2DE sample preparation. The sample preparation for Western blotting is more denaturing and the method itself more sensitive in detecting proteins. Accordingly, a high concentration of reducing agent may be required also or reduction times need to be increased several-fold to allow separation of the

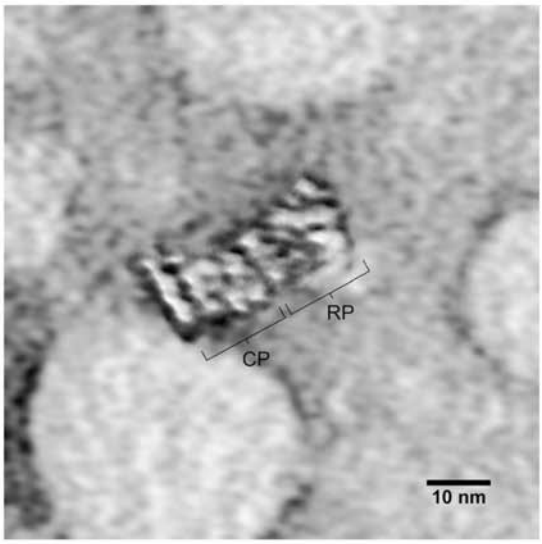

Fig. 3. Transmission electron micrographs of negatively stained *T. reesei* 26S proteasome and subunits. Proteasomes were examined at a magnification of 73,000. In the picture the cylindrical 20S proteasome exhibits four stacked ($\alpha_7\beta_7\beta_7\alpha_7$) rings (CP) and has the 19S RP associated in one end.

subunits of the 19S proteasome. Alternatively, another type of analysis method not requiring application of reducing agents, such as LC MS/MS may be used [28].

TEM

The purified proteasome sample was analysed by electron microscopy after negative staining to investigate the intact structure of *T. reesei* proteasome. Images taken from preparations of the fungal 26S proteasomes showed that the majority of particles represented the 20S proteasome complex alone. However, there were also 20S particles capped at one end by the 19S particle (Fig. 3). Dissociation of 20S proteasome from the 19S complex might be due to the dilution of the sample concentration to a level suitable for microscopy; this action resulted in a lower concentration of ATP than necessary to keep the complex intact [27]. *In vivo*, additional factors probably facilitate the association between the 20S core particle and the 19S regulatory particle. Homology searches showed that the *T. reesei* genome contains as many proteasome genes as *S. cerevisiae*. Therefore, one would expect a similar subunit composition, which is different from the human 11S proteasome complex, for example.

In conclusion, we have developed a rapid and facile purification for the proteasome of *T. reesei*. The proteins associated with the complex have been identified following MALDI-TOF–MS/MS procedures by comparison to the protein databases available and enhanced by comparison with proteins predicted from knowledge of the genomic map of fungus. Most proteins have orthologs in other well-characterized examples such as *S. cerevisiae* and some proteins are associated with the unfolded protein response and co-purified with the proteasome. Electron microscopy allowed the identification of the characteristic structures of the base and lid structures along with core particle as seen in other well-characterized examples. The rapidity of the purification procedure and largely intact nature of the complex suggest that similar procedure may be applicable to the isolation and purification of the other protein complexes.

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Appendix 3.

Table A. Proteins identified by cross species identification from the purified 20S proteasome map of *Trichoderma reesei.*

Protein name	Species	Accession no.	Number of spots identified	MW (Da)	pI	No. of peptides matched	Coverage	Conserved domain present
Conserved hypothetical protein	*Gibberella zeae*	gi|42545254	1	21209	4.98	7	39 %	Proteasome beta
Hypothetical protein	*Yarrowia lipolytica*	gi|50556048	1	23592	5.55	11	43 %	Proteasome beta
Hypothetical protein	*G. zeae*	gi|42553892	1	26488	7.71	7	29 %	Proteasome beta
Hypothetical protein	*Neurospora crassa*	gi|32423127	2	32151	5.68	9-10	35%	Proteasome beta
Hypothetical protein	*Magnaporthe grisea*	gi|39977139	1	29338	7.66	12	36 %	Proteasome alpha
Conserved hypothetical protein	*G. zeae*	gi|42551634	3	27980	5.89	11	39-60 %	Proteasome alpha
Conserved hypothetical protein	*G. zeae*	gi|42544646	3	29147	5.93	6-10	36-56 %	Proteasome beta
Hypothetical protein	*N. crassa*	gi|32410633	1	27917	6.12	14	70%	Proteasome alpha
Hypothetical protein	*N. crassa*	gi|32411629	2	29584	6.92	8-13	36-37 %	Proteasome alpha
Conserved hypothetical protein	*G. zeae*	gi|42552615	4	27913	5.81	9-11	31-45 %	Proteasome alpha
Probable proteasome component C2	*N. crassa*	gi|28949952	1	29020	5.21	9	34 %	Proteasome alpha
Probable proteasome subunit alpha type 2	*G. zeae*	gi|42549948	1	30543	4.99	8	34 %	Proteasome alpha
Hypothetical protein	*N. crassa*	gi|32408341	1	26857	4.85	9	36 %	Proteasome alpha
Hypothetical protein	*G. zeae*	gi|42544315	1	31985	4.71	8	30 %	Proteasome alpha
14-3-3-like protein 2	*Paracoccidioides brasiliensis*	gi|38569374	1	29624	4.68	5	26 %	PIP
14-3-3-like protein	*Hypocrea jecorina*	gi|12054274	1	29579	4.79	14	42 %	PIP
14-3-3-like protein	*H. jecorina*	gi|12054276	1	30404	4.89	8	36 %	PIP
Glyceraldehyde 3-phosphate dehydrogenase	*Trichoderma koningii*	gi|422228	1	35952	6.28	6	18 %	PIP

Ketol acid reductoisomerase	*N. crassa*	gi|18376395	1	44596	8.52	10	28 %	N/A
Actin	*Emericella nidulans*	gi|113277	1	41622	5.64	12	40 %	PIP
Putative serine/threonine phosphatase 2C ptc2	*H. jecorina*	gi|33087518	3	47457	4.57	10-18	34-50 %	UPR
Eukaryotic initiation factor 4α	*N. crassa*	gi|32414453	1	47937	5.9	8	23 %	PIP
Hypothetical protein	*G. zeae*	gi|42551306	1	53193	5.5	16	28 %	N/A
Enolase	*G. zeae*	gi|42545184	1	47367	5.01	6	20 %	PIP
Enolase	*Penicillium chrysogenum*	gi|34392443	2	47207	5.14	5-9	14-21 %	PIP
Hypothetical protein F06655.1	*G. zeae*	gi|42554748	5	53916	5.37	7-10	18-22 %	M18 peptidase and LAP4 domain
78 kDa glucose-regulated protein	*G. zeae*	gi|42553353	1	74524	5.08	15	29 %	UPR / HSP70
78 kDa glucose-regulated protein	*N. crassa*	gi|45645170	1	72287	5.01	14	22 %	UPR / HSP70
Heat shock protein 70	*H. jecorina*	gi|30961863	3	73319	5.17	8-21	18-35 %	HSP70
Heat shock protein 60	*G. zeae*	gi|42550894	1	61430	5.57	12	20 %	HSP60
Hypothetical protein AN0858.2	*Aspergillus nidulans*	gi|67517159	1	103464	5.54	32	29 %	HSP98
Hypothetical protein	*N. crassa*	gi|32402982	2	90515	5.01	26-40	25-47 %	ATPase domain

PIP = Proteasome interacting protein
UPR = unfolded protein response
N/A = No applicable proteasome related domain or function known.

Table B. Proteins identified by matching PMF data to the translated *Trichoderma reesei* cDNA database.

Protein name	Gene reference number	Number of spots identified	MW (Da)	pI	No. of peptides matched	Coverage	Conserved domain present
Proteasome Alpha-1 subunit	6837	7	31645	4.82	7-13	33-49 %	Proteasome alpha
Proteasome Alpha-2 subunit	1126	3	32165	5.7	7-11	23-44 %	Proteasome alpha
Proteasome Alpha-3 subunit	482	6	31696	4.79	6-12	22-40 %	Proteasome alpha
Proteasome Alpha-4 subunit	6123	4	27835	6.14	8-12	31-49 %	Proteasome alpha
Proteasome Alpha-5 subunit	2768	1	29091	4.62	12	45 %	Proteasome alpha
Proteasome Alpha-6 subunit	8317	4	27862	6.16	8-18	31-55 %	Proteasome alpha
Proteasome Alpha-7 subunit	8176	7	29309	7.61	10-17	48-63 %	Proteasome alpha
Proteasome Beta-1 subunit	6377	2	29171	6.88	7-13	27-51 %	Proteasome beta
Proteasome Beta-2 subunit	2077	4	31001	5.16	6-11	27-38 %	Proteasome beta
Proteasome Beta-3 subunit	5333	3	26542	5.1	4-11	24-39 %	Proteasome beta
Proteasome Beta-4 subunit	1749	1	31939	8.62	11	31 %	Proteasome beta
Proteasome Beta-5 subunit	5857	2	32232	6.09	10	34 %	Proteasome beta
Proteasome Beta-6 subunit	2563	4	27607	5.35	5-11	24-43 %	Proteasome beta
14-3-3-like protein	5837	2	29551	4.49	4-9	13-28 %	PIP
Glyceraldehyde-3-phosphate dehydrogenase	168	1	36247	7.14	7	24 %	PIP
Alcohol dehydrogenase	3678	2	37637	6.85	10-11	35-42 %	PIP
Ketol-acid reductoisomerase	4266	1	44749	9.09	11	33 %	N/A
Deoxyhypusine synthase	105	1	39159	5.29	7	21 %	N/A
Transaldolase	1855	2	35584	5.16	6-16	21-48 %	PIP
Actin	2112	1	41595	5.44	8	26 %	PIP
Translation elongation factor	5185	6	45927	6.53	13-21	38-59 %	PIP
Enolase	5647	3	47286	4.84	9-13	29-36 %	PIP
ATP citrate lyase	706	1	52985	5.35	20	37 %	N/A
Aspartyl aminopeptidase	622	8	54325	6.03	14-25	25-43 %	N/A
Histone acetyltransferase	2072	1	59831	4.13	13	25 %	N/A
Histone binding protein	3622	1	55843	4.12	19	43 %	N/A
Heat shock protein 70 kD	3072	2	73697	4.88	9-15	17-25 %	Chaperone
Heat shock protein 70 kD	7312	2	66584	5.03	6-22	17-35 %	Chaperone

Heat shock protein 60 kD	179	2	61105	5.26	16-22	32-43 %	Chaperone
Phosphoglycerate mutase	8591	1	58272	4.82	20	38%	N/A
Transketolase	5330	1	75108	6.21	22	31 %	N/A
SEC23-like protein	5122	1	85729	6.24	17	19 %	Role in proteasomal sorting
Cobalamin-independent methionine synthase	702	2	85621	6.48	21-25	31-34 %	N/A
Heat shock protein 98 kD	1905	1	91807	5.34	35	32 %	Chaperone
ATPase in endoplasmic reticulum (CDC48)	3274	2	89647	4.67	22-35	25-38 %	PIP
Hypothetical protein	1276	1	133773	4.02	18	21 %	N/A

PIP = Proteasome interacting protein

N/A = No applicable proteasome related domain or function known.

Appendix 4.

Proteins identified from the 26S proteasome preparation of *Trichoderma reesei* using in-house *T. reesei* database and cross species identification (CSI).

Protein name	**Species**	**Accession no.**	**MW (Da)**	**pI**	**No. of peptides matched**	**Coverage**	**MALDI or MS/MS**	**Relation to 26S proteasome**
Proteasome alpha 1 subunit	*Trichoderma reesei, Chaetomium globosum CBS 148.51*	6837, gi|116199241	31645	4.82	16-19	48-57%	MALDI and MS/MS	20S proteasome alpha 1 subunit
Proteasome alpha 2 subunit	*T. reesei, Gibberella zeae PH-1*	1126, gi|46117136	32165	5.7	12-15	33-40%	MALDI and MS/MS	20S proteasome alpha 2 subunit
Proteasome alpha 3 subunit	*T. reesei, Saccharomyces cerevisiae*	482, gi|3114275	31696	4.49	5-10	14-34%	MALDI and MS/MS	20S proteasome alpha 3 subunit
Proteasome alpha 4 subunit	*T. reesei, G. zeae PH-1*	6123, gi|46121975	27835	6.14	4	14%	MALDI and MS/MS	20S proteasome alpha 4 subunit
Proteasome alpha 5 subunit	*T. reesei, Coccidioides immitis RS*	2768, gi|119178760	29090	4.62	6	32%	MALDI and MS/MS	20S proteasome alpha 5 subunit
Proteasome alpha 6 subunit	*T. reesei, Botryotinia fuckeliana B05.10*	8317, gi|154296309	27862	6.16	14-18	28-43%	MALDI and MS/MS	20S proteasome alpha 6 subunit
Proteasome alpha 7 subunit	*T. reesei*	8176	29309	7.61	4	18%	MALDI	20S proteasome alpha 7 subunit
Proteasome beta 1 subunit	*T. reesei, G. zeae PH-1*	6377, gi|46108556	29171	6.88	9-11	28-33%	MALDI and MS/MS	20S proteasome beta 1 subunit
Proteasome beta 2 subunit	*T. reesei, Encephalitozoon cuniculi GB-M1*	2077, gi|19173354	31001	5.16	6-8	21-30%	MALDI	20S proteasome beta 2 subunit
Proteasome beta 3 subunit	*T. reesei, G. zeae PH-1*	5333, gi|46108708	26542	5.1	9	30%	MALDI	20S proteasome beta 3 subunit

Proteasome beta 4 subunit	*T. reesei*	1749	31939	8.62	4-5	16%	MALDI	20S proteasome beta 4 subunit
Proteasome beta 5 subunit	*T. reesei*	5857	32232	6.09	4-8	18-24%	MALDI	20S proteasome beta 5 subunit
Proteasome beta 6 subunit	*T. reesei, Phaeosphaeria nodorum SN15*	2563, gi|111067693	27606	5.35	6-10	24%	MALDI and MS/MS	20S proteasome beta 6 subunit
Proteasome beta 7 subunit	*E. cuniculi GB-M1*	gi|85014261	24751	6.65	9	36%	MALDI	20S proteasome beta 7 subunit
HSP70 (fragment)	*Hypocrea jecorina, T. reesei*	gi|30961863, 3072	73319 (fragment)	5.17	3-19	4-32%	MALDI and MS/MS	Chaperone, UPR
Vesicular fusion protein sec17	*T. reesei*	194	33455	4.98	8	27%	MALDI	N/A
14-3-3-like protein	*H. jecorina*	gi|12054274	29579	4.79	12-22	46-75%	MALDI and MS/MS	PiP
Vacuolar protease A	*T. reesei*	8529	42404	4.5	4-9	11-15%	MALDI and MS/MS	N/A
Thioredoxin	*T. reesei*	4055	35053	4.4	7-9	25-38%	MALDI	PiP
Norsolorinic acid reducatase	*T. reesei*	507	45783	6.41	12	23%	MALDI	N/A
Eukaryotic translation initiation factor 3	*T. reesei*	8266	37674	6.01	15	35%	MALDI	PiP
Hypothetical protein (with PCI domain)	*T. reesei*	5787	48501	5.01	15	32%	MALDI	N/A
Actin	*T. reesei, C. globosum CBS 148.51*	2112, gi|116198005	41595	5.44	8-15	24-44%	MALDI and MS/MS	PiP
Septin	*T. reesei*	2371	43260	4.74	10	33%	MALDI	N/A
Eukaryotic initiation factor 4a	*T. reesei, B. fuckeliana B05.10*	5842, gi|154324134	44846	5.13	16	35%	MALDI and MS/MS	PiP

Eukaryotic initiation factor 3 (fragment)	*T. reesei*	5363	65026 (fragment)	5.00	20	28%	MALDI	PiP
Porphyromonas-type peptidyl-arginine deiminase	*T. reesei*	3630	39158	4.33	13	44%	MALDI	N/A
Hypothetical protein	*T. reesei*	4413	52066	4.07	7	13%	MALDI	N/A
Hypothetical protein	*T. reesei*	3622	55843	4.12	10	19%	MALDI	N/A
Histone acetyltransferase type B subunit 2	*T. reesei*	2072	59832	4.13	13	29%	MALDI	Non-PiP
Hypothetical protein FG11597.1 (RPT1)	*G. zeae PH-1*	gi|46140165	50864	4.93	7	21%	MALDI	19S proteasome ATPase subunit
Probable 26S proteasome regulatory particle chain RPT1	*Neurospora crassa*	gi|11265294	48972	6.35	10	20%	MALDI	19S proteasome ATPase subunit
26S proteasome regulatory subunit RPN9	*S. cerevisiae*	RPN9_ YEAST	45754	5.51	7	20%	MALDI	19S proteasome non-ATPase subunit
Elongation factor 1 gamma	*T. reesei*	5185	45927	6.53	8-23	27-64%	MALDI	PiP
Dihydrolipoyl dehydrogenase	*T. reesei, C. globosum CBS 148.51*	8362, gi|116179598	54075	7.17	23-26	47-50%	MALDI and MS/MS	N/A
Aspartyl aminopeptidase	*T. reesei*	622	54326	6.03	14-19	26-32%	MALDI	N/A
RPN5	*T. reesei, C. globosum CBS 148.51*	4412, gi|116193225	55532	6.53	7-12	16-23%	MALDI and MS/MS	19S proteasome non-ATPase subunit
Hypothetical protein SNAO_01062 (RPN7)	*P. nodorum SN15*	gi|111071437	52390	6.93	8-10	22-24%	MALDI	19S proteasome non-ATPase subunit

Acetyl coA synthetase	*T. reesei, G. zeae PH-1*	318, gi|46105404	81201	6.82	7-20	12-25%	MALDI and MS/MS	N/A
SEC23	*T. reesei, N. crassa OR74A*	5122, gi|157071286	85729	6.24	5-14	5-18%	MALDI and MS/MS	Non-PiP
SEC24	*T. reesei, G. zeae PH-1*	8518, gi|46138569	113434	6.43	11-12	11-14%	MALDI and MS/MS	Non-PiP
HSP98	*T. reesei*	1905	91807	5.34	7-17	9-21%	MALDI	Chaperone, UPR
HSP90	*T. reesei, N. crassa*	2288, gi|6979704	80012	4.65	29-35	46-48%	MALDI and MS/MS	Chaperone, UPR
BIP	*N. crassa OR74A*	gi|85080590	72287	5.01	16-18	25-27%	MALDI and MS/MS	Chaperone, UPR
HSP70	*T. reesei, H. jecorina*	3072, gi|30961863	73697	4.88	10-30	20-48%	MALDI and MS/MS	Chaperone, URP
Eukaryotic translation initiation factor 3	*T. reesei*	1032	85232	4.83	32	45%	MALDI	PiP
HSP88	*T. reesei, N. crassa*	7151, gi|14285475	77637	4.78	26	35%	MALDI and MS/MS	Chaperone, UPR
CDC48 (AAA family of ATPases)	*T. reesei, Magnaporthe grisea 70-15*	3274, gi|39940094	89647	4.67	24	29%	MALDI and MS/MS	Non-PiP
Predicted protein	*T. reesei*	3798	88771	4.22	11	13%	MALDI	N/A
Sulphite reductase alpha	*T. reesei*	8319	115208	4.83	20	21%	MALDI	N/A
Histone Chaperone ASF1 (anti-silence protein)	*T. reesei, G. zeae PH-1*	8602, gi|46105444	27911	3.72	4-6	19-30%	MALDI and MS/MS	Chaperone, N/A

PiP = Proteasome interacting protein
Non-PiP = Known to have interaction with proteasome, but not yet classified as PiP.
UPR = Unfolded protein response
N/A = No applicable proteasome related domain or function known.