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THE APPEARANCE OF A 34,000-DALTON INHIBITOR OF CALPAIN (Ca^{2+}-DEPENDENT CYSTEINE PROTEINASE) IN RAT LIVER AFTER THE ADMINISTRATION OF PHENYLHYDRAZINE

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Summary: A 34,000-dalton inhibitor of calpain (Ca^{2+}-dependent cysteine proteinase) was found in the cytosol of anemic rat liver. When phenylhydrazine hydrochloride was continuously administered to rats, a 280,000-dalton calpain inhibitor that existed originally in the liver gradually disappeared within two weeks and, concomitantly, a 34,000-dalton inhibitor appeared. The purified 34,000-dalton inhibitor resembles 280,000-dalton inhibitor in that both are heat-stable proteins and do not inhibit papain and trypsin. Unlike the protomers of a 280,000-dalton inhibitor, 34,000-dalton inhibitor does not show any sign of self-association.

Calpain (Ca^{2+}-dependent cysteine proteinase) [EC 3.4.22.17] and its specific inhibitor are widely distributed in various tissues and cells (1-7). Generic name, calpastatin, was given to calpain-specific inhibitor proteins (5). We described previously that calpastatin exists as a 280-kDa protein in the cytosol of rat liver (6). Similar high molecular weights were estimated for calpastatins in rat brain (300,000, ref. 7), human erythrocytes (280,000, ref. 8) and bovine cardiac muscle (260,000-270,000, ref. 9). These higher values now seem to indicate self-aggregation of monomers. A 280-kDa calpastatin from human erythrocytes was found to dissociate into 70-kDa monomers (8), while 68-kDa calpastatin was purified from rabbit (10) and chicken (11) skeletal muscle. Rabbit muscle calpastatin (10) was shown to be a dimer of identical 34-kDa subunits. However, no previous report has shown the presence of natural calpain inhibitors having a molecular weight lower than 48,000 (12).

Abbreviations: kDa, kilodaltons; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PHH, phenylhydrazine hydrochloride.

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Phenylhydrazine hydrochloride (PHH) has been known as one of the useful drugs in the study of experimental hemolytic anemia produced in animals (13). This report describes dynamic changes of the inhibitors in rat liver injured by PHH, which resulted in the appearance of 34-kDa calpain inhibitor. The inhibitor protein that newly appeared in anemic rat liver was purified and characterized.

MATERIALS AND METHODS

Materials: Male Wistar rats with starting body weight of 200-230 g were used. PHH was obtained from Wako Pure Chemical Industries, Osaka, Japan, casein (Hammarsten grade) from E. Merck, Darmstadt, Germany, papain and trypsin from Sigma Chemical Co., St. Louis, USA, DEAE-cellulose (DE 52) from Whatman, Springfield, UK, Ultrogel AcA34 from LKB, Bromma, Sweden, and Sephadex G-100 from Pharmacia, Uppsala, Sweden.

PHH-induced anemic rats: PHH dissolved in sterile saline was injected subcutaneously in a daily dose of 2.5 mg/100 g body weight on several consecutive days. Three rats were sacrificed at a definite period of days from the administration of PHH. They were fasted overnight before killing. Rats were decapitated and the blood was collected in a heparinized tube, and then reticulocytes were stained with New Methylene Blue (14). Reticulocyte values of more than 90% were obtained after continuous injection over 7 days.

Calpain from normal rat liver: Calpain I (with low Ca^{2+}-requirement) and calpain II (with high Ca^{2+}-requirement) were partially purified from the liver of untreated rats according to the method described previously (5), and used as the reference preparations.

Inhibitor assay: The conditions for the inhibitor assay were essentially the same as those described in the previous report (15). Test sample containing inhibitor(s) was preincubated with the reference preparation of calpain II at 30°C for 10 min and then the reaction was started by the addition of buffered casein containing cysteine and $CaCl_2$. Final Ca^{2+} concentrations were 4.5 mM for calpain II and 0.1 mM for calpain I assays. The acid-soluble products formed were colorimetrically determined at 750 nm by Lowry's method as modified by Ross and Schatz (16). One unit of inhibitor activity was defined as the amount of inhibitor that inhibited one unit of calpain II from rat liver (15).

Extraction and purification of inhibitor from rat liver: A 7-g (wet wt) portion of the liver tissue from PHH-treated rats was washed in saline, cut into pieces with scissors and homogenized in 4 vol of 20 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 1 mM EGTA and 5 mM 2-mercaptoethanol using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 105,000 g for 60 min. The supernatant (20 ml) was applied to an Ultrogel AcA34 column (3.2 x 90 cm) previously equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EGTA and 5 mM 2-mercaptoethanol, and 6-ml fractions were collected. The inhibitor fractions (Nos. 80 to 100) obtained from 7 cycles of the procedures were combined and applied to a DE 52 column (2.8 x 14 cm) equilibrated with the same buffer. The proteins were eluted with the same buffer having a linear 0 - 0.5 M NaCl gradient (total volume, 500 ml). The inhibitor fractions that appeared at 0.15 M NaCl were collected and salted-out with 70% saturation of ammonium sulfate. The protein precipitated was collected and dissolved in 5 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EGTA and 5 mM 2-mercaptoethanol, and the solution was dialyzed overnight against the same buffer. After removing the precipitates by centrifugation, the supernatant was heated at 100°C for 20 min, and the precipitates were again

eliminated. The supernatant was chromatographed successively on a Sephadex G-100 column (2.2 x 62 cm) and on a Sephadex G-75 column (1.8 x 68 cm) both at pH 7.5; the peak fractions from the latter were collected and used as the final product.

Protein: Protein was measured by Lowry's method as modified by Ross and Schatz (16) with bovine serum albumin as a standard.

RESULTS

Dynamic changes of calpain inhibitors in the liver of rats before and after administration of PHH: The rat liver extracts (105,000-g supernatant) obtained from control and PHH-treated animals were chromatographed on Ultrogel AcA34 columns as described in MATERIALS AND METHODS. The proteolytic activity of each fraction was determined with a fixed amount of the reference preparation of calpain II, which had previously been added to each assay tube. Positive and negative deviations from the base line indicate elution of calpain and the inhibitor, respectively. As shown in Fig. 1A, the extract from untreated rat liver gave a positive peak over the base line value at around fraction No. 75, indicating the elution of calpain and, well ahead of it, a large negative trough which must correspond to 280-kDa calpastatin (6-8). With the anemic rats that had received increasing amounts of PHH, the elution profiles gradually changed so that 280-kDa calpastatin decreased and finally disappeared, while a new negative trough at around fraction No. 90 appeared, increased and predominated (Figs. 1B-1F).

Purification and characterization of the inhibitor that newly appeared in anemic rat liver: The anemic rats that received daily administration of PHH for 7 days were used. As shown in Table I, 0.50 mg of the inhibitor protein was obtained from 49 g of the liver. The specific activity of the final product, 212 units/mg protein, is lower than the reported value for human erythrocyte 280-kDa calpastatin, 747 units/mg protein (8). The apparent molecular weight was estimated to be 34,000 by chromatography on a Sephadex G-100 column using bovine serum albumin, ovalbumin, chymotrypsinogen and myoglobin as the standards (graphic data not shown). Fig. 2 shows the inhibitory activity of the newly found inhibitor from anemic rat liver on several proteases. It was effective on liver calpains I and II but not

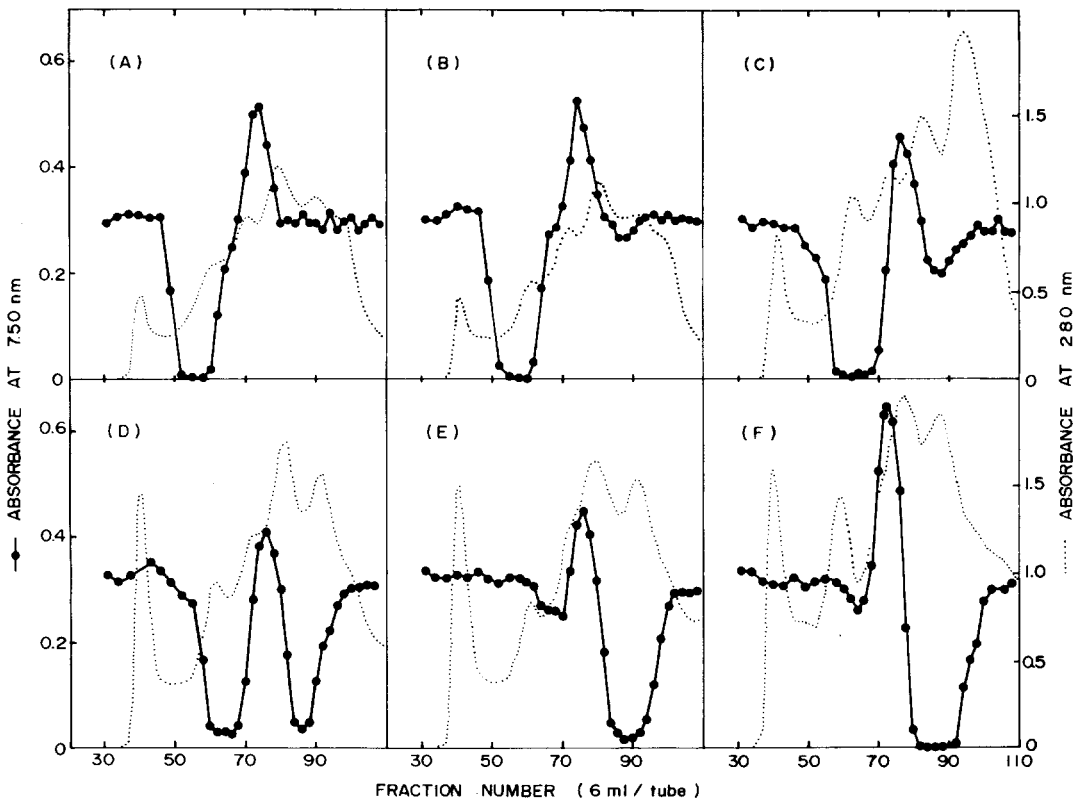

Fig. 1. Dynamic changes of calpain inhibitors as revealed by Ultrogel AcA34 column chromatography. Rat liver was perfused with saline containing 1 mM EGTA before removing the liver. A 7-g (wet wt) portion of the liver was homogenized, and aliquots (20 ml) of the supernatant at day 0 (A), 1 (B), 3 (C), 5 (D), 7 (E), and 14 (F) after the administration of PHH were applied to an Ultrogel AcA34 column. ●, calpain activity; ...., protein. To each effuluent fraction was added a fixed amount of the reference preparation of calpain II, and then calpain assay was carried out in the presence of 4.5 mM Ca^{2+}. The base line corresponds to the level of calpain added previously to each tube. The reticulocyte counts in blood were 1.5, 1.7, 33.0, 70.6, 88.0, 97.7 and 98.3% on day 0, 1, 3, 5, 7, 10 and 14, respectively.

TABLE I. Purification of 34-kDa inhibitor from anemic rat liver[a]

Step	Total protein (mg)	Total activity (units)	(%)	Specific activity (units/mg protein)	(fold)
Homogenate (supernatant)	2,210				
Ultrogel AcA34	1,200	1,040	100	0.87	1
DEAE-cellulose	155	365	35.1	2.35	2.7
Ammonium sulfate	103	254	24.4	2.47	2.8
Heat treatment (100°C, 20 min)	2.98	267	25.6	89.6	103
Sephadex G-100	0.79	148	14.2	187	215
Sephadex G-75	0.50	106	10.2	212	244

[a]The inhibitor activity of the homogenate could not be determined due to the presence of calpain in the same extract. Therefore, the recoveries of activity and purification folds were calculated from the data for step of Ultrogel AcA34 column chromatography.

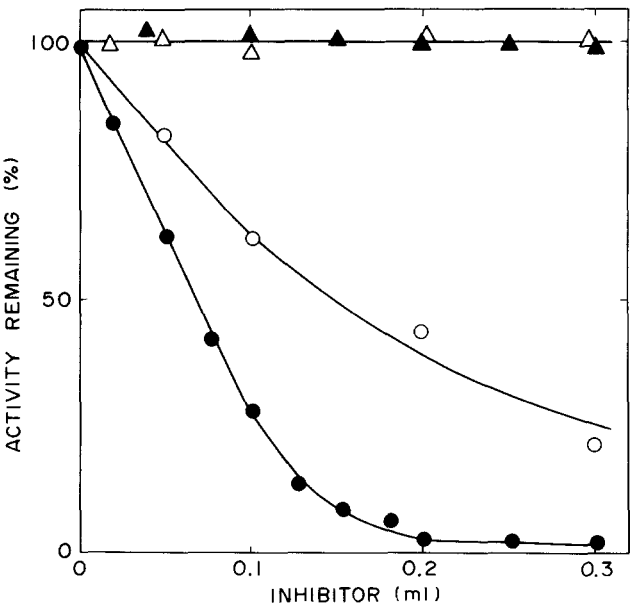

Fig. 2. Effect of 34-kDa inhibitor on several proteases. Increasing amounts of the inhibitor (10.6 μg protein/ml) were preincubated for 10 min at pH 7.5 and 30°C with calpain I (○, 0.3 unit/tube) and calpain II (●, 0.3 unit/tube), and at pH 7.0 and 37°C with trypsin (▲, 5 μg/tube) and papain (△, 1.25 μg/tube). The activity remaining was then determined.

on papain and trypsin. The inhibitor was heat-stable; 100% of the inhibitory activity remained even after the treatment at 100°C for 20 min at pH 7.5.

Analysis on plasma and blood cells of PHH-induced anemic rats: Blood samples from 14-day anemic rats were analyzed on an Ultrogel AcA34 column in the same manner as that for the liver samples. As shown in Fig. 3, there appeared no calpain inhibitor fractions having a molecular weight lower than that of calpain.

DISCUSSION

Phenylhydrazine hydrochloride (PHH) has been known to be a useful drug for studying red cell sequestration and destruction in reticuloendothelial organs such as spleen and liver (17-19). In the present study we have demonstrated that dynamic changes of endogenous calpain inhibitors are induced in rat liver by PHH. During a two-week period of continuous administration of PHH, the 280-kDa calpastatin disappeared and, conversely,

a 34-kDa inhibitor protein appeared (Fig. 1A-1F). The newly induced 34-kDa inhibitor was found to share such properties in common with 280-kDa calpastatin as heat stability, inhibitory effectiveness on calpains I and II and non-effectiveness on papain and trypsin (Fig. 2). However, the 34-kDa inhibitor not only differs from 280-kDa calpastatin in the much lower molecular weight, but also it differs from 34-kDa subunit (10) or 70-kDa monomer of calpastatin (8) in showing no signs of self-association. The newly induced inhibitor is also distinct in molecular weight from any other reported proteinase inhibitors so far reported (20-23). We have noticed that in the final step of the purification on Sephadex G-75 (Table 1), the 34-kDa calpain inhibitor was separated from an approximately 15-kDa inhibitor fraction which was effective on papain but not on calpain. Reticulocytes in blood increased gradually after the administration of PHH, but the absence of an inhibitor smaller than calpain in such blood samples (Fig. 3) excludes the possibility that 34-kDa inhibitor originates from PHH-induced reticulo-

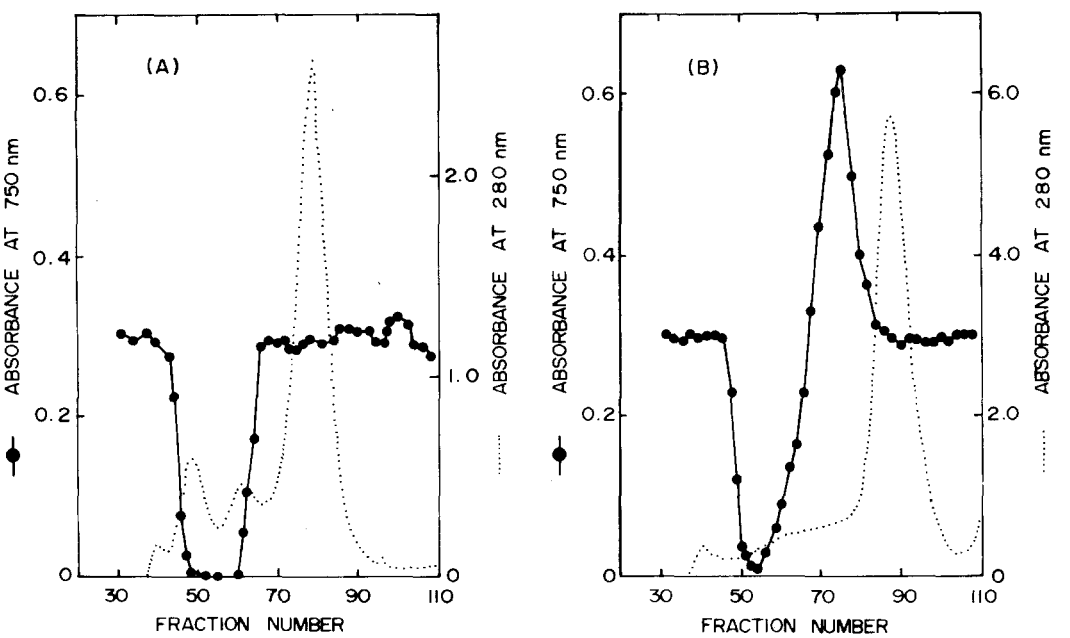

Fig. 3. Elution profile of rat blood samples from Ultrogel AcA34 column. Plasma (A) and the supernatant of blood cell homogenate (B) from rats after 14-day continuous injection of PHH were fractionated and assayed in a manner similar to that for Fig. 1. ●, calpain activity; ...., protein.

cytes. Although it is reasonable to assume that both 34-kDa and 280-kDa inhibitors are synthesized in hepatic cells, it is not known whether 34-kDa inhibitor is a portion of the 280-kDa calpastatin or it is a new protein synthesized entirely independently of the synthesis of normal calpastatin. Studies are in progress on the immunological relationship between 34-kDa and 280-kDa inhibitor proteins.

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