# α5 subunit in *Trypanosoma brucei* proteasome can self-assemble to form a cylinder of four stacked heptamer rings

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The proteasomes have a central role in catalysing protein degradation among both prokaryotes and eukaryotes. The 20 S proteasome constitutes their catalytic core. In studying the structure of *Trypanosoma brucei* 20 S proteasomes, we isolated by two-dimensional (2D) gel electrophoresis a 27 kDa subunit protein with an estimated pI of 4.7 and subjected it to mass spectrometric analysis. A tryptic peptide sequence from the protein was found identical with that of the rat α5 subunit. With the use of antiserum against *T. brucei* 20 S proteasomes to screen a *T. b. rhodesiense* λ expression cDNA library, we obtained a cDNA clone encoding a full-length protein of 246 amino acid residues with a calculated molecular mass of 27174 Da and a pI of 4.71. It bears 50.0 % and 46.3 % sequence identity with rat and yeast proteasome subunit α5 respectively, and matches all the peptide sequences derived from MS of the 2D gel-purified protein. The protein is thus designated the α5 subunit of *T. brucei* 20 S proteasome (TbPSA5). The recombinant protein, expressed in plasmid-transformed *Escherichia coli*, was found in a 27 kDa monomer form as well as polymerized forms with estimated molecular masses ranging from 190 to 800 kDa. Under the electron microscope, the most highly polymerized forms bear the appearance of cylinders of four-stacked heptamer rings with an estimated outer diameter of 14.5 nm and a length of 18 nm, which were immunoprecipitable by anti-(*T. brucei* 20 S proteasome) antiserum. In view of the documented self-assembly of the archaeon proteasome α subunit into double heptamer rings and the spontaneous assembly of the two α subunits from the 20 S proteasome of *Rhodococcus erythropolis*, the self-assembly of the *T. brucei* α subunit might reflect a common feature of proteasome biogenesis shared by prokaryotes and primitive eukaryotes such as the trypanosomes but apparently lost among the higher forms of eukaryote such as the yeast and the mammals.

Key words: proteasome α subunit, spontaneous assembly, trypanosome.

## INTRODUCTION

Proteasomes have been widely recognized as the major proteases performing highly regulated protein degradation in both prokaryotes and eukaryotes [1–3]. In eukaryotes, the 20 S proteasome is the 'catalytic core' of the 26 S proteasome, which is responsible for the ATP-dependent degradation of ubiquitinated proteins [4,5]. The eukaryotic 20 S proteasome is composed of four stacked heptamer rings in the form $\alpha_7\beta_7\beta_7\alpha_7$, with seven structurally similar but different α subunits and seven distinct β subunits in each of the α- and β-rings respectively [2,6]. In the archaebacterium *Thermoplasma acidophilum*, there is only one type of α and one type of β subunit in the stacked ring structure [7,8]. The N-terminal threonine residues of the β subunit in the *Th. acidophilum* 20 S proteasome act as the nucleophile in catalysing the hydrolysis of peptide bonds in the polypeptide substrates [8,9]. Expression of the *Th. acidophilum* proteasome α subunit in *Escherichia coli* revealed that this subunit can polymerize spontaneously in its purified form into pairs of heptamer rings, whereas the pro-β-subunit protein expressed in *E. coli* by itself is present only in the monomeric form [10]. Co-expression of these two subunits in *E. coli* resulted in the correct N-terminal processing of the β subunits, and the subsequent assembly and formation of the functional 20 S proteasome [10,11]. Thus spontaneous formation of the α-rings is most probably the first pivotal step in the assembly of 20 S proteasomes in *Th. acidophilum*. A similar 20 S proteasome was also identified in the actinomycete *Rhodococcus erythropolis* [12]. It is constituted of two α-type and two β-type subunit proteins and can be assembled spontaneously and efficiently *in vitro* with any combination of an α subunit and a β subunit [13]. Apparently, each of the two α subunits from *R. erythropolis* can form α-rings spontaneously, either on its own or in combination with the other α subunit. It is therefore tempting to postulate that, among the relatively simple proteasomes from prokaryotes, self-assembly among the α subunits could provide the initial driving force for proteasome biogenesis.

Several steps in the assembly of eukaryotic 20 S proteasomes have been revealed. A 13–16 S intermediate protein complex containing most of the α-type subunits and some β-type subunits, apparently a half proteasome, has been identified in yeast and humans [7,14–18]. The assembly of these intermediates into a 20 S proteasome is accompanied by proteolytic processing of three of the β-type subunits for proteolytic activity [6]. A box of approximately 25 amino acid residues at the N-terminus, highly conserved among all the α-type subunits, is apparently essential for α-ring formation. However, essentially none of the eukaryotic α subunits have been found capable of self-assembly except for the recombinant human 20 S proteasome α subunit HsC8 (α7), which was recently found to form a complex of approx. 540 kDa,

Abbreviations used: 2D, two-dimensional; DE, delayed extraction; EST, expressed sequence tag; MALDI, matrix-assisted laser desorption ionization; PSD, post-source decay; TOF, time-of-flight.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF140353.

consisting of double heptamer ring structures [19]. The role of this HsC8 ring structure in the assembly process is apparently different from that of the *Th. acidophilum* $\alpha$ subunit, because the HsC8 ring does not form a 20 S complex with the recombinant human $\beta$ subunits [19]. However, the co-expression of HsC8 with either of its two naturally neighbouring $\alpha$ subunits, HsC2 ($\alpha$6) and HsIota ($\alpha$1), which do not form ring-like complexes by themselves *in vitro*, results in the formation of hetero-oligomeric ring complexes resembling the HsC8 ring-like structure [20]. HsC8 might therefore be one of the $\alpha$ subunits that has, like the $\alpha$ subunits from *Th. acidophilum* and *R. erythropolis*, retained the capability of self-assembly and might provide the impetus for $\alpha$-ring formation in the human proteasome.

We have recently isolated and characterized the 20 S proteasome [21] and an activated 20 S proteasome species [22] from both the bloodstream form and the procyclic form of *Trypanosoma brucei*, a protozoan pathogen causing African sleeping sickness in man, and nagana in cattle [23]. *T. brucei* is apparently far removed from either human or yeast in the phylogenetic pedigree, and can therefore be regarded as a relatively primitive eukaryote [24]. It will be interesting to use this primitive eukaryote as a model for dissecting the mechanism of its 20 S proteasome assembly. A main interest will be focused on whether its $\alpha$ subunits are, like the bacterial counterparts, capable of self-assembly or, alternatively, resemble the $\alpha$ subunits of the more advanced eukaryotes, most of which are incapable of self-assembling. The purified *T. brucei* 20 S proteasome exhibits 14 major and seven minor proteins in two-dimensional (2D) gel electrophoresis [25], suggesting that the 14 major proteins might represent seven distinctive $\alpha$ subunits and seven distinctive $\beta$ subunits. For three of the major proteins, partial peptide sequences were determined by MS; full-length encoding genes were recently cloned and sequenced [25]. The sequence data show that these are an $\alpha$6-type, a $\beta$1-type and a $\beta$2-type proteasome subunit protein. The gene encoding the $\alpha$6-type protein, *T. brucei* proteasome subunit $\alpha$6 (*TbPSA6*), was recently expressed in transformed *E. coli* cells and found to polymerize spontaneously [25], suggesting that it might share this property of a bacterial $\alpha$ subunit. In the present study, we tried to identify one or more other $\alpha$ subunits in the *T. brucei* 20 S proteasome that might bear the closest resemblance to the $\alpha$ subunit in *Th. acidophilum* and to examine its potential role in proteasome biogenesis in *T. brucei*. We started out by pursuing the $\alpha$5 counterpart, because among the $\alpha$ subunits in eukaryotes the sequence of $\alpha$5 most closely resembles that of the $\alpha$ subunit of the 20 S proteasome from *Th. acidophilum*. Although there has so far been no specific report on the potential self-assembly of mammalian $\alpha$5 subunits, their relatively high percentage in monomeric forms in mammalian cells might argue against such a possibility [2]. It would make an examination of possible *T. brucei* $\alpha$5 self-assembly interesting.

The subunit from the 20 S proteasome of *T. brucei* with the lowest pI value (4.7), a common feature among all the $\alpha$5 subunits [14,25,27], was isolated from a 2D gel and subjected to mass spectrometric analysis. The protein was shown to possess peptide sequences similar to those in the other eukaryotic $\alpha$5-subunit proteins. A partial cDNA apparently encoding the same protein was previously identified in a screen of expressed sequence tags (ESTs) from a *T. b. rhodesiense* cDNA expression library [28]. Using a rabbit antiserum against *T. brucei* 20 S proteasome [22] to screen the same library, we succeeded in isolating a full-length cDNA encoding the *T. brucei* $\alpha$5 homologue. The recombinant $\alpha$5 homologue, designated TbPSA5, expressed and isolated from transformed *E. coli*, demonstrated spontaneous self-assembly into four stacked heptamer rings, thus suggesting for $\alpha$5 the potential role of one of the initiators in the 20 S proteasome assembly in *T. brucei*.

## MATERIALS AND METHODS

### Materials

*T. brucei* strain 427 procyclic-form and bloodstream-form cells were cultivated and harvested as described previously [21]. Immobilon-P PVDF membrane was from Millipore. Molecular mass standards for SDS/PAGE were a group of broad-range protein markers from Bio-Rad. Molecular mass markers for calibrating the gel-filtration column were purchased from Sigma. The rabbit antiserum against purified *T. brucei* 20 S proteasome was prepared by Animal Pharm Service (Healdsburg, CA, U.S.A.) and characterized in our previous studies [22]. Horseradish peroxidase-conjugated donkey antiserum against rabbit IgG, the random primer labelling system and Redivue™ [$\alpha$-$^{32}$P]dCTP were from Amersham. Reagents for electrophoresis were obtained either from Sigma or Amersham Pharmacia Biotech. All HPLC-grade solvents were obtained from Fisher. The rest of the chemicals used in the present study were all of the highest purity commercially available.

### Protein in-gel digestion and mass spectrometric analysis of the tryptic peptides

Protein spots were identified and excised from silver stained 2D gels and digested (details are available from the authors). In brief, minced gels were washed with 25 mM $NH_4HCO_3$ and 50 % (v/v) acetonitrile, dried in a Speedvac and rehydrated in 25 mM $NH_4HCO_3$ solution containing trypsin (12.5 ng/$\mu$l) and incubated overnight at 37 °C. Peptides were extracted by washing with HPLC-grade water followed by three washes in 50 % (v/v) acetonitrile/5 % (v/v) trifluoroacetic acid. The combined supernatants were dried with a Speedvac and redissolved in 50 % (v/v) acetonitrile/5 % (v/v) trifluoroacetic acid before analysis of the unseparated digest by MS. The peptide extracts were further separated by reverse-phase HPLC on a Vydac microbore $C_{18}$ column (1.0 mm × 15 cm). Each of the HPLC fractions was collected, concentrated and analysed by MS.

### Molecular mass measurements on unseparated and separated tryptic digests

The molecular masses of the tryptic peptides were determined by analysing 1/20 of the unfractionated digest and 1/10 of each HPLC fraction with a matrix-assisted laser desorption ionization (MALDI) delayed extraction (DE) reflection time-of-flight (TOF) instrument (Voyager-DE STR Biospectrometry Workstation; PerSeptive Biosystems, Framingham, MA, U.S.A.) equipped with a nitrogen laser (337 nm), which has a typical mass resolution ($M/\Delta M$) of approx. 8000. Peptides were co-crystallized with equal volumes of matrices consisting of a saturated solution of $\alpha$-cyano-4-hydroxycinnamic acid in 50 % (v/v) acetonitrile/1 % (v/v) trifluoroacetic acid. All MALDI spectra were internally calibrated with trypsin autolysis products to obtain accurate monoisotopic masses of all the tryptic peptides (less than 20 p.p.m.). Peptide masses were submitted for protein mass database searching.

### Peptide sequencing *de novo* by MALDI–post-source decay (PSD)-DE

The peptides displaying the highest pseudomolecular ion abundance in MALDI spectra of the HPLC fractions were subjected to PSD analysis on the same MALDI instrument to determine

the peptide sequences. All the PSD spectra were interpreted manually; a detailed description will be reported elsewhere and is available from the authors on request.

### Screening the cDNA library with anti-(*T. brucei* 20 S proteasome) antiserum

A *T. b. rhodesiense* cDNA λ expression library was obtained from Dr. John Donelson (University of Iowa, Iowa City, IA, U.S.A.). In their previous generation of ESTs from randomly chosen clones of this cDNA library, el-Sayed et al. [28] identified an EST no. 68676 (accession no. S17521) that produced the highest-scoring segment pair with the human multicatalytic endopeptidase complex ζ chain, i.e. the human 20 S proteasome α5 subunit [30]. The EST has a sequenced length of 68 nt and bears a 58.8 % sequence identity and 79.4 % sequence similarity to the corresponding human cDNA segment.

Approx. $4 \times 10^4$ plaque-forming units of the expression library were plated with *E. coli* Y1090 cells on 150 mm agar plates; a total of approx. $8 \times 10^5$ phage clones were screened with the rabbit antiserum against *T. brucei* 20 S proteasome. The plaques were overlaid with isopropyl β-D-thiogalactoside-soaked nitrocellulose filters to induce expression. The filters were screened with the antiserum and stained with anti-rabbit IgG coupled to alkaline phosphatase (Promega). Positive plaques were isolated and the screening was repeated until the clones were purified to homogeneity. The phages were converted to plasmid DNA by the Rapid Excision Kit (Stratagene), and the plasmid clones were sequenced with an automated sequencer (ABI Prism Version 2.1.1).

### Genomic Southern blotting

The genomic DNA from the procyclic forms of *T. brucei* was isolated as described [31]. A 3.5 μg sample of purified genomic DNA was digested with various restriction enzymes and subjected to electrophoresis on a 0.8 % agarose gel. The digested DNA sample was transferred to Immobilon-P PVDF membrane [32], prehybridized for 2 h at 42 °C in 5 × Denhardt's solution containing 5 × SSC (SSC is 0.15 M NaCl/15 mM sodium citrate), 50 % (v/v) formamide, 0.1 % SDS, 0.1 mg/ml denatured, fragmented salmon-sperm DNA, then hybridized overnight at 42 °C in fresh hybridization solution containing $10^6$ d.p.m./ml [α-$^{32}$P]dCTP-labelled DNA probe. The membrane was washed twice at room temperature for 15 min with SSC containing 0.1 % SDS, once at 42 °C for 15 min with 0.1 × SSC containing 0.1 % SDS and once at 65 °C for 15 min with 0.1 × SSC containing 0.1 % SDS, before autoradiography overnight at −70 °C.

### Expression of recombinant protein and fractionation of cell lysate

The full-length cDNA encoding the α5 subunit of *T. brucei* 20 S proteasome was amplified by PCR with two primers, one with a flanking *Nde*I site and the other with a flanking *Xba*I site. The PCR fragment was ligated to the *Nde*I/*Xba*I-digested plasmid pBAce [33] to make the new construct pBAα5tb.

*E. coli* Sφ606 cells [34] transformed with pBAα5tb were grown in low-phosphate medium to a $D_{600}$ of approx. 1.6. The bacterial cells were harvested by centrifugation at 3000 ***g*** for 20 min. The cell pellets were resuspended in 1/40 vol. of 5 × TSD buffer [TSD is 10 mM Tris/HCl (pH 7.4)/25 mM KCl/10 mM NaCl/1 mM $MgCl_2$/0.2 mM EDTA/1 mM dithiothreitol] containing 1 mM tosyl-lysylchloromethane ('TLCK') and 1 mM PMSF. After a brief sonication, the crude lysate was centrifuged at 10000 ***g*** for 20 min and divided into two fractions by Amicon-100 filtration: a high-molecular-mass fraction (more than 100 kDa) and a low-molecular-mass fraction (less than 100 kDa). Each fraction was further filtered through a calibrated Superose-12 HPLC column. The fractions collected from the column were analysed by Western immunoblotting with the rabbit antiserum against *T. brucei* 20 S proteasome [22].

### Electrophoresis, immunoblotting and immunoprecipitation

SDS/PAGE [12.5 % (w/v) gel] was performed as described [21]. For 2D gel electrophoresis, a 10 μg sample of purified *T. brucei* 20 S proteasome was rehydrated overnight in 400 μl of rehydration buffer [9 M urea/4 % (w/v) CHAPS/65 mM dithiothreitol/1:50 Pharmalyte (pH 3–10)] at room temperature. It was then subjected to isoelectric focusing electrophoresis in a Multiphor II system with an EPS 3500 XL electrophoresis power supply (Amersham Pharmacia Biotech). The gel was run with the following voltage gradient programme: 0–300 V, 300 V·h; 300–1000 V, 1000 V·h; 1000–3500 V, 3500 V·h; 3500 V, more than 50 kV·h. The gel strip containing the proteins was equilibrated in equilibration buffer [50 mM Tris/HCl (pH 6.8)/1 % (v/v) SDS/35 % (v/v) glycerol] containing 1 % (v/v) dithiothreitol for 10 min, and then in the same buffer containing 1.4 % (w/v) iodoacetamide for a further 10 min. The equilibrated gel strip was loaded on a second-dimension SDS/11 % (w/v) polyacrylamide gel. After electrophoresis, the bands were revealed by staining with silver. For immunoprecipitation, the salt concentration in protein samples was maintained at 50 mM and the protein sample was incubated with anti-(*T. brucei* 20 S proteasome) antiserum (1:100 dilution) at 4 °C for 2 h. A protein A–Sepharose bead (20 μl) was added and incubated for a further 1 h with intermittent inversions of the tube to resuspend the Sepharose. The recombinant α5 protein remaining in the supernatant was monitored by immunoblotting with the same antiserum as described previously [22].

### Electron microscopy and image averaging

Protein samples were negatively stained with 1 % (w/v) uranyl acetate and imaged in a Philips (Eindhoven, The Netherlands) EM400 transmission electron microscope at a magnification of × 70000. Enlarged prints of selected micrographs were digitized in a flat-bed scanner at a resolution of 260 dpi. Digitized images were ported to a Silicon Graphics (Mountainview, CA, U.S.A.) workstation for processing in the SUPRIM image-processing environment [35]. Particles were extracted from the digitized micrograph images by eye; the criteria for selection were a symmetrically round shape and the absence of debris. A total of 22 particles were excised; one particle was arbitrarily chosen as the initial reference. The other particles were aligned to the reference by cross-correlation, then the aligned particles were averaged together [36]. Two more averaging iterations were performed, each using the average from the previous round. The final average was symmetrized seven-fold to emphasize the symmetry of the averaged image. In contrast, six-fold, eight-fold and five-fold symmetrization smeared the density peaks that were visible in the unsymmetrized average.

## RESULTS

### 20 S proteasome from *T. brucei* is likely to contain seven distinctive α subunits and seven distinctive β subunits

In our previous study, approximately eight visible protein bands, ranging from 23 to 34 kDa, were observed in the SDS/PAGE analysis of purified 20 S proteasomes from the procyclic forms and the bloodstream forms of *T. brucei* [21,22]. For a more

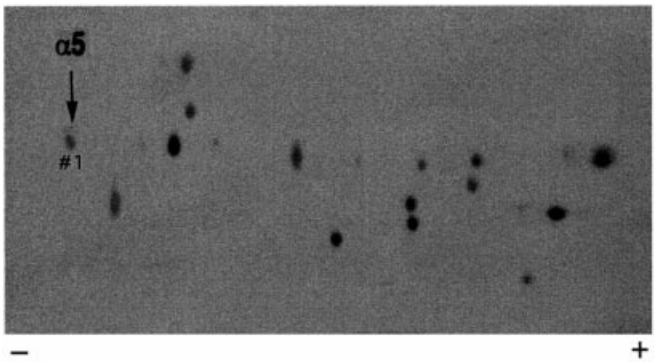


**Figure 1 Two-dimensional gel electrophoresis of purified *T. brucei* 20 S proteasome**

Approx. 10 $\mu$g of protein was subjected to two-dimensional gel electrophoresis followed by staining with silver. The $\alpha$5 subunit (spot 1) is indicated with an arrow.

thorough analysis of the subunit composition of the *T. brucei* 20 S proteasome, 2D gel electrophoresis of highly purified *T. brucei* 20 S proteasomes was performed [21]. The *T. brucei* 20 S proteasome reproducibly exhibited 14 major and seven minor protein spots on the 2D gel (Figure 1). These results suggest that there are most probably seven distinctive $\alpha$ subunits and seven distinctive $\beta$ subunits in the 20 S proteasome of *T. brucei* that could be represented by the 14 major protein spots. The pattern of the 14 putative subunit proteins in *T. brucei* 20 S proteasome is quite different from that of the 14 major subunit proteins in the rat 20 S proteasome [25] but shows some similarity to that of the yeast 20 S proteasome in 2D gel electrophoresis [14]. The identity and potential significance associated with the seven minor protein spots in the 2D gel remain unclear at present. One possibility is that they might be the isoforms of some of the regular 20 S proteasome subunits that might be induced and expressed only under certain specific conditions [37]. Further investigations will be necessary to test this conjecture.

**Table 1 Percentage identities between $\alpha$5 subunits of proteasome from different sources**

| | Rat | Human | *S. cerevisiae* | *Th. acidophilum* |
|---|---|---|---|---|
| *T. brucei* | 51.0 | 51.0 | 46.3 | 37.0 |
| Rat | | 98.3 | 56.9 | 38.6 |
| Human | | | 61.0 | 35.6 |
| *S. cerevisiae* | | | | 36.4 |

## Identification and designation of the $\alpha$5 subunit from *T. brucei* 20 S proteasome by MS

Because the single $\alpha$ subunit present in the 20 S proteasome of the archaeon *Th. acidophilum* is capable of spontaneous self-assembly, a search for its closest counterpart in the *T. brucei* 20 S proteasome could be useful in identifying the $\alpha$ subunit involved in initiating the assembly of the *T. brucei* proteasome. The rat, human and yeast 20 S proteasome $\alpha$5 subunits bear the highest sequence identities with the *Th. acidophilum* $\alpha$ subunit, though none has been shown to be involved in initiating 20 S proteasome assembly [38]. The rat, human and yeast $\alpha$5 subunits have the lowest pI values (4.6, 4.5 and 4.47 respectively) in comparison with the rest of the proteasome subunits [14,25,27]. This drew our attention to the most acidic protein on the 2D gel in Figure 1, spot 1, which had a pI of 4.7. Spot 1 protein, as shown in Figure 1, was excised from the gel and digested with trypsin. The tryptic peptides of spot 1 protein were analysed by

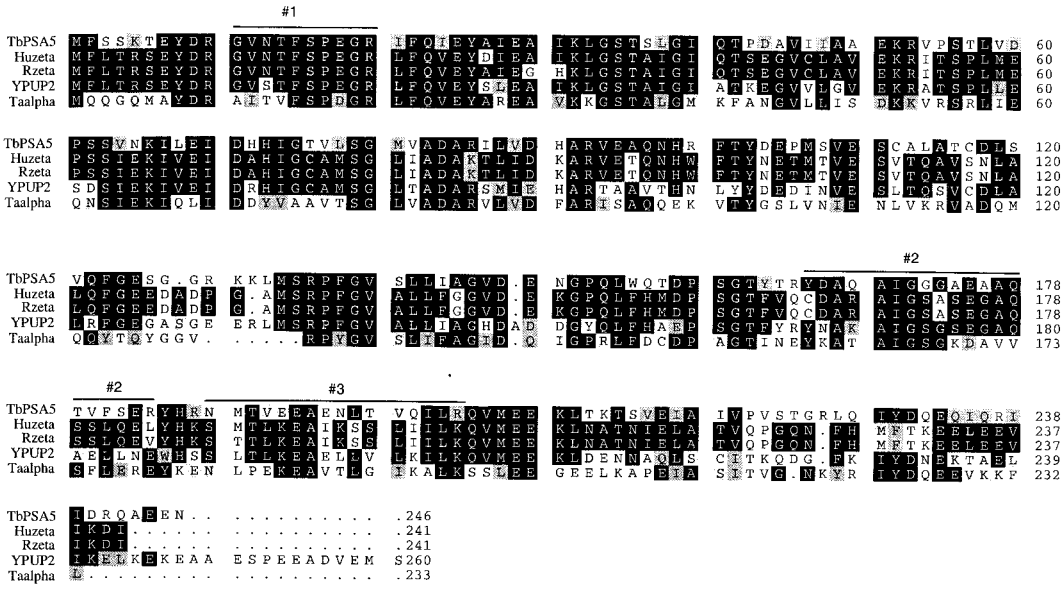


**Figure 2 Multiple sequence alignments between eukaryotic $\alpha$5 proteasomal subunit proteins**

TbPSA5, *T. brucei* $\alpha$5 subunit; Huzeta, human $\alpha$5 subunit (accession no. S17521); Rzeta, rat $\alpha$5 subunit (accession no. P34064); YPUP2, yeast $\alpha$5 subunit (accession no. P32379); Taalpha, *Th. acidophilum* $\alpha$-chain subunit (accession no. X59507). The sequences of the three peptides in TbPSA5 determined by MS are indicated by horizontal bars at the top of its sequence. The ClusterW multiple sequence alignment program from the Baylor College of Medicine was used via the BCM Web Server.

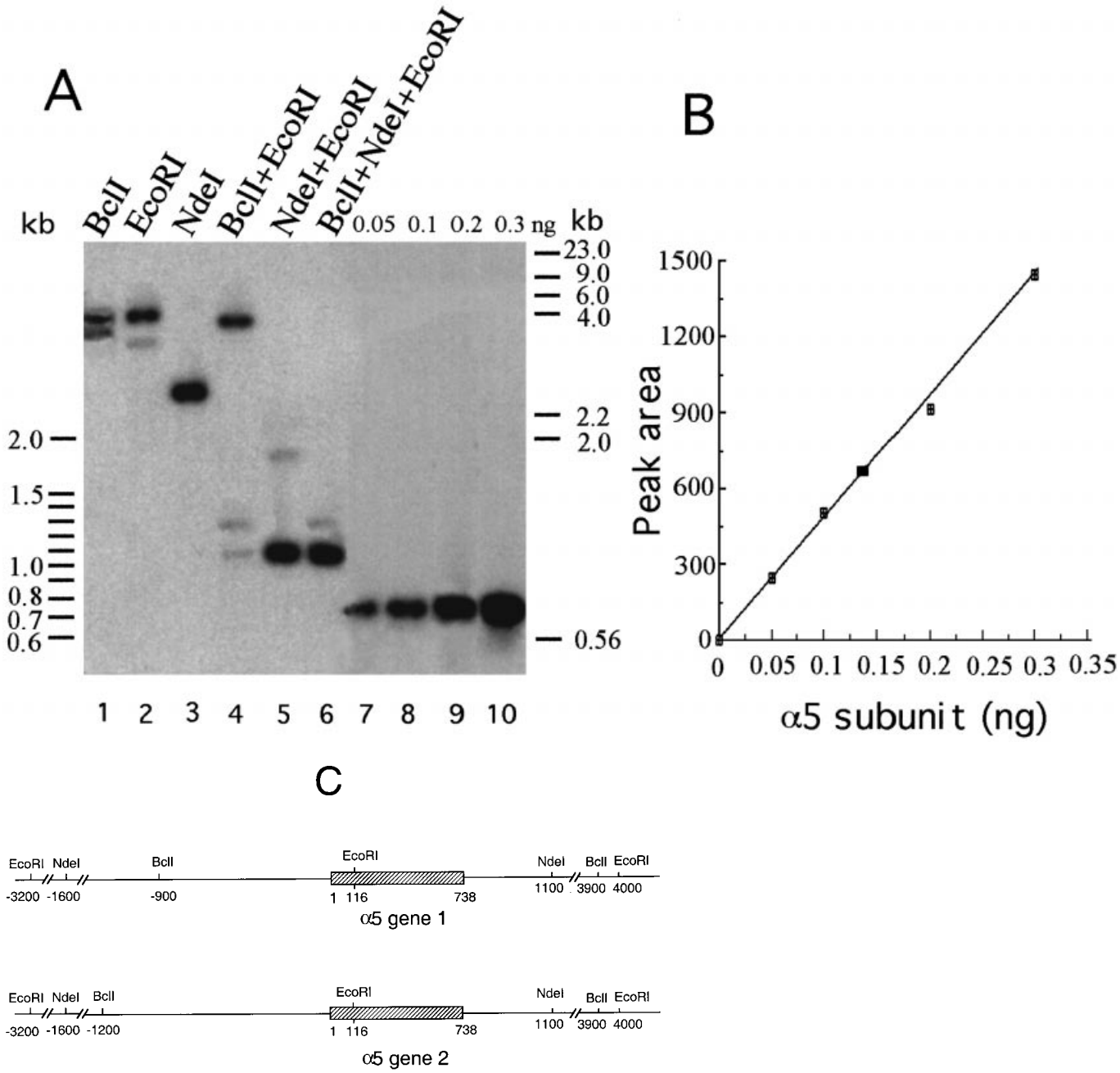


**Figure 3 Determination of the copy number of the α5 gene in *T. brucei* by genomic Southern blotting**

The genomic DNA (3.5 μg) of *T. brucei* was digested with different restriction enzymes and subjected to electrophoresis on a 0.8% agarose gel. The DNA bands were then transferred to an Immobilon-P PVDF membrane and hybridized with a $^{32}$P-labelled probe generated from the 738 bp TbPSA5 full-length gene by random priming (**A**). Various quantities of the 738 bp full-length TbPSA5 genomic DNA were run in the same agarose gel and blotted to the same membrane as the standards from lanes 7–10 in (**A**). The positions of molecular mass markers are indicated. The hybridization intensities of the bands of TbPSA5 genomic DNA of different quantities from lanes 7–10 were traced with an LKB densitometer and plotted against the original quantities of the samples applied to the agarose gel to construct a standard curve (**B**). The hybridization intensity of the 2.7 kbp band in the *Nde*I digest in lane 3 of (**A**) was traced and quantified on the standard curve in (**B**) (the solid square symbol). The result indicates 135 pg of hybridizing DNA in 3.5 μg of total genomic DNA in the sample (see the text). The results from (**A**) were analysed, resulting in a tentative restriction map of two similar but distinctive TbPSA5 genes, 1 and 2, shown in (**C**). The open reading frames are represented as hatched boxes in (**C**).

MS. Peptide mass values obtained from MALDI–TOF-MS were employed for protein database searching with MS-Fit [39]. The protein was identified as the counterpart of the rat proteasome α5 subunit. To confirm the protein identity, MALDI–PSD–DE was performed to obtain peptide sequence information. One of the tryptic peptides with $MH^+$ at 1063.5136 had the sequence (single-letter codes) of GVNTFSPEGR, identical with that of peptide 11-20 in the rat α5 protein (see Figure 2). Two other peptide sequences in the spot 1 protein were also obtained from peptide sequencing by *de novo* MALDI–PSD–DE. They were NMTVEEAEN[I/L]TVQ[I/L][I/L]R and YDA[Q/K]A[I/L]-GGGAEAA[Q/K]TVFSER (see Figure 2). These sequences were

aligned with the α5 sequences from rat, human and yeast (Figure 2) and demonstrated significant sequence similarity to each of them. These results suggest that the spot 1 protein is an α5 counterpart in the 20 S proteasome from *T. brucei*.

### Cloning of full-length cDNA encoding the α5 subunit counterpart in *T. brucei* 20 S proteasome

To verify whether the spot 1 protein is a genuine α5 subunit of *T. brucei* 20 S proteasome, we used a rabbit antiserum against the purified *T. brucei* 20 S proteasome [22] to screen a *T. b. rhodesiense* expression cDNA λ library. A full-length cDNA encoding a *T. brucei* α5 counterpart was identified and isolated. This cDNA consisted of an open reading frame coding for 246 residues with a calculated molecular mass of 27174 Da and a predicted pI of 4.71. It contained the entire sequence of EST no. 68676, originally found to produce the highest-scoring segment pair with the rat 20 S proteasome α5 subunit [40] (see Figure 2). It also contained all of the peptide sequences identified in the spot 1 protein by MS (Figure 2). Alignment of this protein sequence with those of rat, human and yeast α5 proteins as well as the *Th. acidophilum* α subunit protein showed high sequence similarities (Figure 2). It bore 50%, 51%, 46.3% and 37% sequence identities with the rat, human and yeast α5 subunit and the *Th. acidophilum* α subunit respectively (Table 1). There is therefore little doubt that the full-length cDNA clone encoded the 20 S proteasome α5 subunit protein of *T. brucei* and that the spot 1 protein identified in Figure 1 was the α5 subunit protein. This protein has been designated TbPSA5 (for *Trypanosoma brucei* proteasome subunit α5) [25].

### There are two copies of the α5 gene in *T. brucei*

The number of α5 gene copies in *T. brucei* was determined by genomic Southern blotting with the full-length α5 cDNA as a probe (Figure 3). Genomic DNA of the procyclic form of *T. brucei* was digested with *Bcl*I, *Eco*RI, *Nde*I, *Bcl*I/*Eco*RI, *Nde*I/*Eco*RI or *Bcl*I/*Nde*I/*Eco*RI. The full-length cDNA open reading frame of 738 bp was used as a hybridization probe. Within the 738 bp region, there is an *Eco*RI site at nt 116, whereas no *Bcl*I or *Nde*I site is present in the 738 bp stretch (Figure 3C). However, two hybridization bands of 3.5 and 3.8 kbp were observed in the *Bcl*I digest (Figure 3A, lane 1), suggesting that there might be two copies of the α5 gene with different flanking regions in the *T. brucei* genome. There was a strong hybridization band of 3.9 kbp and a much weaker band of 3.3 kbp in the *Eco*RI digest (Figure 3A, lane 2). Assuming that the weaker hybridization could be attributed to the shorter hybridizing DNA segment (116 bp) in the 3.3 kbp fragment, the latter might correspond to a piece of the α5 gene between −3200 and +116, whereas the 3.9 kbp fragment might represent the portion of the α5 gene from +117 to +4000. This 3.9 kbp *Eco*RI fragment was decreased to 3.8 kbp in the *Eco*RI/*Bcl*I double digest (Figure 3A, lane 4), indicating that the downstream *Bcl*I site might be closer to the 3′ end of the open reading frame than the *Eco*RI site by approx. 100 bp and that the two α5 genes might not differ in the positions of *Eco*RI and *Bcl*I sites on the downstream side. However, the upstream 3.3 kbp *Eco*RI fragment is digested by *Bcl*I to yield a 1.0 kbp and a 1.3 kbp fragment, which could mean a *Bcl*I site at position −900 in one α5 gene (gene 1) and at −1200 in the other gene (gene 2) (see Figure 3C). The *Nde*I digest contained one hybridization band of 2.7 kbp, whereas the *Nde*I/*Eco*RI digest consisted of a strong 1.0 kbp and a weak 1.7 kbp band, suggesting the presence of two *Nde*I sites at positions −1600 and +1100 respectively. This weak 1.7 kbp *Nde*I/*Eco*RI band was further decreased to 1.3 kbp in the *Nde*I/*Eco*RI/*Bcl*I digest, thus confirming the presence of a *Bcl*I site at position −1200 in gene 2. (A second predicted 1.0 kbp weakly hybridizing band produced by *Nde*I/*Eco*RI/*Bcl*I digestion of gene 1 was presumably hidden by the more strongly hybridizing downstream 1.0 kbp band with *Nde*I/*Eco*RI termini at positions 116 and 1100.) The restriction map of the two α5 genes in *T. brucei* inferred from these results is depicted in Figure 3(C).

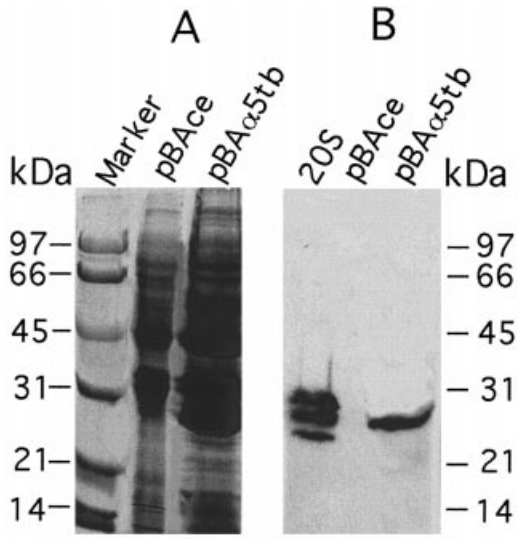


**Figure 4 SDS/PAGE and immunoblotting of TbPSA5**

*E. coli* cells transformed with pBα5tb were lysed by sonication in 5 × TSD buffer. The crude lysate was subjected to SDS/PAGE [12.5% (w/v) gel]. The separated protein bands were either stained with Coomassie Blue (**A**) or transferred to PVDF membrane and immunostained with anti-(*T. brucei* 20 S proteasome) antisera (**B**). Protein size markers are demonstrated on both sides of the figure. The purified 20 S proteasome from *T. brucei* stained with the antiserum is labelled 20 S in (**B**). The molecular masses of markers are indicated.

The copy number of the α5 gene in the genome of *T. brucei* was also determined by titrating the 738 bp α5-encoding cDNA fragment on the same genomic Southern blot with the bulk DNA digests (Figure 3A). The standard curve thus constructed from the quantitative data via densitometer (LKB) tracing of the hybridization bands in lanes 7–10 in Figure 3(A) is presented in Figure 3(B). The quantity of α5 cDNA in the *Nde*I fragment in lane 3 of Figure 3(A) was estimated to be 135 pg (out of 3.5 μg of total genomic DNA) on the standard curve in Figure 3(B). Because the haploid genome size of *T. brucei* is estimated to be $4 \times 10^7$ bp [41], the copy number for the α5 gene in a haploid genome of *T. brucei* can be calculated as 135 pg/3.5 μg × $4 \times 10^7$/738, which is 2.1, a number in good agreement with that inferred from the Southern blot mapping data.

### Recombinant α5 subunit protein forms large complexes spontaneously

Because the recombinant α subunit of the *Thermoplasma* 20 S proteasome has been found capable of spontaneous self-assembly *in vitro* [10,19], we also tried to monitor the potential self-assembly of the recombinant *T. brucei* 20 S proteasome α5 subunit protein. The coding region of the α5 cDNA from *T. brucei* was ligated into the expression vector pBAce to make a new plasmid pBAα5tb. *E. coli* Sϕ606 cells transformed with pBAα5tb were found to express a recombinant protein of approx. 27 kDa (Figure 4A). The antiserum against *T. brucei* 20 S

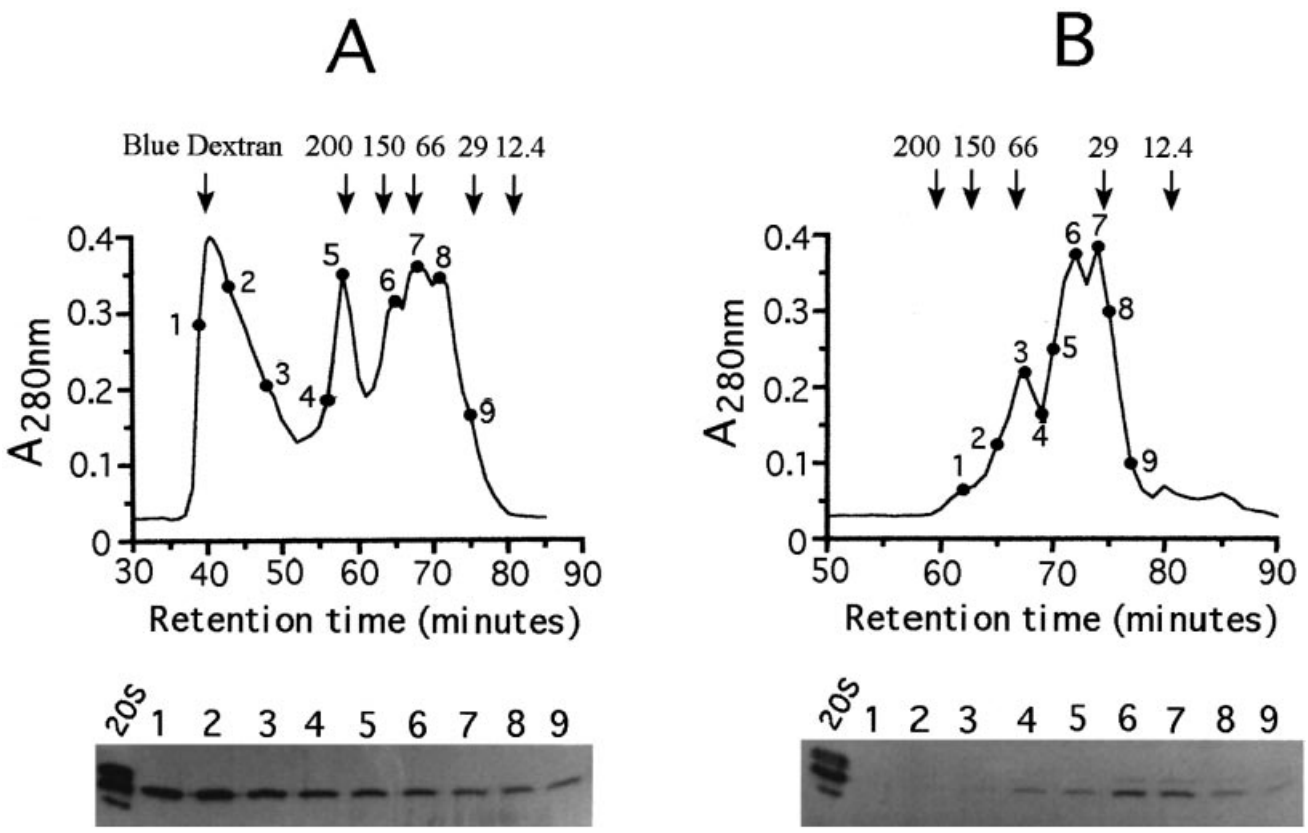


**Figure 5 Gel-filtration analysis of recombinant TbPSA5 in *E. coli* crude lysate**

The crude lysate of pBAα5tb-transformed *E. coli* cells, divided into two fractions by using Amicon-100: a high-molecular-mass fraction (more than 100 kDa) (**A**) and a low-molecular-mass fraction (less than 100 kDa) (**B**), were each fractionated on a calibrated Superose-12 column. The positions of molecular mass markers are indicated (in kDa) above each trace. The collected fractions, labelled by corresponding numbers in the elution profile and the immunoblot in each panel, were each analysed by immunoblotting for the presence of TbPSA5 as described previously. Samples of purified 20 S proteasome from *T. brucei*, used as markers, are labelled 20 S on the left side of each blot in (**A**) and (**B**).

proteasome reacted with this protein (Figure 4B), confirming that the expressed protein was the α5 protein from *T. brucei*. To determine whether the recombinant α5 protein could self-assemble into a polymerized form, the crude lysate of pBAα5tb-transformed *E. coli* cells was divided into two fractions by filtering them through Amicon-100. A high-molecular-mass fraction (more than 100 kDa) (Figure 5A) and a low-molecular-mass fraction (less than 100 kDa) (Figure 5B) were each further fractionated on a Superose-12 column. The collected fractions were each analysed by immunoblotting. Among the Superose-12 fractions generated from the Amicon-100 filtrate (molecular mass less than 100 kDa), fractions 6 and 7, with an estimated molecular mass range of 25–40 kDa, cross-reacted with the rabbit antiserum against *T. brucei* 20 S proteasome (Figure 5B), indicating that these fractions contained the α5 monomer. However, among the fractions generated from the material retained on the Amicon-100 column (molecular mass more than 100 kDa), those in the estimated molecular mass range of 190 to 800 kDa showed the strongest cross-reactions with the antiserum. In every fraction, Western blotting revealed an immunoreactive 27 kDa protein (Figure 5A), demonstrating that the high-molecular-mass forms observed by Superose-12 chromatography were not the result of covalent associations between the α5 molecules. Crude lysate from *E. coli* transformed with the pBAce vector plasmid showed no protein band that cross-reacted with the anti-α5 serum in any of the fractions collected from the Superose-12 column (results not shown). Therefore the recombinant α5 protein monomer might indeed have polymerized spontaneously while either inside the *E. coli* cells or in the crude lysate. The molecular mass of 190 kDa could result from the formation of a heptamer ring of the 27 kDa α5 monomer. However, it was somewhat difficult to envisage what form of aggregation could result in an apparently heterogeneous population of α5-containing complexes with molecular masses of up to 800 kDa.

### Recombinant α5 subunit protein can self-assemble into cylinders of four stacked heptamer rings

The macromolecules collected in fractions 1–9 in Figure 5(A) that cross-reacted with the antisera against *T. brucei* 20 S proteasome were each analysed under the electron microscope. All the fractions were found to contain ring structures with an estimated diameter of $14.5 \pm 0.6$ nm. The fractions containing the highest molecular mass of 800 kDa (Figure 5A, lanes 1 and 2) were found to contain cylinders of four stacked rings with the same diameter and a longitudinal length of $18 \pm 0.8$ nm (Figure 6A). These cylinder-like protein complexes are highly similar to the morphology of the *T. brucei* 20 S proteasome [21] but the latter has smaller dimensions both in diameter (11 nm) and length (16 nm) (Figure 6B). Apparently, this recombinant α5 protein can polymerize spontaneously into the ring form and the

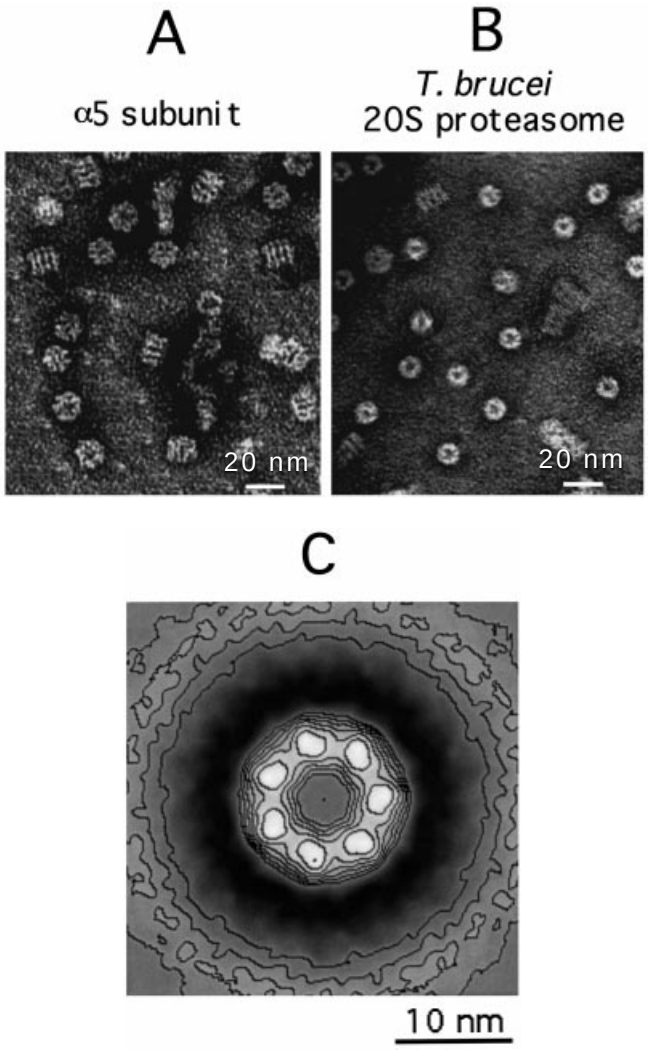


**Figure 6 Electron micrographs of the recombinant TbPSA5 (A) and the purified *T. brucei* 20 S proteasome (B), and symmetrized cylindrical top view after image averaging (C)**

Samples were negatively stained with 3% (w/v) uranyl acetate and examined under a transmission electron microscope at a magnification of × 70 000. Scale bars, 20 nm. The mass density of the symmetrized cylindrical top view after image averaging (**C**) shows that the ring formed by TbPSA5 is a heptamer.

cylinder form of four stacked rings. The gel-filtration results shown in Figure 5(A), indicating a minimum molecular mass of 190 kDa for the polymer, suggest a heptamer ring structure for the 27 kDa protein. To verify this point we performed image averaging of the electron micrographs showing the top view of the cylinders. The averaged image presented in Figure 6(C) indicates quite clearly that the ring structure was indeed a heptamer.

### Cylindrical protein complex is immunoprecipitable by the antisera against *T. brucei* 20 S proteasome

Because the samples containing the cylinders of four stacked heptamer rings were collected from the crude lysate of transformed *E. coli* through a gel-filtration column, contamination by bacterial protein complexes could not be ruled out. To verify that these cylindrical structures contained recombinant α5 subunit protein, the cylinder-containing sample was immunoprecipitated with the rabbit anti-(*T. brucei* 20 S proteasome) antiserum. The supernatant fractions thus left behind were examined by electron microscopy for the cylindrical structures and analysed by immunoblotting with the same antisera for the presence of α5 protein. The results from electron microscopy suggested substantial decreases in the population of cylindrical structures in the supernatants after immunoprecipitation, whereas no apparent decrease in the cylindrical structures resulted from the use of preimmune serum (results not shown). The outcome from immunoblotting shows that the amount of α5 protein in the supernatant decreased significantly after immunoprecipitation with the antiserum (Figure 7, lane 3) in comparison with the original amount of α5 before immunoprecipitation (Figure 7, lane 1) and that immunoprecipitated with preimmune serum (Figure 7, lane 2). This result confirms that the cylindrical structures contain the recombinant α5 protein.

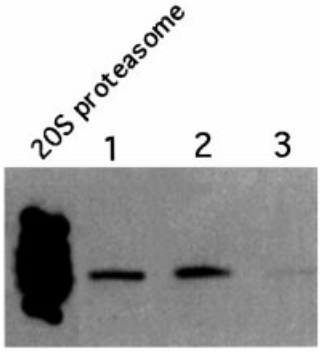


**Figure 7 Immunoprecipitation of the recombinant TbPSA5 from the crude lysate of transformed *E. coli***

The *E. coli* crude lysate was incubated with the anti-(*T. brucei* 20 S proteasome) antiserum followed by immunoprecipitation with the Protein A–Sepharose bead. The TbPSA5 protein remaining in the supernatant was monitored by immunoblotting with the same antiserum. Lane 1, the original *E. coli* lysate before immunoprecipitation; lane 2, immunoprecipitation with the preimmune serum; lane 3, immunoprecipitation with the anti-(*T. brucei* 20 S proteasome) antiserum. The purified 20 S proteasome from *T. brucei*, included as a marker for the antiserum, is shown at the left of the immunoblot.

### Purified recombinant α5 subunit protein is not a ribonuclease

To facilitate purification of the recombinant TbPSA5, an 18 bp oligonucleotide encoding hexahistidine was introduced at the 3′ end of the open-reading-frame of full-length TbPSA5 cDNA by PCR. The PCR fragment was integrated into the plasmid pBAce and expressed in transformed *E. coli* as described in the Materials and methods section. The C-terminal hexahistidine-tagged TbPSA5 was purified through a $Ni^{2+}$/agarose column as described previously [42] and the purified sample was found in an essentially homogeneous state on SDS/PAGE analysis (results not shown). However, this C-terminally tagged protein failed to polymerize *in vitro* to form heptamers and was therefore unsuitable for studying the self-assembly of this protein. It also suggested an essential role of the C-terminus of native TbPSA5 protein in its self-assembly. The availability of purified TbPSA5 nevertheless raised the possibility of examining potential ribonuclease activity in this protein, because proteasomal α5 subunit protein purified from calf liver cells has been demonstrated to possess ribonuclease activity [43]. The purified TbPSA5 samples (0, 0.5, 1.0, 2.5, 5.0 and 10.0 μg of protein) were each incubated with 1 μg of luciferase mRNA (Promega) in 20 μl of

TSD buffer at 37 °C for 20 min. The incubation products were analysed in 1.2 % (w/v) agarose gel containing 2.2 M formaldehyde. The results showed that intact luciferase mRNA resulted from the absence of TbPSA5; however, degraded RNA fragments were observed in the presence of this protein. The extent of RNA degradation, monitored by the distance of migration of RNA fragments, is proportional to the amount of TbPSA5 added to the incubation mixture. Two similarly hexahistidine-tagged recombinant protein samples (His-PA26V and His-PA26T from *T. brucei* [42]) that had been expressed in *E. coli* and purified on a Ni-agarose column were also tested in an experiment similar to that with TbPSA5 and demonstrated similar results (results not shown). Because PA26V and PA26T are two isoform proteins from *T. brucei* that can bind to 20 S proteasomes to activate the peptidase activities of the latter, they are unlikely also to possess ribonuclease activity. Furthermore, a similar demonstration of ribonuclease activity by all three tested proteins strongly suggests the presence of a contaminating ribonuclease activity from *E. coli* in the protein samples that was undetected by SDS/PAGE. Our final conclusion is thus that there is no ribonuclease activity in TbPSA5.

## DISCUSSION

In this study we have isolated a *T. brucei* 20 S proteasome subunit protein on a 2D gel and determined some of its peptide sequences by MS. The results from these preliminary studies agreed perfectly with the sequence of a full-length cDNA clone obtained by screening a *T. b. rhodesiense* λ expression library with rabbit antiserum prepared against the *T. brucei* 20 S proteasome. The protein sequence translated from the full-length cDNA shares significant identity with those of the α5 subunits from rat, human and yeast (see Table 1) and is therefore most probably the *bona fide* α5 subunit of the 20 S proteasome from *T. brucei*. Table 1 also indicates that all the α5 subunits from rat, human, yeast and *T. brucei* share a sequence identity with the sole α subunit from *Th. acidophilum* within a similar extent of 35.6–38.6 %. However, only TbPSA5 has apparently been found capable of self-assembly so far. Although this self-assembly probably took place in the cytoplasm of transformed *E. coli*, the ring structure is most probably a polymer of TbPSA5, because (1) the rings can be immunoprecipitated by antiserum against *T. brucei* 20 S proteasome, (2) the ring structure is not found in the crude lysate of *E. coli* transformed by the empty vector plasmid, and (3) *E. coli* does not have a 20 S proteasome equivalent but instead a high-molecular-mass protein complex, ClpP [44], which bears little morphological resemblance to the 20 S proteasome. The heptamer ring shown in Figure 6(C) is therefore primarily a polymer of TbPSA5, although the possible inclusion of *E. coli* protein cannot be excluded at present.

The TbPSA5 heptamer ring has a similar diameter (14.5 nm) to that of the heptamer ring of HsC8 (14.2 nm) [19]. Both the TbPSA5 and the HsC8 rings differ from the *Th. acidophilum* α-ring by lacking the small, negative-stain-filled hole in the centre of the latter [10]. Another major distinction between the TbPSA5 ring and the others is that the former forms primarily four stacked ring cylinders, whereas the others form mainly dimers. The tetramer formation suggests similar binding conformations and properties on both sides of the TbPSA5 ring that are permissive for stacking with another TbPSA5 ring. However, this interpretation cannot explain why the process of ring stacking should stop at four instead of continuing indefinitely. One possibility is that the bacterial cell lysate might adventitiously contain a scaffolding constituent that limits assembly to four rings. More detailed assembly studies with purified α5 will be required to resolve this question.

Because the *Th. acidophilum* α [10], HsC8 [19], TbPSA6 [25] and TbPSA5 are the only α-subunit proteins known that each polymerize spontaneously, a comparison between the four proteins was made in trying to identify potential common traits. There are relatively poor sequence identities between TbPSA5 and *Th. acidophilum* α (37.0 %) and between TbPSA5 and HsC8 (32.6 %). The sequences of TbPSA6 and TbPSA5 bear even a lower percentage identity (32.3 %). *Th. acidophilum* α, HsC8, TbPSA6 and TbPSA5 have pI values of 5.3, 5.2, 5.2 and 4.7 and molecular masses of 25.8, 28.4, 29.3 and 27.2 kDa respectively. These two features are shared by many other α subunits in eukaryotes that have not been known to have the capability of self-assembly. The yeast 20 S proteasome α5 subunit (PUP2), which has not been shown to form spontaneous heptamer rings by itself, was found to share the same three-dimensional structural feature with the other subunits α1, α3, α4 and α7 [6]. They all have a long helix H5 protruding from the 20 S proteasome particle surface into the surrounding solution [6]. Among all the yeast α subunits, the N-terminal loop segment helix H0, loop L, the loop connecting helix H2 and strand S5, and strand S7 in each α protein are the structural elements most prominently involved in α *cis* interactions. In addition, mutational analyses of *Th. acidophilum* α [10] and HsC8 [19] both indicated that the highly conserved N-terminus, which is also shared by TbPSA5, is essential for self-assembly. Therefore the current consensus seems to emphasize the role of the N-terminus and minimize the potential importance of the C-terminus in the self-assembly of these α subunits. However, in our attempt to tag the C-terminus of TbPSA5 with hexahistidine, the tagged recombinant protein failed to form the heptamer rings. Therefore the C-terminus of TbPSA5 might also have an important role in self-assembly instead of sticking out into the surrounding solution from the ring structure as occurs with yeast α5 [6]. TbPSA5 is 17 residues shorter than yeast α5, and 14 residues shorter than *Th. acidophilum* α at the C-terminus, suggesting that the truncated C-terminus of TbPSA5 does not protrude as much and that this relative truncation might facilitate the formation of four stacked rings.

The fact that TbPSA5 can form a heptamer ring without the participation of other α subunits does not necessarily suggest the natural presence of homogeneous α5 heptamer rings among the 20 S proteasomes from *T. brucei*. The latter have a uniformly smaller diameter, suggesting the inclusion of other α subunits of smaller sizes or a tighter fit than TbPSA5 in the mature structure of the proteasome. There are probably seven distinctive α subunits in *T. brucei* 20 S proteasome (see Figure 1), among which only two have been cloned and sequenced so far: TbPSA5 and TbPSA6. However, each protein is capable of polymerizing on its own. It is therefore highly tempting to postulate that many, if not all, of the α subunit proteins from *T. brucei* proteasome might resemble the α subunit proteins from archaeon and actinomycete in their capability of self-assembly. It will be important to understand the essential structural features that could be shared by the α-subunit proteins from *T. brucei* and those from bacteria that enable them to self-assemble, which the other eukaryotic α subunits, with HsC8 the only exception, apparently do not share, in spite of close sequence similarities.

If we assume that some of the α subunits such as TbPSA5 and TbPSA6 in *T. brucei* are capable of self-assembling, it is likely that they can first form *cis* contacts within the spontaneously formed homogeneous or heterogeneous rings that are structurally similar to those present in the natural hetero-heptameric α rings in 20 S proteasomes. However, that polymerization might result

in a looser structure with a consequently larger diameter. Although it is not itself present in the final proteasome assemblage, an $\alpha$-homoheptameric precursor might serve as an intermediate of the assembly process. It could, for example, serve as a loosely structured matrix that is formed initially, followed by an exchange process in which additional specific subunits move in as most of the initial placeholder subunits shuttle out. Because TbPSA5 and TbPSA6 are apparently both present in the *T. brucei* 20 S proteasome (see Figure 1), there might also be heterogeneity in the population of 20 S proteasomes in *T. brucei*. The potential regulatory mechanisms in controlling proteasome biogenesis in this protozoan pathogen will pose challenging questions for future analysis.

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