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RNA degrading activity is tightly associated with the multicatalytic proteinase, ingensin

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The multicatalytic proteinase, ingensin, was purified to homogeneity from chicken liver. rRNA-degrading activity was co-eluted with the purified multicatalytic proteinase from a TSK-3000SW column. This RNA-degrading activity was inactivated by heat treatment and the addition of a low concentration of SDS. Therefore, the RNA-degrading activity co-eluted with the multicatalytic proteinase was not due to contamination by low-molecular-mass RNases. These results strongly suggest that this RNA-degrading activity was tightly associated with the multicatalytic proteinase, ingensin.

Proteinase; RNA; Ingensin; RNA-degradation

1. INTRODUCTION

The multicatalytic proteinase, ingensin, is a cytosolic protease which has an unusually high molecular mass, 650–750 kDa, and is composed of multiple subunits of 25–35 kDa [1–5]. One of the important characteristics of the enzyme is the multiplicity of its activity. The protease cleaves peptide substrates with both aromatic and basic amino acid residues at the P1 site with different pH optima. These activities are inhibited by chymostatin and leupeptin, and stimulated by SDS and 2-mercaptoethanol. The inhibition profile is complicated. The chymotryptic activity is strongly inhibited by chymostatin but not by leupeptin. The trypsin-like activity, on the other hand, is suppressed by leupeptin. Chymostatin did not strongly inhibit the activity, So, this enzyme was thought to have at least two catalytic sites and to play multiple roles in vivo. But the physiological roles and functions of the multicatalytic proteinase are not well-known at present.

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Recently, it was shown that the multicatalytic proteinase was identical to the 19 S ribonucleoprotein 'prosome'. Not only did the purified prosome hydrolyze synthetic peptide substrates and [^{14}C]methylcasein, but antibodies raised against 19 S prosome specifically reacted with the multicatalytic proteinase and inhibited its protease activity [6,7]. The prosome is known to be a ubiquitous ribonucleoprotein and was identified as mRNA-protein complexes, including globin and other repressed mRNAs [8,9]. Their functions were regarded to be related to inhibition of protein synthesis, processing of tRNA and repression of mRNA. However, no direct evidence that prosome has RNase activity has been reported.

In this paper, we demonstrate that a highly purified multicatalytic proteinase sample degrades RNA in vitro.

2. MATERIALS AND METHODS

2.1. *Purification of the multicatalytic proteinase, ingensin, from chicken liver*

The proteinase was purified from chicken liver to homogeneity as previously described [10,11].

The chicken liver homogenate was fractionated with ammonium sulfate (30–70% saturation), and then applied to a

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DE-52 column and eluted with a linear, 0.1–0.5 M, NaCl gradient. The active fractions were subjected to hydroxylapatite column chromatography (eluted with a 5–300 mM phosphate buffer gradient). The active fractions were applied to a poly-L-lysine agarose column. Elution was performed with a stepwise NaCl gradient, 0.1, 0.3, 0.6 and 1 M. The enzyme eluted at 0.6 M NaCl from the poly-L-lysine agarose column was subjected to HPLC gel filtration on a TSK-G4000SW column. The purified multicatalytic proteinase, ingensin, was obtained by heparin Sepharose column chromatography, with elution with a linear, 0–1 M NaCl gradient.

2.2. *Preparation of rRNA*

rRNA was prepared from mouse kidney as previously described [12]. Frozen tissue was broken into pieces, which were then homogenized in a GT stock solution with Polytron. The homogenate was centrifuged at 25000 × *g* for 10 min. 2-Mercaptoethanol was added to the supernatant up to 1 M, which was then laid on CsCl buffer. Samples were ultracentrifuged at 200000 × *g* for 15 h at 20°C. The pelleted RNA was rinsed, and then purified with ethanol, phenol and chloroform according to the protocol of RNA purification methods [12].

In our preparation, the major 28 S rRNA was lost because of degradation during the purification procedure. But the 18 S rRNA remained intact. There was no problem with the use of the 18 S rRNA as a substrate for RNA hydrolytic activity (see fig.2).

2.3. *Assay for RNase activity*

To prevent contamination by low-molecular-mass RNases, the purified enzyme was applied to a TSK-3000SW HPLC column equilibrated with autoclaved (therefore, RNase-free) 0.1 M potassium phosphate buffer, pH 7.0, before use. Aliquots (5 μl) of the separated fractions were immediately mixed with 0.1 μg of rRNA (total volume: 10 μl). After incubation at 37°C for 8 h, the reaction mixtures were denatured by heat treatment (65°C, 10 min) and then cooled in an ice-water bath. 2 μl of gel-loading buffer was added to each 10-μl sample, and the mixtures were subjected to 1% agarose gel electrophoresis [12]. rRNA was visualized by ethidium bromide-UV transillumination. RNase activity was monitored for the fragmentation of rRNA during incubation.

All laboratory ware and solvents were sterilized in an autoclave or we used brand-new ware to avoid contamination by RNases.

2.4. *Assay for the multicatalytic proteinase activity*

The proteinase activity was determined with succinyl-leucyl-leucyl-valyl-tyrosine-methylcoumarinamide (SLLVY-MCA) as the substrate, as previously described [11].

2.5. *Electrophoresis*

SDS-polyacrylamide electrophoresis was performed by the method of Laemmli in 12% gel [13]. Electrophoresis under non-denaturing conditions was performed in a Tris-glycine, 7% polyacrylamide gel system. The gels were fixed and stained with methanol-acetic acid (50:7, by vol.) and 0.02% Coomassie brilliant blue R-250, respectively.

Agarose gel electrophoresis was performed with a Mupid-2 system (Advance Co., Ltd) in 1% agarose gel. Ethidium bromide-stained RNA bands were photographed using a UV-transilluminator [12].

3. RESULTS

3.1. *Confirmation of the purity of the multicatalytic proteinase, ingensin, from chicken liver*

First, we confirmed the purity of the enzyme, which was used in the RNase assay, by non-denaturing- and denaturing-gel electrophoresis. As shown in fig.1a, only one band was detected in the non-denatured gel. And SDS gel elctrophoresis of the purified protease demonstrated its multisubunit structure, which was the same as that of the multicatalytic proteinase, ingensin, from other sources [2–5,11] (fig.1b). These results indicated that the enzyme sample was highly purified, i.e. homogeneous.

3.2. *Assay for RNA-degrating activity of the purified multicatalytic proteinase*

As low-molecular-mass RNases are present in most laboratory ware, it is very difficult to exclude possible natural RNase contamination of samples. We succeeded in the exclusion of RNase contamination by the method described under section 2.3. As shown in fig.2b (right), there was no RNA-degrading activity in heat-treated samples. Because low-molecular-mass RNases (for instance, RNase A) were stable after heat treatment at 100°C for 10 min and degraded rRNA (data not shown), the absence of RNA-degrading activity in heated samples indicates no contamination by general RNases of gel-filtered samples. Although low-

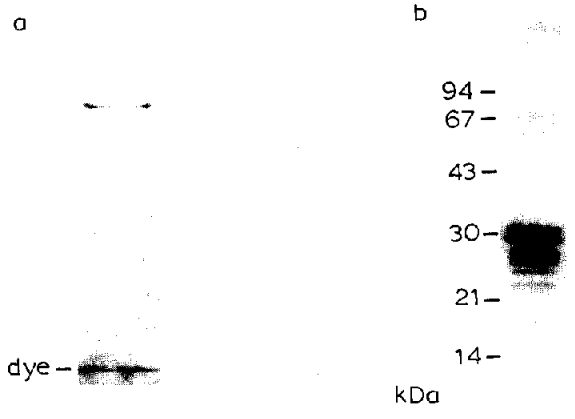

Fig.1. Polyacrylamide electrophoresis of the purified multicatalytic proteinase. The purified chicken liver multicatalytic proteinase was subjected to electrophoresis under non-denaturing (a) and denaturing (b) conditions as described under section 2.5.

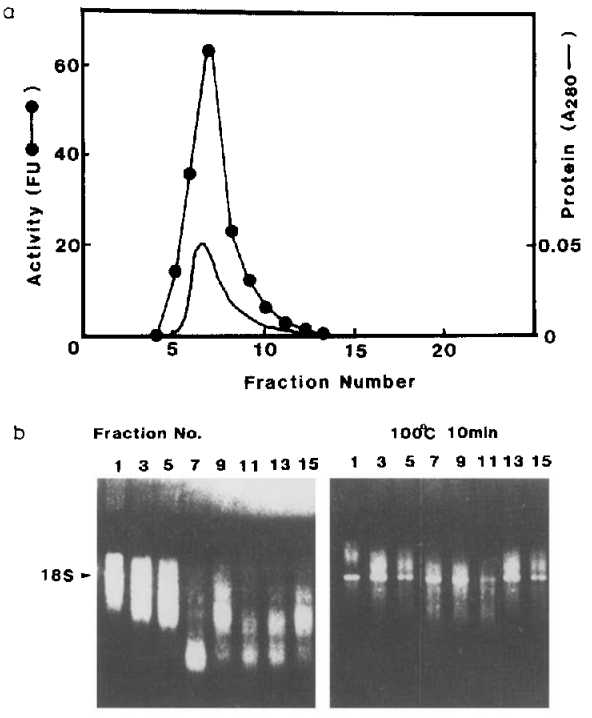

Fig.2. Assaying of RNA-degrading activity of the purified multicatalytic proteinase. Immediately before use, the purified multicatalytic proteinase (0.3 mg) was applied to an RNase-free TSK-G3000SW column. 5-μl aliquots of eluted fractions were assayed for RNase activity as described under section 2.3. 50-μl aliquots were heated at 100°C for 10 min and then cooled. 5-μl aliquots of heat-treated samples were also subjected to RNase-activity assaying as described above. The proteinase activity was assayed with S-LLVY-MCA as a substrate.

molecular-mass RNases were absent in the samples, rRNA-degrading activity was found in the samples with no heat treatment. rRNAs were degraded into low-molecular-weight fragments by high-molecular-weight fractions which coincided with the multicatalytic proteinase activity. Both the highest proteinase and RNase activities were eluted in the same fraction, number 7 (fig.2a, and b, left). These results strongly suggest that rRNA-degrading activity is tightly associated with the high-molecular-weight multicatalytic proteinase, ingensin.

3.3. *Characteristics of RNase activity associated with the multicatalytic proteinase*

To ensure that the degradation of rRNA is catalyzed by an enzyme reaction, the temperature dependency of rRNA hydrolysis was examined. As a control, we added fraction number 1 to rRNA. As shown in fig.3, rRNA was digested only at 37°C, i.e. not at 0°C, on the addition of fraction number 7. RNA was not degraded by the heat-inactivated sample, even at 37°C, and also not by fraction number 1.

Next, we investigated the effects of some reagents on the rRNA-degrading activity. The multicatalytic proteinase was inhibited by PMSF, DFP, chymostatin and pCMB [2,3]. But these protease inhibitors did not affect the RNase activity at all (fig.4). Degradation of RNAs by fraction 7 was observed with chymostatin and DFP. This may be due to contaminating RNase in the inhibitor solution because the same degradation pattern was observed on the addition of control fraction 1. Also, RNase A inhibitor from calf liver did not inhibit the activity.

Among the reagents we examined, only SDS inhibited RNase activity associated with the multicatalytic proteinase sample. The inhibitory effect of SDS was dose-dependent and observed in the presence of more than 0.01%.

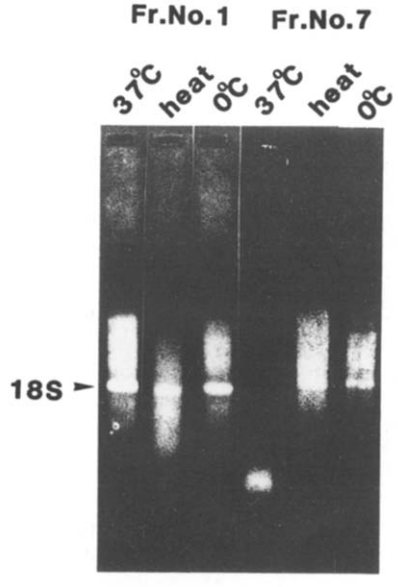

Fig.3. Temperature dependence of RNase activity. The RNase activity was assayed as described in fig.2 at 37°C and 0°C. Heat-treated samples were also assayed at 37°C (heat). An aliquot of fraction number 1 was used as a blank.

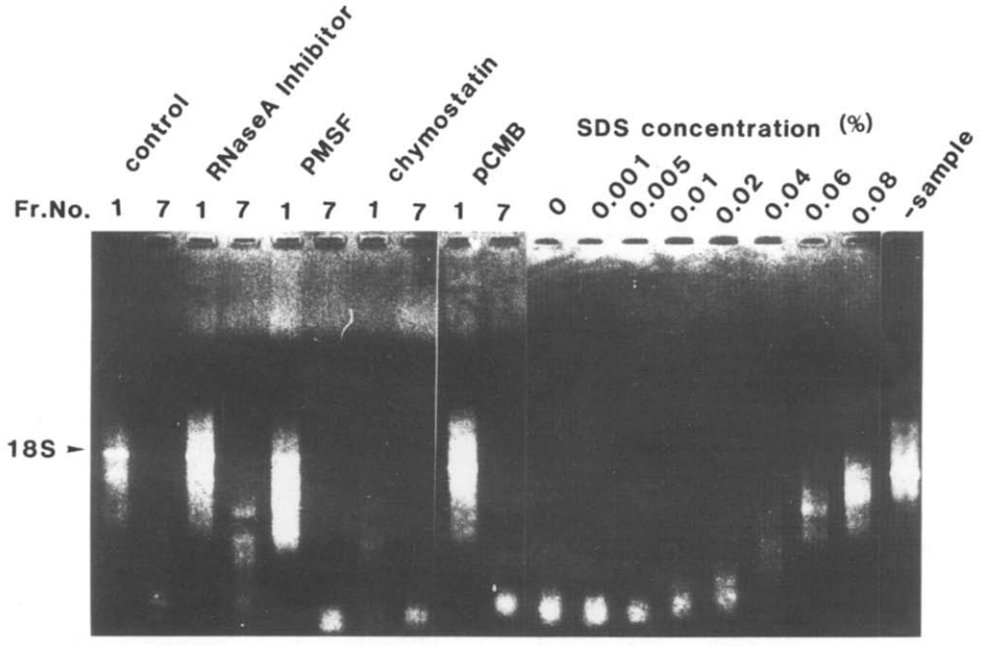

Fig.4. Effects of various reagents on the RNase activity. The rRNA-degrading activity was assayed in the presence of the indicated reagents, as described under section 2.3. The concentrations of reagents were as follows: RNase A inhibitor (calf liver), 600 U/ml; PMSF, 1 mM; chymostatin, 100 μg/ml; pCMB, 0.1 mM. An aliquot of fraction number 1 was used as a blank. 0.08% SDS dit not affect the mobility of rRNA (-sample).

4. DISCUSSION

We found that rRNA hydrolytic activity was co-eluted with the highly purified multicatalytic proteinase, ingensin. The shift of rRNA to low molecular weight was due to an enzymatic reaction, not to a change in electrophoretic mobility after protein-RNA interaction (fig.3). This RNA-degrading activity ws distinguishable from that of known low-molecular-mass RNases by its biochemical characteristics; the RNase activity which accompanied the multicatalytic proteinase was inactivated on heat treatment at 100°C for 10 min and inhibited on the addition of a low concentration of SDS (figs 2 and 4). Therefore, we concluded that this RNA-degrading activity was tightly associated with the multicatalytic proteinase, ingensin. Initially, we tried to obtain direct proof that rRNA hydrolysis was catalyzed by the multicatalytic proteinase itself, by using an immunological method, i.e. the addition of antibodies against the multicatalytic proteinase accelerated the breakdown of rRNA (data not shown). However, this may be explained by the RNase activity in IgG fractions. It is necessary to prepare RNase-free IgG to examine the involvement of the multicatalytic proteinase in RNA digestion. There is so far no evidence allowing a decision as to whether the RNase activity is catalyzed by one of the subunits of the proteinase.

Recently, we and other investigators demonstrated that the multicatalytic proteinase was involved in the ATP-dependent protein degradation system [14,15]. The ATP-dependent proteolytic activity in K562 or BHK21/C13 cells was inhibited on the addition of antibodies against the multicatalytic proteinase. Furthermore, McGuire et al. have shown that this proteinase plays a key role in both ubiquitin-mediated and ubiquitin-independent ATP-stimulated proteolysis [16]. The multicatalytic proteinase is undoubtedly involved in the ATP-dependent proteolytic pathway in mammalian cells and tissues.

Falkenburg et al. reported that the rat liver multicatalytic proteinase contains RNA molecules in the size range of 80–100 nucleotides [6]. If the multicatalytic proteinase has RNase activity like prosomes, small RNAs present in the proteinase

preparation may be hydrolytic products of the enzyme itself. Small RNAs are involved in unknown functions, regardless of RNase activity, because it was demonstrated that prosomes inhibited the in vitro translation of polysomal mRNA from adenovirus-infected HeLa cells by hybridizing with mRNA [17]. This enzyme may play a physiological role in controlling both the post-transcriptional and post-translational modulation of cytoplasmic proteins, as a proteinase or a ribonuclease.

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