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PURIFICATION AND PROPERTIES OF GLYCYLPROLYL β-NAPHTHYLAMIDASE IN HUMAN SUBMAXILLARY GLAND

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SUMMARY

Glycylprolyl β-naphthylamidase was found to be the most active among various amino acid β-naphthylamidases in human submaxillary gland. Approximately 50% of the enzyme was localized in the microsomal fraction. The enzyme was solubilized from the microsomal fraction by autodigestion, and subsequently purified by $(NH_4)_2SO_4$ fractionation (55–80% saturation) followed by Sephadex G-200 chromatography. The purified enzyme was nearly homogeneous as judged from acrylamide disc electrophoresis. The purified enzyme had an optimum pH between 8.5–9.0. The K_m value toward glycylprolyl β-naphthylamide was about $3 \cdot 10^{-4}$ M. The purified enzyme preferentially hydrolyzed glycylprolyl β-naphthylamide, and liberated N-terminal glycylproline from glycylprolyl β-naphthylamide, glycylprolylalanine or glycylprolylleucine.

INTRODUCTION

In 1966, HOPSU-HAVU AND GLENNER[1] discovered a new dipeptide naphthylamidase hydrolyzing glycylprolyl β-naphthylamide (glycylprolyl β-naphthylamidase) in rat liver. The enzyme was subsequently solubilized and purified from rat liver microsomes by HOPSU-HAVU AND SARIMO[2]. In the course of our studies on enzymes in human saliva, glycylprolyl β-naphthylamidase activity in human parotid saliva was found to be distinctly higher than other amino acid β-naphthylamidase activities[3]. Glycylprolyl β-naphthylamidase appears to be secreted into saliva from the salivary glands. The enzyme was also found mainly in the microsomal fraction of bovine parotid gland[4]. The present communication describes solubilization, purification and properties of glycylprolyl β-naphthylamidase from human submaxillary gland.

MATERIALS AND METHODS

Glycyl-L-prolyl β-naphthylamide, glycylprolylalanine and glycylprolylleucine

were kindly synthesized by Dr. S. Sakakibara and Dr. K. Takada. β-Naphthylamides of glycylphenylalanine, cystine, norleucine and norvaline, which were synthesized by the method of GLENNER *et al.*[5], were kindly supplied by Dr. G. G. Glenner. Other amino acid β-naphthylamides were obtained from Mann Research Laboratories; Fast Garnet GBC from Sigma Chemical Co.; and Sephadex G-200 and blue dextran from Pharmacia. Human submaxillary glands were obtained at autopsy, and bovine submaxillary and parotid glands from the slaughterhouse. Salivary glands were stored frozen. Human saliva from submaxillary and sublingual glands was collected aseptically by means of a canula as described previously[3].

The enzyme activity for hydrolysis of glycylprolyl β-naphthylamide or of other amino acid β-naphthylamides was measured as described before[6], either by colorimetry[7] or by fluorimetry[8]. The incubation mixture contained: 90 μmoles of Tris–maleate buffer, pH 7.0; 0.45 μmole of glycylprolyl β-naphthylamide or other amino acid β-naphthylamides; an appropriate amount of the enzyme; and water to 0.90 ml. Incubation was carried out at 37° for 30 min. In colorimetry, the incubation was stopped by adding 0.3 ml of 10% Tween 20 in 1 M acetate buffer, pH 4.2, containing 0.45 mg of stabilized diazonium salt Fast Garnet GBC. After 30 min, absorbance at 530 nm was measured. In fluorimetry, the fluorescence intensity of β-naphthylamine released by enzymatic hydrolysis of the substrate was determined at 410 nm with the excitation light at 355 nm in an Aminco-Bowman spectrophotofluorometer. All values were corrected for spontaneous hydrolysis of amino acid β-naphthylamide[5] by subtracting the value of a control solution containing water instead of the enzyme sample.

One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmole of β-naphthylamide per min at 37°.

The enzymatic hydrolysis of glycylprolyl β-naphthylamide, glycylprolylalanine, and glycylprolylleucine were determined by using either paper chromatography or an amino acid analyzer (Hitachi KLA-3B). Incubation mixtures employed for the detection by paper chromatography contained (in μmoles): substrate, 0.9; potassium phosphate buffer (pH 7.5), 10; an appropriate amount of the purified enzyme; and water to 0.1 ml. Incubation was carried out at 37° for 1 h. An aliquot (20 μl) of the supernatant was applied to Toyo Roshi No. 51 paper. Paper chromatography was carried out with the solvent system *n*-butanol–formic acid–water (20:6:5, by vol.). The chromatograms were developed with 0.2% ninhydrin in acetone–pyridine (94:6, v/v). When the hydrolysis of the substrates was assayed by means of an amino acid analyzer, the same incubation mixture as in the assay of glycylprolyl β-naphthylamide hydrolysis was used. The substrate concentration was 1.7 mM in a total volume of 0.9 ml. Incubation was carried out at 37° for 30 min. The reaction was stopped by adding 4.5 ml of 1% picric acid. After light centrifugation, the supernatant was passed through a column of Dowex-2 X8 (Cl^- form) to remove picric acid. The column was washed with 15 ml of 0.02 M HCl. The eluate and washings were combined and lyophilized. The residue was dissolved in 1.0 ml of 0.2 M citrate buffer (pH 2.2). The amino acids were analyzed by the method of SPACKMAN *et al.*[9].

The approximate molecular weight was determined by gel filtration on Sephadex G-200 according to the method of WHITAKER[10]. The molecular weights of apoferritin, γ-globulin, bovine albumin, ovalbumin, chymotrypsinogen A, and cytochrome *c* were taken as 480 000, 160 000, 67 000, 45 000, 25 000, and 12 400, respectively.

Disc electrophoresis was carried out as described by DAVIS[11]. After electrophoresis, protein on one gel was located by staining with naphthalene black. The enzyme activity on the other gel was located by incubating it in the incubation mixture for the assay of glycylprolyl β-naphthylamidase, and β-naphthylamine liberated was located on the gel by the reaction with Fast Garnet GBC as described above. Protein was measured by the method of LOWRY *et al.*[12] using bovine serum albumin as standard.

Subcellular fractionation of salivary glands was carried out by differential centrifugation. Tissues were homogenized in 9 vol. of 0.25 M sucrose using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 700 × *g* for 10 min, then at 5000 × *g* for 10 min, and finally at 100 000 × *g* for 60 min, to separate nuclear, mitochondrial, and microsomal fractions, respectively.

TABLE I

DISTRIBUTION OF GLYCYLPROLYL β-NAPHTHYLAMIDASE AND LEUCYL β-NAPHTHYLAMIDASE IN SALIVARY GLANDS

Salivary glands	*Glycylprolyl β-naphthylamidase (units/mg)*	*Leucyl β-naphthylamidase (units/mg)*
Human submaxillary		
Microsomes	0.091	0.010
Soluble fraction	0.011	0.009
Bovine submaxillary		
Microsomes	0.022	0.025
Soluble fraction	0.004	0.004
Bovine parotid		
Microsomes	0.031	0.014
Soluble fraction	0.004	0.004

RESULTS

Distribution of glycylprolyl β-naphthylamidase in bovine submaxillary gland, bovine parotid gland and human submaxillary gland

Glycylprolyl β-naphthylamidase activity was found in bovine submaxillary gland, bovine parotid gland, and human submaxillary gland (Table I). Leucyl β-naphthylamidase activity was also examined in comparison. The enzyme activity was localized both in microsomal and soluble fractions, but the specific activity in microsomes was much higher than that in the soluble fraction. The microsomal fraction of human submaxillary gland had the highest activity. As shown in Table II, 55% of the activity in human submaxillary gland was present in the microsomal fraction, as compared with 20% in the soluble fraction. The activity in mitochondria was small. The activity in the nuclear fraction (18%) may be due to contamination with microsomes.

Purification of glycylprolyl β-naphthylamidase from human submaxillary gland

Purification of glycylprolyl β-naphthylamidase was carried out using the microsomal fraction of human submaxillary gland. All procedures were carried out at 0–4°.

Step 1. Isolation of microsomes. 50 g of human submaxillary glands were homo-

TABLE II

SUBCELLULAR DISTRIBUTION OF GLYCYLPROLYL β-NAPHTHYLAMIDASE IN HUMAN SUBMAXILLARY GLAND

Subcellular fractions were separated from 17.5 g of human submaxillary gland as described in MATERIALS AND METHODS.

Subcellular fraction	*Total protein (mg)*	*Glycylprolyl β-naphthylamidase*		
		Total activity		*Specific activity (units/mg)*
		(units)	*(%)*	
Homogenate	988	27.9	100	0.028
Nuclear fraction	298	5.0	18	0.017
Mitochondrial fraction	101	2.3	8	0.023
Microsomal fraction	167	15.2	55	0.091
Soluble fraction	498	5.6	20	0.011

genized in 9 vol. of 0.25 M sucrose using an Ultra-Turrax homogenizer. Microsomes were isolated by differential centrifugation as described in MATERIALS AND METHODS. The microsomes were suspended in 50 ml of water and stored frozen at −20°.

Step 2. Solubilization. Solubilization of the enzyme in microsomes was achieved by autodigestion, as described by HOPSU-HAVU AND SARIMO[3] for rat liver. The microsomal suspension stored frozen at −20° for more than 24 h was incubated at 37° for 24 h, then centrifuged at 100 000 × *g* for 60 min. The supernatant was carefully removed. The residue was resuspended in the original volume of water and stored frozen at −20° for 24 h. This autodigestion procedure was repeated twice, and the three supernatant fractions were combined (88 ml). As shown in Table III, about 70% of the enzyme activity was solubilized by repeated autodigestion.

Step 3. $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to 88 ml of the extract from the microsomal fraction to 55% saturation. After mixing for 60 min, the solution was centrifuged at 8000 × *g* for 20 min. The supernatant was removed and the precipitate discarded. To the supernatant, solid $(NH_4)_2SO_4$ was added to 80% saturation. After mixing for 60 min, the solution was centrifuged at 8000 × *g* for

TABLE III

SOLUBILIZATION OF GLYCYLPROLYL β-NAPHTHYLAMIDASE FROM THE MICROSOMAL FRACTION OF HUMAN SUBMAXILLARY GLAND

The microsomal fraction was isolated from 17.5 g of human submaxillary gland, and glycylprolyl β-naphthylamidase was solubilized by autodigestion as described in RESULTS.

Preparation	*Total protein (mg)*	*Glycylprolyl β-naphthylamidase*		
		Total activity		*Specific activity (units/mg)*
		(units)	*(%)*	
Microsomal fraction	167	15.2	100	0.091
Supernatant				
after 1st autolysis	10.2	5.40	36	0.529
after 2nd autolysis	7.5	3.60	24	0.480
after 3rd autolysis	2.3	1.13	7	0.491
Combined supernatant	20.0	10.13	67	0.507

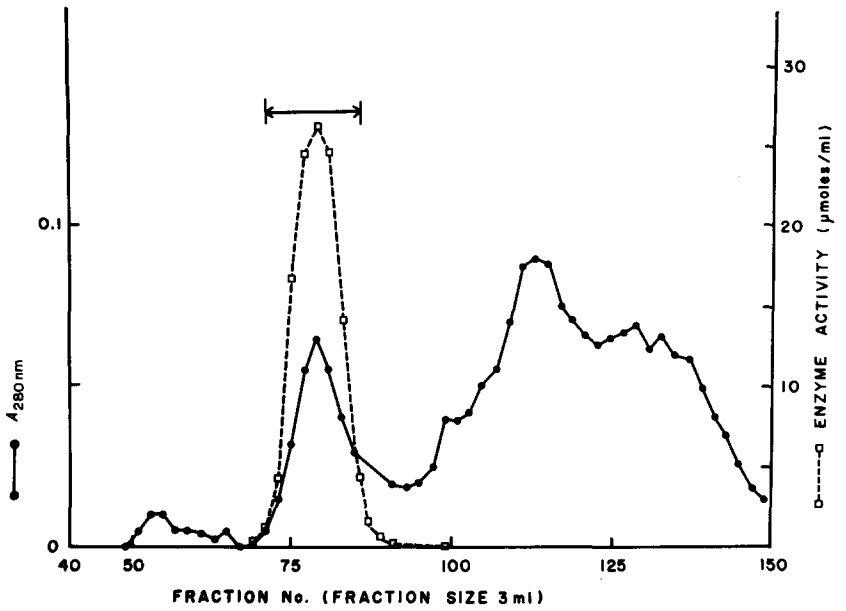


Fig. 1. Chromatography of glycylprolyl β-naphthylamidase from human submaxillary gland on Sephadex G-200. The conditions are described in RESULTS. |←→|, fractions pooled.

20 min. The supernatant was discarded, and the precipitate dissolved in 9.0 ml of 20 mM Tris–HCl buffer, pH 7.5. The solution was dialyzed against a large volume of the same buffer. The dialyzed solution could be stored frozen at −20°.

Step 4. Sephadex G-200 chromatography. The enzyme at Step 3 (9 ml) was concentrated to 5 ml by ultrafiltration. The enzyme solution was passed through a column of Sephadex G-200 equilibrated previously with 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl. For 16.9 mg of protein in 5 ml, a 2.5 cm × 86 cm column was used. Elution was carried out using the same buffer at a flow rate of 5–6 ml/h, and fractions of 3 ml each were collected. As shown in Fig. 1, the enzyme activity was eluted together with the second protein peak between the 71st and 86th fractions. These fractions were combined. The entire purification is shown in Table IV. The purified enzyme could be stored at −20° for several months without appreciable loss in activity.

TABLE IV

PURIFICATION OF GLYCYLPROLYL β-NAPHTHYLAMIDASE FROM HUMAN SUBMAXILLARY GLAND

Step	*Protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification*
Homogenate*	4035	73.3	0.018	100	1
Microsomes	605	36.7	0.060	50	3
Extract	178.5	25.2	0.141	34	8
$(NH_4)_2SO_4$ (50–80%)	16.9	19.7	1.17	27	65
Sephadex G-200	5.0	18.7	3.74	26	208

* Human submaxillary gland, 50 g.

Properties of purified glycylprolyl β-naphthylamidase

The purity of the purified glycylprolyl β-naphthylamidase was examined by polyacrylamide disc electrophoresis. As shown in Fig. 2, a major protein band which represents 95% of the total protein was observed, and the enzyme activity coincided with this protein band.

The approximate molecular weight was estimated by gel filtration on Sephadex G-200 in 20 mM Tris–HCl buffer, pH 7.5. As shown in Fig. 3, plots according to the

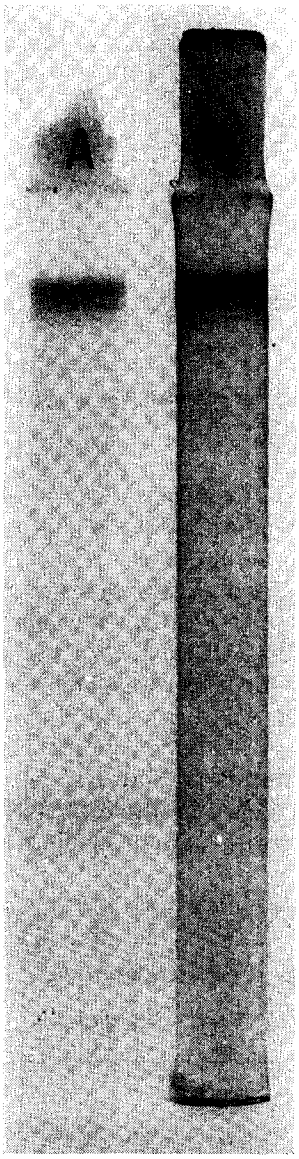


Fig. 2. Disc electrophoresis of purified glycylprolyl β-naphthylamidase preparation from human submaxillary gland. A, protein was located by staining with naphthalene black; B, enzyme activity was located by incubating the gel in a reaction mixture containing glycylprolyl β-naphthylamide and by visualizing β-naphthylamine released with Fast Garnet GBC.

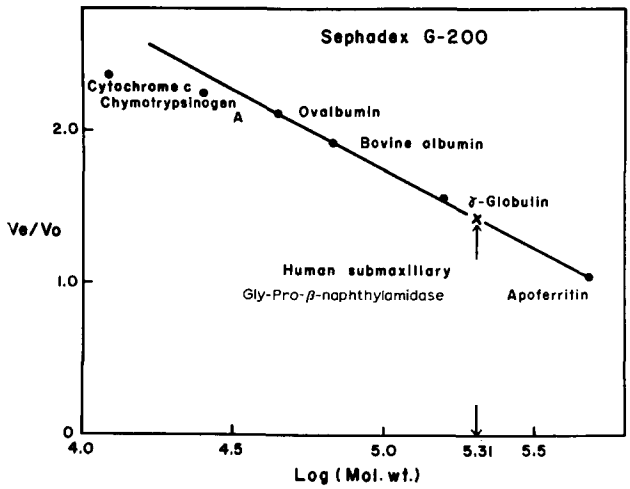


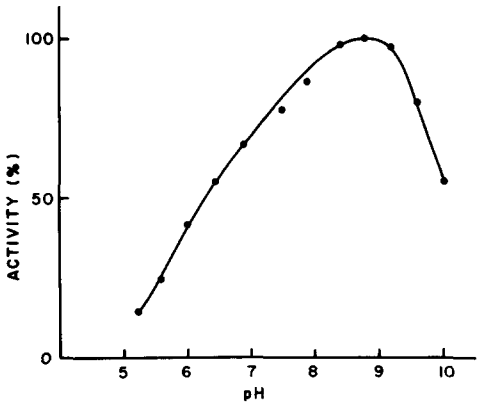
Fig. 3. Estimation of molecular weight of purified glycylprolyl β-naphthylamidase from human submaxillary gland by Sephadex G-200 gel filtration.

method of WHITAKER[10] indicated an approximate molecular weight of 225 000.

The pH–activity relationship was examined in 0.1 M Tris–maleate buffer. The ionic strength was not constant. An optimum pH was observed at 8.5–9.0 (Fig. 4).

The K_m value towards glycylprolyl β-naphthylamide obtained from a Lineweaver–Burk plot was $3 \cdot 10^{-4}$ M (Fig. 5). The incubation mixture was the same as described in MATERIALS AND METHODS except for variation in the concentration of substrate. The activity was measured fluorimetrically.

The effects of metals on the purified enzyme were examined. Ca^{2+}, Co^{2+}, Cu^{2+},



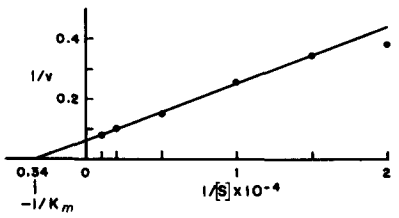


Fig. 4. pH–activity curve of purified glycylprolyl β-naphthylamidase from human submaxillary gland.

Fig. 5. Lineweaver–Burk plot of glycylprolyl β-naphthylamide concentration against the activity of purified glycylprolyl β-naphthylamidase from human submaxillary gland.

Mn^{2+}, Mg^{2+}, and Fe^{2+} had almost no effect at 1 mM. Zn^{2+} and Hg^{2+} were inhibitory at 1 mM.

Various compounds which might affect the purified enzyme were examined. EDTA, KCN and hydroxylamine had no effect. *p*-Chloromercuribenzoate was slightly inhibitory at 1 mM, but another SH-blocking agent, *N*-ethylmaleimide had no effect. Various SH compounds such as glutathione and cysteine had no effect. β-Aminopropionitrile, which inhibits the cross-linking formation of collagen, did not affect the activity.

Substrate specificity of purified glycylprolyl β-naphthylamidase

The products obtained after incubating glycylprolyl β-naphthylamide with purified glycylprolyl β-naphthylamidase were examined by paper chromatography. Glycylproline was identified, but neither glycine nor proline was detected on the chromatogram. When glycylprolylalanine or glycylprolylleucine was incubated with purified enzyme, glycylproline and alanine or leucine were identified, but neither glycine nor proline was detected.

These results from paper chromatography indicated that purified glycylprolyl β-naphthylamidase hydrolyzes N-terminal glycylproline from glycylprolyl β-naphthylamide, glycylprolylalanine or glycylprolylleucine. The results from paper chromatography were confirmed quantitatively using an amino acid analyzer. Equivalent amounts of alanine and glycylproline after incubating glycylprolylalanine with the enzyme, and equivalent amounts of leucine and glycylproline after incubating glycylprolylleucine with the enzyme, were identified quantitatively. The purified enzyme hydrolyzed both peptide substrates at rates of 69.5% and 65.0% of the rate towards glycylprolyl β-naphthylamide, respectively. The hydrolysis of various amino acid β-naphthylamides was examined. It was shown that the purified enzyme hydrolyzed glycylprolyl β-naphthylamide almost specifically. Glycylphenylalanyl β-naphthylamide was not hydrolyzed at all. No simple amino acid β-naphtyhlamide including β-naphthylamides of alanine, methionine, leucine, glycine, proline and hydroxyproline had more than 10% activity.

Glycylprolyl β-naphthylamidase activity in human submaxillary and sublingual saliva

The activities of glycylprolyl β-naphthylamidase and of other amino acid β-naphthylamidases in human saliva collected from submaxillary and sublingual glands were measured. It was found that glycylprolyl β-naphthylamidase activity was distinctly higher than the other amino acid β-naphthylamidase activities.

DISCUSSION

We have found that glycylprolyl β-naphthylamidase activity in human submaxillary gland is very high and that the specific activity of the enzyme in microsomes of human submaxillary gland is about 150 times higher than that of rat liver microsomes as reported by HOPSU-HAVU AND SARIMO[2]. The enzyme can be solubilized from microsomes of human submaxillary gland and purified. The purified enzyme was nearly homogeneous as judged from polyacrylamide gel electrophoresis. The enzyme did not show any requirement of divalent metals, and metal-chelating agents did not inhibit the enzyme. It may not have essential SH group(s) since SH reagents

had no significant effect on the activity. These properties are similar to those of the rat liver enzyme[2]. From the substrate specificity studies, the enzyme appears to hydrolyze N-terminal glycylproline specifically, and may be a specific dipeptidyl aminopeptidase.

The physiological significance of the presence of this enzyme in salivary gland and saliva remains unknown. Since the amino acid sequence glycylproline is predominant in collagen, the enzyme may act on collagen metabolism. Collagen is a major protein in oral tissue including dentine, and the physiological and pathological role of the enzyme in salivary gland and saliva remains to be elucidated. The enzyme appears to be secreted from the salivary gland. Human duct saliva from submaxillary and sublingual glands was found to contain significant activity of the enzyme. We have found that glycylprolyl β-naphthylamidase activity in human mixed saliva is also the highest among amino acid β-naphthylamidase activities (H. ŌYA, I. NAGATSU AND T. NAGATSU, unpublished results).

The enzyme activity was also present in the soluble fraction of human submaxillary gland. Since the microsomal enzyme appears to be tightly bound to the particles, the soluble enzyme and the particulate enzyme may exist in the cells *in vivo*. Since the activity of the soluble enzyme was low, it has not been purified yet. However, the comparison of the properties of soluble and particulate enzymes as well as the properties of the enzyme in saliva may be important, especially for the elucidation of the mode of secretion of the enzyme into saliva.

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