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Addition of ATP increases the apparent molecular mass of the multicatalytic proteinase, ingensin

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A high-molecular mass ATP-dependent proteinase was shown to be identical to a multicatalytic proteinase, ingensin [(1988) Eur. J. Biochem. 177, 261–266]. The molecular mass of this proteinase increased in crude extracts of the rat liver and porcine brain, but not in the purified sample, only when the proteinase was extracted with ATP. The higher-molecular form of ingensin may be the intact one, because the concentration of ATP in vivo never decreases below 1 mM. This form of the proteinase is latent and it requires a high concentration of detergent for activation. On chromatography, it was found that the high-molecular form corresponds to the previously reported minor isoenzyme of ingensin [(1986) Biochim. Biophys. Acta 882, 297–304], ingensin A, or possibly to the ATP/ubiquitin-dependent 26S protease [(1987) J. Biol. Chem. 262, 8303–8313], and the low-molecular form to major ingensin B or the ATP/ubiquitin-independent 20 S protease.

Multicatalytic proteinase; Isoenzyme; ATP; (Rat liver)

1. INTRODUCTION

A multicatalytic proteinase, ingensin, is present in the cytosol of various species including mammals [1], insects [2] and plants [3], but its physiological role remains nuclear. The enzyme has been found to have a high molecular mass of about 600 000 kDa and to contain 20 subunits with molecular masses of 25–35 kDa [4–7]. The enzyme has recently been shown to be the same as the 19 S ribonucleoprotein particle (prosome) [8] and also as the eukaryotic ATP-dependent proteinase, possibly mediating the ubiquitin conjugation pathway [9–12]. Previously, we found that this proteinase gave two peak materials, ingensins A and B, on hydroxyapatite column chromatography [13]. Ingensin A often appeared in the breakthrough fraction or was eluted with a low concentration of phosphate. The major ingensin B activity, however, was tightly bound to the column and eluted at 0.2 M phosphate. The molecular properties of the two isoforms of ingensin were quite different. For example, ingensin A was activated by sonication, but ingensin B was not. Ingensin A gave a CBB stainable high molecular weight band corresponding to a molecular mass of 90 kDa on SDS gel electrophoresis in addition to a low molecular mass, 25–35 kDa complex, which is a typical feature of the multicatalytic proteinase, ingensin. It is possible that the two isoforms share several low molecular weight subunits encoded by the same set of genes, and the differences in their properties are attributable to post-translational modifications or proteolytic processing. The fact that these two isoforms were found to be immunologically related favors the above idea [13].

In the course of a study on the extraction efficiency for the enzyme, we found that the addition of ATP in the homogenization buffer altered the column elution profile on hydroxyapatite. On quick isolation, it was found that ATP increases the apparant molecular mass of the multicatalytic proteinase, ingensin, suggesting that the ATP-dependent association of unidentified proteins with the native ingensin molecule occurs in vivo.

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2. MATERIALS AND METHODS

2.1. *Materials*

The substrates, succinyl-Leu-Leu-Val-Tyr-MCA (SLLVY-MCA) and Boc-Leu-Arg-Arg-MCA (BLRR-MCA), were purchased from Peptide Inst. Co., Ltd, Osaka.

2.2 *Extraction of tissue*

The homogenization buffers were as follows: 5 mM phosphate, pH 7.0 (buffer A), 5 mM phosphate / 5 mM ATP / 5 mM Mg, pH 7.0 (buffer B), and 5 mM phosphate / 10% glycerol, pH 7.0 (buffer C). Rat liver (1 g) was homogenized in 5 vols of each buffer with a glass homogenizer, and then the nuclear and mitochondrial fractions were removed by centrifugation at 10 000 × *g* for 20 min. The postmitochondrial supernatant was directly applied to a 5 ml hydroxyapatite column equilibrated with each buffer. Unadsorbed proteins were washed out with 30 ml of each buffer and then the phosphate concentration was increased to 0.1 M without changing the other conditions. After eluting the protein with the same buffer (30 ml), the phosphate concentration was further increased to 0.3 M. Almost all the proteins adsorbed to the column were eluted at this step. Fractions of 5 ml were collected.

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2.3. *Assay method*

The multicatalytic proteinase was quantitated by determining the release of aminomethylcoumarin (AMC) from a synthetic substrate, SLLVY-MCA or BLRR-MCA, as reported previously [1,6]. The incubation mixture comprised 50 mM Tris-HCl, pH 9.0, and 0.1 mM substrate, in a total volume of 0.1 ml. Samples were incubated at 37°C for 30 min and AMC was determined with a Hitachi F-3000 spectrofluorometer.

3. RESULTS AND DISCUSSION

Fig.1 shows the elution profiles of the rat liver multicatalytic proteinase, ingensin, from a hydroxyapatite column under two different sets of conditions. In the absence of ATP, ingensin was strongly adsorbed to the column and only eluted on the addition of 0.3 M phosphate. 10% glycerol did not affect the elution profile (data not shown). On the other hand, when the liver was homogenized and chromatographed with 5 mM ATP and 5 mM Mg, almost all of the proteinase was eluted in both the flowthrough and 0.1 M phosphate fractions. The addition of 0.3 M phosphate did not cause elution of the enzyme at all. Since the addition of ATP and Mg did not affect the overall yield of the proteinase, the result indicates changes in the molecular properties of the proteinase. The ratio of the relative amounts of ingensin eluted in the presence of ATP and Mg was 9:4:1 (flowthrough: 0.1 M:0.3 M) in 3 independent experiments.

The multicatalytic proteinase, ingensin, exhibits the special property of SDS activation. The addition of SDS drastically increases the chymotryptic activity of the proteinase. If this activation is observed in the crude extract of an uncharacterized sample, we can conclude that the sample undoubtedly contains the multicatalytic proteinase, ingensin. Fig.2 shows the results with SLLVY-MCA for the enzymes eluted from a hydroxyapatite column. The most abundant species isolated with 5 mM ATP-Mg, which was eluted in the breakthrough fraction, was activated by SDS, the maximum activation being observed at 0.06% SDS (fig.2a). The activation was marked (above 10-fold) at 37°C. The second enzyme, eluted at 0.1 M phosphate with ATP-Mg, was also activated by SDS, but the optimal SDS concentration was 0.03% (fig.2b). The third one, eluted at 0.3 M phosphate with ATP-Mg, was activated at the lowest concentration of SDS (0.02%) (fig.2c). With a high concentration of SDS (0.08%) the enzyme was reactivated, but the extent of the activation was low. This dual activation pattern was also observed for the 0.3 M phosphate eluate obtained without ATP (data not shown). Therefore, we conclude that the two-phase activation with different concentrations of SDS is a common property of the enzyme eluted from a hydroxyapatite column at 0.3 M phosphate.

SDS is generally used to denature proteins. Since no dissociation of subunits was observed in a 0.1% SDS solution, the effect of SDS is limited to either a conformational change of each subunit or subunit interaction. The enzymes eluted at 0.3 M phosphate with and without ATP may both have a relatively loose structure, because with a small amount of SDS the catalytic site of the enzyme could gain access to the substrate. On the other hand, the enzyme, which weakly interacted with hydroxyapatite in the presence of ATP, had a tighter structure than the former, i.e. the latter exists as a latent form in vivo. Other biochemical properties, such as substrate specificity, pH optimum, heat stability, etc., were the same for the 3 enzymes. Mg ions are not indispensable for this ATP-dependent transition of the enzyme to a high-molecular-mass form.

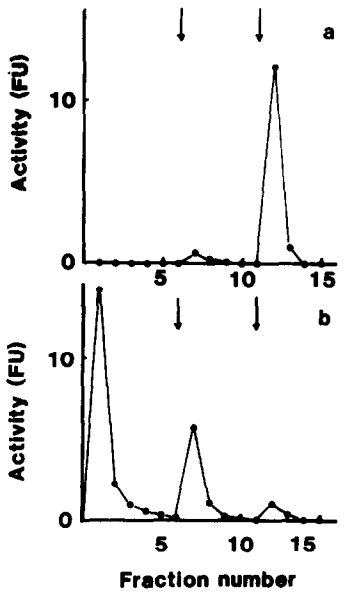

Fig.1. Hydroxyapatite chromatography of the multicatalytic proteinase, ingensin, from rat liver. Samples of liver extract (25 mg) were applied directly to 5 ml of hydroxyapatite equilibrated with ATP, Mg-free buffer A (a) or ATP, Mg-containing buffer B (b). Bound proteins were eluted with a stepwise gradient (each 25 ml) of 0.1 M (left arrow) and 0.3 M (right arrow) phosphate with (b) or without (a) ATP-Mg. Fractions (5 ml) were collected, and 20 μl portions were assayed for the multicatalytic proteinase, ingensin, with SLLVY-MCA for 30 min at 37°C. Portions were also assayed with BLRR-MCA but the activity was proportional to that with SLLVY-MCA. 1FU (fluorescence unit) = nmol/h.

We then applied these active fractions to an analytical HPLC gel filtration column (TSK G4000SW, 0.8 × 65 cm) to determine the native molecular weight. Contrary to our expectation, the enzyme eluted in the flowthrough fraction in the presence of ATP was separated into two peaks (fig.3a). The first corresponds to a molecular mass of above 1000 kDa and the second to 600 kDa. The second peak coincided with the purified multicatalytic proteinase, ingensin, from rat liver [14]. When the enzyme was dialyzed against an ATP-free neutral solution overnight and then chromatographed on the same column, the first peak disappeared or sharply decreased in amount. Since the purified ingensin never moved to the high-molecular-

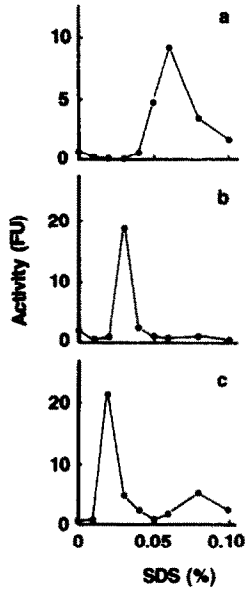

Fig.2. Effect of SDS concentration on the SLLVY-MCA hydrolyzing activities of ingensin in each fraction. Aliquots of ingensins eluted from a hydroxyapatite column were incubated with SDS in a total volume of 100 μl and the liberation of AMC was determined spectrophotometrically. (a) fraction 1 of fig.1b; (b) fraction 7 of fig.1b; (c) fraction 12 of fig.1a.

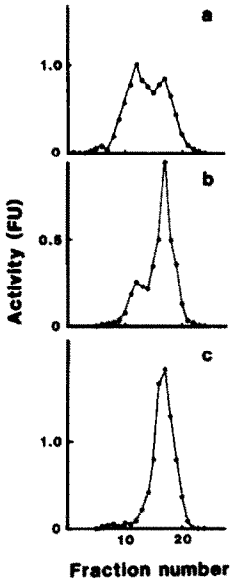

Fig.3. Gel filtration chromatography of the isolated proteinase fraction. Samples (0.5 ml) of the peak materials from fig.1 were applied to a TSK G4000 SW HPLC gel filtration column (0.8 × 60 cm) equilibrated with ATP, Mg-containing buffer A (a,b) or ATP, Mg-free buffer B (c) at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected. Applied samples are: (a) fraction 1 of fig.1b; (b) fraction 7 of fig.1b; (c) fraction 12 of fig.1a. Activity was determined with SLLVY-MCA as the substrate.

weight region, even after preincubation with ATP and Mg, the possibility that ingensin aggregated into a high molecular form by itself was ruled out. The relative amounts of high molecular form decreased when the 0.1 M eluate was applied to the gel filtration column. The 0.3 M eluate did not contain the high molecular form. These results indicate that the multicatalytic proteinase, ingensin, is converted from a pure 600 kDa proteinase form (20 S) to a 1000 kDa complex (26 S) in the presence of ATP, possibly through binding to another cytosolic component. Substrate specificity of these enzymes is shown in table 1. Both enzymes hydrolyzed synthetic substrates at the same rate.

The multicatalytic proteinase, ingensin, is thought to comprise 20–30 low-molecular-weight subunits. In the experiments shown in fig.3, the molecular masses of the proteinases in different column fractions were compared with that of the purified one. An increase in molecular mass was observed especially in that of the enzyme showing no interaction with hydroxyapatite. The finding is in agreement with our next observation for the molecular mass of the 0.1 M eluate enzyme. This material is a mixture of the flowthrough enzyme and the ingensin isolated in the absence of ATP.

The column elution profile of the former seems to be the same as that of ingensin A previously reported by us [13]. The appearance of ingensin A is not reproducible from preparation to preparation. Since we previously never employed the purification method involving ATP, we may have missed the intact form of the multicatalytic proteinase, ingensin. Goldberg and coworkers reported that in the presence of glycerol in the buffer the enzyme remains intact [12,15]. Their enzyme still exhibited ATP-dependency as to peptide hydrolysis after purification. However, we have already demonstrated that ATP only stabilized the enzyme to

Table 1

Substrate specificity of the isolated enzymes

Substrate	Relative activity (%)		
	Mg F.17	ATP F.13	ATP F.17
(a) Trypsin substrate			
Boc-Leu-Arg-Arg-MCA	(100)	(100)	(100)
Boc-Gln-Arg-Arg-MCA	38	36	28
Boc-Leu-Gly-Arg-MCA	36	29	39
Boc-Phe- Ser-Arg-MCA	33	33	40
Boc-Leu- Ser-Thr-Arg-MCA	29	27	31
Boc-Gln-Gly-Arg-MCA	17	16	19
Boc- Val-Pro-Arg-MCA	10	8	5
Boc- Ile-Glu-Gly-Arg-MCA	3	8	6
Boc-Gly-Arg-Arg-MCA	0	0	0
(b) Chymotrypsin substrate			
Suc-Leu-Leu- Val-Tyr-MCA	(100)	(100)	(100)
Suc-Ala- Ala-Phe-MCA	57	35	47
Suc- Ser- Ser-Leu-Tyr-MCA	34	27	27

Hydrolysis of both trypsin and chymotrypsin substrates were measured independently. 0.1 μg of the enzyme was incubated with 50 mM Tris-HCl, pH 9.0, and 0.1 mM of each substrate for 30 min. The activity is expressed as a percentage hydrolysis compared with that of the best substrate.

protect it from inactivation at 37°C [16]. This effect of ATP is unrelated to the ubiquitin-dependent system reported by other investigators [9–11], where another intact high-molecular-mass 26 S proteinase requires ATP and Mg for the hydrolysis of the radiolabeled ubiquitinated proteins. Our ingensin A or the hydroxyapatite flowthrough enzyme looks similar to this high-molecular-mass ATP/ubiquitin-dependent 26 S proteinase. Currently, we are investigating the ATP-dependency of the purified enzyme as to catalysis.

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