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PURIFICATION AND CHARACTERIZATION OF THE RABBIT INTESTINAL BRUSH-BORDER AMINOPEPTIDASE A

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Summary

The papain form of rabbit intestinal brush-border aminopeptidase A has been purified to homogeneity. It is a monomeric enzyme of molecular weight 170 000. It represents 3.5% of the total proteins of the membrane. Its specificity slightly overlaps with that of aminopeptidase N.

Enterocyte brush-border peptidases play an important role in protein digestion. Their specificities are complementary to those of pancreatic enzymes. We are interested in the comparative study of specificities of the two aminopeptidases of the brush border: the aminopeptidases N and A. Until now, no aminopeptidase A has ever been purified. We have now purified the pig intestinal brush-border aminopeptidase A solubilized by detergent treatment (the detergent form) or protease digestion (the protease form) [1]. It has been shown to be a symmetrical dimer as the aminopeptidase N from the same membrane [2]. In contrast both forms of the rabbit aminopeptidase N are monomeric [3]. We report here on the purification and characterization of the protease form of the rabbit intestinal aminopeptidase A.

The procedure for the full purification of the above enzyme is summarized in Table I. The detergent form was partially purified and converted into the protease form. Then, this latter was further purified to chemical and immunological homogeneity.

As previously reported [3], the Emulphogen-solubilized detergent forms of the rabbit aminopeptidases N and A copurified up to the Sepharose 6B filtration. After this step in which the two enzymes were clearly separated (emergence at 1.7 and 1.96 void volume, respectively) the major contaminant

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

TABLE I

PURIFICATION OF THE PROTEASE FORM OF RABBIT AMINOPEPTIDASE A. BEFORE PAPAIN TREATMENT, ALL BUFFERS CONTAINED 1% EMULPHOGEN

Steps	Yield (%)	Specific activity (units/mg protein)
Gauze filtration of homogenate	100	40
3000 × *g* supernatant	65	40
30 000 × *g* pellet	55	120
Emulphogen extract	45	420
$(NH_4)_2SO_4$ precipitation (0.5 satn.)	35	850
$(NH_4)_2SO_4$ fractionation (0.2—0.5 satn.)	25	1 700
Sepharose 6B filtration	20	2 800
Concentration and $(NH_4)_2SO_4$ precipitation (0.2—0.5 satn.)	18	3 500
Sephadex G-200 filtration	16	14 500
Immunoabsorbant chromatography (see text)	11	—
DEAE-cellulose concentration	10	18 000
Papain treatment	10	18 000
ACA34 Ultrogel filtration	8	25 000
Hydroxyapatite chromatography	7	32 000

of the aminopeptidase A fractions was the sucrase-isomaltase complex [4]. This latter was removed by filtration through a Sephadex G-200 column (4 × 60 cm) equilibrated and eluted with a 10 mM HEPES buffer (pH 7.0), 1% Emulphogen, 0.1 mM NaCl and 5 mM $CaCl_2$. The aminopeptidase was eluted immediately after the void volume whereas the sucrase-isomaltase was strongly retarded [4]. Traces of remaining sucrase-isomaltase and aminopeptidase N were carefully eliminated using the already reported principle of reverse immunoabsorbant chromatography. For this purpose, the solutions were passed through columns filled with immobilized immunoglobulins [5] raised in guinea pigs against the sucrase-isomaltase after Sephadex G-200 filtration (see above), or with goat anti-aminopeptidase N [3]. In both cases, the detergent form of aminopeptidase A migrated unretarded with an excellent yield while the two contaminants were successively retained.

After dialysis against a 10 mM phosphate buffer (pH 6.0) the solutions were concentrated on a DEAE-cellulose column (1 × 5 cm) equilibrated with the buffer and eluted by a small volume of 0.15 M NaCl. They were again dialysed against a 50 mM phosphate buffer pH 6.2), 5 mM cysteine and 30 μM dithiothreitol, and incubated at 37°C for 45 min with 0.1 mg/ml activated papain [6] to insure conversion into the protease form. Hydrolysis was stopped by *N*-ethylmaleimide (50 mM) and the aminopeptidase was further purified by filtration through an Ultrogel ACA-34 column (1 × 200 cm), equilibrated and eluted with a 10 mM Tris-HCl buffer (pH 7.0), 5 mM $CaCl_2$ (emergence at 1.45 void volume). The last step was chromatography through a hydroxyapatite column (1.5 × 10 cm) equilibrated with a 10 mM phosphate buffer (pH 6.0), 0.2 M KCl. The column was washed with the buffer and the enzyme was eluted as a symmetrical peak by a linear phosphate concentration gradient (2 × 200 ml) from 10 to 100 mM.

The preparations thus obtained yielded a single band by polyacrylamide gel electrophoresis in the presence or absence of SDS. They also gave a single immunoprecipitate line by rocket immunoelectrophoresis against anti-total membrane proteins [3] and anti-aminopeptidase A.

As already stressed earlier [3], full purification of the rabbit aminopeptidases N and A met with unusual difficulties mainly due to the abundance of mucus in the intestine of this species. In fact, the anti-'impurities' immunoabsorbant chromatography, successfully applied, for the complete purification of detergent forms of rabbit aminopeptidase N [3], and pig aminopeptidase A [1], partially failed in the case of rabbit aminopeptidase A. This explains why full purification of the detergent form of this latter enzyme has not yet been achieved.

Using the equilibrium ultracentrifugation method of Yphantis [7] applied to 0.6 mg/ml enzyme samples (15 000 rev./min for 28 h at 200°C, a linear log Y/r^2 plot was obtained which, led to an apparent M_F of 185 000 ± 5000. The average (0.700 ml/mg) of all the partial specific volume values already determined for similar brush-border hydrolases was used for the calculation. An absorption coefficient ($E^{1\%}_{1\,cm}$ at 280 nm) of 12.1 was measured on 1.3 mg/ml solutions with the aid of the interferential optics of the ultracentrifuge.

In a Sepharose 6B column (1.5 × 300 cm) equilibrated with the 10 mM phosphate buffer (pH 6.0) and calibrated with the protease forms of porcine and rabbit aminopeptidase N (M_r: 245 000 and 125 000, respectively [3—8]), the enzyme migrated as a protein weighing 180 000. Polyacrylamide gel electrophoresis of the reduced carboxymethylated enzyme in the presence of SDS yielded a single band corresponding to an M_r of 170 000 ± 5000. These two latter results demonstrate that the protease form of rabbit aminopeptidase A is monomeric.

In pig [2], rat [9] and man [10], the intestinal aminopeptidase N has been described as a dimeric enzyme. Pig aminopeptidase A is also dimeric [1]. Thus, the rabbit appears to be an exception since both aminopeptidases N and A in this species are monomeric. The two forms (detergent and protease) of rabbit aminopeptidase N have even been shown to be monomeric [3]. Information is presently lacking about the detergent form of rabbit aminopeptidase A.

A preliminary comparison of the enzymatic specificities of the rabbit aminopeptidase A and N was carried out with the aid of four chromogenic substrates: the α-*p*-nitroanilides of glutamic acid, leucine, alanine and lysine. The results obtained with the pure enzymes and the total brush border are presented in Table II.

TABLE II

SPECIFIC ACTIVITY (NMOL SUBSTRATE HYDROLYSED PER MIN/MG PROTEIN) OF RABBIT AMINOPEPTIDASES A AND N AND BRUSH-BORDER VESICLES [6] TOWARDS FOUR SYNTHETIC SUBSTRATES

Hydrolysis of glutamic acid α-*p* nitroanilide 1.5 mM in 0.1 M Tris-HCl buffer (pH 7.0) containing 25 mM $CaCl_2$, and of alanine, leucine and lysine *p*-nitroanilides 1.5 mM in 50 mM phosphate buffer (pH 7.0) were performed at 37°C.

	α-*p*-nitroanilide of			
	Glu	Ala	Leu	Lys
Aminopeptidase A	32 000	5 500	2 500	2 500
Aminopeptidase N	2 400	15 000	21 500	4 500
Brush-border membrane vesicles	1 100	850	750	600

The enzymatic specificities of the aminopeptidases N and A are similar in the pig [1] and the rabbit. The best substrates for the enzymes are, respectively, the α-*p*-nitroanilides of leucine and glutamic acid. Both enzymes are involved in the hydrolysis of alanine and lysine *p*-nitroanilides. The amounts of aminopeptidases N and A are approximately the same in the rabbit brush border (about 3.5% of the total proteins of the membrane), whereas the aminopeptidase N is largely predominant in the pig [1, 8].

Comparison of the enzymatic properties of dimeric pig aminopeptidases and monomeric rabbit enzymes is now in progress.

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