**19. The "ATP-Dependent Protease" in Human
Erythroleukemia (K562) Cells is Identical
to a High-Molecular-Mass Protease,
Ingensin**

By Toshifumi TSUKAHARA, Shoichi ISHIURA, and Hideo SUGITA

Division of Neuromuscular Research, National Institute of
Neuroscience, NCNP, Kodaira, Tokyo 187

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Rieder *et al.* reported that an ATP-dependent proteolytic activity is present in human erythroleukemia (K562) cells.¹⁾ Recently, we showed that the Suc-Leu-Leu-Val-Tyr-MCA hydrolytic activity in K562 lysate at pH 9 rose more than 10-fold on the addition of 1 mM ATP, and this ATP-dependent increase in the protease activity was not due to stimulation of the protease activity by ATP but to stabilization of the protease activity by ATP during incubation.^{2),3)} Characteristics of the ATP-dependent protease in K562 cells are quite similar to those of a high-molecular-mass protease, ingensin, previously reported by us.^{4),5)} Through inhibition studies of the crude protease activities and the purified ingensin from K562 cells by various protease inhibitors, we concluded that the "ATP-dependent" SLLVT-MCA hydrolytic activity in K562 lysate was due to the K562 ingensin.³⁾ But ATP had no effect on the ingensin purified from K562 cells as well as from other sources.

Here we give the direct proof of the identity of two enzymes, showing that anti-rat liver ingensin IgG inhibited K562 ATP-dependent protease activity.

Materials and methods. K562 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. The cultured cells were collected by centrifugation, washed 4 times with phosphate-buffered saline (PBS), lysed by sonication and then centrifuged. Proteolytic activity was assayed with succinyl-leucyl-leucyl-valyl-tyrosine-methylcoumarinamide (SLLVT-MCA) as a substrate at pH 9.0 as described previously.⁴⁾

Antiserum against ingensin was raised by repeated injection of purified rat liver ingensin B⁵⁾ into a New Zealand white rabbit with Freund's complete adjuvant. Anti-rat ingensin IgG was prepared from antiserum by protein A-Sepharose column chromatography.

Gel filtrated samples were mixed with various concentrations of anti-ingensin IgG or ovalbumin as controls. After incubation at 4°C overnight, sample mixtures were centrifuged at 10,000 g for 20 min. SLLVT-MCA hydrolytic activities of the supernatant were assayed with or without 2 mM ATP. Precipitates were washed with PBS containing 0.1% Triton X-100 and then subjected to SDS-polyacrylamide gel electrophoresis.⁶⁾

Results. Mammalian ATP-dependent proteolytic activity is generally undetectable or becomes less detectable after chromatographic procedures. This indicates the possibility that some factor(s) were separated from the enzyme or that ATP-dependent protease system is too labile to maintain its ATP-dependency. To know whether the ATP-dependency of the proteolytic activity

in K562 lysate is retained after gel filtration or not, we first added 2 mM ATP to the crude lysate and then applied it to a rapid HPLC gel filtration column. Fig. 1 shows that the SLLVT-MCA hydrolytic activity in K562 cells was still regulated by ATP after separation of the lysate by a TSK-G3000SW column. The activity was "stimulated" 5-folds by the addition of ATP, and eluted as a single peak just after V_0 as described previously.^{2),3)} These results indicate that ATP-dependent activity of K562 ingensin resides in protease itself, not in other low-molecular-weight proteins.

Next, we demonstrated that the ATP-dependent protease in K562 cells is identical to ingensin, previously reported by us.^{4),5)} As shown in Table I, anti-ingensin IgG inhibited the SLLVT-MCA hydrolytic activity both in the presence and absence of ATP. The protease activity was decreased in proportion to increase in IgG concentration, and 80% of the activity was inhibited in the presence

Fig. 1. Gel filtration of K562 lysate. Two mM ATP was added to K562 lysate and then the mixture was applied to a TSK-G3000SW HPLC column equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. Fractions were assayed SLLVT-MCA hydrolytic activities with or without 2 mM ATP as described under "Materials and methods". The molecular weight of the protease was estimated to be about 700 kD.

Table I. Effect of anti-ingensin IgG on the protease activities in K562 lysate

Treatment	Concentration (mg/ml)	Relative activity*	
		-ATP	+ATP
None	—	100	100
Anti-ingensin IgG	0.2	85.7	80.7
	0.6	44.3	38.6
	1.0	21.9	18.1

50 μ l of gel filtrated samples were mixed with 50 μ l of anti-ingensin IgG or the same concentration of ovalbumin (50 μ l) solution. Immunoabsorbed fraction were removed and the supernatants were assayed as described under "Materials and methods".

* Values represent relative residual activities against controls treated with the same concentrations of ovalbumin. SLLVT-MCA hydrolytic activities of 0.2, 0.6 and 1.0 mg/ml of ovalbumin treated samples were stimulated to 1.5, 1.8 and 1.8 folds, respectively.

Fig. 2. Analysis of immunoprecipitates on 12% SDS-polyacrylamide gel electrophoresis. Immunoprecipitated samples were prepared as described in Table I, washed with PBS containing 0.1% Triton X-100, then dissolved in SDS sample buffer containing 2-mercaptoethanol.

of 1 mg/ml of IgG. In spite of increase in protease activities of ovalbumin treated controls to about double with ATP treatment, inhibitory effects of anti-ingensin IgG were similar both with and without ATP.

Fig. 2 shows the analysis of immunoprecipitates by SDS-PAGE. More than ten protein bands were observed at the positions of 30–20 kDa except a heavy chain of IgG (about 50 kDa). They are quite similar to those of ingensin from rabbit reticulocyte and other sources.

Discussion. There have been little information about the physiological roles of the high-molecular-mass protease, ingensin. It has been suggested that ingensin is involved in ATP-dependent protein degradation,^{4),7),8)} and we recently demonstrated that “ATP-dependent protease” in K562 cells may be identical to ingensin.³⁾ In this paper, we directly showed that K562 ATP-dependent protease activity was inhibited by the addition of anti-ingensin IgG (Table I). No protein except ingensin was precipitated with the IgG (Fig. 2). Therefore, we concluded that the ATP-dependent protease in K562 cells is identical or immunologically identical to ingensin.

An ATP-dependency of the proteolytic activity of K562 lysate decreased and disappeared during the purification procedure.³⁾ It is possible that an unknown regulatory protein(s) of ingensin, if exists, was removed or inactivated during the purification procedure. In this experiment, the protease activity was still stimulated by ATP after separation of the lysate by a TSK-G3000SW column (Fig. 1). This suggests that the regulatory protein(s) has the same molecular weight or is associated with ingensin itself. More reasonable explanation of the results is as follows: The ATP-dependent mechanism of protein breakdown resides in the ingensin multisubunits. But only the ATP-dependency is inactivated during preservation at 4°C due to its lability. In the immunoabsorption experiment, the decrease in ATP-dependency of the protease activity during overnight incubation was shown to be prevented by the addition of ovalbumin (data not shown), indicating that high concentration of proteins in ingensin solution is necessary to maintain its ATP-dependency. Further study is required to clarify these problems.

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