Isolation of two forms of the high-molecular-mass serine protease, ingensin, from porcine skeletal muscle

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Two forms of a neutral protease that catalyzed the hydrolysis of succinyl-Leu-Leu-Val-Tyr-MCA were isolated from porcine skeletal muscle cytosol by fractionation on DEAE-cellulose, hydroxyapatite and Sephadex G-100. The native enzyme had a molecular mass of above 1000 kDa. Peak A, which was eluted from hydroxyapatite at 50 mM phosphate, was activated 37-fold by the detergent, SDS, while peak B which was eluted at 150 mM phosphate, was activated only 2-fold. After dialysis against water, the B form showed restored ability to be activated by SDS (9.6-fold with 0.04% SDS). The activated peak B was extremely sensitive to divalent and monovalent cations such as Ca^{2+}, Mg^{2+}, Na^+, K^+ and NH_4^+ as well as protease inhibitors such as leupeptin, chymostatin and DFP. These results suggest that these proteases are generally latent in the cells and may be regulated by changes in the concentrations of cations in the cytosol. We call this new type of protease, ingensin.

Porcine muscle *Protease* *SDS-activation*

1. INTRODUCTION

Several reports have indicated that the mammalian cytosol contains neutral proteolytic activity which plays major roles in physiological protein turnover or regulation of enzyme activities. Ca^{2+}-activated neutral protease is a typical intracellular cytosolic protease responsible for initiating the breakdown of a variety of cellular components [1–6]. A 230 kDa high-molecular mass neutral protease digested oxidized proteins rapidly [7].

One of the major problems encountered in assessing the physiological significance of these cytosolic proteases in the cell is that they require some factor(s) such as calcium ions, in the case of Ca^{2+}-activated neutral protease, for their activity, otherwise all the cellular components would be randomly degraded without effectors.

In the course of studying the role of cytosolic neutral proteases, two forms of succinyl-Leu-Leu-Val-Tyr-MCA-degrading enzymes from porcine skeletal muscle were separated and partially purified. The molecular masses of the two proteases were high and they were unique in their activation by SDS. In the presence of a high salt concentration both forms were inactive towards the synthetic peptide. A low concentration of SDS in the absence of salt highly activated both forms, but the activation was reversed by the addition of 10 mM cations. Here we present evidence that many factors regulate this newly-found cytosolic protease, named 'ingensin'.

2. MATERIALS AND METHODS

2.1. *Materials*

Succinyl-Leu-Leu-Val-Tyr-MCA, leupeptin, chymostatin and bestatin were from the Peptide Research Institute, Osaka. Ep475 (E-64-c) was a gift from Taisho Pharm., Ohmiya. DEAE-cellulose and Sephadex G-100 were obtained from Pharmacia. Hydroxyapatite was from Seikagaku Kogyo.

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2.2. *Assay of succinyl-Leu-Leu-Val-Tyr-MCA-degrading activity*

Succinyl-Leu-Leu-Val-Tyr-MCA-degrading activity was determined by measuring the fluorescence of liberated aminomethylcoumarin (AMC) from the peptide. The incubation mixture (100 μl) contained 20 mM Tris-HCl, pH 8, and 0.5 mM substrate dissolved in dimethylsulfoxide (DMSO). The concentration of DMSO never exceeded 5%. After incubation at 37°C for 60 min, the reaction was terminated by the addition of 100 μl of 10% SDS and 1.3 ml of 0.1 M Tris-HCl, pH 9.

2.3. *Isolation of enzymes*

Porcine skeletal muscle (200 g) was minced and homogenized in a Waring Blender with 5 vols of 5 mM phosphate/0.1 M NaCl, pH 7.0. The homogenate was centrifuged at 8000 × *g* for 30 min and the sediment was discarded. Cold acetone (1:1 v/v) was added gradually to the cytosol while stirring, and the suspension was then centrifuged at 8000 × *g* for 20 min. The pellet was redissolved in distilled water and dialyzed against water overnight. The dialyzed sample was applied to a 200 ml column of DEAE-cellulose equilibrated with 5 mM phosphate/0.1 M NaCl, pH 7.0. Elution was performed with the same buffer until the breakthrough fractions appeared, and then with a linear gradient of 0.1–0.4 M NaCl in the same buffer (total 1200 ml). Fractions containing enzyme activity were pooled and diluted 3-fold with water. The sample was applied to a 30 ml column of hydroxyapatite equilibrated with 5 mM phosphate, pH 7.0. The column was developed with a linear gradient of 5–300 mM sodium phosphate buffer, pH 7.0.

3. RESULTS

3.1. *Separation of two forms of proteolytic activity on hydroxyapatite*

Fig.1 shows the elution pattern of the enzymes from a hydroxyapatite column. Two activities were detected eluting separately from the column. The first peak (peak A) was eluted at 50 mM phosphate, while the second one (peak B) was at 150 mM. As shown in table 1, peak A was activated 37-fold by the addition of SDS (optimal concentration: 0.04%).

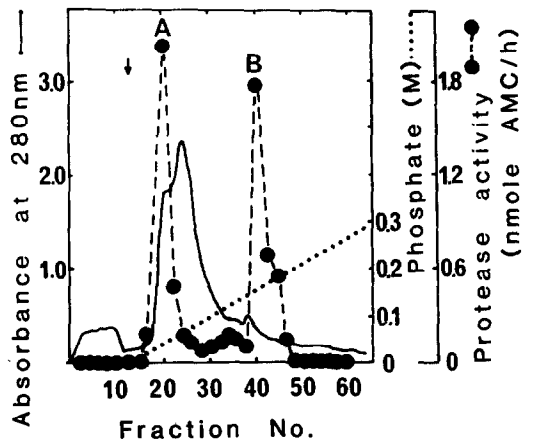

Fig.1. Separation of porcine skeletal muscle succinyl-Leu-Leu-Val-Tyr-MCA-degrading activity into two peaks on a hydroxyapatite column. Active fractions from a DEAE-cellulose column were chromatographed on a hydroxyapatite column (2 × 10 cm) as described in section 2. Aliquots (50 μl) were assayed for protease activity (●---●) at pH 8. The protein concentrations of column fractions (——) were determined from the absorbance at 280 nm.

Table 1

Stimulation of succinyl-Leu-Leu-Val-Tyr-MCA-degrading activity by 0.04% SDS

Enzyme	Activity		Activation (-fold)
	No addition (nmol AMC/h)	+0.04% SDS (nmol AMC/h)	
Peak A	0.805	30.3	37
Peak B	0.580	0.967	1.7

Peaks A and B from Sephadex G-100 columns of fig.2 were incubated as described in section 2. After incubation (37°C, 60 min), the liberated AMC was determined fluorometrically

3.2. *Molecular mass determination*

For further purification and molecular mass determination, the peak fractions from the hydroxyapatite column were applied to a column of Sephadex G-100 equilibrated with 5 mM phosphate, pH 7.0, with 0.1 M NaCl (fig.2). The succinyl-Leu-Leu-Val-Tyr-MCA-degrading activi-

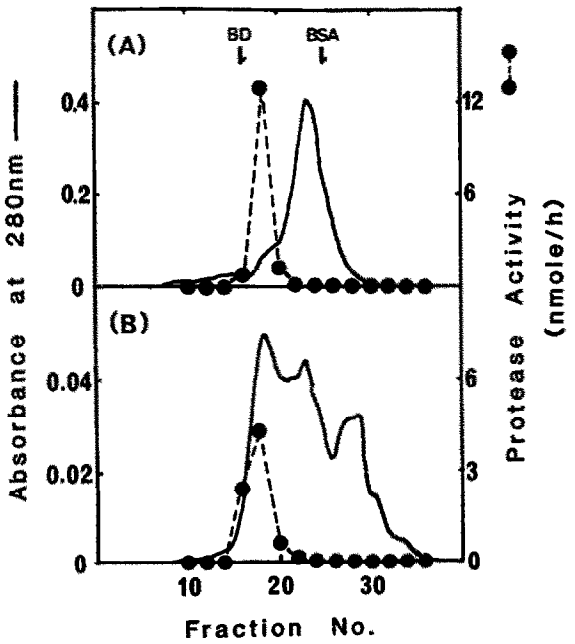

Fig.2. Chromatography of succinyl-Leu-Leu-Val-Tyr-MCA-degrading activity on a Sephadex G-100 column. Fractions 20 (peak A) and 40 (peak B) shown in fig.1 (5.5 ml) were chromatographed on a Sephadex G-100 column (2 × 50 cm). Fractions of 5.5 ml were collected and aliquots (50 μl) were assayed for protease activity (●---●).

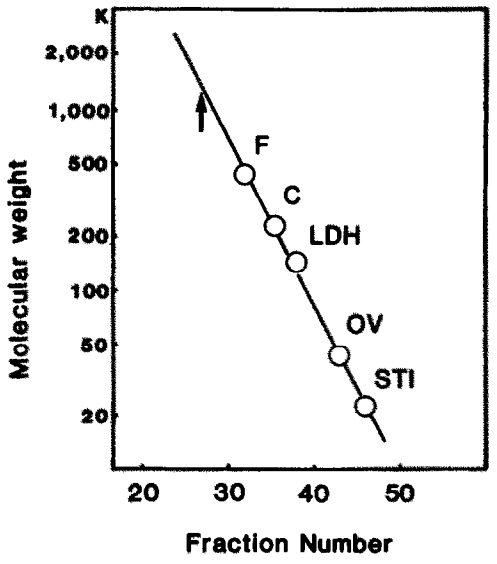

Fig.3. Molecular mass determination of the protease, ingensin, by HPLC (TSK 3000SW). Ferritin (F), catalase (C), lactate dehydrogenase (LDH), ovalbumin (OV) and soybean trypsin inhibitor (STI) were used as standards. Blue dextran was eluted at fraction 20.

ty was eluted near the void fraction, suggesting that the native molecular masses of these protease forms were above 100 kDa. The molecular mass of this protease, ingensin, was determined by HPLC on a TSK C3000SW column (fig.3). A value of between 1000 and 1300 kDa was obtained. The pH optimum of the peptide-hydrolyzing activity was between 8 and 10.

3.3. *Effect of protease inhibitors and cations*

To further characterize the nature of the SDS activation, the effect of various protease inhibitors on the peak B protease was investigated.

As shown in table 2, chymostatin as well as leupeptin at 0.1 mg/ml significantly inhibited the SDS-activated form. A cysteine protease inhibitor, E-64-c, and an aminopeptidase inhibitor, bestatin, at 0.1 mg/ml did not inhibit ingensin B both in the presence and absence of SDS. However, DFP at the concentration of 5 mM strongly inhibited both

Table 2

Percentage activity in the presence of various protease inhibitors

Additions	Suc-Leu-Leu-Val-Tyr-MCA-degrading activity (%)	
	− SDS	+ SDS
No addition	(100)	961
Leupeptin (100 μg/ml)	78	98
Antipain (100 μg/ml)	25	4
Chymostatin (100 μg/ml)	15	3
Pepstatin (100 μg/ml)	100	960
Bestatin (100 μg/ml)	100	960
E-64-c (100 μg/ml)	100	955
DFP (5 mM)	0	0

Peak fractions of ingensin B from a G-100 column were incubated in the presence of the indicated inhibitors. Enzyme activity was measured under the same conditions as described in table 1. Final concentration of SDS was adjusted to 0.04%

forms. Thus, this peak B protease appears not to be a cysteine protease but a serine protease.

Monovalent and divalent cations tested at 10 mM and 1 mM, respectively, inhibited the SDS-activated form of peak B (table 3). The inhibition was greater with divalent cations such as Ca^{2+} and Mg^{2+} than with monovalent ones. EDTA did not have an inhibitory effect. However, these cations rather enhanced the latent peak B activity. At physiological concentrations of the ions, the activity of peak B was twice that of the latent form. ATP added as the Na^+ inhibited the SDS-activated form. This inhibition can be explained on the basis of the increased concentration of Na^+ in the assay mixture. So far we have not found evidence that ATP itself activates the enzyme. Phospholipids and related compounds such as phosphatidylserine, phosphatidylinositol, 1,2-diolein and phosphatidylethanolamine (not shown) did not affect the activity of ingensin in the absence of SDS. However, linoleic acid added at above 100 μg/ml activated ingensin 6.5-fold. Table 3 also shows that proteins such as bovine serum albumin, ovalbumin, etc. and detergents other than SDS tested had no stimulatory effect on peptide hydrolysis by ingensin. Peak A showed essentially the same activity profile.

Table 3

Effects of various salts, lipids, proteins and detergents on Suc-Leu-Leu-Val-Tyr-MCA-degrading activity of ingensin B

Additions	Percentage activity	
	− SDS	+ SDS (0.04%)
No addition	100	961
2-Mercaptoethanol (10 mM)	100	1730
Dithiothreitol (10 mM)	100	1730
ATP (2 mM)	81	672
NaCl (10 mM)	115	298
KCl (10 mM)	113	278
NH_4Cl (10 mM)	135	63
$CaCl_2$ (1 mM)	203	153
$MgCl_2$ (1 mM)	135	254
$SrCl_2$ (1 mM)	149	203
EDTA (1 mM)	126	953
Phosphatidylserine (100 μg/ml)	110	N.D.
Phosphatidylinositol (100 μg/ml)	113	N.D.
1,2-Diolein (100 μg/ml)	100	N.D.
Sphingomyelin (100 μg/ml)	100	N.D.
Palmitic acid (100 μg/ml)	100	N.D.
Linoleic acid (100 μg/ml)	650	N.D.
Bovine serum albumin (100 μg/ml)	100	917
Ovalbumin (100 μg/ml)	100	979
Histone H2A (100 μg/ml)	44	679
Insulin (100 μg/ml)	47	562
Soybean trypsin inhibitor (100 μg/ml)	100	960
Deoxycholate (0.04%)	46	N.D.
N-Lauroylsarcosine Na (0.04%)	67	N.D.
Triton X-100 (0.04%)	95	N.D.
Triton X-303 (0.04%)	110	N.D.
Triton X-405 (0.04%)	100	N.D.
Triton N-101 (0.04%)	65	N.D.
Nonidet P-40 (0.04%)	77	N.D.

Peak fractions of ingensin B from a Sephadex G-100 column were incubated as described in table 1. Ingensin activity was expressed as a percentage of the control in the absence of effectors. N.D., not determined

4. DISCUSSION

An N-terminus-blocked tetrapeptide-MCA, succinyl-Leu-Leu-Val-Tyr-MCA, has been shown to be a substrate for a number of neutral proteases. For example, chymotrypsin and Ca^{2+}-activated neutral protease can hydrolyze this peptide-MCA [6]. It thus became apparent that this substrate is a sensitive indicator for chymotrypsin-type endoprotease [8].

Here we found for the first time that porcine skeletal muscle contains two succinyl-Leu-Leu-Val-Tyr-MCA-degrading activities. The preliminary results indicated that human placenta and uterus and rat lung, liver and brain also contained this new protease, ingensin. Therefore the presence of ingensin in the mammalian cytosol is ubiquitous. In human placenta and rat liver, we could detect two ingensin peaks on hydroxyapatite columns (unpublished). The differences in physicochemical properties between the two peaks were very small except for the SDS-activation as shown in table 1. A proteinase with properties similar to that reported here (ingensin B) has been recently identified in the rat skeletal muscle [9,10]. Those authors described an SDS-activated enzyme. However, in contrast with our enzymes from porcine skeletal muscle, the enzyme from rat skeletal

muscle is a cysteine proteinase. Although some further differences exist with respect to the catalytic properties, the rat and the porcine enzymes seem to be essentially similar.

Evidence that the peak B protease activity is influenced by a small amount of SDS and cations may be important as to the regulation of protein breakdown within living cells. The effect of SDS suggests that a slight conformational change activates the enzyme, but the active conformation may be labile because a small alteration of the ionic environment immediately disactivates the enzyme. Muscle cells contain about 36 mM Na^+ and 92 mM K^+. Therefore the enzyme in physiological conditions remains in a less active form which may be reasonable for the cells. Inhibition of the proteolytic activity by cations could be of physiological significance if some factor(s) like a linoleic acid in the cell act as a substitute for SDS. We are currently searching for such substances.

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