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RAPID CHROMATOGRAPHIC PURIFICATION OF DIPEPTIDYL PEPTIDASE IV IN HUMAN SUBMAXILLARY GLAND

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SUMMARY

Pure dipeptidyl peptidase IV (X-prolyl dipeptidyl aminopeptidase), which did not contain aminopeptidase activity at all, was rapidly prepared from the human submaxillary gland by chromatography with concanavalin A–Sepharose and Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose. The entire purification took only 3 days. Aminopeptidase, which was very difficult to separate from dipeptidyl peptidase IV by various chromatographic procedures, could be completely removed by chromatography with Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose. On SDS gel electrophoresis the purified enzyme gave a single band with a molecular weight of 116,000. The apparent molecular weight of the enzyme was estimated to be 225,000 by gel filtration. Therefore, the enzyme consists of two identical subunits. It did not hydrolyze Ala *p*-nitroanilide at all, but the hydrolysis of the *p*-nitroanilides of Gly-Pro, Lys-Pro and Arg-Pro at pH 8.0 was nearly specific.

INTRODUCTION

In 1966, Hopsu-Havu and Glenner[1] discovered a new arylamidase, using a newly synthesized chromogenic substrate, Gly-Pro β-naphthylamide. They designated the enzyme glycylproline β-naphthylamidase. The enzyme was purified from porcine kidney[2–5], lamb kidney[6], porcine small intestine[7] and human submaxillary gland[8], and was shown to hydrolyze N-terminal glycylproline from a peptide such as Gly-Pro-Leu or Gly-Pro-Ala. We synthesized various dipeptide *p*-nitroanilides having a sequence of X-proline *p*-nitroanilide (X = Gly, Ala, Lys, Arg, Glu and Asp) as new chromogenic substrates, and showed that the human submaxillary enzyme hydrolyzes various X-Pro *p*-nitroanilides to produce X-Pro and *p*-nitroaniline. Gly-Pro *p*-nitroanilide had the highest activity among the substrates at an optimum pH of 8.7, followed by *p*-nitroanilides of Ala, Lys, Arg, Glu and Asp in a decreasing order of activity; at pH 7.0 the enzyme had similar high activities towards *p*-nitroanilides of Lys-Pro, Arg-Pro and Gly-Pro[9]. We designated the enzyme X-prolyl dipeptidyl aminopeptidase[9], whereas Yoshimoto and Walter[6] referred to the enzyme as post-proline dipeptidyl aminopeptidase. McDonald *et al.*[10] referred to the enzyme as

dipeptidyl peptidase IV (E.C. 3.4.14.–), which is now recommended by the Enzyme Commission. The enzyme is highly specific for the second amino acid proline, but alanine[3,4,11,12] and hydroxyproline[11] can also be the second amino acid. Preferential hydrolysis of a N-terminal glycylproline sequence by the enzyme suggests some involvement of this enzyme in the degradation of peptides derived from collagen. However, since a lysylproline or arginylproline sequence can be found in various biologically active peptides, the enzyme could have some physiological roles in the degradation of bioactive peptides. In fact, Kato *et al.*[13] showed that the human submaxillary enzyme cleaved N-terminal dipeptide Arg^1-Pro^2 and the subsequent dipeptide Lys^3-Pro^4 from substance P.

The enzyme hydrolyzes the peptide bond between N-terminal glycylproline and adjacent amino acids or peptides (except proline or hydroxyproline[14], β-naphthylamine[1], *p*-nitroaniline[9] or 7-amino-4-methylcoumarin[15,16].

Although the enzyme purified from human submaxillary gland had been reported to be 95% pure based on disc gel electrophoresis[8], the presence of *ca.* 5% activity towards Ala β-naphthylamide as compared with the activity towards Gly-Pro β-naphthylamide suggests that there is still a contamination of aminopeptidase in the apparently single enzyme band on the disc gel. We have therefore further purified the enzyme from the human submaxillary gland by chromatographic procedures including chromatography with concanavalin A–Sepharose and with Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B. The use of concanavalin A–Sepharose was adopted because the enzyme from pig kidney was reported to contain *ca.* 18% of carbohydrate[5]. Separation of the contaminating aminopeptidase from dipeptidyl peptidase IV was very difficult by various purification procedures, but the activity towards Ala *p*-nitroanilide was completely removed from the activity towards Gly-Pro *p*-nitroanilide by chromatography with Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B. The purified enzyme revealed a single band upon sodium dodecyl sulphate polyacrylamide-gel electrophoresis. The homogeneous enzyme purified by the chromatographic procedures is useful for polypeptide sequencing.

MATERIALS AND METHODS

Materials

p-Nitroanilides of Gly-Pro, Gly-Hyp, Gly-Ala, Gly-sarcosine, Arg-Pro, Lys-Pro, Ala-Ala and Ala were synthesized at Protein Research Foundation (Minoh, Osaka, Japan), as reported previously[9,11]. Human submaxillary gland was obtained at autopsy. Concanavalin A–Sepharose was from Pharmacia (Uppsala, Sweden). Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B was prepared as described by Fukasawa *et al.*[5] based on the method of Cuatrecasas[17].

General methods

The activity of dipeptidyl peptidase IV or aminopeptidase was assayed with Gly-Pro *p*-nitroanilide or Ala *p*-nitroanilide as substrate, as reported previously[9]. The incubation mixture (total volume 0.55 ml) contained 114 m*M* Tris–maleate buffer, pH 8.0, 1.4 m*M* Gly-Pro *p*-nitroanilide or Ala *p*-nitroanilide and enzyme. Incubation was carried out at 37° for 30 min and the reaction was stopped by adding 1.5 ml of 1 *M* acetate buffer (pH 4.2). Determination of *p*-nitroaniline, liberated by

the enzyme reaction, was based on the absorbance at 380 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of *p*-nitroaniline per min at 37°. Polyacrylamide-gel electrophoresis was performed by the method of Davis[18]; 7% separation gel in 0.37 *M* Tris–HCl buffer (pH 8.9) was prepared using ammonium persulphate as the catalyst. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis was performed based on the method of Weber and Osborn[19].

Purification of dipeptidyl peptidase IV by chromatography with concanavalin A–Sepharose and with Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B

The enzyme was partially purified from the microsomal fraction of human submaxillary gland according to Oya *et al.*[8] with some modifications, and complete purification was further achieved by two successive chromatographic runs with concanavalin A–Sepharose and Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B. All procedures were carried out at 0–4°. A 59-g amount of human submaxillary gland was homogenized in nine volumes of water using a Polytron homogenizer. Microsomes were isolated by centrifuging the homogenate at 7500 *g* for 15 min, then at 100,000 *g* for 60 min. The microsomes were resuspended in 150 ml of water, the suspension was adjusted to pH 3.8 with 3 *M* H_2SO_4, incubated at 37° for 24 h to solubilize the enzyme by autolysis and then centrifuged at 100,000 *g* for 60 min. To the supernatant which contained the solubilized enzyme, solid $(NH_4)_2SO_4$ was added to 50% saturation. After mixing for 30 min, the solution was centrifuged at 20,000 *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 30 min, the solution was centrifuged at 20,000 *g* for 20 min. The supernatant was discarded, and the precipitate dissolved in 4 ml of 5 m*M* Tris–HCl buffer, pH 7.4. The solution was dialyzed against a large volume of the same buffer, and then concentrated to 5 ml by immersing the dialysis tubing in sucrose crystals. The enzyme solution was passed through a column of Sephadex G-200 equilibrated previously with 5 m*M* Tris–HCl buffer (pH 7.4). For 30.5 mg of protein in 5 ml, a 96 × 2.6 cm column was used. Elution was carried out using the same buffer at a flow-rate of 5–6 ml/h, and 3-ml fractions were collected. The active fractions were combined.

For further chromatographic purification, the enzyme solution was first dialyzed against a large volume of 15 m*M* potassium phosphate buffer, pH 7.4, containing 0.15 *M* NaCl. This dialyzed enzyme solution was then applied on a column of concanavalin A–Sepharose (4 × 2.6 cm), previously equilibrated with 15 m*M* phosphate buffer, pH 7.4, containing 0.15 *M* NaCl (Fig. 1). After the enzyme had been adsorbed to the column, it was washed extensively with the same buffer, and the enzyme eluted with the same buffer containing 5% α-methyl-D-mannoside. The pooled eluate containing the enzyme was dialyzed against 20 m*M* sodium acetate buffer, pH 6.0, and concentrated by ultrafiltration. The enzyme solution was further purified by chromatography on Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B (3 × 1.9 cm), equilibrated with 20 m*M* acetate buffer (pH 6.0) (Fig. 2). The column was washed with the same buffer and the enzyme was eluted with a linear gradient of NaCl. The purified enzyme was stable at least for 6 months at −80°.

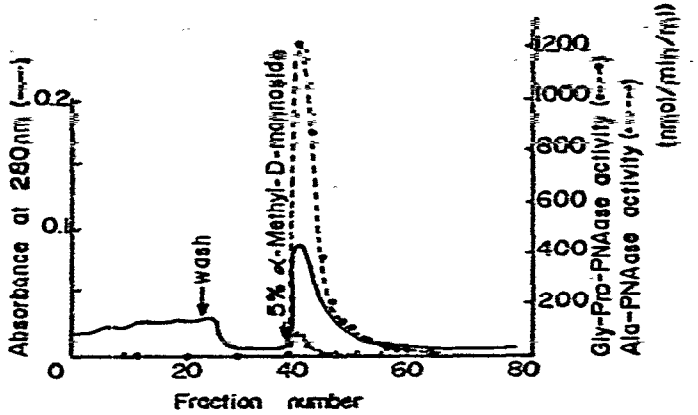

Fig. 1. Chromatography of dipeptidyl peptidase IV on a column of concanavalin A–Sepharose. The column (4 × 2.6 cm) was equilibrated with 15 m*M* potassium phosphate buffer, pH 7.4, containing 0.15 *M* NaCl. After washing until the absorbance of the effluent at 280 nm was less than 0.005, the enzyme was eluted with the same buffer containing 5% α-methyl-D-mannoside. Flow-rate was 0.3 ml/min. Absorbance was measured at 280 nm (———). Enzyme activities were determined as described in the text using Gly-Pro *p*-nitroanilide (⊙—⊙) and Ala *p*-nitroanilide (△—△) as substrates, respectively.

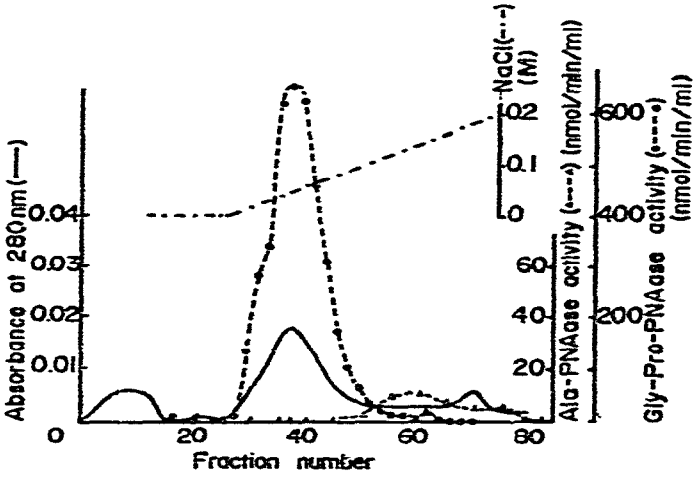

Fig. 2. Chromatography of dipeptidyl peptidase IV on a column of Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B. The column (3 × 1.9 cm) was equilibrated with 0.02 *M* acetate buffer (pH 6.0). The column was washed with 50 ml of the same buffer and the enzyme was eluted with a linear gradient of NaCl (0–0.2 *M*) in the same buffer. Other details as in Fig. 1.

RESULTS

Purification of dipeptidyl peptidase IV by chromatography with concanavalin A–Sepharose and with Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B

Although at the Sephadex G-200 stage of the purification procedure a single symmetrical protein peak which coincided with the activity was observed as reported previously[8], contaminated proteins could be removed by further chromatography on

concanavalin A–Sepharose (Fig. 1). Most of the containing protein remained bound to the column even in the presence of 5% α-methyl-D-mannoside and was eluted with 1 *M* NaCl. However, as shown in Fig. 1, the active fraction in the eluate from concanavalin A–Sepharose still contained a very low activity of aminopeptidase with Ala *p*-nitroanilide as substrate. This contaminating aminopeptidase could not be separated from dipeptidyl peptidase IV by various column chromatographic methods but was found to be completely removed by chromatography with Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B (Fig. 2). A small amount of other contaminating proteins was also removed by this column procedure. By the two column procedures, the activity of dipeptidyl peptidase IV was increased about 1300-fold with a yield of 10% starting from the homogenate (Table I). The specific activity of purified enzyme was 57.4 units/mg protein (37°) using Gly-Pro *p*-nitroanilide at pH 8.0.

TABLE I

PURIFICATION OF DIPEPTIDYL PEPTIDASE IV FROM HUMAN SUBMAXILLARY GLAND

Step	*Protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification*
Homogenate*	3280	147	0.045	100	1
Microsomes	1589	143	0.090	97	2
Extract	384	55.0	0.143	37	3
$(NH_4)_2SO_4$ (50–80%)	30.5	34.0	1.11	23	25
Sephadex G-200	6.63	41.7	5.29	28	140
Concanavalin A–Sepharose	1.00	37.4	37.3	25	829
Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B	0.283	16.2	57.4	11	1280

* Human submaxillary gland, 59 g.

Criteria for the purity of dipeptidyl peptidase IV

Upon polyacrylamide-gel and SDS–polyacrylamide gel electrophoreses the enzyme revealed only a single, sharp band. The approximate molecular weight was estimated to be 116,000 based on SDS–polyacrylamide gel electrophoresis (Fig. 3). Since the approximate molecular weight of the enzyme was estimated to be 225,000 by Sephadex G-200 gel filtration, the enzyme may be a dimer composed of identical subunits.

Properties of homogeneous dipeptidyl peptidase IV

The optimum pH of enzyme activity towards Gly-Pro *p*-nitroanilide as substrate was 8.7. The enzyme activity was higher in 0.1 *M* Gly–NaOH buffer than in 0.1 *M* Tris–maleate buffer. Glycine activated the enzyme at an optimum concentration of 100 m*M*, but the activation was more significant at pH 8.7 than at pH 8.0 in 0.1 *M* Tris–maleate buffer. The enzyme was strongly inhibited by diisopropyl fluorophosphate, indicating that it is a serine enzyme.

Fig. 3. Sodium dodecyl sulphate–polyacrylamide disc gel electrophoresis of dipeptidyl peptidase IV.

Catalysis by the enzyme of the hydrolysis of the peptide bond at the carboxyl site of a Pro residue in the penultimate position was nearly specific (Table II). As we had previously reported with the partially purified enzyme[9], *p*-nitroanilides of Lys-Pro, Arg-Pro and Gly-Pro gave similar activities at pH 8.0. Far lesser but still significant activities were found with substrates containing alanine, sarcosine and hydroxyproline in the penultimate position. However, Ala *p*-nitroanilide was not hydrolyzed at all. The K_m value to give half the maximum velocity for Gly-Pro *p*-nitroanilide was determined as $3.6 \cdot 10^{-4}$ *M* from the Lineweaver–Burk plots.

TABLE II

SUBSTRATE SPECIFICITY OF DIPEPTIDYL PEPTIDASE IV

Activities were measured at pH 8.0 in 114 m*M* Tris–maleate buffer and at a substrate concentration of 1.4 m*M*. The values are the mean of duplicate experiments. pNA = *p*-nitroanilide.

Substrate	*Activity (μmol/min·mg enzyme)*	*Relative activity (%)*
Gly-Pro pNA	57.4	100
Lys-Pro pNA	60.8	106
Arg-Pro pNA	60.0	101
Ala-Ala pNA	3.20	5.57
Gly-sarcosine pNA	1.85	3.22
Gly-Ala pNA	1.32	2.30
Gly-Hyp pNA	0.28	0.48
Ala pNA	0.00	0.00

DISCUSSION

Chromatography with concanavalin A–Sepharose and Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B are highly effective for the purification of dipeptidyl peptidase IV.

The presence of 18% of carbohydrate in the enzyme from pig kidney reported by Fukasawa *et al.*[5] suggested the possible use of affinity chromatography using concanavalin A, since this plant lectin was shown to specifically bind α-D-mannosyl, α-D-glucosyl and sterically similar residues that are located on the free terminal of a carbohydrate chain[20].

Chromatography of Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B[5] was highly effective for the purification of dipeptidyl peptidase IV, especially for separating contaminating aminopeptidase activity which could not be removed by other chromatographic procedures. Although the Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B column was considered to be an affinity column[5], this may not be the case since the enzyme can be eluted with a low concentration of NaCl (0.05 *M*) and actually elutes before aminopeptidase activity.

Although we had previously reported that the dipeptidyl peptidase IV preparation from human submaxillary gland at the Sephadex G-200 stage showed a major protein band, which represented 95% of the total protein by ordinary polyacrylamide disc gel electrophoresis, the present results clearly showed that this apparently single protein band still contained several contaminating proteins besides the enzyme. In fact, SDS disc gel electrophoresis of this previously reported preparation showed contaminating protein bands besides the main band of the enzyme. However, the final preparation in this study showed a single band in ordinary and SDS–polyacrylamide gel electrophoreses.

Dipeptidyl peptidase IV purified from the human submaxillary gland rapidly hydrolyzed *p*-nitroanilides of Gly-Pro, Arg-Pro and Lys-Pro, but also very slowly hydrolyzed *p*-nitroanilides of Gly-Ala, Gly-sarcosine and Gly-Hyp. These results confirmed our previous, preliminary findings on the substrate specificity of dipeptidyl peptidase IV, namely that the enzyme catalyzes the hydrolysis of amide bonds of peptides with a proline, alanine, sarcosine and hydroxyproline residues in the penultimate position[11]. On the other hand, the separation of the activity for Ala *p*-nitroanilide from dipeptidyl peptidase IV by chromatography of Gly-Pro-NH-$(CH_2)_6$-NH–Sepharose 4B (Fig. 2) and the complete absence of the activity for Ala *p*-nitroanilide in the purified enzyme indicate that dipeptidyl peptidase IV does not cleave a single N-terminal amino acid.

The present purification procedure of the enzyme from human submaxillary gland using the two chromatographic procedures is simple and reproducible, and the specific activity of the resulting enzyme is high. Therefore, the purified enzyme free from aminopeptidase may be useful for structural studies of peptides[21], as has already been shown on the degradation of substance P by the enzyme[13].

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