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A Peptidase (Aminopeptidase B) from Cat and Guinea Pig Liver Selective for N-Terminal Arginine and Lysine Residues

I. Purification and Substrate Specificity

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An enzyme selectively hydrolyzing L-arginyl- and L-lysyl-β-naphthylamides has been purified about 20-fold from cat and guinea pig liver and its characteristics have been studied. In all experiments comparisons were made with a highly purified rat liver aminopeptidase B preparation previously described. The enzyme preparation of each of the above species was shown to be free of esterase activity. L-Arginyl- and L-lysyl-β-naphthylamide were hydrolyzed at similar rates by the three preparations. The substrate specificity and hydrolytic characteristics described are similar to those of the previously reported rat liver aminopeptidase B preparation.

Aminopeptidase B (APB), an enzyme selectively catalysing the hydrolysis of peptides of the N-terminal basic amino acids, arginine and lysine, has been described.[1] The enzyme hydrolyzed only L-arginyl- and L-lysyl-β-naphthylamide (L-Arg- and L-Lys-β-NA) of a number of amino acid β-naphthylamides tested. It has been purified about one thousand fold from rat liver and its characteristics and specificity have been further analysed.[2,3] One of the singular properties of the rat liver APB was its prominent activation by certain monovalent anions, notably chloride ion. No metal ion activator was found, nor did any metal chelating agent affect the activity of APB. The marked activation by physiologic concentrations of chloride ions suggested that the enzyme was physiologically active only in the extracellular space or in biological events producing significant concentrations of chloride ions within the cell.[4]

This report deals with the occurrence and properties of APB in the liver of other mammalian species, *viz.* cat and quinea pig. For comparison all experiments were performed simultaneously with rat liver APB, the purification and properties of which have been previously described. The results obtained indicate the presence of an enzyme with similar properties in these three species.

MATERIAL AND METHODS

The reagents and general methods were the same as described in earlier reports on rat liver APB.[2,3] Any variations in the methods will be noted.

As previously described the estimation of APB activity was performed in an incubation mixture containing the following ingredients: 1.5 ml 0.1 M TRIS—HCl buffer, pH 7.0, 0.5 ml 1 mM L-Arg- or L-Lys-β-NA, 0.5 ml enzyme preparation and 0.5 ml water, which could be replaced by various modifier solutions. The mixture was incubated 60 min at 37°C and the reaction stopped by adding 1 ml 0.1 % fast Garnet GBC salt in 1 M acetic acid buffer containing 10 % Tween 20. The color intensity was read with a Klett-Summerson colorimeter, with a No. 52 filter.

RESULTS

1. Purification of the *cat* liver enzyme

Preparation of the homogenate. 35 g of fresh cat liver was homogenized with Waring Blendor for 45 sec in 60 ml 0.1 M TRIS—HCl buffer, pH 7.0. The thick suspension was centrifuged (30 min, 34 000 *g*) and the sediment discarded. All these and the following procedures were performed at 0 to 4°C.

Precipitation at pH 5.5. The pH of the resulting centrifugate (35 ml) was adjusted to 5.5 by the slow addition of 0.2 N HCl with constant and cautious mixing. After standing for 2 h the suspension was centrifuged (10 min, 34 000 *g*) and the precipitate discarded. After an additional period of 24 h the pH was carefully adjusted to 7.0 with 8 N NaOH. The mixture was centrifuged (10 min, 34 000 *g*) and the precipitate discarded.

Gel filtration. 30 ml of the resulting centrifugate was applied to a Sephadex G-200 Superfine column (3.6 × 65 cm). The gel was originally packed at a hydrostatic pressure of 35 cm. Elution was performed using 0.1 M TRIS—HCl buffer, pH 7.0, with the hydrostatic pressure and the flow rate adjusted to 35 cm and 0.2 ml/min, respectively. A typical chromatographic pattern obtained on the cat liver enzyme by gel filtration with Sephadex G-200 Superfine is represented in Fig. 1. The most active fractions (Nos. 65—75) were pooled.

First ammonium sulfate treatment. To the pooled fractions (25 ml) was added 6.95 g ammonium sulfate (45 % saturation). After 60 min standing the mixture was centrifuged (10 min, 25 000 *g*) and the precipitate discarded.

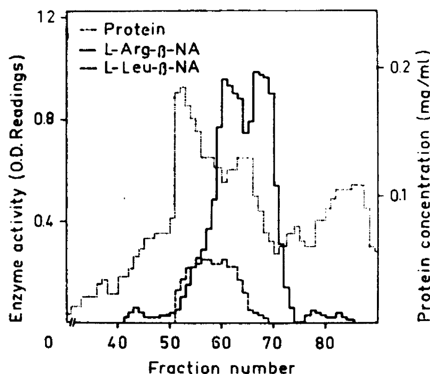

Fig. 1. Gel filtration of the centrifugate obtained after the pH 5.5 precipitation step in the purification of the *cat* liver enzyme. For details see text.

Table 1. Purification of aminopeptidase B from *cat* liver. For determination of the APB unit, see Ref. 2.

Purification step	Volume (ml)	Conc. (units/ ml)	Total units	Protein (mg/ml)	Specific activity units/ mg)	Yield (%)	Purification
Supernatant of the homogenate	35	0.98	34.3	44.5	0.02	100	1
After pH 5.5 sedimentation	30	0.90	27.0	31.0	0.03	92	1.3
Pooled fractions after Sephadex	25	0.34	8.2	2.0	0.17	35	8
55 % ammonium sulfate precipitation	10	0.30	3.0	0.7	0.43	30	20

Second ammonium sulfate treatment. To the resultant centrifugate (28 ml) was added 1.75 g ammonium sulfate (55 % saturation). After 60 min standing the mixture was centrifuged (10 min, 25 000 *g*) and the supernatant was discarded. The precipitate was dissolved in 0.1 M TRIS—HCl buffer, pH 7.0, to a final volume of 10 ml.

The progress of the purification is recorded in Table 1. For experiments on the characterization of the enzymes the enzyme preparation was diluted 1:100 with 0.1 M TRIS—HCl buffer, pH 7.0. In modified studies with monovalent anions, the preparation was diluted in the same ratio with water.

2. Purification of the *guinea pig* enzyme

Preparation of the homogenate. 30 g of fresh guinea pig liver was homogenized in 60 ml of 0.1 M TRIS—HCl buffer, pH 7.0, in the same manner as the cat material. The mixture was centrifuged (30 min, 34 000 *g*) and the sediment discarded. All procedures were performed at 0 to 4°C.

Precipitation at pH 5.5. The pH of the resulting centrifugate (35 ml) was adjusted to 5.5 by the addition of 0.2 N HCl. This mixture was treated in a manner similar to the treatment of the cat liver.

Gel filtration. 31 ml of the resulting centrifugate was applied to a Sephadex G-200 Superfine column (3.7 × 65 cm). The flow rate was adjusted to 2.2 ml/h. All other details were similar to those used in the fractionation of cat liver. The most active fractions (Nos. 58—63) were pooled (34 ml). A typical result of gel filtration with Sephadex G-200 Superfine of the guinea pig liver enzyme is shown in Fig. 2.

First ammonium sulfate treatment. To the pooled fractions was added 8.85 g ammonium sulfate (42 % saturation). After 60 min standing the mixture was centrifuged (10 min, 25 000 *g*) and the precipitate discarded.

Second ammonium sulfate treatment. To the resulting centrifugate (36 ml) was added 3.25 g ammonium sulfate (56 % saturation). After 60 min standing

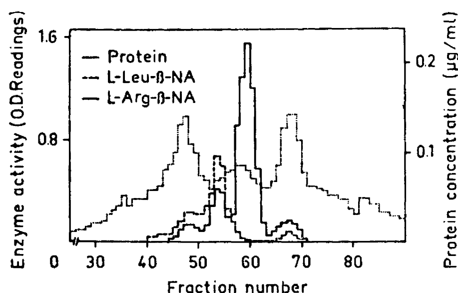

Fig. 2. Gel filtration of the centrifugate obtained after the pH 5.5 preparation step in the purification of the *guinea pig* liver enzyme. For details see text.

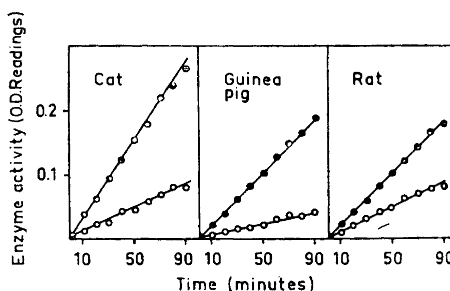

Fig. 3. Hydrolysis of L-Arg-(●) and L-Lys-β-NA (○) by the three enzyme preparations. For details see text.

the mixture was centrifuged (10 min, 25 000 *g*) and the centrifugate discarded. The precipitate was dissolved in 0.1 M TRIS—HCl buffer, pH 7.0 to a final volume of 10 ml. The progress of the purification is recorded in Table 2.

3. The hydrolysis of L-Arg- and L-Lys-β-naphthylamide

Fig. 3 shows the hydrolysis of L-Arg- and L-Lys-β-NA as catalyzed by the three enzyme preparations. It is seen that L-Arg-β-NA was hydrolyzed more rapidly than L-Lys-β-NA by all the enzymes preparations. The ratio of the hydrolysis rates with these substrates (L-Arg-β-NA/L-Lys-β-NA) was about 2:1, 4:1, and 2:1 with the cat, guinea pig, and rat enzyme, respectively.

Table 2. Purification of aminopeptidase B from *guinea pig* liver. For determination of the APB unit, see Ref. 2.

Purification step	Volume (ml)	Conc. (units/ml)	Total units	Protein (ml/mg)	Specific activity (units/mg)	Yield (%)	Purification
Supernatant of the homogenate	35	0.87	30.5	50	0.017	100	1
After pH 5.5 treatment	31	0.82	25.4	33	0.025	80	1.5
Pooled fractions after Sephadex	34	0.31	10.5	2.5	0.124	34	7
56 % ammonium sulfate precipitate	10	0.27	2.7	0.9	0.300	9	17

Table 3. Hydrolysis of various amino acid β-naphthyl amides by three different enzyme preparations. For each enzyme the hydrolysis rate of L-Arg-β-NA has taken as 100.

Substrate	Cat	Guinea pig	Rat
Gly-β-NA	—	—	—
Gly-Gly-β-NA	—	—	—
L-Ala-β-NA	—	—	—
L-Val-β-NA	—	—	—
L-norVal-β-NA	—	—	—
L-Leu-β-NA	—	—	—
L-norLeu-β-NA	—	—	—
L-Thr-β-NA	—	—	—
L-Phe-β-NA	—	—	—
L-Tyr-β-NA	—	—	—
L-Trp-β-NA	—	—	—
L-Cystinyl-β-NA	—	—	—
L-Met-β-NA	—	—	—
L-Glu-β-NA	—	—	—
L-His-β-NA	—	—	—
L-Arg-β-NA	100	100	100
L-Lys-β-NA	50	25	50
L-Orn-β-NA	—	—	—
Benzoyl-DL-Arg-β-NA	—	—	—
γ-Amino butyryl-β-NA	—	—	—

4. Substrate specificity

Methods for the determination of substrate specificity were similar to those described with the rat liver enzyme.[3] The results of the hydrolysis of a number of amino acid naphthylamides are given in Table 3. It appeared that only the naphthylamides of basic amino acids, arginine and lysine, were hydrolyzed. Substrates with a blocked α-amino group, *e.g.* N-α-benzoyl-DL-arginine β-naphthylamide, were not cleaved.

The hydrolysis of the following compounds was also studied: β-naphthyl acetate, β-naphthyl propionate, β-naphthyl butyrate, β-naphthyl caprylate, β-naphthyl laurate, β-naphthyl acid phosphate, naphthol-AS acetate, and L-arginine and L-lysine methyl ester. No detectable hydrolysis by the three enzyme preparations occurred with any of these substrates. The results indicate that these enzymes possess a marked specificity for N-terminal arginine and lysine residues.

DISCUSSION

The main purpose of this study was to determine whether enzymes similar to the aminopeptidase B of rat liver also exist in other mammalian species. The enzyme activity hydrolyzing only L-Arg-β-NA therefore was followed during the purification procedure and characteristics of the final enzyme preparations were compared to the known properties of the rat liver enzyme. The differences in the degree of purification of the preparations, (the rat liver enzyme being the most highly purified) made comparisons somewhat difficult. As a result, absolute relationships could not be expected.

All three preparations behaved quite similarly in the pH 5.5 precipitation step, *i.e.* the enzyme did not sediment out during this procedure. In the Sephadex gel filtration procedure it was more difficult to separate the cat enzyme from other enzymes also capable of hydrolyzing the same substrates.

It was possible to obtain all preparations free from esterase activity by surprisingly simple fractionation procedures. Gel filtration either prior to or proceeding ammonium sulfate precipitation appeared to be the decisive steps in eliminating the esterase activities.

The hydrolysis rates by the three preparations of the basic amino acid β-naphthylamides were in general similar. L-Arg-β-NA was rapidly hydrolyzed, L-Lys-β-Na was cleaved much more slowly and L-Orn-β-NA and benzoyl-DL-Arg-β-NA were not hydrolyzed at all. It would appear that both the α- and ε-amino groups are a prerequisite for hydrolysis and the distance of the amino group farthest removed from the susceptible bond seems to be strictly limited, since even a substrate with a δ-amino group is not hydrolyzed.

The few properties of the three preparations described here are sufficient to indicate the presence in other species of enzymes with substrate specificities roughly similar to aminopeptidase B of rat liver. Further characterization of these enzymes is presented in the subsequent paper.[6]

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