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Purification and refolding of human α5-subunit (PSMA5) of the 20S proteasome, expressed as inclusion bodies in *Escherichia coli*

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Abstract

The 20S proteasome is the central enzyme of nonlysosomal protein degradation in both the cytosol and nucleus. It is composed of 28 protein subunits which are arranged into four staggered heptameric rings. The outer rings consist of α-subunits which are responsible for binding of proteasome activators, inhibitors, and regulators. To better characterize human α5-subunit (PSMA5) of the 20S proteasome, we have established a high-efficiency *Escherichia coli* expression system. The DNA-coding sequence for the human PSMA5, which was subcloned into the vector pET-22b (+), has been expressed as inclusion bodies in *E. coli* BL21 (DE3). To produce the native PSMA5, straightforward protocols have been developed for refolding the human PSMA5 in the presence of surfactants using dilution refolding and size-exclusion chromatography matrix refolding methods. After refolding, recovery yields of about 20% were obtained, respectively, with purity above 95%. The human PSMA5 was detected by dynamic light scattering in refolding process, and the molecular weight of the final refolded product was measured using gel filtration chromatography, which indicates that the human PSMA5 exists mainly as tetramer.

Keywords: PSMA5; Expression; Purification; Refolding; Dynamic light scattering; Gel filtration

The 20S proteasome is a high molecular weight proteolytic enzyme, present in the cytoplasm and the nucleus of eukaryotic cells as well as in archaebacteria and eubacteria. It is the catalytic core of the 26S proteasome that represents the proteolytic machinery of the ubiquitin pathway, able to degrade ubiquitinated proteins to peptides and ubiquitin chains [1,2]. Within the cells, the 20S proteasome exists either in a free state or associated with large regulatory complexes. It can, thus, bind one or two 19S complexes responsible for the ATP-ubiquitin-dependent hydrolytic activity of the resulting 26S complex [3]. It can also bind one or two 11S activator complexes. Recent findings further suggest a possible simultaneous binding of PA700 and PA28 activators to the 20S proteasome. Such a hybrid proteasome complex is probably more efficient for the generation of peptide antigens [4].

Crystal structure of 20S proteasome of the yeast *Saccharomyces cerevisiae* [5] shows that the 20S proteasome is composed of 28 protein subunits, which are built of 2 copies of each of 7 distinct α- and β-type subunits and arranged into four staggered heptameric rings. Some of the β-type subunits are known to contain the catalytically active sites for proteolysis which are situated within the central chamber, while the α-type subunits, which are responsible for binding of proteasome activators, inhibitors, and regulators subunits, play a role in the assembly of the proteasomes, and are involved in interactions with components that cap the 20S proteasome at each end to yield the 26S complex [6]. It is known that human α-type subunit HsC8 (PSMA7) plays a critical role in hypoxia-inducible factor-1α regulation [7]. Recently, it has been reported that PSMA7 assembles into a heptameric ring-like structure by itself [8]. However, further studies have found that two naturally neighboring subunits of PSMA7 do not form ring-like complexes by themselves, but only dimers. This indicates that the propensity to form homo-oligomeric rings is not a general feature

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among human α-type subunits [9,10]. As for the human PSMA5, very little is known about its function and self-aggregation up to now. To study its structures and functions, we have established an *E. coli* expression system for the human PSMA5 and developed a straightforward protocol to refold the recombinant protein in the presence of surfactants using dilution and size-exclusion chromatography. The refolded products were tested with the dynamic light scattering and by gel filtration chromatography. The results suggest that the human PSMA5 exists mainly as tetramer.

Materials and methods

Expression of PSMA5

The plasmid was provided by Shanghai Institute of Hematology, Rui jin Hospital, Shanghai Second Medical University. As shown in Fig. 1, the gene encoding the human PSMA5 was subcloned into the vector pET-22b (+) at the sites cut by *Nde*I and *Xho*I and sequenced. The sequence encoding 6× histidine is downstream to the sequence encoding the human PSMA5, which was expressed as a 6× His-tagged fusion protein. The induction procedure proved critical to achieve the highest recombinant quantity or activity yields. Isolated colonies of transfected *E. coli* BL21 (DE3) were inoculated into LB medium (2.5 mL, 100 μg/mL ampicillin) and grown at 37 °C. When the OD_{600} reached 0.6, the cell suspension was transferred to 15 mL LB medium and grown at the same conditions. Finally, cells were transferred to 500 mL LB medium and grown until the OD_{600} reached 0.6. 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was subsequently added and the suspension was incubated at 37 °C for 3 h.

Isolation and solubilization of inclusion bodies

Total cell paste was harvested and then suspended in buffer (50 mM Tris–HCl, 1% Triton X-100, pH 8.0). After applied repeated rounds of sonication, solution was centrifuged at 12,000*g* and then washed in buffer (8% sucrose, 10 mM Tris–HCl, and 2% Triton X-100, pH 8.0) with sonication three times through a high-pressure homogenizer (Cole–Parmer). After centrifugation, the supernatant was decanted and the precipitate washed in 2 M urea for 20 min. Inclusion bodies were collected by centrifugation at 6000*g* for 30 min. The supernatant was decanted and the precipitate pellet was solubilized in 20 mL of denaturation buffer (50 mM Tris–HCl, 300 mM NaCl, 5 mM imidazole, and 8 M urea, pH 8.0). The solubilized inclusion bodies were incubated in the denaturation buffer with 2 mM DTT as the reducing agent for 1 h at 30 °C to ensure complete reduction of cysteine residues.

Purification of denatured PSMA5

Before loading, a polyethersulfone microfiltration membrane of 0.45 μm pore size was used to remove particulates. Recombinant inclusion bodies (PSMA5) were purified by affinity chromatography using Ni–NTA His-bind resin [11]. The column was equilibrated with 3–5 column volumes denaturation buffer. The sample was loaded slowly at the rate of 2 mL/min and then the column was washed with washing buffer (50 mM

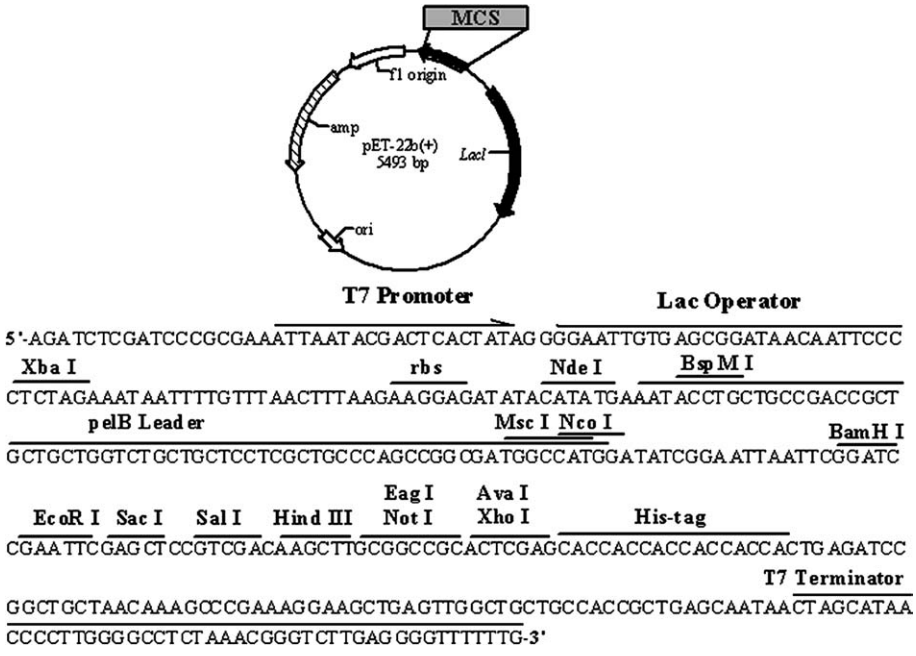

Fig. 1. pET-22b(+) vector map.

Tris–HCl, 300 mM NaCl, 10 mM imidazole, 8 M urea, and 2 mM DTT, pH 8.0) at the rate of 10 mL/min until the absorbance at 280 nm reached 0. The column was eluted with a series of elution buffers (50 mM Tris–HCl, 300 mM NaCl, 8 M urea, and 2 mM DTT, pH 8.0) that contain a step gradient of imidazole from 50 to 250 mM. The human PSMA5 was finally eluted by the buffer containing 250 mM imidazole and then dialyzed against buffer (50 mM Tris–HCl, 8 M urea, 1% glycerol, 5 mM EDTA, 2 mM DTT, and 0.1 mM PMSF, pH 8.0) for 12 h at 4 °C. After dialysis, the protein solution was concentrated to 4 mg/mL by superfiltration for later refolding experiments.

Refolding of PSMA5

Dilution refolding

With reference to the procedure given by Batas and Chaudhuri [12], denatured and reduced sample (4 mg/mL) was one-step diluted 40-fold into refolding buffer (5 mM EDTA, 2 mM DTT, 1 mM oxidized glutathione, 5 mM reduced glutathione, 0.1 mM PMSF, and 50 mM Tris–HCl, pH 7.3) at 30 °C for 12–24 h.

Size-exclusion chromatography refolding

The experimental procedures for chromatography refolding have special virtues [13]. Denatured recombinant protein was refolded using Sephadex 75 HR 10/30 column (Pharmacia). The human PSMA5 was first filtered through a 0.45 μm Millipack cellulose acetate filter (Millipore) to remove particulates. Four milliliters of denatured protein was injected onto the column, which was previously equilibrated with 3–5 column volumes refolding buffer. The human PSMA5 was eluted at the rate of 1 mL/min using refolding buffer as the mobile phase. Absorbance at 280 nm was monitored with a UV detector connected to a data acquisition package. Experiments were carried out at room temperature and in duplicate.

Purification of renatured PSMA5

Refolded PSMA5 was further purified using 10 mL DEAE Sephadex A-25 filled column. 5 ml of refolded protein was injected onto the column, which was previously equilibrated with 3–5 column volumes refolding buffer. Recombinant protein was eluted at the rate of 5 mL/min using the refolding buffers which contain NaCl from 0.1 to 0.6 M with a gradient of 0.1 M. Absorbance at 280 nm was monitored with a UV detector connected to a data acquisition package. Experiments were carried out at 4 °C.

Dynamic light scattering

An analysis of light scattering signal provides quantitative information about the behavior of protein in solution [14]. All DLS measurements were performed on a DynaPro DLS instrument with a 781.8 nm wavelength laser (Protein Solutions). The fluctuations in light intensity, due to the Brownian motion of the molecules, were measured by a photodiode at a 90° angle. Photons were counted and the time dependence of the light intensity fluctuations was analyzed by autocorrelation. Assumptions include a solution viscosity equal to 1.019 and that the proteins are spherical in nature. The human PSMA5 solution was centrifuged for 10 min with 12,000*g* prior to collecting scattering data. Experiments were run at 20 °C and at least 20 measurements were taken at each measurement. Regularization histogram analyses of samples were carried out using the software DYNAMICS Version 5.25.44.

Gel filtration chromatography

Gel filtration chromatography was performed in a column with a diameter of 1 cm, packed with Superdex 200 gel (Pharmacia) to a height of 37 cm. The gel filtration column was equilibrated with 200 mL elution buffer (50 mM Tris–HCl, 2 mM DTT, and 5 mM EDTA, pH 8.0). 0.5 mL (10 mg/mL) mixture of standard proteins (i.e., catalase 232,000 Da, serum albumin 66,200 Da, ovalbumin 45,000 Da, and lysozyme 14,400 Da) was loaded very slowly and gently on top of the gel bed. After collecting all the elution volumes, the same kind of elution process was repeated with the human PSMA5. Based on the inverse logarithmic relationship between the size of the molecule and the volume eluted, the molecular weight of the human PSMA5 could be determined from the curve, log molecular weight vs. elution volume.

Results and discussion

Expression of PSMA5

As shown in Fig. 2, the plasmid constructed with pET-22b (+) exhibited a high level of the human PSMA5 over-expression in BL21 (DE3), which after 3 h of induction with 1 mM IPTG accounts for approximately 33% of total cellular proteins. The human PSMA5 was expressed in the form of inclusion bodies and analyzed by SDS–PAGE using 8–12% polyacrylamide gels (Invitrogen). Based on the amino acid sequence, the calculated molecular weight of the human PSMA5 is 26,409 Da, consistent with the result of SDS–PAGE measurement.

Heterologous expression of foreign genes in *E. coli* often leads to production of the expressed proteins in insoluble inclusion bodies. The level of expression of the human PSMA5 was optimized by 1 mM IPTG induction at 37 °C for 3 h. It has been reported that the human PSMA7 was expressed as soluble protein [8], so we have tried several strategies to improve the solubility of the expressed protein but made little progress.

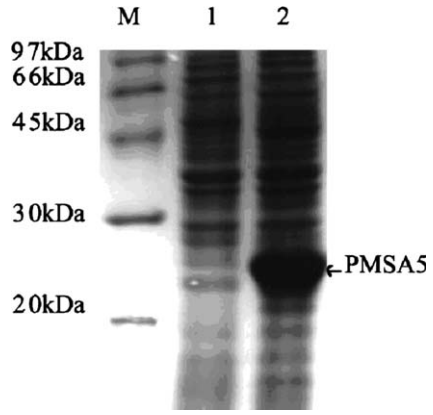

Fig. 2. SDS–PAGE analysis of the human PSMA5 expressed in *E. coli* BL21 (DE3). Lane M, high molecular weight marker; lane 1, extracts of whole cells of BL21 (DE3) transformed without induction; and lane 2, extracts of whole cells of BL21 (DE3) transformed after 3 h of IPTG induction. The gel was developed by $CBBR_{250}$ staining.

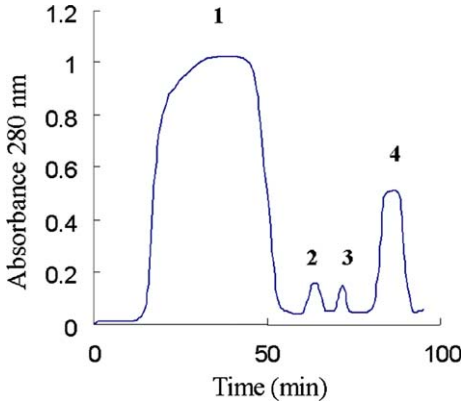

Fig. 3. Purification profile of the denatured PSMA5 using affinity chromatography with Ni–NTA His · bind column. Peak 1, proteins not bound; peaks 2, 3, proteins with less affinity; and peaks 4, 6× His-tagged fusion proteins.

Purification of denatured PSMA5

As shown in Fig. 3, the first flow-through peak contains proteins that have no affinity with Ni–NTA His · bind. The peak 2 eluted by 50 mM imidazole contains proteins that have a less affinity with Ni–NTA His · bind. The peak 3, which was eluted by 100 mM imidazole, contains less bound proteins. The major peak, i.e., peak 4, eluted by 250 mM imidazole, represents 6× His-tagged fusion proteins that have a high affinity with Ni–NTA His · bind. All eluted fraction proteins were stained with $CBBR_{250}$ and the 6× His-tagged fusion protein (PSMA5) had a purity of at least 85%.

Since there is a potential for binding background contaminant in the process, low concentration of imidazole in the lysis buffer and washing buffers (5–10 mM) are added. At low imidazole concentration nonspecific, low-affinity binding of background proteins is prevented, while 6× His-tagged proteins still bind strongly to the Ni–NTA matrix. Therefore, adding imidazole to the lysis buffer leads to greater purity in fewer steps. EDTA is not used in inclusion body solubilization process because it can remove Ni from the NTA matrix.

Refolding and purification of PSMA5

Various redox conditions were evaluated during development the human PSMA5 refolding under neutral or basic pH conditions in Tris-based buffers. The addition of 1 mM oxidized glutathione and 5 mM reduced glutathione (1:5) to the refolding buffer at pH 7.3 maximally increased the yield of refolding efficiency. Recoveries were optimal at a final protein concentration of 0.1 mg/mL if the refolding temperature was maintained between 28 and 30 °C. In this experiment, maybe because the human PSMA5 contains only one disulfide bond which makes it easy to refold, with the optimal conditions, as shown in Table 1, a refolding yield of about 20% was obtained.

After refolding, the human PSMA5 was further purified by ion exchange chromatography. The major peak (PSMA5) in Fig. 5, which was eluted by the refolding buffer containing 0.6 M NaCl, had a purity of about 100% as indicated by Fig. 6.

Examination by DLS and gel filtration chromatography

DLS was applied to detect samples during the refolding process at intervals of 6 h. Before refolding, the denatured PSMA5 could aggregate as shown in Fig. 4A.

Table 1
Process summary and yields

Process step	Total protein (mg)	Concentration (mg/mL)	PSMA5[c](mg)	Purity[a] (%)	Recovery yield (%)
Total cell paste	320[b]		105	33	100
Solubilized proteins	86	25	52	60	45
Ni-column purified protein	35	20	30	86	29
Dilution refolding	12	0.1	10	85	19[d]
Sephadex 75 refolding	13	0.8	11	86	21[d]
DEAE A-25 purified	9.5	1.0	9.5	>99	18[d]

[a]The purity of the human PSMA5 was estimated from protein band intensity in SDS–PAGE.
[b]Total cell paste 320 mg was obtained from 1000 mL cell culture.
[c]The amount of the human PSMA5 was estimated from the protein band in SDS–PAGE with known amounts of BSA.
[d]The recovery yields of 19 and 21% were calculated based on the half quantities of purified inclusion bodies.

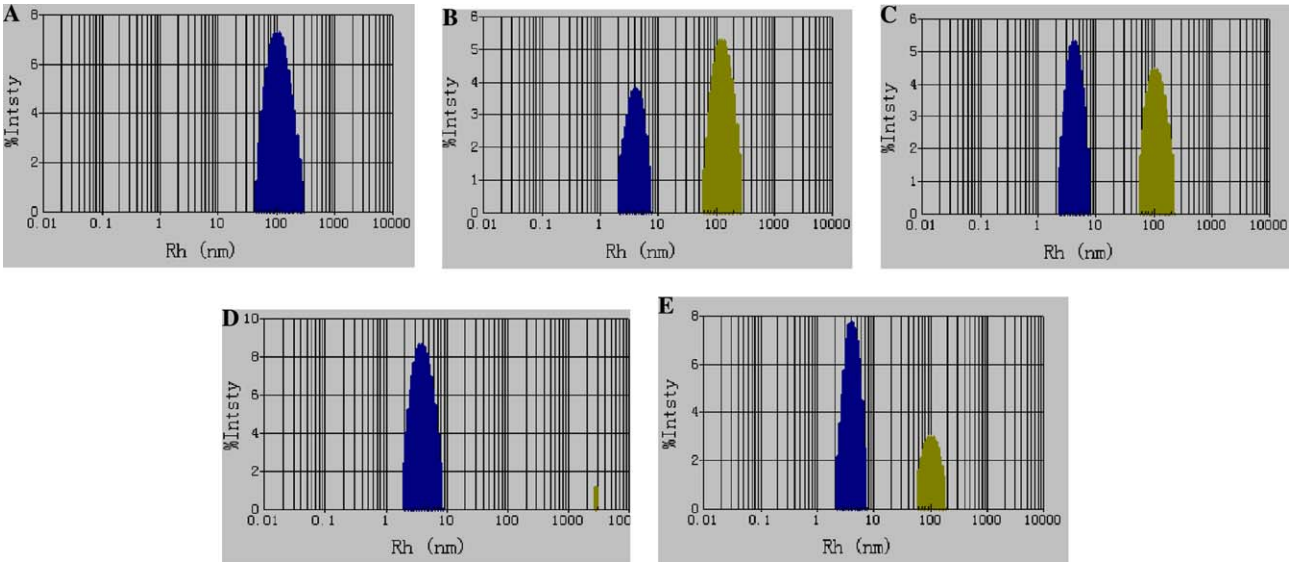

Fig. 4. DLS regularization histograms for samples at different times during dilution refolding process. (A) Control (denatured sample); (B) refolded at 6 h; (C) refolded at 12 h; (D) refolded at 18 h; and (E) refolded at 24 h.

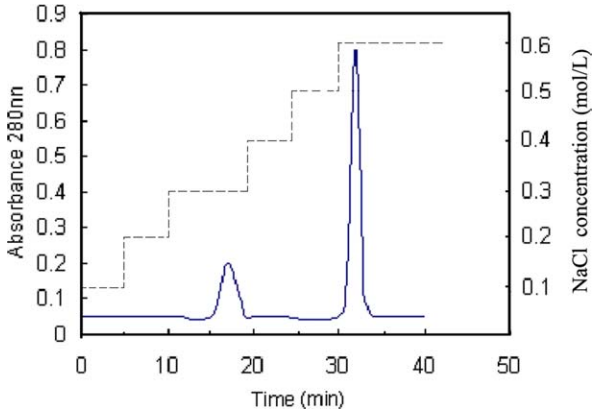

Fig. 5. Purification profile of the refolded PSMA5 using ion exchange chromatography with DEAE Sephadex A-25. (– – –) NaCl concentration; (——) absorbance of 280 nm.

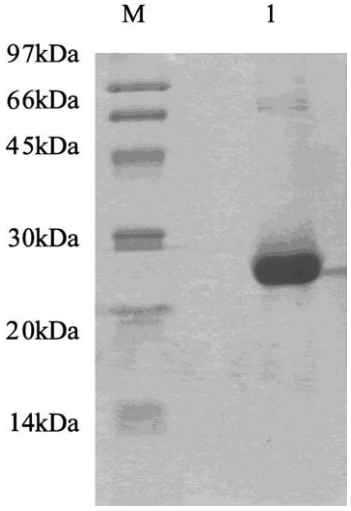

Fig. 6. SDS–PAGE of the refolded PSMA5 purified by ion exchange chromatography with DEAE Sephadex A-25. Lane M, high molecular weight marker; lane 1, proteins eluted by buffer containing 0.6 M NaCl.

During the initial 18 h (see Fig. 4B,C, and D), the intensity of peak 2 approximately at 110 nm decreased while the intensity of peak 1 at about 4 nm increased gradually, implying that during the refolding process large aggregates, composed of denatured proteins, deaggregated along with more and more refolded PSMA5 forming smaller particles. On the other hand, as shown in Table 2, the refolded PSMA5, corresponding to peak 1 with a Rh of about 4 nm, has a molecular weight of about 100 kDa, nearly four times the molecular weight of PSMA5 monomer. This suggests that the refolded PSMA5 exists as tetramer. Gel filtration chromatography has given the semi-log graph (Fig. 7), indicating that the molecular weight of the PSMA5 particle is about 108,000 Da. This result confirms the above suggestion. Furthermore, a corresponding calculation has shown that the PSMA5 monomer with molecular weight of 26 kDa has a Rh of about 2.45 nm which is about half of the measured Rh (4.27 nm) of PSMA5 tetramer.

Our result further justifies the theory that human α-type proteasomal subunits do not generally form ring structures [10]. Why different human α-type subunits present individual self-assembly behaviors remains unclear due to lack of structural information of these oligomers. However, structural implications for their self-assemblies might be derived from the solvent accessible surface area (SASA) analysis with the software Surface Racer based on the PDB data (code 1iru) [15]. For example, the SASA analysis shows that the SASA of mammalian PSMA5 and PSMA7 is 55 and 54% hydrophobic, respectively. The interactions between the

Table 2
The results of DLS measurements during refolding process at different times

Peak 1				Peak 2		
Time (h)	Rh (nm)	MW[a] (kDa)	Intensity (%)	Rh (nm)	MW[a] (kDa)	Intensity (%)
0				118	2.1×10^5	100
6	4.1	90	38	131	3.0×10^5	62
12	4.3	100	50	112	2.0×10^5	51
18	4.0	90	99			
24	4.2	95	71	105	1.8×10^5	29

[a]The molecular weights were estimated from the hydrodynamic radius using the software DYNAMICS Version 5.25.44.

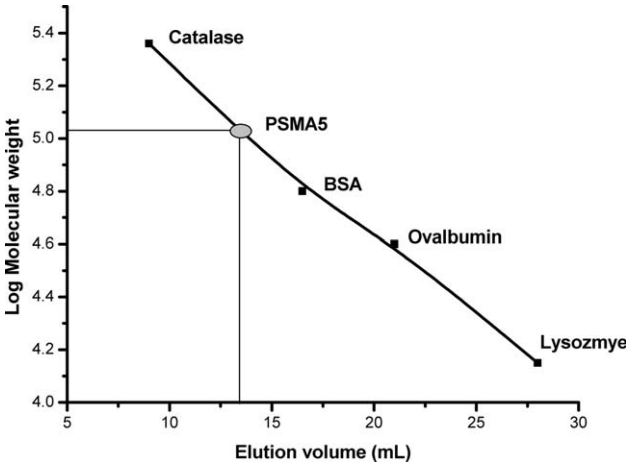

Fig. 7. Semi-log graph of molecular weight standards and the refolded PSMA5 detected with a column filled with Sephadex G-200.

overwhelming hydrophobic surfaces may probably be the main driving force for self-assembling. To elucidate the mechanism of PSMA5 self-assembly as well as its structure–function relationship, our efforts including crystallization trials are under way.

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