Vol. 178, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1991 Pages 256-262

PROTEASOME AND ITS NOVEL ENDOGENEOUS ACTIVATOR IN HUMAN PLATELETS

Masao Yukawa, Masato Sakon, Jun-ichi Kambayashi, Eiichi Shiba, Tomio Kawasaki, Hideo Ariyoshi, and Takesada Mori

Hematology Research Unit, Department of Surgery II, Osaka University Medical School
1-1-50, Fukushima, Fukushima-ku, Osaka 553, Japan

Received May 28, 1991

Proteasome, a high molecular weight multicatalytic protease, was purified from the cytosolic fraction of human platelets for the first time. The biochemical properties of the enzyme including substrate specificity, optimal pH and effects of various inhibitors were almost identical with those of other cells. During the purification with a Heparin-Sepharose chromatography, a novel endogenous activator of the protease was identified and was partially purified. The activator enhanced both chymotrypsin or trypsin like activities of the proteasome in a dose related manner and was inactivated by heating at 56 °C for 30 min. This newly identified activator may serve as an important regulator or cofactor of intracellular activities of the proteasome. © 1991 Academic Press, Inc.

Calpain is a sole nonlysozomal protease identified so far in platelets(1-3). Recently, another cytosolic protease, i.e., proteasome (multicatalytic proteinase complex), has been found in various cells(4). This high molecular weight protease has been found to be identical with prosome (19S ribonucleoprotein particle)(5,6), and has been also reported to be a component of the ubiquitin conjugates degrading enzyme(7-9). As the protease degrades various natural substrates in vitro, it has been considered to play an important role in intracellular protein turnover in eukaryotic cells (10-12). However, the presence of proteasome in platelets has never been reported until present. To clarify its physiological roles in platelets, attempts were made to identify and to purify the proteasome from

Abbreviations: EGTA, ethylene glycol bis (beta aminoethylether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide-gel electrophoresis; MCA, 4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin.

0006-291X/91 $1.50
Copyright © 1991 by Academic Press, Inc.
All rights of reproduction in any form reserved.

human platelets and during the purification a novel endogeneous activator of the enzyme was separated from the crude preparation. In the present study, the occurence and the biochemical properties of platelet proteasome and its activator were reported.

MATERIALS AND METHODS

PURIFICATION OF PLATELET PROTEASOME

Washed platelets prepared from 30 units of platelet concentrates ($1.2x10^{11}$/50ml) were sonicated and centrifuged as described in (13). The cytosolic fraction of human platelets thus obtained was precipitated with ammonium sulfate (45-75%) and the resultant precipitates were dissolved in 5ml of buffer A (10mM Tris-HCl, 0.25M sucrose, 5mM EGTA, 1mM DTT, pH 7.5). After overnight dialysis against buffer A, the sample was subjected to Sephacryl S 300 HR column chromatography (2.6x90cm) and eluted with the same buffer (15ml/hr, 4.5ml/fraction). The active fractions (#46-56) were collected and applied on a Heparin Sepharose CL-6B column(1.5x10 cm) equilibrated with buffer A. Bound proteins were eluted with a linear gradient concentration of NaCl(0-0.5 M, total volume 120ml, 1ml/fraction). Active fractions (#40-46, 7ml) dialyzed against buffer A were subjected to DEAE Mem Sep 1000 ion exchange chromatography (bed volume 1.4ml, Millipore, Bedford, USA) and eluted with a linear concentration gradient of NaCl(0-0.5M, total volume 25ml). All the procedures were performed at 4 °C unless otherwise described.

ASSAY OF PROTEASOME AND ACTIVATOR ACTIVITY

The activity of platelet proteasome was measured according to the modified method of Rivett et al (14) except that the reaction was carried out for 60 min using Suc-Leu-Leu-Val-Tyr-MCA (100μM, Peptide Institute, Osaka, Japan) as a substrate. When the effect of SDS on proteasome activity was examined, it was added immediately after the reaction was started. Other fluorometric peptides (Boc-Phe-Ser-Arg-MCA, Boc-Leu-Thr-Arg-MCA, Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, Arg-MCA, from Peptide Institute) were also used to study the substrate specificity. The activity was expressed as pmole of liberated AMC in 1hr.

ELECTROPHORESIS

PAGE under nondenaturing conditions was carried out by the method of Davis(15), on 5% gels. SDS-PAGE was performed as described by Laemmli(16) using 10-20% gradient gels.

RESULTS

A single peak of proteasome activity was eluted at 0.25M NaCl on DEAE Mem Sep 1000 ion exchange chromatography(Fig 1) and the activity was increased (about 2 fold) by an addition of 0.05% SDS. The active fractions(#44-46) yielded a single band on non-denaturing PAGE (Fig 1, inlet). Platelet proteasome consisted of nine subunits (21-31kDa) on SDS-PAGE, although its molecular weight was estimated to be about 650 kDa by Sephacryl S 300 HR column chromatography. The purified proteasome cleaved Suc-Leu-Leu-Val-

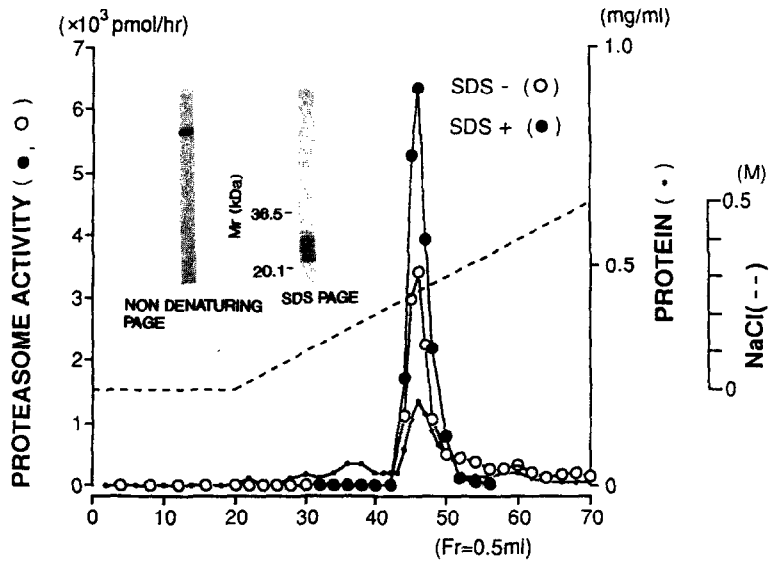

Fig. 1. Purification of platelet proteasome by DEAE Mem Sep 1000 chromatography.
Active fractions of Heparin Sepharose column chromatography were subjected to DEAE Mem Sep 1000 chromatography and eluted by a linear gradient concentration of NaCl as described in the text.
Inlet; Non denaturing PAGE (left) and SDS-PAGE (right) of fraction # 45 (5μg).

Tyr-MCA (chymotrypsin-like activity) and Boc-Phe-Ser-Arg-MCA or Boc-Leu-Thr-Arg-MCA (trypsin-like activity) but not substrates for cathepsins(Table 1). Alpha casein was not degraded either (data not shown). The optimal pH was around 8.5-9.0 when Suc-Leu-Leu-Val-Tyr-MCA was used as a substrate. The proteasome activity was inhibited by chymostatin (chymotrypsin-like activity) and leupeptin (trypsin-like activity). ATP did not potentiate but rather suppressed the activity (data not shown).

Table 1. Substrate specificity of human platelet proteasome

	ACTIVITY (pmol / hr)		
	NONE	+SDS *	+ACTIVATOR**
Suc Leu Leu Val Tyr - MCA	**808**	**1523**	**2628**
Boc Phe Ser Arg - MCA	**141**	**29**	**251**
Boc Leu Thr Arg - MCA	**225**	**68**	**560**
Z Arg Arg - MCA	**23**	**23**	**23**
Z Phe Arg - MCA	**41**	**33**	**41**
Arg - MCA	**17**	**16**	**46**

*) SDS; 0.05% **)activator; 10μg

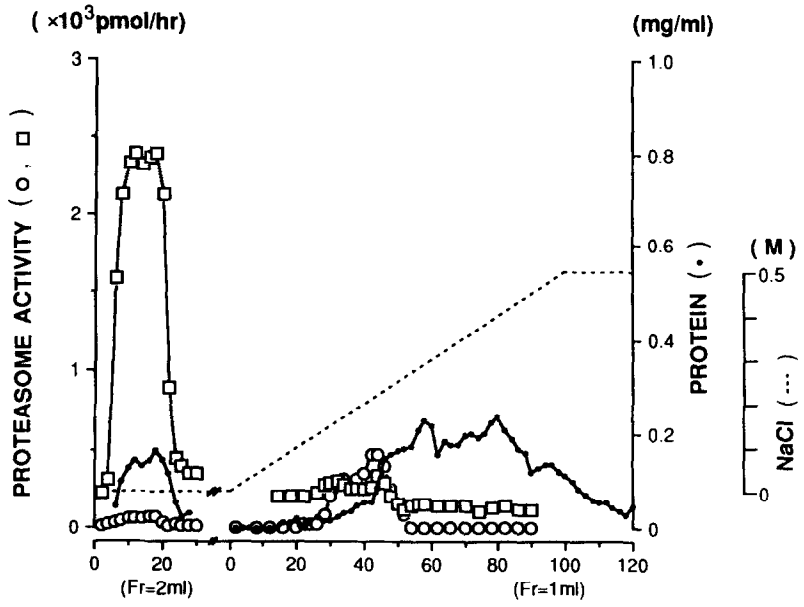

Fig. 2. Resolution of platelet proteasome and its endogenous activator by Heparin Sepharose column chromatography.
Active fractions of Sephacryl S-300 HR column chromatography were subjected to Heparin Sepharose column chromatography and eluted with a linear gradient concentration of NaCl as described in the text. The proteasome activity of each fraction was assayed in the presence (□) and absence (○) of purified proteasome (0.2μg).

After Heparin Sepharose CL-6B chromatography, the total amount of proteasome activity was significantly decreased from 16087 to 119 nmole/hr, suggesting that the substance activating proteasome activity might be removed from the sample by this procedure. To confirm this, the proteasome activity of each fraction was assayed in the presence of the purified enzyme (0.2 μg), as shown in Fig 2. The significant activator activity was observed in the breakthrough fraction, in which proteasome activity was not detected. This activator fraction was further purified by Mem Sep 1000 ion exchange chromatography (Fig. 3). A single peak of activator activity was eluted at 0.3M NaCl and the fractions (#38-40) were collected and used for the biochemical examination. The partially purified activator enhanced the proteasome activity in a dose related manner (Fig. 3, inlet). It potentiated both chymotrypsin- and trypsin- like activities (Table 1), while SDS enhanced the chymotrypsin-like activity only. The activator activity was lost completely by incubating at 56 °C for 30 min.

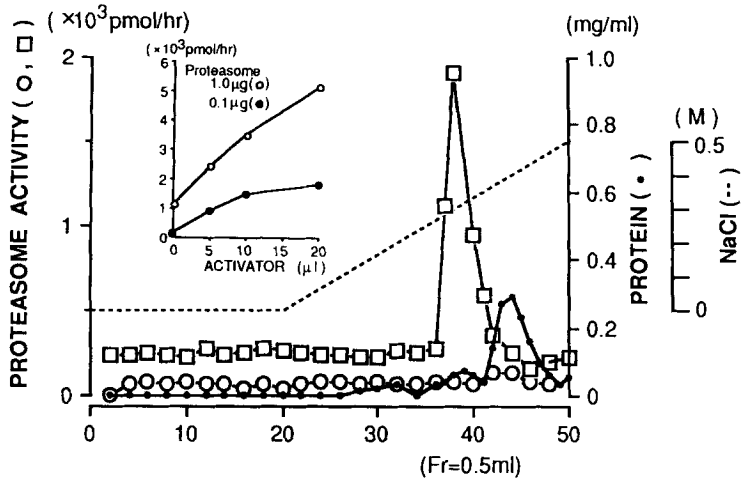

Fig. 3. DEAE Mem Sep 1000 ion exchange chromatography of platelet proteasome activator.
The breakthrough fractions (#6-20, 30ml) of Heparin Sepharose column chromatography were subjected to DEAE Mem Sep ion exchange chromatography and eluted with a linear gradient concentration of NaCl (0-0.5M), in the same way that platelet proteasome was purified.
Proteasome activity of each fraction was assayed in the presence (□) and absence (○) of purified proteasome (0.2μg).
Inlet; The effect of partially purified activator on platelet proteasome. The partially purified activator was preincubated with purified proteasome for 1 min at 37 °C and then the proteasome activity was assayed.

DISCUSSION

The biochemical properties of proteasome purified from the cytosol of human platelets were almost identical with those of other tissues (10-12). In contrast to the results of previous studies(17,18), the purified proteasome did not degrade alpha casein. A similar proteasome without caseinolytic activity was identified in human erythrocytes and it was speculated that its endopeptidase activity might be differentiated according to the cell types(19). The purified enzyme was a latent form because its chymotrypsin-like activity was significantly enhanced by SDS but not by ATP, another well known activating substance of proteasome (10-12). The activation of proteasome by ATP is also controversial. According to Driscoll and Goldberg, ATP-activated form of proteasome was identified in rabbit liver but not in rabbit reticulocytes even by the identical method of purification (20).

Platelet proteasome was activated in a dose related manner by the partially purified activator, in which proteasome activity was not detected. There have been a considerable number of reports on

proteasome and its purification(17-22). However, the endogeneous activator of proteasome, presented herein, has never been reported so far. It is probably because in these studies the crude extract (or ammonium sulfate fraction) was subjected to DEAE ion exchange chromatography for the first step of purification, during which the activator was potentially removed from the enzyme preparation. The possible presence of the activator in these cells may not be ruled out, until the same procedure is employed in them. The intracellular regulation of proteasome activity has not been well clarified yet. Proteasome is generally considered to require ATP and Mg^{++} for its activity (10-12). Fatty acids are also known to potentiate proteasome activity(21,22). Another pathway of activation might be through cAMP-dependent protein kinase (A-kinase), which was co-purified with proteasome even after five steps of purification including DEAE Sepharose ion exchange chromatography(23). However, the activator identified in this study is apparently different from those reported so far because it was heat labile and was easily separated from proteasome activity by Heparin Sepharose or ion exchange chromatography. Thus, this endogenous activator might be involved in a novel, distinct pathway of proteasome activation. Since proteasome is considered to be involved in protein turnover through its multicatalytic peptidase activities, the proteasome-endogenous activator system might play an important role in the intracellular regulation of platelets.

REFERENCES

1. Murachi,T., Tanaka,K., Hatanaka,M. & Murakami,T. (1981) Adv. Enzyme Regul. 19, 407-424
2. Sakon,M., Kambayashi,J., Ohno,H. & Kosaki,G. (1981) Thrombos. Res. 24, 207-214
3. Kambayashi,J. & Sakon,M. (1989) *in* Methods in Enzymology, Vol.169, pp. 442-455, Academic Press, San Diego
4. Tanaka,K., Yoshimura,T., Kumatori,A. & Ichihara,A. (1988) J. Biol. Chem. 263, 16209-16217
5. Falkenburg,P.E., Haass,C., Kloetzel,P.M., Niedel,B., Kopp,F., Kuehn,L. & Dahlmann,B. (1988) Nature 331,190-192
6. Arrigo,A.P., Tanaka,K., Goldberg,A.L. & Welch,W.J. (1988) Nature 331, 192-194
7. Waxman,L., Fagan,J.M. & Goldberg,A.L. (1987) J. Biol. Chem. 282, 2451-2457
8. Driscoll,J. & Goldberg,A.L. (1990) J. Biol. Chem. 265, 4789-4792
9. Armon,T., Ganoth,D. & Hershko,A. (1990) J. Biol. Chem. 265, 20723-20726
10. Rivett,A.J. (1989) Biochem. J. 263, 625-633
11. Tanaka,K. & Ichihara,A. (1990) Cell Struct. Funct. 15,127-132
12. Orlowski,M. (1990) Biochemistry 29, 10289-10297

13. Kajiwara,K., Tsujinaka,T., Sakon,M., Kambayashi,J., Ohshiro,T., Murachi,T. & Mori,T. (1987) Biochem. Int. 15, 935-944
14. Rivett,A.J. (1989) J. Biol. Chem. 264, 12215-12219
15. Davis,J.B. (1964) Ann. N. Y. Acad. Sci. 121,404-427
16. Laemmli,U.K. (1970) Nature 227,680-685
17. Tanaka,K., Ii,K., Ichihara,A., Waxman,L. & Goldberg,A.L. (1986) J. Biol. Chem. 261, 15197-15207
18. Hough,R., Pratt,G. & Rechsteiner,M. (1987) J. Biol. Chem. 262, 8303-8313
19. Kinoshita,M., Hamakubo,T., Fukui,I., Murachi,T. & Toyohara,H. (1990) J. Biochem. 107, 440-444
20. Driscoll,J. &Goldberg,A.L. (1989) Proc. Natl. Acad. Sci. USA 86, 787-791
21. Dahlmann,B., Rutschmann,M., Kuehn,L. & Reinauer,H. (1985) Biochem. J. 228, 171-177
22. Ishiiura,S., Nojima,M., Yamamoto,T., Fuchiwaki,T., Okuyama,T., Furuya,H. & Sugita,H. (1986) Int. J. Biochem. 18, 765-769
23. Pereira,M.E. & Wilk,S. (1990) Archiv. Biochem. Biophys. 283, 68-74