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Source: Zoological Science, 15(1) : 43-49

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.15.43

ZOOLOGICAL SCIENCE **15**: 43–49 (1998)

Irreversible Potent Activation and Reversible Inhibition of Trypsin-Like Activity of 20S Proteasome Purified from *Xenopus* Oocytes by Fatty Acid

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ABSTRACT—The 20S proteasome purified from animal cells has various latent peptidase activities. Fatty acids such as linoleic, linolenic and oleic acids strongly activate both the chymotrypsin-type and peptidylglutamylpeptide (PGP) hydrolase-type activities, but have been reported to have little activation or inhibition of the trypsin-type activity. We show here that an increase of the fatty acid concentration produces activation of chymotrypsin-type and PGP hydrolase-type in a biphasic fashion: no effect until the threshold concentration and then a sharp activation. In contrast, the trypsin-type activity was markedly inhibited at low concentrations of fatty acid, slightly activated at higher concentrations, and inhibited again at even higher concentrations. The inhibition was removed when the concentration of fatty acid was reduced by dilution after pre-incubation with the fatty acid. As a result, the activation pattern became biphasic, which was identical to that of chymotrypsin-type and PGP hydrolase-type activities. These results suggest that in the chymotrypsin-type and PGP hydrolase-type peptidases fatty acids bind first to a class of sites without direct effect on the peptidase activity, but after saturation of this class it permits more fatty acid to bind to another class of sites involved in the activation. In the trypsin-type peptidase an additional class of fatty acid binding sites is uniquely present, which is involved in the enzyme inhibition. The dilution procedure described above removes the fatty acid molecules bound to the inhibition sites, but not the fatty acid molecules bound to the activation sites; this results in the fatty acid activation profile indistinguishable from that of the chymotrypsin- and PGP hydrolase-type peptidases.

INTRODUCTION

In recent years, a considerable amount of new information has become available about the mechanisms of intracellular protein degradation. In eukaryotic cells, most intracellular proteins are degraded by the proteasome (for review, see Coux *et al*., 1996). This enzyme is the major contributor to the neutral proteolytic activity in animal cells, accounting for up to 1% of the cell proteins (Tanaka *et al*., 1988). The 26S proteasome (2000-kDa), which ATP-dependently degrades ubiquitinated proteins, consists of the 20S proteasome (700-kDa) and two 19S regulatory complexes having several ATPase functions (for review, see Hershko and Ciechanover, 1992).

The 20S proteasome shows multiple peptidase activities controlled by a new type of proteolytic mechanism involving a threonine active site (Löwe *et al*., 1995). The enzyme has at least three distinct endopeptidase activities, cleaving bonds on the carboxyl side of basic, hydrophobic or acidic amino acid residues, as well as a protein-degrading activity (for review, see Rivett, 1993). The three peptidase activities have been referred to as “trypsin-like”, “chymotrypsin-like” and “peptidylglutamylpeptide (PGP) hydrolase” activities, respectively. The activities of purified 20S proteasome are essentially latent. Therefore, activities of the enzyme have usually been measured in the presence of some activator such as SDS or fatty acid (for review, see Orlowski, 1990; Rivett, 1993). However, the activation mechanism is still unclear. Fatty acids such as linoleic, linolenic and oleic acids, which are the commonly used activators, strongly activate the chymotrypsin-like and peptidylglutamylpeptide hydrolase activities of the enzyme, but have different effects (little activation or sometime strong inhibition) on the trypsin-like activity (Dahlmann *et al*., 1985; Ishiura and Sugita, 1986; Orlowski and Michaud, 1989; Saitoh *et al*., 1989). The reason why the mode of func-

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tion of fatty acids on the trypsin-like activity is distinctly different from that on the other types of peptidase activity remains unresolved. In this study, therefore, the effects of fatty acid on various peptidase activities of 20S proteasome purified from *Xenopus* oocytes were examined in detail. Here, we report that potent activation of the trypsin-like activity of fatty acid-pretreated 20S proteasome occurs when the concentration of fatty acid in the reaction solution is reduced by dilution.

The results presented in this paper suggest several new mechanisms by which the three peptidase activities of the 20S proteasome are regulated by fatty acids.

MATERIALS AND METHODS

Materials

Succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Suc-LLVY-MCA), *t*-butyloxycarbonyl-Leu-Arg-Arg-4-methylcoumaryl-7-amide (Boc-LRR-MCA), *t*-butyloxycarbonyl-Phe- Ser-Arg-4-methylcoumaryl-7-amide (Boc-FSR-MCA), *t*-butyloxycarbonyl-Gln-Ala-Arg-4-methylcoumaryl-7-amide (Boc-QAR-MCA),and 7-amino-4-methylcoumarin (AMC) were purchased from Peptide Institute (Osaka, Japan). Benzyloxycarbonyl-Leu-Leu-Glu-2-naphthylamide (Cbz-LLE-2NA) and 2-naphthylamide (2NA) were from Sigma (St. Louis, MO, U.S.A.). These fluorogenic peptides were dissolved in dimethylsulfoxide (DMSO) and used. Sepharose CL-6B, phenyl-Sepharose CL-4B and Mono Q were from Pharmacia (Uppsala, Sweden) and DEAE-cellulose (DE52-cellulose) was from Whatman (Maidstone, U.K.). Specially purified SDS and analytical-grade fatty acids (linoleic, linolenic and oleic acids) were obtained from Wako Pure Chemicals (Osaka, Japan) and Sigma. All other reagents were of analytical grade.

Preparation of 20S proteasome

The 20S proteasome was prepared from oocyte cytosol of *Xenopus laevis* ovaries as described previously with slight modifications (Tokumoto and Ishikawa, 1993; Yamada *et al*., 1995). The ovaries (60-80 g) of adult female *Xenopus* were washed six times with 200 ml of 15 mM Tris-HCl (pH 7.5), 20% glycerol and 10 mM 2-mercaptoethanol (TGM buffer). The resultant ovaries were centrifuged without the TGM buffer (80,000 g, 50 min) and the supernatant (~10 ml) was applied to a Sepharose CL-6B column (2.7 × 98 cm) in TGM buffer. The fractions with Suc-LLVY-MCA-hydrolyzing activity (active fractions) were pooled and applied again to a DE52-cellulose column (1.2 × 10 cm) in TGM buffer containing 100 mM KCl. The column was washed with the same buffer and then eluted with a linear gradient of 100-300 mM KCl in TGM buffer. The active fraction was applied to a phenyl-Sepharose CL-4B column (1.2 × 5 cm) in TGM buffer containing 1.5 M KCl. The adsorbed materials were eluted with a linear gradient of 1.5-0.5 M KCl in TGM buffer. The active fraction was collected, diluted to 100 mM KCl with TGM buffer and applied to a second DE52-cellulose column (1.2 × 5 cm). This column was washed with TGM buffer containing 100 mM KCl, and eluted with a linear gradient of 100-300 mM KCl in TGM buffer. The active fraction was pooled and concentrated through a small DE52-cellulose column in TGM buffer. The fraction was dialyzed against 5 mM potassium phosphate (pH 7.6) containing 20% glycerol and 10 mM 2-mercaptoethanol. The dialysate was applied to a column (1.25 × 10 cm) of hydroxylapatite equilibrated with the same buffer, and elution was conducted with a linear gradient of potassium phosphate (5-350 mM: 300 ml). The active fraction was dialyzed against TGM buffer, and the dialysate was applied to a Mono Q column (HR 5/5) equilibrated with TGM buffer and eluted with a linear gradient of KCl in TGM buffer (0-0.35 M KCl: 40 ml). To examine the purity and the subunit composition of the 20S proteasome, the active fraction was subjected to SDS-PAGE and non-denaturing PAGE. This preparation gave at least eight or nine typical 20S proteasome bands in SDS-PAGE and almost a single band in non-denaturing PAGE. The sample was stored at -85°C. No loss of activity was detected for at least 6 months.

Enzyme assays

The activity of peptidyl substrate hydrolysis was measured at 37°C in 50 mM Tris-HCl buffer (pH 8.5) containing 0.28-13.8 μg/ml 20S proteasome, 1-100 μM peptidyl substrate, 1 mM EDTA, 10% DMSO and various concentrations of fatty acids (total volume of 0.1-1.0 ml). After incubation for various times, the reaction was stopped by adding an equal volume of 1% SDS solution and the medium was diluted to 2.2 ml with 100 mM Tris-HCl (pH 9.0) solution. Then, the 7-amino-4-methylcoumarin (AMC) or 2-naphthylamide (2NA) liberated by the reaction was measured fluorometrically (380 nm excitation/460 nm emission for AMC and 335 nm excitation/410 nm emission for 2NA) with a fluorescence spectrophotometer (F-3000, Hitachi, Japan). The activity of peptidyl substrate hydrolysis was presented as nmol of substrate hydrolyzed per min per mg protein of 20S proteasome.

Electrophoreses

Electrophoresis followed the method of Laemmli (1970) in the presence or absence of SDS. The gels were stained for protein with either Coomassie Brilliant Blue or silver.

Protein concentration

Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as a standard. The concentration of the purified enzyme was determined by measuring the absorbance at 280 nm, assuming that $E^{1\%}_{1cm}$ value is 12.3 (Tanaka *et al*., 1988).

RESULTS

Activation profile of various peptidase activities by linoleic acid

The chymotrypsin-like, PGP-hydrolase and trypsin-like activities of the 20S proteasome have generally been determined using the fluorogenic substrates, Suc-LLVY-MCA, Cbz-LLE-2NA and Boc-LRR-MCA, respectively. By using these substrates, we examined the effects of several fatty acids, well-known activators, on the activities of the three peptidases of 20S proteasome purified from *Xenopus* oocytes. In the experiments shown in Fig. 1, we added appropriate concentrations of the above substrates to the purified 20S proteasome, and the peptidase reaction was carried out for 30 min at 37°C in the presence of various concentrations of the activator linoleic acid. Figure 1 depicts the activation profiles of hydrolysis of Suc-LLVY-MCA (A), Cbz-LLE-2NA (B) and Boc-LRR-MCA (C) by linoleic acid. As seen, the overall pattern of the fatty acid activation of chymotrypsin-type (A) and PGP hydrolase-type (B) peptidase activities is distinctly different from that of trypsin-type activity (C). In cases of chymotrypsin-type and PGP hydrolase-type peptidases, the activation occurred in a biphasic fashion. At concentrations of the fatty acid below 60 μg/ml, there was no appreciable effect on the Suc-LLVY-MCA (A) and Cbz-LLE-2NA (B) hydrolyses. Upon increasing the linoleic acid concentration above this threshold level, there was a sharp increase in the hydrolysis of these substrates, then leveled off at 100-120 μg/ml. In contrast, the fatty acid activation pattern of the trypsin-type peptidase is very complex. At the zero concentration of linoleic acid, there was an

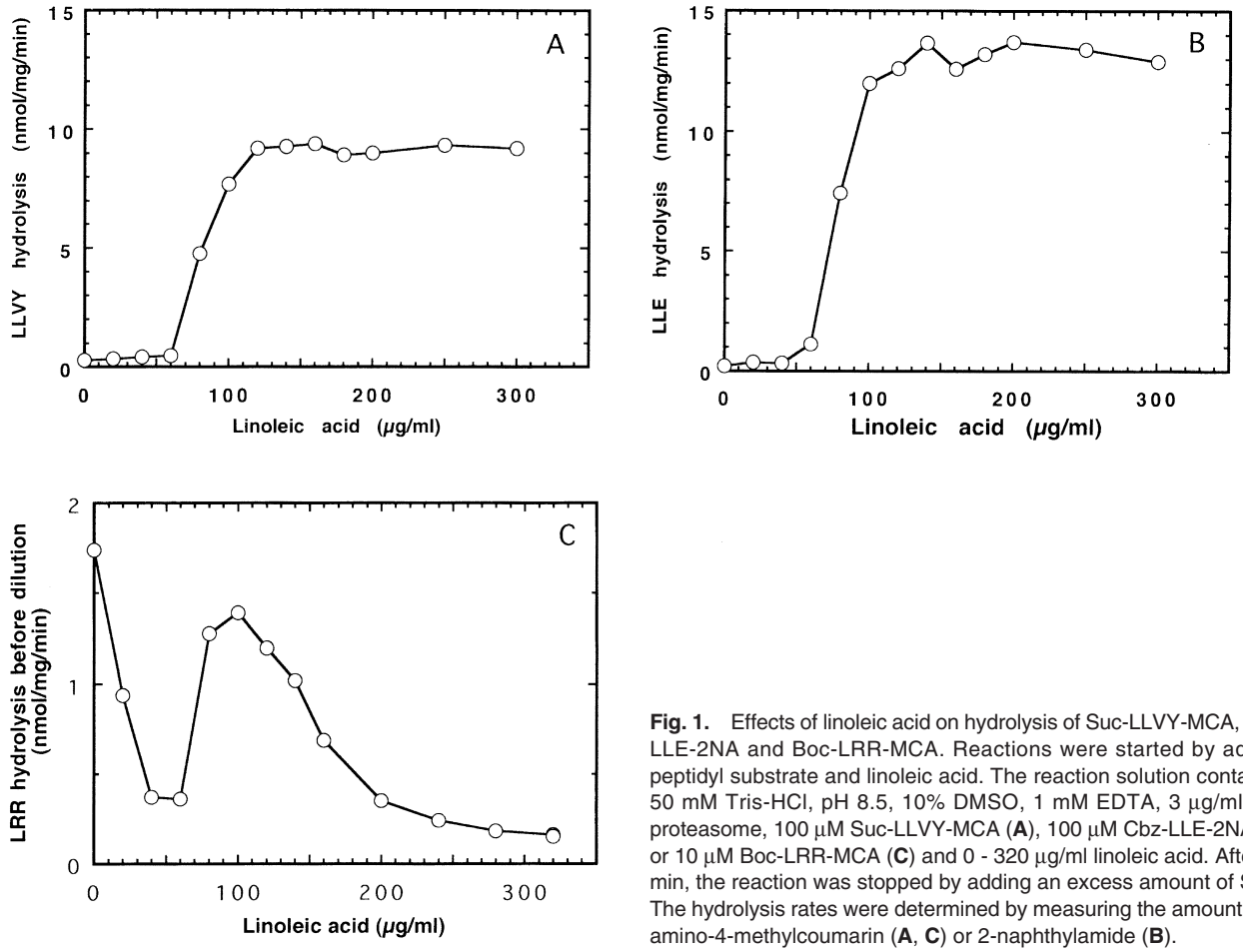

Fig. 1. Effects of linoleic acid on hydrolysis of Suc-LLVY-MCA, Cbz-LLE-2NA and Boc-LRR-MCA. Reactions were started by adding peptidyl substrate and linoleic acid. The reaction solution contained 50 mM Tris-HCl, pH 8.5, 10% DMSO, 1 mM EDTA, 3 μg/ml 20S proteasome, 100 μM Suc-LLVY-MCA (**A**), 100 μM Cbz-LLE-2NA (**B**) or 10 μM Boc-LRR-MCA (**C**) and 0 - 320 μg/ml linoleic acid. After 30 min, the reaction was stopped by adding an excess amount of SDS. The hydrolysis rates were determined by measuring the amount of 7-amino-4-methylcoumarin (**A**, **C**) or 2-naphthylamide (**B**).

appreciable level of activity. An increase of the linoleic acid concentration to a level of 50-60 μg/ml produced a sharp inhibition of the peptidase activity. Interestingly, this inhibition phase coincided approximately with the "latency" phase (0-60 μg/ml) observed in the chymotrypsin- and PGP hydrolase-type activities. An increase of the linoleic acid concentration above this level produced an appreciable activation of the trypsin-type activity. Further increase of the linoleic acid concentration resulted in the inhibition of the activity. Thus, the maximal level of activation of trypsin-type peptidase that occurred at 100 μg/ml was significantly lower than those of chymotrypsin- and PGP hydrolase-type peptidases. These results suggest that linoleic acid has dual functions on the trypsin-type activity, inhibition and activation, although it has only activating effect on the other types of peptidase activity.

The inhibition of the peptidase activity in a low concentration range of linoleic acid is a phenomenon characteristic for the trypsin-type peptidase as described above. We found that inhibition of Boc-LRR-MCA hydrolysis by linoleic acid was dependent on the concentration of the substrate (Boc-LRR-MCA) in the reaction solution as shown in Table 1. At the lowest concentration of the substrate Boc-LRR-MCA tested, 1 μM, the concentration for half maximal inhibition ($K_{0.5}$) of linoleic acid was very low (0.7 μg/ml). Upon increasing the substrate concentration, the $K_{0.5}$ increased markedly. Similar results were also obtained in hydrolysis reactions of other sub-

Table 1. Concentration of linoleic acid giving half-maximum inhibition of trypsin-like activity at various concentrations of the substrate

	Boc-LRR-MCA (μM)				
	1	3	10	25	100
$K_{0.5}$ (μg/ml)	0.7 ± 0.1(5)*	1.5 ± 0.3 (3)*	18 ± 3.5 (5)*	33 ± 4.9 (3)*	65 ± 7.1 (2)*

Boc-LRR-MCA hydrolysis was performed under the same conditions as in Fig. 1C, except that various concentrations of Boc-LRR-MCA were used. Values are means ± SD of two to five experiments.

*Numbers in parenthesis are the numbers of determination.

strates specific for trypsin-like activity, Boc-FSR-MCA and Boc-QAR-MCA (data not shown). These results show that higher concentrations of the substrate overcome the inhibition of trypsin-type peptidase by low concentration of linoleic acid.

Removal of fatty acid inhibition of trypsin-type peptidase

In the experiment shown in Fig. 2, Boc-LRR-MCA hydrolysis was performed in the presence of a relatively high concentration of linoleic acid (300 μg/ml), which strongly inhibited the trypsin-like activity as shown in Fig. 1C. Then, the reaction solution was mixed with larger volumes of a fatty acid-free solution to reduce the fatty acid concentration. Surprisingly, the hydrolysis reaction was strongly activated by this dilution procedure. As shown in Fig. 2, when 6- or 8-fold dilution happened after the enzyme had been treated with 300 μg/ml of linoleic acid for 11 min, the rate of hydrolysis increased to the levels 32 or 82 times as high as the value before the dilution. This activation occurred immediately after the dilution and the increased hydrolysis rate was maintained at a constant value during the time examined. This activation can not be explained by the reduction of the fatty acid concentration *per se*, since 8-fold dilution of 300 μg/ml for example resulting in 37.5 μg/ml would produce virtually no activation on the basis of the fatty acid activation pattern shown in Fig. 1C. Rather, it appears that the activation induced by dilution is due to the removal of the fatty acid inhibition.

In the experiment shown in Fig. 3, the extent of activation

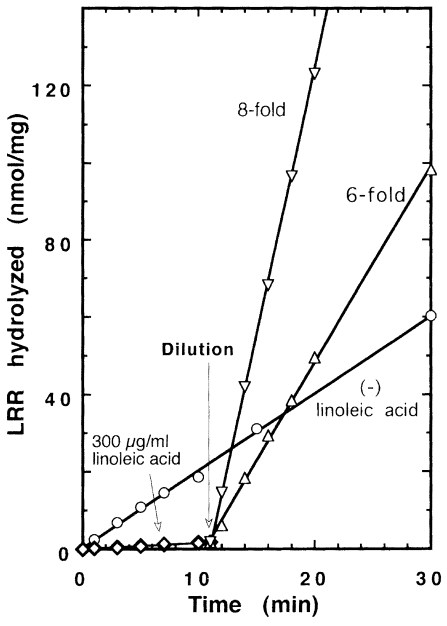

Fig. 2. Inhibition of trypsin-like activity by linoleic acid and powerful activation by dilution of the fatty acid. Reactions were started by adding Boc-LRR-MCA and linoleic acid. The reaction solution contained 50 mM Tris-HCl, pH 8.5, 10% DMSO, 1 mM EDTA, 3 μg/ml 20S proteasome, 10 μM Boc-LRR-MCA and 0 (○) or 300 μg/ml (◇) linoleic acid. In case of 300 μg/ml linoleic acid, at 11 min after the start, the reaction solution was diluted 6-fold (△) or 8-fold (▽) by adding appropriate volumes of the same reaction solution devoid of linoleic acid and the enzyme.

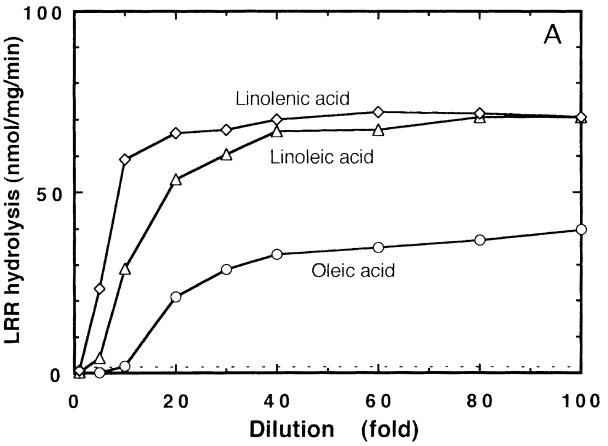

Fig. 3. Effects of dilution after pre-reaction with various fatty acids (300 μg/ml) on trypsin-like activity. Pre-reactions were carried out for 10 min in the presence of 300 μg/ml of oleic (○), linoleic (△) or linolenic (◇) acid under the same conditions as for Fig. 1C. After the pre-reaction period, the reaction solution was diluted 1- to 100-fold by adding appropriate volumes of the same reaction solution devoid of fatty acid and the enzyme, and the reaction was continued for 30 min. Dilutions of the fatty acid giving half-maximum activation were about 12- and 6-fold for linoleic and linolenic acid, respectively, and that for oleic acid was over 20-fold.

of Boc-LRR-MCA hydrolysis by linolenic acid was examined as a function of the degree of dilution. After the 10 min pre-reaction in the presence of 300 μg/ml of various fatty acids, the fatty acid in the reaction medium was diluted to different degrees ranging from 1- to 100-fold, and the reaction was continued for 30 min. As seen here, the extent of activation increased as the degree of dilution increased up to about 20- to 40-fold, and leveled off. Importantly, two different fatty acid, linolenic acid and oleic acid, showed similar dilution-dependent activation pattern, rationalizing the use of linoleic acid as a fatty acid representative in the present study.

In order to investigate the above hypothesis that the dilution-induced activation of trypsin-type peptidase activity is due to the removal of the fatty-acid inhibition characteristic for this type of peptidase, we carried out the Boc-LRR-MCA hydrolysis reaction for 10 min in the presence of various concentrations of linoleic acid in the same way as done in the experiment of Fig. 1C. Then, the reaction solution was diluted 20-fold (the degree of dilution that produces a near maximal activation, see Fig. 3) with the linoleic acid-free solution and the hydrolysis reaction was carried out for another 30 min. The results of the experiment are shown in Fig. 4. Interestingly, the overall pattern of fatty acid activation of trypsin-type peptidase has now become identical to those of other peptidases obtained with Suc-LLVY-MCA or Cbz-LLE-2NA (cf. Fig. 1A and B). Importantly, the maximal level of the peptidase activity after dilution (50-55 nmol/mg/min at linoleic acid ≥ 120 μg/ml) is significantly higher than the maximal level before dilution.

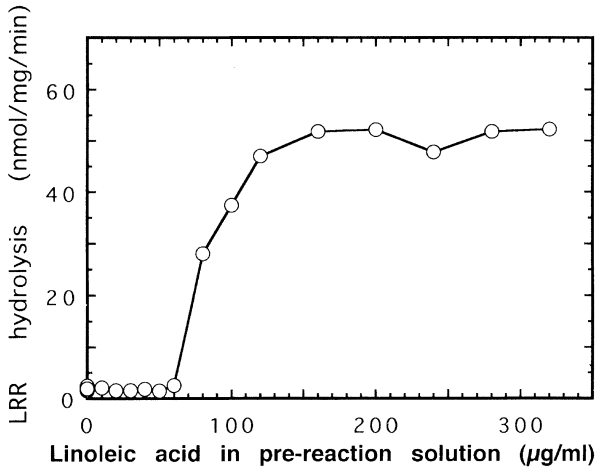

Fig. 4. Potent activation of trypsin-like activity by 20-fold dilution of the fatty acid. Pre-reactions were carried out in the presence of various concentrations of linoleic acid (0-320 μg/ml) for 10 min under the same conditions as for Fig. 1C. After the pre-reaction period, the reaction solutions were diluted 20-fold by adding the same reaction solution devoid of linoleic acid and the enzyme, and the reaction was continued for 30 min.

Activation profiles at various concentrations of the enzyme

In all experiments described above, the peptidase reactions were carried out at the enzyme concentration of 3.0 μg/ml, because most peptidase assays reported in the literature were carried out in the enzyme concentration range of 2-3 μg/ml. Whether changes in the enzyme concentration in a broad range have any effect on the fatty acid activation pattern has not yet been investigated. We investigated the linoleic acid concentration-dependent activation of the three types of peptidase at different concentrations of the 20S proteasome (Fig. 5): A, chymotrypsin-type; B, PGP hydrolase-type; C, trypsin-type. As described above (cf. Fig. 1A and B, and Fig. 4), activation by linoleic acid occurs only above the threshold level, producing an appreciable "latency" phase in the activation curve. As seen in Fig. 5, the threshold value was found to increase as the enzyme concentration increased for all types of peptidase activity examined. Interestingly, the "latency" phase of the linoleic acid activation virtually disappeared when the enzyme concentration was reduced to a very low value. Figure 5 also shows that the specific activities of the three types of peptidase at maximally activating concentrations of linoleic acid tend to decrease upon the increase of the enzyme concentration.

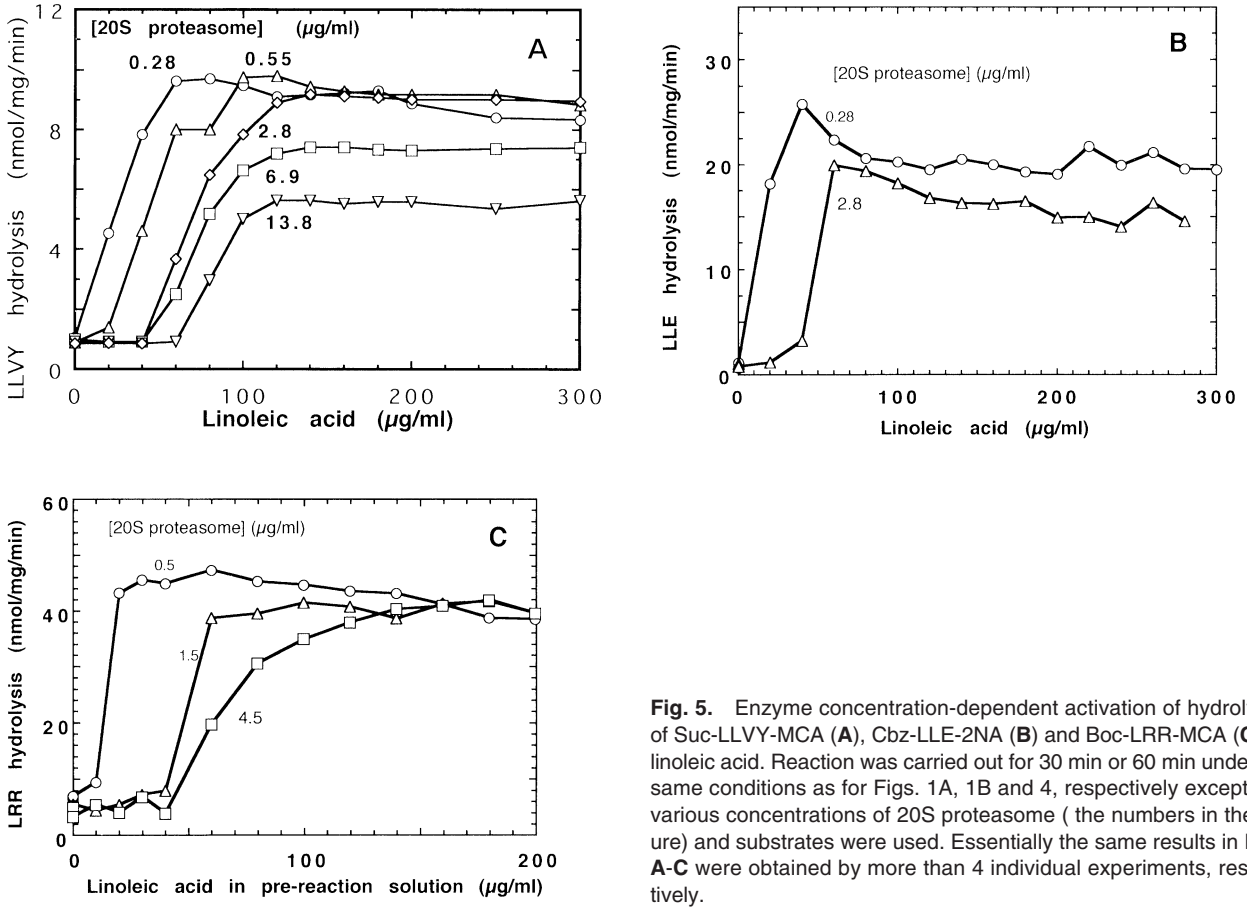

Fig. 5. Enzyme concentration-dependent activation of hydrolyses of Suc-LLVY-MCA (**A**), Cbz-LLE-2NA (**B**) and Boc-LRR-MCA (**C**) by linoleic acid. Reaction was carried out for 30 min or 60 min under the same conditions as for Figs. 1A, 1B and 4, respectively except that various concentrations of 20S proteasome (the numbers in the figure) and substrates were used. Essentially the same results in Figs. **A**-**C** were obtained by more than 4 individual experiments, respectively.

DISCUSSION

The 20S proteasome shows peptidase activities only in the presence of appropriate activators such as SDS and fatty acids, and hence it is known as the latent enzyme (for review, see Coux *et al*., 1996). The enzyme has at least three endopeptidase activities, which is referred to as chymotrypsin-type, PGP hydrolase-type and trypsin-type peptidases (Orlowski, 1990; Rivett, 1993). According to several reports, fatty acids (e.g. linoleic, linolenic and oleic acids) produce an appreciable activation of the chymotrypsin-type and PGP hydrolase-type peptidases, but has rather inconsistent effects on the trypsin-type peptidase: little activation (Dahlmann *et al*., 1985; Orlowski and Michaud, 1989) or strong inhibition (Ishiura and Sugita, 1986; Saitoh *et al*., 1989). However, little is known about how the mode of fatty acid activation of the trypsin-type peptidase is differentiated from that of the other types of peptidase.

In the present study, we investigated the details of the modes of fatty acid activation of the three types of peptidase activity. One of the most important aspects of this study is the finding that the chymotrypsin-type and PGP hydrolase-type peptidases share common features of fatty acid activation, but the trypsin-type peptidase is governed by a more complex mechanism. The trypsin-type peptidase, which is suppressed by linoleic acid, is activated concomitantly with the dilution of linoleic acid in the reaction solution and the resultant high activity is maintained during the experimental period (Fig. 2). After prereaction in the presence of linoleic acid, the reaction solution was diluted by excess volume of the linoleic acid free solution and the hydrolysis reaction was carried out in experiment of Fig. 4. The resultant activation pattern of the trypsin-type became identical to those of the chymotrypsin-type and PGP hydrolase-type peptidases. These findings suggest that all of the three peptidases share a common mechanism of fatty acid activation, except that the trypsin-type peptidase has an additional unique mechanism as discussed below.

The common feature of fatty acid activation of the chymotrypsin- and PGP hydrolase-types is that the activation occurred in two clearly distinguishable phases. As shown in this study, no activation occurs until the concentration of the added fatty acid reached to certain threshold level, producing a “latency” phase. Upon increasing the fatty acid concentration above this threshold level, fatty acid became a strong activator of these peptidases. This biphasic activation pattern can be explained by the existence of two classes of fatty acid binding sites: (a) high-affinity sites that are not involved in the activation of the peptidases (designated as latency sites), and (b) other sites that are involved in the enzyme activation (activation sites). In view of the abrupt transition from the latency phase to the activation phase at the threshold concentration of fatty acids, it appears that binding of fatty acids to the latency sites is prerequisite to the binding of the fatty acid molecules to the activation sites. The number of the latency sites seems to be some definite stoichiometric number per mole of the 20S proteasome, since the threshold value for activation varied with the enzyme concentration in a parallel manner as demonstrated in Fig. 5. However, the precise stoichiometry remains to be determined.

An other important finding in this study is that the pattern of fatty acid activation of the trypsin-type peptidase is distinctly different from those of chymotrypsin- and PGP hydrolase-types described above. Fatty acid activation in this case occurs in a tri-phasic manner: inhibition in a low concentration range, activation in a middle concentration range, and inhibition again in a high concentration range (cf. Fig. 1C). Interestingly, however, the biphasic activation pattern which is identical to those of the chymotrypsin- and PGP hydrolase-type peptidases was produced when the fatty acid was diluted after the enzyme had reacted with the substrate in the presence of various concentrations of fatty acid (cf. Fig. 4). The simplest explanation for such a phenomenon is as follows. The third class of fatty acid binding sites characteristic for the trypsin-type is present, and the fatty acid binding to this class produces inhibition of the peptidase (inhibition sites). We propose that the dilution procedure removes fatty acids that have been bound with the inhibition sites, but does not remove the fatty acids that have been bound with the activation sites. The present data also suggest that the fatty acid moiety bound to the activating sites is occluded upon their binding, since the maximum activation persisted even after a large dilution.

In conclusion, this study revealed several intricate mechanisms by which the three peptidase activities of the 20S proteasome are regulated by fatty acids. It appears that all of these peptidases are controlled by at least two classes of fatty acid binding sites; latency sites and activation sites. We propose that the fatty acid binding to the former produces conformational change in the enzyme, now allowing fatty acids to bind to the activation sites. The additional class of fatty acid binding (inhibition sites) is involved in the regulation of the trypsin-type peptidase. The fatty acid moiety bound to the inhibition sites is readily removed from the sites by dilution, while that bound to the activation sites is occluded upon binding. Correlation of the fatty acid activation mechanism described in this paper with the mechanism of activation by physiological activators will be one of the important problems to be investigated in the future studies.

ACKNOWLEDGMENTS

We wish to thank Dr. Noriaki Ikemoto, Department of Muscle Research, Boston Biomedical Research Institute and the Department of Neurology, Harvard Medical School for valuable comments on the manuscript. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and grants from the Saito-Chion Foundation, Shizuoka, Japan.

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(Received February 3, 1997 / Accepted October 28, 1997)