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PLASMA HIPPURYL-L-LYSINE HYDROLASE INHIBITION BY LOW MOLECULAR WEIGHT SUBSTANCE IN LYSOSOMES OF SKELETAL MUSCLE

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Abstract—Low molecular weight inhibitor of plasma hippuryl-L-lysine (HLL) hydrolase was partially purified by chromatography on CM-Sephadex C-25 from lysosomal fraction of the dog skeletal muscle. The molecular weight of this inhibitor was approximately 2000. It was stable to boiling 10 min at neutral pH and acidification at pH 2 for 10 min at 37°. The inhibitory activity of this substance was not affected by the action of α-chymotrypsin. The inhibitor could cause complete inhibition of plasma HLL hydrolase but not plasma bradykininase.

IT HAS been reported that the sera or plasma of many animals including human contain a carboxypeptidase which cleaves basic C-terminal amino acids and hydrolyses hippuryl-L-lysine (HLL), hippuryl-L-arginine and other peptides.[2] This enzyme was named carboxypeptidase *N*, which can be distinguished from the pancreatic carboxypeptidase. Furthermore, the level of this enzyme was measured in the serum under various conditions.[3] During the study of the kininase activities in blood following the application of a tourniquet, the author noticed the presence of high and low molecular weight inhibitors of plasma HLL hydrolase in the lysosomal fraction of the skeletal muscle.

This paper describes the partially purified low molecular weight inhibitor which could inhibit HLL hydrolase activity completely.

MATERIALS AND METHODS

Preparation of lysosomal extract

Twenty mongrel dogs weighing 14–18 kg were used for the experiments. The whole thigh muscles were excised under pentobarbital sodium (Nembutal) anesthesia and cut into small pieces with scissors. Dices of the muscle were minced and mixed with 4 times its weight of ice-cooled 0·25 M sucrose solution containing 0·001 M ethylendiaminetetra-acetic acid (EDTA) and sufficient K_2HPO_4 to bring the pH to 7·0. The suspension was homogenized for 1 min at moderate speed in a Waring blender. The homogenate was subject to the differential centrifugation method of Stagni and De Bernard.[4] The supernatant obtained after the centrifugation at 2500 *g* was centrifuged at 8500 *g* for 10 min. This precipitate represented the mitochondrial fraction. Furthermore the supernatant obtained after the centrifugation at 8500 *g* was centrifuged at 14,000 *g* for 20 min to obtain a lysosomal fraction. Lysosomal fraction was washed

twice with the sucrose–EDTA solution described above and suspended in 0·15 M KCl containing 0·1% (v/v) Triton X-100. The resulting suspension was homogenized using a glass Potter–Elvehjem homogenizer with a motor-driven glass pestle and centrifuged at 15,000 *g* for 40 min. The supernatant was stored at −20° and used as lysosomal extract. Heparinized blood was taken by venepuncture from the femoral vein and plasma was separated by centrifugation at 2000 *g* for 15 min. All procedures were performed at 0–3°.

Column chromatography on CM-Sephadex C-25. Five ml of lysosomal extract (5 mg protein/ml) were subjected to column chromatography on a 2·8 × 50 cm column of CM-Sephadex C-25. The column had been previously equilibrated with 0·05 M NaCl in 0·05 M tris buffer, pH 7·4, Elution was performed by a linear gradient of increasing concentrations of NaCl starting at 0·05 increasing it to 0·5 M. To produce the NaCl gradient, three containers connected in series were used. A volume of 250 ml of 0·05 M NaCl in Tris buffer, pH 7·4, was placed in the first container, an equal volume of 0·25 and 0·5 M NaCl in Tris buffer, pH 7·4, in the second and third containers of the mixing chamber, respectively. The flow rate was 45 ml/hr and 3-ml fractions were collected. The effluent was assayed by measuring the u.v.-absorption at 280 nm and by Ninhydrin reaction.[5]

Gel filtration on Sephadex G-25. After column chromatography on CM-Sephadex C-25, the tubes corresponding to inhibitor of the plasma HLL hydrolase were combined, lyophilized, dissolved in water and subjected to gel filtration on Sephadex G-25 to remove salts. The tubes contained inhibitor of the plasma HLL hydrolase were pooled and lyophilized again.

Protein determination. The protein content of the solutions was determined by applying the formula of Kalckar.[6]

Enzyme assays. Acid phosphatase and β-glucuronidase activities were measured according to the methods in Sigma Technical Bulletin Nos. 104 and 105, respectively.[1]

The hydrolysis of the substrates was measured by the methods of Folk *et al.*[7] and Erdös *et al.*[2] The concentration of HLL was 10^{-3} in an 0·1 M Tris buffer, pH 7·4. Heparinized dog plasma was used as the source of enzyme. The final dilution of the plasma samples was 1:30 (v/v).

For experiments on inhibition of HLL hydrolase, aliquots of each effluent obtained by column chromatography of lysosomal extract on CM-Sephadex C-25, the freeze-dried residue obtained by gel filtration on Sephadex G-25 or lysosomal extract was preincubated with enzyme for 30 min at 37°.

Bioassay. An approximately 2 cm segment of the guinea-pig ileum was suspended in a muscle bath containing 10 ml of oxygenated Tyrode's solution. The response of the muscle was recorded on a recorder by using a strain gauge-transducer. The minimal quantities of bradykinin that were used to obtain standard responses ranged 5–20 ng. The ileum was treated with α-chymotrypsin as described by Edery[8] prior to bioassay.

Three ml of heparinized plasma were shaken for 30 min with 150 mg of silica powder according to the method of Rinvick *et al.*[9] and then used as a source of kininase.

Estimation of molecular weight. The molecular weight of partially purified inhibitor of plasma HLL hydrolase was estimated on a 1 × 110 cm column of Sephadex G-25 previously equilibrated with 0·05 M ammonium acetate, pH 3·0 by the method of

Greenbaum *et al.*[10] After elution the presence of the inhibitor was determined by inhibitory activity for plasma HLL hydrolase.

Chemicals. HLL was obtained from Protein Research Foundation, Osaka, Japan; bradykinin (100 μg/ml) from Sandoz, A.G., Basel, Switzerland and α-chymotrypsin from Eisai Co., Tokyo, Japan.

RESULTS

Comparison of mitochondrial and lysosomal fractions. The specific activities of acid phosphatase and β-glucuronidase for both of the mitochondrial and lysomal fractions are shown in Table 1. There is a 1·7-fold increase in the specific activity of acid phosphatase in the lysosomal fraction over the mitochondrial fraction. In contrast there is a 2·0-fold decrease in the specific activity of β-glucuronidase in the lysosomal fraction.

TABLE 1. SPECIFIC ACTIVITIES OF ACID PHOSPHATASE AND β-GLUCURONIDASE IN MITOCHONDRIAL AND LYSOSOMAL FRACTIONS

	Specific activity*	
	Acid phosphatase	β-glucuronidase
Mitochondrial fraction†	9·5 ± 2·2	0·85 ± 0·06
Lysosomal fraction	16·0 ± 3·1	0·41 ± 0·07

Each result is the mean value ± standard deviation of eight experiments.

* The specific activities of acid phosphatase and β-glucuronidase is defined as μmoles of *p*-nitorophenol and phenolphthalein released for 30 min/mg protein, respectively.

† Mitochondrial fraction was extracted by the same method as that of lysosomal extraction in Materials and Methods, except for centrifugation at 8500 *g*.

HLL hydrolysis by heparinized plasma and lysosomal extract. HLL was hydrolyzed by heparinized dog plasma. One ml of plasma cleaves 0·95 ± 0·09 μmoles (±S.D.) HLL/min. When plasma was activated by 10^{-4} M $CoCl_2$, the hydrolysis of HLL was 1·34 ± 0·26 μmoles (±S.D.)/min/ml. No hydrolysis of HLL was observed in the lysosomal extract of the dog skeletal muscle, when 5–500 μg protein in 0·1 ml were used instead of plasma as a source of enzyme.

Inhibition by lysosomal extract. Heparinized plasma was preincubated for 30 min with the lysosomal extract over a wide range of concentrations in order to assay its inhibitory activity to HLL hydrolase. HLL hydrolysis of plasma was inhibited at the concentration of 100 μg protein of lysosomal extract and decreased with increasing concentrations of the lysosomal extract up to 500 μg protein as shown in Fig. 1.

Aliquots of the lysosomal extract containing 100 μg protein in 0·1 ml were ultrafiltered using an Amicon ultrafiltration chamber with UM-10 membrane. The ultrafiltrate inhibited plasma HLL hydrolase as much as the original extract. Most of the inhibitory activity of lysosomal extract remained unchanged after boiling for 10 min at neutral pH. These results suggest the presence of low molecular weight inhibitor in the lysosomal extract.

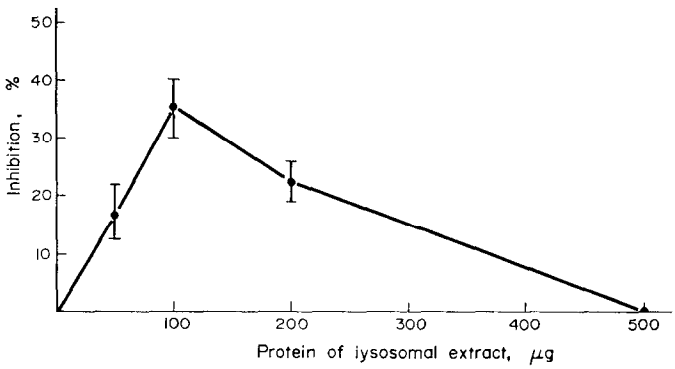

FIG. 1. Inhibition of plasma HLL hydrolase by lysosomal extract 0·1 ml of lysosomal extract containing varied amount of protein was preincubated with 0·1 ml of heparinized plasma for 30 min at 37°.

Purification of inhibitor. In order to separate the inhibitor described previously from lysosomal extract, 3 ml of the lysosomal extract containing 25 mg protein were applied to a CM-Sephadex C-25 column. The typical elution pattern for absorbance measurements at 280 nm and Ninhydrin reaction was shown in Fig. 2. There were two kinds of active fractions indicating that one is high molecular weight substance (tube No. 4–34), which lost its activity during boiling for 10 min at neutral pH and the other low molecular weight substance (tube No. 100–112), which was stable to the heat treatment.

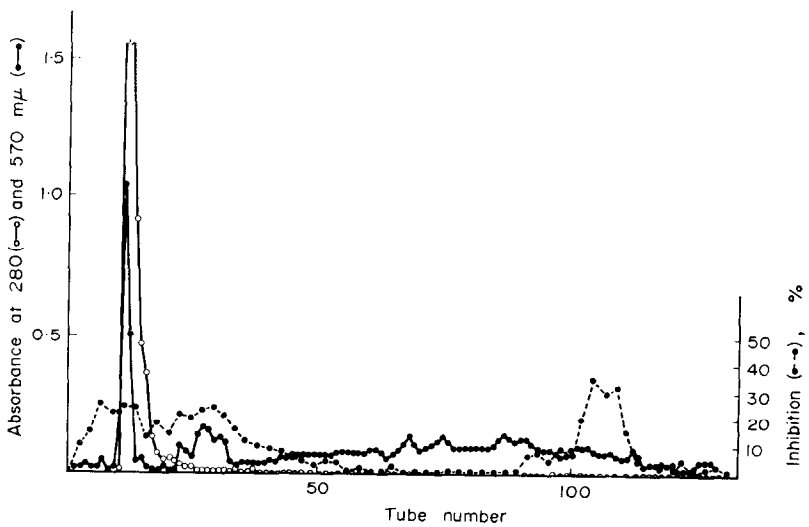

FIG. 2. Chromatography of lysosomal extract on CM-Sephadex C-25. The column was operated as described in Materials and Methods. 0·3 ml of each effluent was used for ninhydrin reaction. 0·2 ml of each effluent was preincubated with 0·1 ml of heparinized plasma for 30 min at 37° and HLL hydrolyzing activity of the mixture was determined.

Tubes corresponding to No. 100–112 were pooled and lyophilized. The freeze-dried residue was rechromatographed on a Sephadex G-25 column and eluted with water. A single peak which gave the inhibitory activity for plasma HLL hydrolase was obtained. Ninhydrin reaction was found in the same fraction. Tubes corresponding to this single peak were pooled and lyophilized again. Approximately 1·0 mg of the final freeze-dried residue was produced from 1 g of fresh muscle. This preparation was used as low molecular weight inhibitor for HLL hydrolase in plasma.

Molecular weight of inhibitor. The molecular weight of partially purified inhibitor of plasma HLL hydrolase was estimated on a 1 × 110 cm column of Sephadex G-25. A single peak which gave the inhibitory activity for plasma HLL hydrolase was obtained and the molecular weight of plasma HLL hydrolase inhibitor was approximately 2000.

Properties of low molecular inhibitor. Low molecular weight inhibitor of plasma HLL hydrolase was preincubated with the enzyme for the assay of inhibitory activity. The relationship of inhibition with length of preincubation period at 37° is shown in Fig. 3. The data indicates that a period of 30 min is satisfactory to obtain a maximum

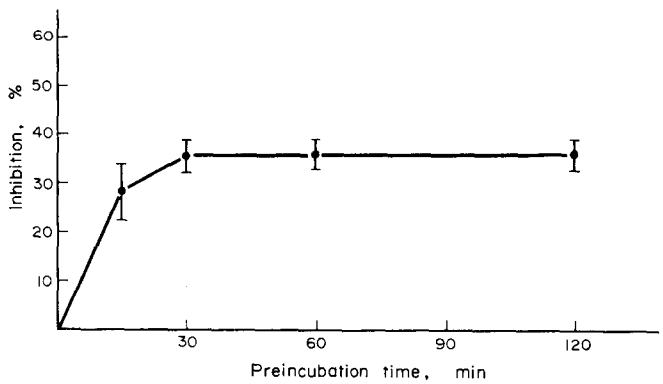

FIG. 3. Effect of the length of preincubation with low molecular weight inhibitor on HLL hydrolase activity of plasma. The mixture of 100 μg of low molecular weight inhibition and 0·1 ml of heparinized plasma was preincubated at various intervals at 37°

inhibition. Low molecular inhibitor was stable to boiling for 10 min at neutral pH. Low molecular weight inhibitor solution was adjusted to pH 2 with HCl. This acidified solution was incubated for 60 min at 37° and then neutralized. Low molecular weight inhibitor did not lose its inhibitory activity by acid-treatment. When low molecular weight inhibitor was incubated with α-chymotrypsin, its inhibitory activity was not affected by the action of α-chymotrypsin as shown in Table 2.

Influence of the concentration of low molecular weight inhibitor on inhibition of plasma HLL hydrolysis is shown in Fig. 4. The rate of hydrolysis of HLL decreased gradually while increasing low molecular weight inhibitor; increasing the concentration of inhibitor to 500 μg could cause complete inhibition of the enzyme.

HLL hydrolase activity of plasma activated by preincubation with 10^{-4} M $CoCl_2$ according to Erdös *et al.*,[2] decreased with increasing concentration of low molecular weight inhibitor up to 125 μg as did in the experiment used the plasma without activation by cobalt, but a plateau was reached with 125 μg of low molecular inhibitor.

TABLE 2. EFFECT OF α-CHYMOTRYPSIN ON LOW MOLECULAR WEIGHT INHIBITOR

Reagents of mixture	HLL hydrolase activity (μmoles/min/ml)	P*	P†
α-Chymotrypsin-treated low molecular weight inhibitor 100 μg + plasma	0·54 ± 0·07	<0·001	
Untreated low molecular weight inhibitor 100 μg + plasma	0·56 ± 0·05		<0·001
Plasma	0·95 ± 0·09		

Each result is the mean value ± standard deviation of seven experiments.

100 μg of low molecular weight inhibitor was incubated with 100 μg α-chymotrypsin in 0·1 M Tris buffer, pH 7·4. Incubation was for 60 min at 37°. Reaction was stopped by placing samples in boiling water bath for 10 min. α-chymotrypsin-treated and untreated low molecular weight inhibitors were preincubated with 0·1 ml of plasma for 30 min at 37°.

* Statistical significance of difference between α-chymotrypsin-treated inhibitor + plasma and plasma.

† Statistical significance of difference between untreated inhibitor + plasma and plasma.

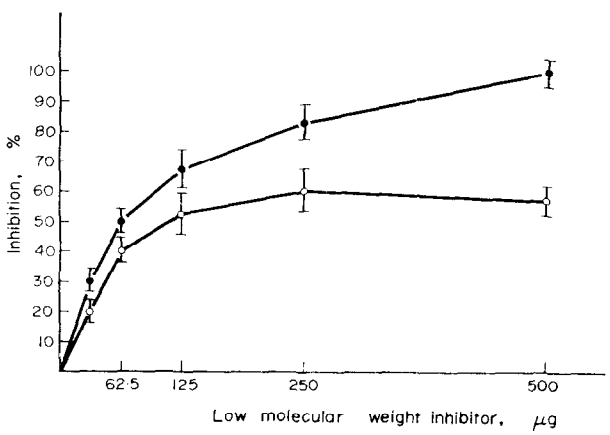

FIG. 4. Inhibition of plasma HLL hydrolase by low molecular weight inhibitor. ●——● heparinized plasma without adding cobalt ions. ○——○ heparinized plasma with adding cobalt ions. 0·1 ml of 0·1 M tris buffer, pH 7·4 containing varied amount of low molecular weight inhibitor was preincubated with 0·1 ml of heparinized plasma for 30 min at 37°.

Low molecular weight inhibitor, 200 μg/ml in the organ bath, did not cause contractions of the guinea-pig ileum.

In order to test whether there was any inhibitory activity of bradykinin to contraction of the guinea-pig ileum, a mixture of 50 ng of bradykinin and 1000 μg of low molecular weight inhibitor was prepared. This mixture gave the same degree of contraction as did 50 ng of bradykinin alone. The result indicated that low molecular weight inhibitor did not interfere the ileum stimulating ability of bradykinin. Effect of low molecular weight inhibitor on inhibition of plasma kininase was shown in Table 3. Low molecular weight inhibitor did not inhibit plasma kininase up to 1500 μg.

TABLE 3. INHIBITION OF PLASMA KININASE BY LOW MOLECULAR WEIGHT INHIBITOR

Reagents of mixture	Inactivated bradykinin (ng)
Bradykinin 100 ng + diluted plasma + low molecular weight inhibitor	50 ± 3·7
Bradykinin 100 ng + diluted plasma	49 ± 4·1

Each result is the mean value ± standard deviation of five experiments.
The silica powder-treated plasma was diluted 1:5 with 0·2 M phosphate buffer, pH 7·4. 0·5 ml of diluted plasma was mixed with 0·5 ml of 100 ng of bradykinin in 0·1 M phosphate buffer 7·4. The mixture was tested on the organ bath after 10 min incubation at 37°. 1·0 mg of low molecular weight inhibitor in phosphate buffer was preincubated with diluted plasma for 30 min at 37°.

DISCUSSION

The results in this paper indicate that a low molecular weight substance in lysosomes of the skeletal muscle caused a marked inhibition of plasma HLL hydrolase activity, but not plasma bradykininase activity.

It is known that human plasma contains two kinds of kininases; kininase I (carboxypeptidase *N*) hydrolyses HLL, hippuryl-L-argininic acid and bradykinin and kininase II inactivates bradykinin only.[11] A low molecular weight substance in this study, may, therefore, be an inhibitor of kininase I in plasma as well as arginine, ϵ-amino-*n*-caproic acid and $CdSO_4$.[11] The inhibitory activity of the low molecular weight inhibitor was lower in the presence of 10^{-4} $CoCl_2$ than that of in the absence of cobalt ions. Thus, the low molecular weight inhibitor in lysosomes exhibits a striking contrast to metals such as $HgCl_2$, $ZnSO_4$ and $CdSO_4$, which were strongly inhibitory in the presence of 10^{-4} M $CoCl_2$ with HLL substrate.[2]

In considering the blood enzyme levels in various pathological conditions, production, release and destruction of enzyme are mainly responsible for the maintenance of blood enzyme level. Erdös *et al.*[3] suggested that from the results of the normal values in pancreatitis and the tendency toward low levels in cirrhosis of the liver, the circulating enzyme may not originate in the pancreas, but may be released by the liver. With our present knowledge of plasma kininases, the finding of a biological HLL hydrolase inhibitor is very interesting.

It may be considered that low molecular weight inhibitor is easily released from lysosomes under some pathological conditions. In fact this inhibitor appeared in the blood after release of tourniquet (unpublished data).

The possibility that HLL hydrolase inhibitor might be present in the blood should be taken into consideration in the study of enzyme levels in muscle disorders.

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