Korean J. Zool. 31:295-299, 1988

Purification of a 17,000-Dalton Inhibitor of Ca^{2+}-Activated Protease from Neoplastic Tissues of Human Stomach

Jae H. Seol, Sang C. Park*, Doo B. Ha and Chin H. Chung

*Department of Zoology, College of Natural Sciences; *Department of Biochemistry, School of Medicine, Seoul National Univesity, Seoul 151-742, Korea*

An endogenous 17-kDa inhibitor of Ca^{2+}-activated protease has been purified from neoplastic tissues of human stomach using heat treatment and conventional chromatographic procedures. It appears to consist of a single polypeptide since the same molecular weight was obtained by both gel filtration under nondenaturing condition and gel electrophoresis in the presence of SDS. Since the size of the inhibitor is the smallest among those reported so far, it may represent a functional unit for inhibiting Ca^{2+}-activated protease. This protein is also capable of inhibiting the protease isolated from chick skeletal muscle. Thus, the functional unit of the inhibitor must well be conserved during evolution. However, it remains unclear what may be the physiological significance of the presence of the low-molecular weight form of the inhibitor in neoplastic tissues of human stomach.

KEY WORDS: Calpain, Calpastatin, Stomach tumor

Ca^{2+}-activated thiol protease(s) (calpain) has been isolated from a variety of animal tissues including chick skeletal muscle (Ishiura *et al.*, 1978) and rat brain and muscle (Zimmerman and Schlaepfer, 1984). This enzyme has initially been found to function in the activation of phosphorylase b kinase (Houston and Krebs, 1968). Later, this enzyme has also been found to play a role in specific digestion of myofibrillar Z-lines and contractile components such as tropomyosin and troponins Y and I (Dayton *et al.*, 1976; Reddy *et al.*, 1975). In addition, implications are accumulating for its participation in nonlysosomal pathway of intracellular protein breakdown (Kay, 1983).

In addition to Ca^{2+}, the activity of calpain is regulated by an endogenous inhibitor (often called as calpastatin). Calpastatin has initially been purified by Waxman and Krebs (1978) from bovine cardiac muscle and then by a number of investigators from various mammalian and avian cells (Ishiura *et al.*, 1982; Murachi, 1983). Despite that the properties of calpastatin have extensively been characterized (Parkes, 1986), its reported size has been shown to vary considerably upon the purification procedures used or the sources from which the inhibitors were isolated. For example, the native size of calpastatin in rabbit skeletal muscle is 50 kDa, while that in bovine cardiac muscle is 270 kDa, whose subunit size also varies from 100 kDa to 150 kDa (Parkes, 1986). Such difference in the molecular weight has been attributed to limited proteolysis of calpastatin during purification and/or to a genuine multiplicity of the inhibitor *in vivo* (Lepley *et al.*, 1985; Takano *et al.*, 1984). Very recently, Suzuki and his colleagues (1987) have reported that calpastatins from rabbit and rat contain four internal repeats of about 140 amino acid residues, which are assumed to be a functional unit of the inhibitors. However, calpastain having a size as low as that corresponding to

This work was supported by grants from Daewoo Foundation and Korea Science and Engineering Foundation.

140 amino acids has not yet been found.

In this communication, we report a complete purification of 17 kDa polypeptide from neoplastic tissues of humn stomach, which is capable of inhibiting calpain from the same tissue as well as that from chick skeletal muscle.

Materials and Methods

Materials

Neoplastic tissues of human stomach were obtained from Seoul National University Hospital. During the isolation of the inhibitor of calpain from the cancer tissues, partially purified calpain was obtained by the DEAE-cellulose chromatography (see below). Calpain was also purified from chick skeletal muscle as described by Ishiura *et al.* (1982).

[^{3}H]Methyl-casein was prepared as described previously (Goldberg *et al.*, 1981). [^{3}H]Formaldehyde was purchased from New England Nuclear. All other chemicals were obtained from Sigma.

Assays

Calpain was assayed by its ability to convert [^{3}H]-casein to materials soluble in 10% (w/v) trichloroacetic acid (TCA). Reaction mixtures (0.1 ml) containing 50 mM Tris-HCl (pH 8), 10 mM 2-mercaptoethanol, 1 mM $CaCl_2$, 10 μg of casein and proper amounts of calpain preparations were incubated for 60 min at 30°C. The reaction was then terminated by adding TCA and bovine serum albumin (BSA) to give final concentrations of 10% and 3%, respectively. After a centrifugation, the supernatants were counted for their radioactivity in a liquid scintillation spectrometer (Goldberg *et al.*, 1981). The inhibitor was assayed by its ability to inhibit the casein-hydrolyzing activity of calpain.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described by Laemmli (1970). Protein was assayed by its absorbance at 280 nm or by the method of Bradford (1976) using BSA as a standard.

Preparation of Tissue Extracts

Neoplastic tissues of human stomach were minced, homogenized using a Waring blendor, and centrifuged for 1 hr at 30,000 xg. The supernatant was spun again at 100,000 xg for 2 hr. It was then dialyzed against Buffer A, which contained 20 mM Tris-HCl (pH 7.8), 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 0.5 mM EDTA and 10%(v/v) glycerol. The resulting solution was referred to as Tissue Extract.

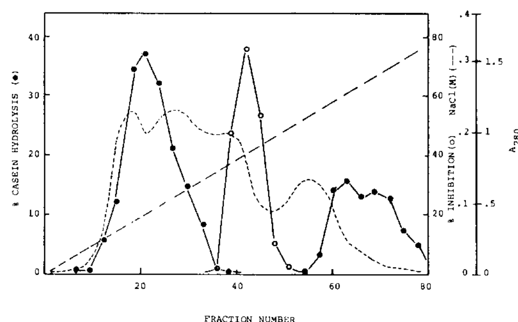

Fig. 1. DEAE-cellulose chromatography of the extract of tumor tissue of human stomach. Chromatography was performed as described in the text. Fractions of 10 ml were collected at a flow rate of 70 ml/hr. Protein, ------; Casein-degradaing activity of calpain, ○; Inhibitory activity against calpain, ●

Results

Purification

The extract (595 mg) obtained from 87 g of neoplastic tissues of human stomach was loaded on a DEAE-cellulose column (3 x 4 cm) that had been equilibrated with Buffer A. After washing the column extensively with the buffer, proteins were eluted with a linear gradient of 0 to 0.3 M NaCl. As showm in Fig. 1, in addition to the casein-degrading activity of calpain, two inhibitor activities against the protease were eluted from the column. The one eluted with 50-150 mM NaCl exhibits common properties shared by calpastatin from normal human tissues (data not shown). However, the other peak eluted at a much higher ionic strength (i.e., 250-350 mM NaCl) than the reported so far (Parkes, 1986). Therefore, we pursued further studies only with the latter peak.

The active fractions from the DEAE-column

Table 1. Summary of Purification Protocol.

Stepts	Protein (mg)	Total Act.(U)*	Speciifc Act.	Recovery (%)	Fold Purif.
Extract	595	—**	—	—	—
DEAE-cellulose	26.5	269	10	100	1
Heating	7.8	600	77	223	8
Sephacryl S-200	0.23	341	1483	127	148

*One unit was defined as the amount of the inhibitor required to inhibit 50% the activity of calpain, whose amount can convert 5 μg of casein completely to acid-soluble materials in 60 min at 30°C.

**Total activity of the inhibitor as well as other parameters in the extract can not be determined because the inhibitor is in association with calpain. Thus, the activity of calpain can neither be seen in the extract.

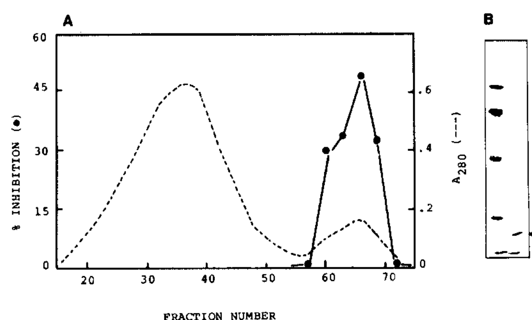

Fig. 2. Gel filtration on a Sephacryl S-200 column of the inhibitor of calpain. Proteins obtained from the heating step was separated by the column by collecting 1 ml fractions at a flow rate of 8 ml/hr. (A) Elution profile of proteins (······) and the inhibitor activity (●). (B) SDS-gel electrophoresis of the pooled inhibitor fraction. Standards used (from top to bottom) were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (68 kDa), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa).

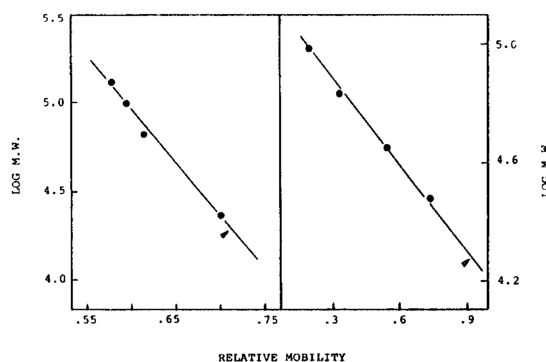

Fig. 3. Estimation of the molecular weight of the purified inhibitor. (A) The data obtained from Fig. 2A were plotted against the ratio of elution volume to bed volume. Markers used (from left to right) were alcohol dehydrogenase (150 kDa), hexokinase (100 kDa), BSA (68 kDa) and trypsin (24 kDa). (B) The results from Fig. 2B were plotted against the relative mobility.

were pooled, concentrated by ultrafiltration using a PM10 membrane (Amicon), and heated at 100°C for 5 min. Precipitates were removed by centrifugation, and the soluble proteins were loaded on a Sephacryl S-200 column(1.3 x 65 cm) that had been equilibrated with Buffer A. As shown in fig. 2A, the inhibitory activity against calpain eluted with a relatively low-molecular mass. The active fractions under the peak was pooled and kept frozen at −30°C for further studies. The summary of the purification protocol is shown in Table 1.

Properties

The purified inhibitor seems to be homogeneous since it migrated as a single band upon the SDS-gel electrophoresis (Fig. 2B). From this result, the molecular weight of the inhibitor was estimated to be 17 kDa (Fig. 3B). The native size of this protein was also determined to be 17 kDa (Fig. 3A) upon the gel filtration under nondenaturing condition (Fig. 2A). Thus, it appears to be consisted of a single polypeptide.

To examine if the inhibitor from the neoplastic tissues of human stomach are also capable of inhibiting calpain from other sources, it was incu-

Table 2. Cross-reactivity of Calpain and Calpastatin from Chick Skeletal Muscle and from Neoplastic Tissues of Human Stomach.

Source of Inhibitor	% Inhibition of	
	Human Calpain	Chick Calpain
Human stomach tumor	69	48
Chick skeletal muscle	75	81

Partially purified human calpain was obtained by the chromatography of the extract on the DEAE-cellulose column (see Fig. 1). Chick calpain and its inhibitor were prepared as described in Materials and Methods.

bated with the protease purified from chick skeletal muscle. Table 2 shows that the human calpastatin inhibits the avian calpain as well. Furthermore, human calpain was also inhibited by chick calpastatin. Thus, the functional sites on the inhibitor as well as on the protease appear to be highly conserved during evolution.

Discussion

In this study, an endogenous 17 kDa inhibitor of calpain was purifed to homogeniety from neoplastic tissues of human stomach. Of note-worth during the purification is the finding that the recovery of the inhibitor's total activity after the heating step is more than 2-fold (Table 1). A large fraction of the inhibitor obtained from the DEAE-cellulose column chromatography (Fig. 1) might still tightly bind with calpain. Thus, the heating seems to dissociate calpain from the inhibitor and selectively inactivate the protease. In fact, calpain is very labile at the temperatures above 40°C (data not shown) unlike the inhibitor, which is completely stable against heating at 100°C for 5 min. Thus, the total activity of the inhibitor from the DEAE-cellulose column is likely to be an underestimated value.

The size of the purifed inhibitor is the smallest as compared to others so far been reported (Parkes, 1986). Furthermore, it differs from other inhibitors in its chromatographic behavior on a DEAE-cellulose column (i.e., it elutes with a much higher NaCl concentration). A comparison of other properties (e.g., amino acid composition and isoelectric point) of the inhibitor from the tumor tissue to those from other sources should be valuable for the clarification of physicochemical chracteristics and for further understanding of the diversity of the inhibitor in nature. However, such studies were not able in the present study due to limitation of the cancer tissue.

A recent report by Suzuki and his colleques (1988) has shown that the inhibitor of calpain in rabbit and rat contains four internal repeats of about 140 amino acids. It is interesting that the size of the inhibitor isolated in this study resembles the sum of the molecular weights of the 140 residues, which may represent a functional unit of the inhibitor. However, it remains to be elucidated if the 17 kDa is the authentic size of the inhibitor in the neoplastic tissues of stomach or if it is resulted from the specific cleavage of a larger inhibitor polypeptide at internal links between the functional units.

References

Bradford, M. M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248-254.

Dayton, W. R., D. E. Goll, M. G. Zeece, R. M. Robson, and W. J. Reville, 1976. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. *Biochemistry* **15:** 2150-2158.

Emori, Y., H. Kawaski, S. Imajoh, S. Ishida, Y. Minami, and K. Suzuki, 1988. Structure and function of an endogenous inhibitor for calcium dependent protease (CANP Inhibitor). *In:* Intracellular Protein Catabolism. (Katunuma, N. ed.), p. 46.

Goldberg, A. L., K. H. S. Swamy, C. H. Chung, and F. S. Larimore, 1981. Proteases in *Escherichia coli. Methods Enzymol.* **80:**680-702.

Houston, R. B. and E. G. Krebs, 1968. Activation of skeletal muscle phosphorylase kinase by Ca^{2+}. II. Identification of the kinase activating factor as a proteolytic enzyme. *Biochemistry* **7:**2116-2122.

Ishiura, S., H. Murofushi, K. Suzuki, and K. Imahori, 1978. Studies of a calcium-activated neutral protease from chick skeletal muscle. *J. Biochem.* **84:**225-230.

Ishiura, S., S. Tsjuji, H. Murofushi, and K. Suzuki, 1982. Purification of an endogenous 68,000 dalton inhibitor of Ca^{2+}-activated neutral protease from chicken

skeletal muscle. *Biochim. Biophys. Acta* **701**:216-223.

Kay, J., 1983. Ca^{2+}-activated proteinases, protein degradation and muscular distrophy. *In:* Proteases, Potential Role in Health and Disease. (Heidland, A. and W. H. Horl, eds.), Plenum Press, New York, pp. 519-531.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

Lepley, R. A., M. Pampusch, and W. R. Dayton, 1985. Purification of a high-molecular-weight inhibitor of the calcium-activated proteinase. *Biochim. Biophys. Acta* **828**:95-103.

Murachi, T., 1983. Calpain and calpastatin. *Trends Biochem. Sci.* **8**:167-169.

Parkes, C., 1986. Calpastatins. *In:* Proteinase Inhibitors. (Barrett, A. J. and G. Salvesen, eds.), Elsevier, New York, pp. 571-587.

Reddy, M. K. J. D. Etlinger, M. Rabinowitz, D. A. Fischman, and R. Zak, 1975. Removal of Z-line and α-actinin from isolated myofibrils by a calcium-activated neutral protease. *J. Biol. Chem.* **250**:4278-4285.

Takano, E., N. Yumoto, R. Knnagi, and T. Murachi, 1984. Molecular diversity of calpastatin in mammalian origans. *Biochem. Biophys. Res. Commun.* **122**: 912-917.

Waxman, L. and E. G. Krebs, 1978. Identification of two protease inhibitors from bovine cardiac muscle. *J. Biol. Chem.* **253**:5888-5891.

Zimmerman, U. P. and W. W. Schlaepfer, 1984. Multiple forms of Ca-activated protease from rat brain and muscle. *J. Biol. Chem.* **259**:3210-3218.

(Accepted August 21, 1988)

사람의 위암조직으로부터 Ca^{2+}-Activated Protease를 저해하는 17 kDa-단백질의 분리

설재홍 · 박상철* · 하두봉 · 성진하(서울대 자연대 동물학과; *의과대 생화학교실)

사람의 위암조직으로부터 Ca^{2+}-activated protease을 저해하는 단백질을 열처리 및 여러 크로마토그라피 방법을 이용하여 순수분리 하였다. 이 저해단백질은 SDS-전기영동과 gel filtration의 방법에 의하여 그 분자량이 17 kDa으로 나타남으로 보아 단일 polypeptide로 구성되어 있음을 알 수 있었다. 이 저해 단백질의 분자량은 지금까지 알려진 Ca^{2+}-activated protease의 저해단백질에 비하여 가장 작은 것이었다. 따라서, 이 단백질은 Ca^{2+}에 의하여 활성화되는 단백질 분해효소의 작용을 저해하는 기능적 단위로 추측되었다. 한편, 이 저해제는 게 골격근에서 추출한 Ca^{2+}-activated protease의 활성도 억제하는 것으로 나타났다. 이러한 결과는 이 저해단백질의 기능적 단위가 진화과정 동안 오래 보존되어 왔음을 시사한다. 그러나, 사람의 위암조직에서 이 저해제가 무슨 이유로 가장 작은 분자량의 단백질로 존재하는지에 대한 생리학적 중요성에 관한 문제는 앞으로 많이 연구되어져야 할 것이다.