Volume 312, number 2,3, 157–160 FEBS 11716 November 1992

Expression of functional *Thermoplasma acidophilum* proteasomes in *Escherichia coli*

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Received 21 September 1992

The two genes encoding the constituent subunits of the *Thermoplasma acidophilum* proteasome were expressed in *Escherichia coli* yielding fully assembled molecules as shown by electron microscopy. The recombinant proteasomes were purified to homogeneity and were shown to have proteolytic activity indistinguishable from proteasomes isolated from *T. acidophilum*.

Proteasome; Gene expression; Archaebacterium; *Thermoplasma acidophilum*

1. INTRODUCTION

The proteasome is a high molecular weight (approx. 700 kDa), non-lysosomal multicatalytic proteinase ubiquitous in eukaryotic cells (for review, see [1] and [2]. While all attempts to detect proteasomes in eubacteria have been unsuccesful so far [3,4] proteasomes were recently found to occur in the archaebacterium, *Thermoplasma acidophilum* [5]. Unlike eukaryotic proteasomes, which are composed of 10–20 different though related subunits, all in the molecular weight range from 20 to 35 kDa, the *T. acidophilum* proteasome is made of multiple copies of two subunits only, α and β, with molecular weights of 25.9 and 22.3 kDa, respectively. All the amino acid sequences of proteasomal subunits from eukaryotes available to date can be related to either the α- or the β-subunit of the *T. acidophilum* 'Urproteasome' [6]. Also the basic molecular architecture is conserved from *Thermoplasma* to higher eukaryotes; four 7-subunit rings collectively form a cylinder- or barrel-shaped structure [7,8]. The α-subunits are located in the two outer rings or disks, while the β-subunits have been mapped to the two juxtaposed inner rings by means of immunoelectron microscopy [9].

In this communication we describe the co-expression of the genes encoding the α- and β-subunits of the *T. acidophilum* proteasome in *Escherichia coli* yielding fully assembled and functional proteasomes. This provides the basis for addressing important questions regarding proteasome assembly and function.

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Abbreviations: FPLC, fast protein liquid chromatography; NMec, 4-methyl-7-coumarylamide; OD_{600}, optical density measured at 600 nm; pI, isoelectric point; RBS, ribosome binding site.

2. MATERIALS AND METHODS

2.1. *Cloning of the α- and the β-gene into the expression vector pT7-5*

Starting from the initial pUC18 clone, which harbours the gene encoding the β-subunit of the *T. acidophilum* proteasome on an *Hin*dIII fragment, the *Dra*I sites at position −17 and 654 [6] were used to clone the β-gene into the *Sma*I-site of pUC19. Clones with the β-gene in the desired orientation were used for cloning into the expression vector, pT7-5 [10], by use of the respective *Eco*RI and *Hin*dIII sites of the pUC19- and the pT7-5 polylinker; this resulted in the plasmid, pT7-5-β.

The gene encoding the α-subunit of the *T. acidophilum* proteasome was amplified by a polymerase chain reaction. Two oligonucleotides, one with a flanking *Pst*I-site (5'-ATTCTGCAGATTATAG-TGGCTGGAGG-3') and one with a flanking *Hin*dIII-site (5'-TA-TAAGCTTGCGATTCAAG-GCGGGG-3') were used to amplify the α-gene coding region. These restriction sites were used for cloning the amplified fragment into the *Pst*I and *Hin*dIII sites of pUC19. The *Kpn*I- and *Hin*dIII-sites of this plasmid were used to clone the α-gene into the pT7-5-β plasmid. The resulting construct, pT7-5-β-α, contained the α- and β-gene with their respective putative ribosome binding site (RBS) under the transcriptional control of the Φ10 promoter (Fig. 1). DNA sequencing according to the dideoxynucleotide method [11] confirmed that the cloning procedures did not change the DNA sequence. *E. coli* BL21(DE3) cells [12], which contain the T7 polymerase gene in their genome under the control of the *lac*UV5 promoter, were transformed with the plasmid pT7-5-β-α. The bacteria were grown in LB-medium to an OD_{600} of approximately 0.8 and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside overnight.

2.2. *Purification of the recombinant proteasomes*

Harvesting of cells from induced overnight cultures, preparation of spheroplasts, sonication and nucleic acid digestion were performed as described by Lin and Cheng [13]. The lysate was further fractionated by ultracentrifugation (100,000 × *g*, 1.5 h) and the resulting supernatant was loaded onto a Sepharose 6B column. Subsequent purification was achieved by DEAE–Sephacel and hydroxlyapatite chromatography as described previously for the isolation of the proteasomes from *T. acidophilum* [7]. Fractions of the columns were assayed for proteolytic activity with the fluorigenic peptide substrate, Suc-Leu-Leu-

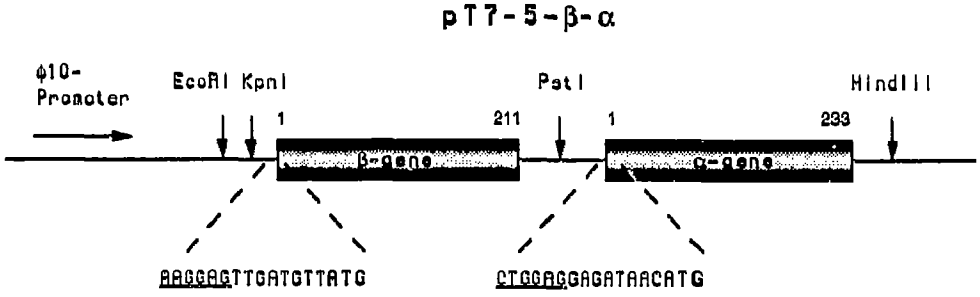

Fig. 1. Schematic drawing of the plasmid pT7-5-β-α used for expressing the proteasome in *E. coli*. Restriction sites for cloning are indicated by vertical arrows. Direction of transcription is indicated by a horizontal arrow. The nucleotide sequence of the putative RBSs' and the initiation codons of the two genes are underlined or written in bold type letters, respectively.

Val-Tyr-NMec. For Western Blot analysis the samples were separated by Tricine–SDS-PAGE, blotted onto nitrocellulose, and probed with antiproteasome antibodies [9]. Two-dimensional gel electrophoresis, electroblotting and microsequence analysis were performed as described by Eckerskorn et al. [14].

3. RESULTS AND DISCUSSION

We expressed the proteasome of *T. acidophilum* in *E. coli*, using the T7 polymerase expression system [10,12], since it was succesfully applied for the expression of several archaebacterial ribosomal proteins [15,16]. The α- and β-gene were subcloned into pT7-5, making use of their own putative ribosome binding sites (RBS) (Fig. 1). Recognition of archaebacterial RBSs' by the *E. coli* translational machinery has been demonstrated [15,16], particularly for the *T. acidophilum* citrate synthase gene [17,18].

We organized the two genes into an operon yielding pT7-5-β-α and demonstrated by Western blot analysis that expression of both proteins in *E. coli* BL21 (DE3) occurs (Fig. 2B). To investigate whether fully assembled and functional recombinant proteasomes were produced, we applied the purification protocol developed for *Thermoplasma* cells to induced cultures of *E. coli* BL21(DE3)/pT7-5-β-α. After final FPLC/Superose 6 chromatography recombinant proteasomes were purified to homogeneity as judged by SDS-PAGE (Fig. 2A). Electron micrographs of negatively stained purified recombinant proteasomes showed barrel-shaped complexes indistinguishable from *T. acidophilum* proteasomes (Fig. 3). Assembly of proteolytically active recombinant proteasomes is demonstrated by their similar rate of degradation of two synthetic substrates, as compared to proteasomes purified from *T. acidophilum* (see Table I).

In contrast to the α-subunit purified from *T. acidophilum*, the recombinant α-subunit was accessible to N-terminal protein sequencing, yielding an amino acid sequence (1-MQQGQMAYDRA-11) which was identical to the sequence derived from the gene [19]. Protein sequencing of the N-terminus of the recombinant β-subunit gave the same amino acid sequence as determined for the *T. acidophilum* protein (9-TTTVGITLKDA-19) [6]. This implies that the processing of the first 8 amino acids of the β-subunit takes place in *E. coli* as it does in *T. acidophilum*. This processing of the β-pro-region is probably effected in an autocatalytic reaction, which is dependent on the presence of the α-subunit. This latter conclusion was based on the observation that expression of the β-subunit alone does not result in the removal of the pro-region (data not shown).

As also reported for proteasomes purified from *T. acidophilum* [6], 2D gel electrophoresis of purified recombinant proteasomes gave rise to multiple protein spots (Fig. 4). N-Terminal protein sequencing of the two major protein spots with approximate pI's of 5.6 and 5.8 gave identical sequences, identifying them unambigously as α-subunits differing in their isoelectric

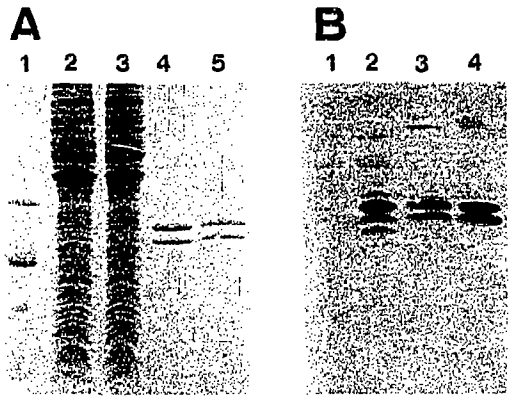

Fig. 2. Tricine–SDS-PAGE and Western Blot analysis of *E. coli* BL21(DE3) cell lysates and of purified proteasomes. Panel A shows Tricine–SDS-PAGE stained with Coomassie blue G: Lane 1, 5 μg molecular mass standards (carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 21 kDa; cytochrome *c*, 12.5 kDa; lung trypsin inhibitor, 6.5 kDa; and insulin, 3.4 kDa. Lanes 2 and 3, cell-lysate of IPTG-induced *E. coli* BL21 (DE3) transformed with pT7-5 or pT7-5-β-α, respectively. Lanes 4 and 5, 10 μg purified recombinant and native *T. acidophilum* proteasomes, respectively. Panel B shows Western blot analysis of Tricine–SDS-PAGE probed with antibody to *T. acidophilum* proteasomes: Lanes 1 and 2, cell lysate of IPTG-induced *E. coli* BL21 (DE3) transformed with pT7-5 or pT7-5-β-α, respectively. Lanes 3 and 4, 10 μg purified recombinant and native *T. acidophilum* proteasomes, respectively.

Table I

Rate of degradation of synthetic substrates by purified recombinant and native *T. acidophilum* proteasomes

Substrate	Activity (nmol/mg proteasomes per min)			
	37°C		60°C	
	Recombinant	Native	Recombinant	Native
Suc-Leu-Leu-Val-Tyr-NMec	0.13	0.10	1.01	0.79
Z-Gly-Gly-Leu-NMec	0.20	0.17	0.61	0.50

Activity was determined by incubating 4.1 μg of protein with 500 pmol of substrate for 1 h at the given temperature and fluorimetric measurement of enzymatically released 4-methyl-7-coumarylamide (NMec) [5].

points; this corroborates our previous conclusions based on amino acid composition analysis [6]. Since it was shown for the *Drosophila melanogaster* α-type subunit PROS28.1 [20] and for two subunits of the bovine multicatalytic proteinase complex (MPC) [21] that they are phosphorylated, and since putative phosphorylation sites exist in the primary sequence of the *T. acidophilum* α-subunit [19], it is quite possible that the isoelectric variants of the α-subunit purified from *T. acidophilum* and *E. coli* result from phosphorylation.

Recently it was shown that the eukaryotic proteasome is involved in the ubiquitin-dependent N-end rule pathway of degradation [22,23]. This N-end rule pathway also exists in *E. coli* and the ATP-dependent protease, Clp (Ti), is required for degradation of N-end rule substrates [24]. Therefore it will be of interest to investigate whether the archaebacterial proteasome can complement this function in the null *clpA*$^-$ mutant [24].

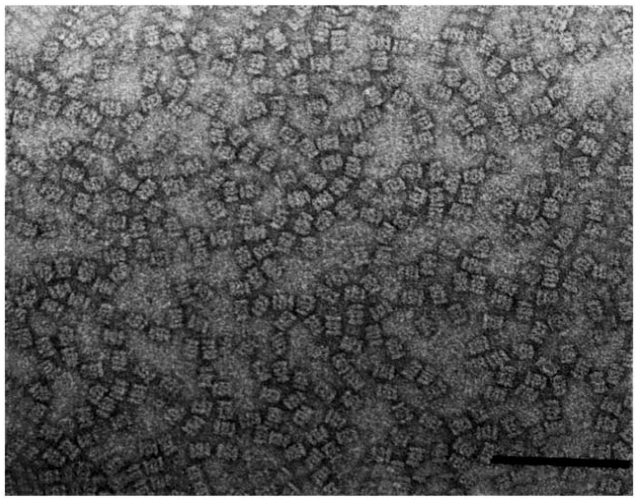

Fig. 3. Electron micrograph of negatively stained purified recombinant *T. acidophilum* proteasomes. Bar = 100 nm.

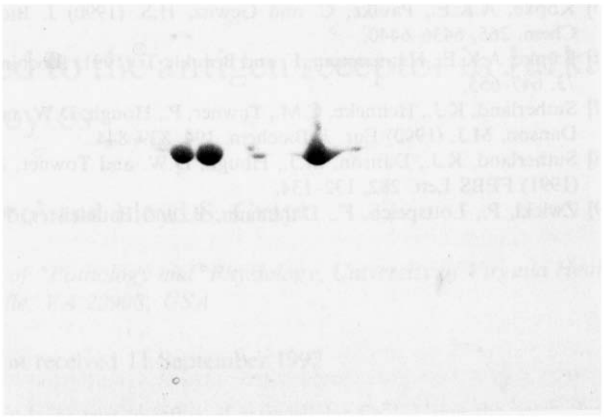

Fig. 4. 2D gel electrophoresis of purified recombinant *T. acidophilum* proteasomes. Horizontal: isoelectric focusing, cathode left and anode right. Vertical: SDS-PAGE. A total of 20 μg of protein was applied. The two major protein spots with approximate pI's of 5.6 and 5.8 were identified as α-subunits by N-terminal protein sequencing. The protein spot with an approximate pI of 6.8 corresponds to the β-subunit.

In conclusion co-expression of the two genes encoding the *T. acidophilum* proteasome in *E. coli* yields correctly folded and assembled proteolytically active proteasomes, which do not influence the viability of their host cells. This allows detailed structural and functional investigation to be performed including site directed and deletion mutagenesis.

Acknowledgements: The authors wish to thank Mrs. U. Santarius for doing the electron microscopy. A Köpke (Göttingen) for providing *E. coli* BL21 (DE3) cells and for advice, S. Tabor (Boston) for providing pT-7 vectors and instruction manuals and Mrs. A. Erdmann and Mr. R. Seimet for experimental help.

REFERENCES

[1] Goldberg, A.L. and Rock, K.L. (1992) Nature 357, 375–379.
[2] Tanaka, K., Tamura, T., Yoshimura, T. and Ichihara, A. (1992) New Biol. 4, 173–187.
[3] Arrigo, A.P., Simon, M., Darlix, J.L. and Spahr, P.F. (1987) J. Mol. Evol. 25, 141–150.
[4] Zwickl, P., Pfeifer, G., Lottspeich, F., Kopp, F., Dahlmann, B. and Baumeister, W. (1990) J. Struct. Biol. 103, 197–203.
[5] Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R. and Baumeister, W. (1989) FEBS Lett. 241, 239–245.
[6] Zwickl, P., Grziwa, A., Pühler, G., Dahlmann, B., Lottspeich, F. and Baumeister, W. (1992) Biochemistry 31, 964–972.
[7] Pühler, G., Weinkauf, S., Bachmann, L., Müller, S., Engel, A., Hegerl, R. and Baumeister, W. (1992) EMBO J. 11, 1607–1616.
[8] Hegerl, R., Pfeifer, G., Pühler, G., Dahlmann, B. and Baumeister, W. (1991) FEBS Lett. 283, 117–121.
[9] Grziwa, A., Baumeister, W., Dahlmann, B. and Kopp, F. (1991) FEBS Lett. 290, 186–190.
[10] Tabor, S. and Richardson, C.C. (1985) Proc. Natl. Acad. Sci. USA 82, 1074–1078.
[11] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[12] Studier, W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Methods Enzymol. 185, 60–89.
[13] Lin, K.H. and Cheng, S.Y. (1991) Biotechniques 11, 748–753.
[14] Eckerskorn, C., Jungblut, P., Mewes, W., Klose, J. and Lottspeich, F. (1988) Electrophoresis 9, 830–838.

[15] Köpke, A.K.E., Paulke, C. and Gewitz, H.S. (1990) J. Biol. Chem. 265, 6436–6440.
[16] Köpke, A.K.E., Hannemann, F. and Boeckh, T. (1991) Biochim. 73, 647–655.
[17] Sutherland, K.J., Henneke, C.M., Towner, P., Hough, D.W. and Danson, M.J. (1990) Eur. J. Biochem. 194, 839–844.
[18] Sutherland, K.J., Danson, M.J., Hough, D.W. and Towner, P. (1991) FEBS Lett. 282, 132–134.
[19] Zwickl, P., Lottspeich, F., Dahlmann, B. and Baumeister, W. (1991) FEBS Lett. 278, 217–221.
[20] Haass, C. and Kloetzel, P.M. (1989) Exp. Cell Res. 180, 243–252.
[21] Pereira, M.E. and Wilk, S. (1990) Arch. Biochem. Biophys. 283, 68–74.
[22] Richter-Ruoff, B., Heinemeyer, W. and Wolf, D.H. (1992) FEBS Lett. 302, 192–196.
[23] Seufert, W. and Jentsch, S. (1992) EMBO J. 11, 3077–3080.
[24] Tobias, J.W., Shrader, T.E., Rocap, G. and Varshavsky, A. (1991) Science 254, 1374–1377.