A 107-kDa INHIBITOR FOR CALCIUM-ACTIVATED NEUTRAL PROTEASE (CANP): PURIFICATION FROM THE HUMAN LIVER

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ABSTRACT

An endogenous inhibitor for Ca^{2+} -activated neutral protease (CANP) was purified to homogeneity from the human liver. One mole of the inhibitor inhibited 5 mol of CANPs from various sources. The purified inhibitor has a molecular weight of 107 K, which is significantly larger than the value previously reported for the CANP inhibitor.

Ca^{2+} -activated neutral protease (CANP), an intracellular thiol protease, plays important roles in various cellular events, e.g. activation of kinases (5, 8, 14), turnover of myofibrillar proteins (3, 4, 17), and regulation of receptors for hormones and neurotransmitters (1, 2, 16, 25). Two kinds of isozymes (μ -CANP and m-CANP) are identified based on the requirement for calcium (4, 6, 9, 11, 12, 19, 28). However, it is not clear how they share the physiological roles. The intracellular activity of CANP is regulated by an endogenous inhibitor specific to CANP found in various tissues (15, 26). Knowledge of the inhibitor is essential to clarify the regulatory system for CANP. However, precise analyses of the inhibitor are few, mainly because of the difficulty in its purification. Further, various values are reported for its molecular weight (7, 13, 18, 20, 22, 23, 27).

The present paper describes a rapid purification method of the inhibitor and some properties of the purified inhibitor including the molecular weight.

MATERIALS AND METHODS

Preparation of CANPs

μ -CANP was purified from the bovine liver by

steps of chromatography on DE-53, Sephacryl S-200, and Phenyl-Sepharose CL-4B to 50% homogeneity (80 units/mg protein). m-CANP was prepared from the human, bovine and chicken liver by ion exchange and gel filtration chromatography to 70% homogeneity. Chicken derived μ -CANP was prepared by autodigestion of m-CANP as previously described (21).

Preparation of Crude Extract from the Human Liver

Human liver (400 g) stored at -80°C was broken into pieces, and homogenized in a mixer in 3 volumes of chilled buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM EDTA, 5 mM 2-mercaptoethanol and 5 μM leupeptin (TEM buffer), supplemented with 5 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was centrifuged at 30,000 rpm for 1 h in a Hitachi RPZ-35T rotor at 2°C . The supernatant was transferred to 1,000-ml flasks, about 500 ml of sample per flask. The flask was soaked in a water bath at 100°C with stirring. The sample was heated up to 70°C , and was kept for 10 min at the temperature. The heat-treated sample was chilled on ice, and centrifuged at 8,000 rpm for 30 min using a Hitachi RP-9 rotor at 2°C . To the super-

natant was added solid ammonium sulfate to a final concentration of 50% saturation at 0°C.

Assay of CANP Inhibitor

An inhibitor sample (below 5 μ l) was added to 290 μ l of assay mixture containing 3 mg/ml casein, 50 mM Tris-HCl, pH 7.6, 5 mM 2-mercaptoethanol and 5 mM CaCl_2 in a 1.5-ml tube. m-CANP (1.8 units) prepared from the bovine liver was added, and the mixture was incubated for 20 min at 30°C. The reaction was stopped by adding 600 μ l of cold 15% (w/v) trichloroacetic acid (TCA), and after 30 min at 0°C, the solution was centrifuged at 10,000 g for 3 min. TCA-soluble casein was measured with a Uvidec 340 spectrophotometer at 280 nm. One unit of inhibitor was defined as the amount that inhibited one unit of bovine m-CANP.

Other Methods

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (10). Protein concentration was estimated by measuring the 280-nm absorbance of fractions during purification of the inhibitor. Concentration of the purified inhibitor was determined by amino acid analysis. Molecular weight of CANP was assumed to be 80 K, when stoichiometry of inhibition was calculated.

RESULTS

Purification of CANP Inhibitor from the Human Liver

Frozen human liver (400 g) was extracted and heat-treated as described in Materials and Methods. Heat-stable protein fraction, collected by ammonium sulfate precipitation, was applied to a Phenyl-Sepharose CL-4B column (3 $\times$ 12 cm) equilibrated with TEM buffer containing 1 M NaCl (Fig. 1A). The flow-through fractions were dialyzed against TEM buffer, and applied to a DE-53 column (1.5 $\times$ 40 cm) equilibrated with the same buffer. After washing with two column volumes of TEM buffer, the column was eluted with a linear gradient of 0–0.2 M NaCl in TEM buffer. Inhibitory activity for CANP was eluted at 0.1 M NaCl. CANP inhibitor fractions were concentrated with ammonium sulfate, and applied to a Phenyl-Sepharose CL-4B column (1.1 $\times$ 120 cm) equilibrated with TEM buffer containing 1 M NaCl. The inhibitory activity for CANP was eluted before most other proteins came out from the column (Fig. 1B). The inhibi-

tor was further purified by gel filtration through Sephacryl S-300 (1.1 $\times$ 150 cm column) in the presence of 8 M urea (Fig. 1C).

Purification steps of CANP inhibitor from the human liver are summarized in Table 1. CANP inhibitor was purified 450-fold with a yield of 20% based on the activity in the heat-treated extract. The preparation showed a single band on an SDS-polyacrylamide gel. In order to identify the inhibitor, we sliced the gel and examined the inhibitory activity in each gel extract. The inhibitory activity corresponded to the band stained with Coomassie blue (Fig. 2).

Properties of the Inhibitor

The molecular weight of CANP inhibitor was estimated to be 107,000 (107 K) by SDS-polyacrylamide gel electrophoresis (Fig. 2). However, the inhibitor was eluted in higher-molecular-weight (200 K–450 K) fractions on Bio-gel A-0.5m gel filtration under non-denaturing condition. This indicates that the inhibitor exists as aggregates. This is the reason why we performed gel filtration in 8 M urea. CANP inhibitor showed somewhat decreased activity in 8 M urea, but it regained the full activity after removal of urea.

As shown in Fig. 3, the inhibitor did not inhibit bromelain or papain even in a very high concentration (10-fold molar excess). During incubation the inhibitor was digested to smaller fragments by these enzymes as observed by SDS-polyacrylamide gel electrophoresis (not shown). The inhibitor did not inhibit trypsin, chymotry-

Fig. 1 Elution profiles of CANP inhibitor from Phenyl-Sepharose CL-4B and Sephacryl S-300 chromatography. A: First Phenyl-Sepharose CL-4B chromatography. Soluble fraction of the human liver extract after heat treatment was concentrated with ammonium sulfate, and applied to a Phenyl-Sepharose CL-4B column (3 $\times$ 12 cm). The column was developed with TEM buffer and fractions of 100 ml was collected. B: Second Phenyl-Sepharose CL-4B chromatography. Inhibitor fraction from the first Phenyl-Sepharose CL-4B was further chromatographed on a DE-53 column, and the inhibitor eluted was applied to a slim column (1.1 $\times$ 120 cm) of Phenyl-Sepharose CL-4B after concentration with ammonium sulfate. Elution was performed with TEM buffer. Fraction volume: 12 ml. C: Sephacryl S-300 gel filtration. CANP inhibitor fraction from the second Phenyl-Sepharose CL-4B chromatography was concentrated with ammonium sulfate and chromatographed through a Sephacryl S-300 column equilibrated with TEM buffer containing 8 M urea. Protein concentration measured by the absorbance at 280 nm (●) and the inhibitor activity (○) are shown.

Fig. 1

Table 1 Purification of a CANP Inhibitor from the Human Liver^a

Purification step	Total protein (A ₂₈₀ units) (mg) ^b	Total inhibitor (units)	Specific activity (units/A ₂₈₀ units)	Yield (%)
Heat treatment	1,250	12,000	9.6	100
First Phenyl-Sepharose CL-4B	98	11,300	120	94
DE-53	12	6,800	570	56
Second Phenyl-Sepharose CL-4B	3.9	5,900	1,500	49
Sephacryl S-300	0.57	2,500	4,400	20
	1.2 ^b			

^aThe preparation was started from 400 g of the liver. ^bDetermined by amino acid analysis

Fig. 2 SDS-polyacrylamide gel electrophoresis of the purified inhibitor. The purified CANP inhibitor was subjected to SDS-polyacrylamide gel electrophoresis (7.5% acrylamide). One of the duplicated gels was stained with Coomassie blue R-250 (panel A), and the other was analyzed for the inhibitor activity (panel B). The gel for the inhibitor assay was sliced in 3 mm thick, and each slice was incubated with 200 μ l of TEM buffer for 12 h at 4°C. Five microliters of the extract was added to the assay mixture and the inhibitor activity was examined (●). Molecular weight standards are indicated by arrows: 1, β -galactosidase (*E. coli*); 2, phosphorylase *a* (rabbit muscle); 3, albumin (bovine serum); 4, albumin (egg white); and 5, lactate dehydrogenase (hog muscle).

psin and subtilisin. On the other hand, when various amounts of the purified inhibitor was added to the assay mixture containing a fixed amount of CANPs from various sources, the

caseinolytic activity of the enzyme was stoichiometrically decreased according to the amount of the added inhibitor. One mole of the inhibitor completely inactivated 5 mol of chicken CANP.

Fig. 3 Inactivation of CANPs by the human liver inhibitor. Various amounts of the purified inhibitor was added to CANPs or other proteases. The remaining activity of each enzyme was measured as described under Materials and Methods, except that the assay mixture for μ -CANP contained 100 μ M CaCl_2 . m-CANPs from human liver (□), chicken liver (■), and bovine liver (○); μ -CANP from bovine liver (●); derived μ -CANP of chicken (×); bromelain (▲); and papain (△)

The same stoichiometric relation was observed for all species of CANPs tested. The inhibitory action was observed in the presence of μ M or mM calcium for μ - and m-CANPs, respectively.

The amino acid composition of the inhibitor is shown in Table 2. The composition of human erythrocyte CANP inhibitor (23) is also indicated for comparison. Higher contents of glutamic acid and aspartic acid are the reflection of the acidic nature of this inhibitor. Most striking feature is the low content of aromatic amino acids. Thus the absorption coefficient of the inhibitor is quite low ($A_{280}^{0.1\%}=0.48$). These features are common to the two human inhibitor preparations.

DISCUSSION

Human CANP inhibitors have been purified from erythrocytes and platelets (20,23). The properties of these inhibitor preparations are similar to those observed with the present inhibitor; *i.e.* aggregation on gel filtration under non-denaturing conditions, heat stability and susceptibility to proteases other than CANP. However, marked difference was found in the molecular weights of the preparations estimated by SDS-polyacrylamide gel electrophoresis. The molecular weight of the present preparation of human CANP inhibitor is 107 K, whereas those reported for hu-

Table 2 Amino Acid Composition of the Human CANP Inhibitor

Amino acid	Source	
	Liver (mol%)	Erythrocyte ^a (mol%)
Asx	11.1	12.6
Thr	5.8	5.0
Ser	8.8	9.6
Glx	15.1	14.9
Pro	8.7	10.1
Gly	6.3	5.3
Ala	8.7	9.5
Cys	0.6	0.1
Val	3.9	3.3
Met	1.0	1.1
Ile	2.7	2.2
Leu	6.7	7.9
Tyr	0.7	0.9
Phe	1.3	1.2
Lys	12.9	12.1
His	3.3	0.9
Arg	2.9	3.1
Trp	N.D. ^b	0

^aCalculated from Reference 23. ^bNot determined

man inhibitor are 63 K and 70 K for platelets and erythrocyte inhibitors, respectively. Values from 34 K to 145 K are reported for inhibitors from other sources (7, 13, 18, 22, 27). The discrepancy in the molecular weight may be due to degradation of the inhibitor during preparation, because the purified inhibitor is very sensitive to proteases. The inhibitor often degraded from 107 K to smaller fragments upon storage without apparent loss of the inhibitory activity. The present preparation method employed protease inhibitors (PMSF and leupeptin) and took only 3 days for the whole process. These must have prevented proteolytic degradation of the inhibitor during preparation. In fact, without such synthetic protease inhibitors, the inhibitor was degraded easily, and the fractions containing CANP inhibitor activity showed a main band with a molecular weight of 70 K rather than 107 K on an SDS-polyacrylamide gel. Furthermore, in another preparation from another human liver specimen, a degraded form of the inhibitor was obtained. Although the preparation gave a single peak on chromatography on Phenyl-Sepharose CL-4B, DE-53 or Sephacryl S-200 in the absence of 8 M urea, it showed multiple bands of molecular weights ranging from 20 K to 107 K on SDS-polyacrylamide gel electrophoresis. In this case, the inhibitor may have been degraded before the liver specimen was stored at -80°C . Recently, Takano *et al.* examined the CANP inhibitor in various tissue extracts by SDS-polyacrylamide gel electrophoresis and immunoblotting with antibody raised against 70 K inhibitor from human erythrocyte (24). They observed a 100 K molecular species that cross-reacted with the antibody in the human heart. This may be an indication that the molecular weight of undegraded CANP inhibitor in human tissues is 100 K.

Mellgren and Carr (13) reported similar stoichiometric value (inhibitor/CANP=0.24 mol/mol) as we obtained in this experiment. The mechanism of how one molecule of inhibitor can inhibit 5 mol of CANP remains to be answered.

More precise studies on the stoichiometry and reversibility of inhibitor are now in progress.

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