A Neutral Trypsin-like Protease from the Rat Submandibular Gland

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The presence of a number of trypsin-like proteases in rat submandibular gland tissue has been described earlier.[1] Two alkaline proteases have been purified and characterized earlier, *i.e.* salivain [2] and glandulain.[3] This report deals with the purification and characteristics of a neutral sulphydryl-dependent protease in the same tissue.

Material and methods. Activity assays were as described earlier [1-3] except that liberated naphthylamine was demonstrated with *p*-dimethylaminobenzaldehyde at pH 2.2.[4] The substrates and other chemicals were those described earlier.[2]

Purification of the enzyme. Submandibular salivary glands of 40 adult male rats were homogenized in 160 ml of 0.88 M sucrose in 0.9 % NaCl. The pH was adjusted to 5.2 by adding 1 N HCl in ice water. The precipitate was removed by centrifugation (16 000 rpm; 30 min) and dissolved in 100 ml of distilled water. The pH was adjusted to 7.0 with 1 N NaOH. The solution was frozen and thawed six times, rehomogenized, and incubated at 37°C for 6 h. The precipitate was removed by centrifugation (3000 rpm; 30 min) and discarded. The supernatant (82 ml) was 20 % saturated with ammonium sulphate and the precipitate removed by centrifugation as before and discarded. Ammonium sulphate was added to 40 % saturation and the precipitate collected by centrifugation as before. The sediment was dissolved in 32 ml distilled water and dialyzed against 20 mM Tris-HCl buffer, pH 7.0, at +4°C for three days. The preparation was chromatographed on a column (2 × 60 cm) of DEAE-cellulose at +4°C, using a 0–50 mM NaCl gradient in 20 mM Tris-HCl buffer pH 7.0 with the hydrostatic pressure 80 cm H_2O, the flow rate 0.5 ml/min, the fraction volume 10 ml, and number of fractions 100. The activity was determined at pH 7.0 in 0.1 M Tris-HCl buffer with N - benzoyl - DL - arginine - β - naphthylamide (BANA) * as substrate with and without monoiodoacetic acid (1 mM). The second peak

* ArgMe, L-arginine methyl ester; ArgNa, L-arginine 2-naphthylamide; BAA, N^{α}-benzoyl-DL-arginine amide; BAEE, N^{α}-benzoyl-DL-arginine ethyl ester; BAPA, N^{α}-benzoyl-DL-arginine *p*-nitroanilide; BANA, N^{α}-benzoyl-DL-arginine 2-naphthylamide; BPANE, N^{α}-benzoyl-DL-phenylalanine 2-naphthyl ester; Hb, human hemoglobin; LysMe, L-lysine methyl ester; LysNa, L-lysine 2-naphthylamide; N-CBZ-L-Tyr. Hydraz., N-carbobenzyloxy-L-tyrosine hydrazide; TAME, N-toluene *p*-sulphonyl-DL-arginine methyl ester.

Table 1. Summary of the purification procedure for neutral protease.

Purification stage	Volume (ml)	Amount of protein (mg)	Activity, mμmole/mg/min	Purification
Homogenate	160	3200	0.85	1.0
pH 5.2 sediment	100	1850	1.55	1.9
Supernatant for the former	82	1340	1.9	3.6
Ammonium sulphate fractionation 20–40 %	32	595	4.7	5.6
DEAE-cellulose	55	45	21	24.7
Sephadex G-100, pooled preparation	70	12.5	76	90
CM-cellulose, pooled preparation	45	2.0	118	138

active toward BANA was sensitive to monoiodoacetic acid (1 mM) and the fractions of this peak were pooled (55 ml). The preparation was concentrated against carbowax to a final volume of 11 ml and subjected to gel filtration on a Sephadex G-100 column (4 × 90 cm) at +4°C. The eluent was 0.1 M NaCl in 20 mM Tris-HCl buffer pH 7.0, flow rate 0.7 ml/min, fraction volume 10 ml, number of fractions 100. The fractions of the first peak active toward BANA (sensitive to 1 mM monoiodoacetic acid) were pooled (70 ml). The preparation was concentrated against carbowax to 9.0 ml and dialyzed against 20 mM Medinal-HCl buffer pH 5.0 for three days and was then subjected to chromatography on a column (2 × 40 cm) of CM-cellulose equilibrated with 20 mM Medinal-HCl buffer pH 5.0, the flow rate 0.4 ml/min, hydrostatic pressure 70 cm H_2O, and the volume and number of fractions 5.0 ml and 100, respectively. The active fractions were pooled (45 ml) and concentrated against carbowax to 20 ml and dialyzed against distilled water for three days. This preparation was used for further studies.

Characteristics of the enzyme. The optimal pH for the hydrolysis of BANA and casein by the preparation was pH 6.8—7.1 in 0.1 M Michael's veronal-acetate buffer. A Linearweaver-Burk plot of the hydrolysis rates at various substrate concentrations gave K_m values 6.5×10^{-3} and 0.65 % for BANA and casein, respectively.

Table 2. Hydrolysis of various substrates by neutral protease.

Substrate	Conc.	Hydrolysis rate (mμmole/mg/min)
BANA	2.0 mM	118
BAPA	1.0 mM	45
LysNa	0.25 mM	0
ArgNa	0.25 mM	0
BAEE	10 mM	310
TAME	10 mM	255
LysMe	10 mM	36
ArgMe	10 mM	54
N-CBZ-L-Tyr-Hydraz.	2.0 mM	12
BPANE	0.08 mM	28
BAA	5.0 mM	174
Casein	0.25 %	235
Hb	0.25 %	108

The hydrolysis rates of various substrates of trypsin and cathepsin B are shown in Table 2. It can be seen that ester substrates are hydrolyzed only 2—3 times more rapidly than the corresponding amides. The rate of BAPA hydrolysis was only one third of that of BANA. The proteolytic nature of the enzyme is demonstrated by hydrolysis of casein and Hb. Chymotrypsin substrates were hydrolyzed only slowly.

The effect of several substances on the hydrolytic reaction is shown in Table 3. The same results were obtained with BANA and casein as substrates. Cystein and

Table 3. Effect of various enzyme modifiers on the hydrolysis of BANA by neutral protease in 0.1 M Tris-HCl buffer pH 7.0.

Affector	Conc.	Percentage change
Cystein	5 mM	+240
Mercaptoethylamine	5 mM	+165
Iodoacetamide	1 mM	− 98
Lima bean	0.1 mg/ml	− 35
Ovomucoid	0.1 mg/ml	0
Tetra-N-butylammonium iodide	20 mM	− 78
	10 mM	− 38
	5 mM	− 25
	1 mM	− 13
Tetra-N-methylammonium iodide	20 mM	− 42
	10 mM	− 26
	5 mM	− 8
	1 mM	+ 18
E-600	0.25 mM	0
DFP	0.25 mM	0
EDTA	2 mM	+ 35
$CuCl_2$	1 mM	− 75
$AlCl_3$	1 mM	− 62
$CoCl_2$	1 mM	− 42
$CrCl_2$	1 mM	− 34
$CaCl_2$	1 mM	− 57
$HgCl_2$	1 mM	−100
$MgCl_2$	1 mM	− 38
$MgCl_2$	1 mM	− 25
$CdCl_2$	1 mM	− 84
NaCl	1 mM	0
KCl	1 mM	0

mercaptoethylamine activated strongly and SH reagents inhibited drastically the enzymatic activity. Bivalent metals were inhibitory and E-600 (diethyl-*p*-nitrophenyl phosphate) and DFP (di-isopropyl fluorophosphate) had no influence even when a pre-incubation of 4 h was allowed. Molecular weight determinations using gel filtration on Sephadex G-100 [2] with albumin, 70 000,[5] and trypsin 23 800,[6] as standards gave a value of 48 000.

Discussion. A 140-fold purification was obtained by the method used. The purified enzyme is clearly a protease with a substrate specificity resembling that of bovine trypsin. Its pH optimum, reaction to a number of modifier substances, particularly sulphydryl reagents, as well as its different behaviour during the purification procedure show that the protease is not identical with either salivain [2] or glandulain.[7] The dependence on SH-groups, preference for BAA and BANA before BAPA [8] as substrate, the relatively slow hydrolysis of ester substrates,[9] and the molecular weight of similar order [10] are features resembling those of cathepsin B. A marked contrast is the neutral pH optimum of the protease (reported here) compared with the pH 5.0—5.5 optimum of cathepsin B.[11] The presence of enzymes, resembling closely the submandibular protease has been demonstrated in other tissues, *e.g.* in thyroid.[12] Also, Curoff [13] has purified from rat brain tissue (32-fold) a neutral SH-dependent protease with characteristics resembling those of the submandibular protease. The possible identity of these enzymes remains to be decided after further purification and characterization of the brain protease has been carried out.

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The Occurrence of Amino Acid Naphthylamidase in Baker's Yeast

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Yeast contains proteolytic enzymes which hydrolyze various proteins and peptides. These enzymes are described in early works by Dernby [1] and by Grassmann, Willstätter, and co-workers.[2-5] Besides proteinase and dipeptidase activity, an amino polypeptidase hydrolyzing DL-leucinamide and various peptides is reported. Another polypeptidase has been purified by Johnson [6] from brewer's bottom yeast. The proteinases in baker's yeast have been studied by Lenney,[7] but his work does not concern exopeptidases.

I have found in extracts of commercial baker's yeast, (*Saccharomyces cerevisiae*), an enzyme which hydrolyzes several amino acid naphthylamides. These chromogenic substrates are often used for the assay of leucine aminopeptidase, although Smith and Hill [8] have called attention to the possibility that these compounds might be hydrolyzed also by other enzymes. No hydrolysis of L-leucinamide, a classical substrate for leucine aminopeptidase determination, was detected with the yeast extract. The enzyme studied might therefore be called an amino acid naphthyl-