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Isolation and Characterization of Dipeptidyl Peptidase IV from Human Placenta

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Human placenta is surprisingly rich in post-proline dipeptidyl peptidase activity. Among various cell fractions, microsomes have the highest specific activity. A homogeneous enzyme preparation is obtained in a six-step purification procedure. The final preparation appears homogeneous upon dodecyl sulfate electrophoresis, but analytical isoelectric focussing reveals various active bands with isoelectric points in the range of pH 3–4. The enzyme is a glycoprotein containing about 30% carbohydrate. Treatment with neuraminidase lowers the isoelectric points but does not reduce the heterogeneity of the band pattern. The subunit molecular weight is 120000 as estimated by dodecyl sulfate electrophoresis, whereas M_r of the native enzyme is >200000, as can be concluded from gel filtration experiments.

The purified dipeptidyl peptidase cleaves various synthetic and natural peptides, including substance P, kentsin, casomorphin and a synthetic renin inhibitor. In general, the specificity of the placenta peptidase is similar to that of post-proline dipeptidyl peptidase from other sources. Phenylalanylprolyl-β-naphthylamide (K_m = 0.02 mM, V = 92 U/mg) is the best substrate among various synthetic peptide derivatives. Only peptides with a free N-terminal amino group and proline, hydroxyproline, or alanine in position 2 of the N-terminal sequence are cleaved. However, X-Pro-Pro-... structures, e. g. as in bradykinin, are not attacked.

1 mM bis-(4-nitrophenyl)phosphate or 1 mM diisopropylfluorophosphate completely inactivate the peptidase within 30 min at 30 °C (pH 8). The peptidase is also completely inhibited by 1 mM Zn^{2+} and by other heavy metals.

An enzyme that rather specifically cleaved naphthylamine from glycylprolyl-2-naphthylamide was first described by Hopsu-Havu and Sarimo [1]. McDonald et al. [2, 3] designated this enzyme dipeptidyl (amino)peptidase IV as it preferably removed dipeptides with a penultimate prolyl residue from the N terminus of peptide chains.

Dipeptidyl peptidase IV has been isolated from many animal tissues, especially from kidney [4–10]. In most cases synthetic substrates, such as synthetic peptides or peptide derivatives, were used to characterize the enzyme. Only recently some natural substrates have been found: the hormone substance P [11, 12], promelittin [13], and casomorphin [14], a peptide surviving the degradation of β-casein. The only human dipeptitidyl peptidases IV that have been purified so far are from submaxillary gland [9] and from kidney [4]. Dipeptidyl peptidase IV has not yet been demonstrated in the placenta. On the other hand, it has recently been reported that the placenta had a high capacity for the degradation of substance P [15]. We assumed that a dipeptidyl peptidase IV might be involved in this process and, therefore, might have an important regulatory function in the placenta. Furthermore, we could show that dipeptidyl peptidase IV was involved in the regulation of blood clotting [16]. To study these regulatory processes it was necessary to have an easily accessible source of human dipeptidyl peptidase IV. The placenta enzyme seemed to be suitable for this purpose.

Abbreviations. Dansyl, 5-dimethylaminonaphthalene-1-sulfonyl-; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AH-Sepharose, aminohexyl-Sepharose; CL-Sepharose, Sepharose cross-linked with 2,3-dibromopropanol; HPTLC, high-performance thin layer chromatography.

Enzymes. Dipeptidyl peptidase IV, postproline dipeptidyl peptidase (EC 3.4.14.5); neuraminidase (EC 3.2.1.18).

MATERIALS AND METHODS

The peptides substance P, kentsin, tuftsin, bradykinin, most of the synthetic peptides, and the peptidylnaphthylamides were purchased from Bachem (Bubendorf, Switzerland). Casomorphin-5 was a gift from A. Barth (Halle). [^{3}H]Diisopropylfluorophosphate was from Amersham/Buchler (Braunschweig, FRG) and bis-(4-nitro[^{14}C]phenyl)phosphate from Hoechst AG (Frankfurt, FRG). The following separation materials and detergents were purchased from the firms listed in parentheses: DEAE-Sephacel, AH-Sepharose, CL-Sepharose, CM-Sephadex (Pharmacia, Freiburg, FRG), Ampholine (LKB, Gräfeling, FRG), high-performance thin-layer plates, kieselgel, saponin (Merck, Darmstadt, FRG), polyamide thin-layer plates (Cheng Chin, Taipeh, Taiwan), Nonidet P40 (Shell), Triton X-100, dodecyl sulfate, dansyl chloride, dansyl-amino acids (Serva, Heidelberg, FRG).

Glycylprolylaminohexyl-Sepharose was synthesized from glycylproline and AH-Sepharose [7]. Rat liver carboxylesterase pI 6.0 was isolated as described [17]. *Vibrio cholerae* neuroaminidase was from Behringwerke (Frankfurt, FRG), ferritin, catalase, bovine serum albumin, and *Escherichia coli* RNA polymerase from Boehringer (Mannheim, FRG).

Enzyme Assays

The release of 2-naphthylamine from peptidylnaphthylamides was recorded with an Aminco fluorimeter equipped with a digital voltmeter (Micrologic 415) and a calculator (CBM 3032). The excitation wavelength was 335 nm and the emission was observed at 410 nm. With 4-methoxynaphthyl-2-amides the corresponding optimal values were 315 nm and 435 nm. All fluorimetric assays were performed in 50 mM Tris/HCl buffer at pH 8.0 and 37 °C, if not otherwise stated.

Carboxylesterase was assayed with methyl butyrate [17]. Glycylprolyl-β-naphthylamide was used as standard substrate for the purification procedure.

Enzymatic Cleavage of Peptides

2 mM peptide solutions in 8 mM Tris/HCl pH 8.0 were incubated with 0.6 µg highly purified dipeptidyl peptidase IV at 37 °C. The peptide fragments were identified by co-chromatography with the expected dipeptides or amino acids on thin-layer plates with kieselgel G or on HPTLC plates.

In addition, the sites of cleavage were identified by dansylation before and after the incubation with the peptidase. The mixtures were dansylated according to Gray [18] and subsequently hydrolyzed in 6 M deaerated HCl (18 h at 105 °C). After evaporation of the HCl the dansyl-amino acids were dissolved in 50% pyridine/water and applied to polyamide thin-layer sheets. The identity of the spots after one or two-dimensional chromatography [19] was confirmed by cochromatography with reference dansyl-amino acids.

Estimation of Protein and Total Hexose

The protein concentration of particulate fractions and crude enzyme solutions was determined with a modified biuret procedure [20], that of purified enzyme fractions with a sensitive procedure according to Folin [21]. Both procedures have been standardized with bovine serum albumin. The hexose content of the highly purified peptidase was estimated with phenol/sulfuric acid [22], this method was standardized with glucose.

Analytical Isoelectric Focussing and Neuraminidase Treatment

Isoelectric focussing was performed in 4.5% polyacrylamide slab gels containing 2% Ampholine pH 3 – 5 before and after the treatment with neuraminidase. Before application to the gels the enzyme samples were desalted on a small column with Sephadex G-50 superfine. The gels were stained with glycylprolyl-4-methoxynaphthylamide [23]. In order to remove sialic acid from the enzyme surface 5 µg highly purified dipeptidyl peptidase IV were incubated (37 °C, 1 – 18 h) with 5 U neuraminidase in 0.1 M sodium acetate, pH 5.5, containing 1 mM Ca^{2+}.

Dodecyl Sulfate Electrophoresis

Polyacrylamide slab gels (10%) containing 0.1% dodecyl sulfate were used for the separation of denatured proteins. Samples of 5 – 100 µg protein were incubated for 2 min at 95 °C in 200 µl 60 mM Tris/HCl buffer, pH 6.8, containing 2.5% sodium dodecyl sulfate and 5% mercaptoethanol. Gel and electrode buffers were prepared according to Laemmli [24]. The gels were stained with 0.1% Coomassie brilliant blue R250 in 50% (w/v) trichloroacetic acid/water and destained with 7.5% (v/v) acetic acid.

The protein samples from varying steps of the purification procedure were labeled with [^{3}H]diisopropylfluorophosphate prior to the denaturation with dodecyl sulfate. All samples were incubated with 0.1 mM organophosphate (6.5 Ci/mmol) for 1 h at pH 8.0 (25 °C). The gels for autoradiography were dried on filter paper *in vacuo* and exposed to X-ray films (Agfa-Gevaert Osray T4) for 4 weeks.

Subcellular Fractionation

Fresh human placenta was minced, diluted with a twofold volume of 0.25 M sucrose/20 mM Tris, pH 7.4 and homogenized in an ice bath with a blender (Ultra-Turrax T 45/6, Janke und Kunkel, Staufen, FRG). Fibers of connective tissue were removed by filtration through a double layer of gauze. Samples for the study of the subcellular enzyme distribution were passed through a nylon screen (80 µm). The homogenate was further diluted with a twofold volume of 0.25 M sucrose and fractionated by differential centrifugation. We separated four fractions, namely nuclei ($500 \times g$), mitochondria ($10000 \times g$), microsomes ($105000 \times g$), and cytoplasm (supernatant liquid). The particulate sediments were resuspended in small volumes of 100 mM Tris/HCl, pH 7.4.

Enzyme Purification

Placenta microsomes were prepared by ultracentrifugation starting with 1400 g fresh human placentas. The $105000 \times g$ sediment was resuspended with 700 ml 0.1 M Tris/HCl buffer, pH 8.5, and kept at – 15 °C. After thawing and rehomogenizing 700 ml 0.1 M Tris/HCl, pH 8.5, saturated at 0 °C with butanol was added. The mixture was stirred for 1 h at 0 °C, then centrifuged for 2 h at $105000 \times g$. The sediment was resuspended in 10 mM Tris/HCl, pH 7.4, containing 1% Nonidet P40, and stirred again for 1 h at 0 °C. All the following procedures were performed at temperatures between 0 and 10 °C.

After centrifuging (2 h, $105000 \times g$), the supernatant fluid containing the bulk of dipeptidyl peptidase IV activity was applied to an anion-exchange column (DEAE-Sephacel, 2.6 × 30 cm) equilibrated with 10 mM Tris/HCl buffer, pH 7.4. The DEAE-Sephacel column was eluted with 1.5 l 10 mM Tris buffer, pH 7.4, containing NaCl in a concentration that gradually increased from 0 to 1 M. Solid ammonium sulfate was dissolved in the pooled fractions containing dipeptidyl peptidase IV from this column until 80% saturation was achieved. The floating precipitate was collected and redissolved in 25 ml 20 mM sodium acetate buffer, pH 6.0. After dialysis with 5 l of the same buffer (2 × 12 h) the enzyme solution was applied to an affinity column containing 20 ml glycylprolyl-AH-Sepharose equilibrated with the acetate buffer. The affinity column was washed with 120 ml buffer and then eluted with a linear gradient (150 ml) of 0 – 0.5 M NaCl in 20 mM acetate, pH 6.0. The enzyme-containing fractions of the eluate were pooled and directly chromatographed on a column with CL-Sepharose 4B (2.6 × 90 cm) pre-equilibrated and eluted with 10 mM Tris buffer, pH 8.0.

The next purification step was cation-exchange chromatography with CM-Sephadex (column 2.6 × 30 cm, equilibrated with 10 mM sodium acetate buffer, pH 4.2). The pooled enzyme fractions of the gel chromatography were dialyzed with 5 l of this buffer and applied to the CM-Sephadex column, which was then washed with 300 ml acetate, pH 4.2, and eluted with a linear buffer gradient made of 750 ml 10 mM acetate, pH 4.2, and 750 ml 200 mM acetate, pH 5.8. The enzyme-containing fractions were pooled, adjusted to pH 7 with a small volume of 1 M Tris and dialyzed overnight with 10 mM Tris/HCl buffer, pH 7.4.

The last purification step was a rechromatography on a small column (1 × 7 cm) with DEAE-Sephacel, equilibrated with 10 mM Tris, pH 7.4. This column was eluted with 100 ml 0.5 M NaCl in 10 mM Tris buffer. The purified enzyme was stored in this buffer at – 18 °C.

RESULTS

SUBCELLULAR DISTRIBUTION

A subcellular fractionation study demonstrated that dipeptidyl peptidase IV was membrane-bound in human placenta. The soluble fraction only contained 4% of the total enzyme activity, probably because of some contamination with microsomes. The microsomal fraction had the highest specific activity (44.5 mU/mg) and contained 48% of the total activity. The specific activities (percentage of total activities) of the other fractions were: nuclei 6.0 mU/mg (5%), mitochondria 25.9 mU/mg (42%), cytoplasm 0.8 mU/mg (4%). These values correspond to a single representative experiment.

SOLUBILIZATION OF THE ENZYME

We used the microsomal cell fraction for the enzyme purification experiments. The solubilization experiments are summarized in Table 1. Most authors who reported a purification of dipeptidyl peptidase IV used autolysis conditions [4–7, 9] to solubilize the membrane-bound enzyme. It is remarkable that an autolysis step was not effective in the case of placenta microsomes (Table 1). Saponin, which solubilized about 25% of the dipeptidyl peptidase IV from rat liver microsomes [11, 25], also was without effect. Recently Triton X-100 has been proposed for the solubilization of dipeptidyl peptidase IV from kidney [8] and small intestine [26]. With placenta microsomes, Nonidet P40 acted quite as well as Triton X-100. We decided to use a combined treatment with 10% butanol followed by 1% Nonidet P40 for the enzyme solubilization at 0 °C. The butanol treatment removed much protein from the microsomal membrane, while the loss of dipeptidyl peptidase IV was moderate (Tables 1, 2).

ENZYME PURIFICATION

Dipeptidyl peptidase IV was isolated in five chromatography steps from the microsomal raw extract. In contrast to Kenny's procedure for the isolation of detergent-solubilized dipeptidyl peptidase IV from kidney [8], it was not necessary to add detergent to the elution buffers in any of our chromatography steps. The first DEAE-Sephacel chromatography and an affinity chromatography with glycylprolyl-AH-Sepharose (Fig. 1) were the most effective purification steps (Table 2).

Extended washing of the loaded affinity column before starting the gradient was not possible, probably because dipeptidyl peptidase slowly removed the gel-bound glycylprolyl groups. The same reason made it impossible to reutilize the affinity gel.

Three additional steps, namely gel filtration, cation-exchange chromatography, and rechromatography on a small column with DEAE-Sephacel, were necessary to obtain an enzyme preparation that appeared homogeneous in the dodecyl sulfate electrophoresis (Fig. 2). In some preparations a minor low-molecular-weight band was present after completion of the isolation procedure.

All samples applied to the gel shown in Fig. 2 had previously been labeled with [^{3}H]diisopropylfluorophosphate (60 min, 25 °C, pH 8.0), which was irreversibly coupled to the active site of dipeptidyl peptidase IV (11). The autoradiograph of the dried gel showed the same broad enzyme band in all fractions, including the microsomes. Thus, the occurrence of this broad band was not an artefact produced by any step of the isolation procedure. The broad enzyme band could not be resolved into sharp lines on dodecyl sulfate gels with lower acrylamide concentration.

Neuraminic-acid-containing glycoproteins often occurred as such diffuse zones in the dodecyl sulfate electrophoresis [27].

Table 1. *Solubilization of dipeptidyl peptidase IV from human placenta microsomes*
Phospholipase A_2 was from *Crotalus durissus terrificus* (Boehringer, Mannheim); 100 mU/ml. The yield represents the activity in the supernatant fluid after centrifugation at 105000 × *g* for 1 h. The sum of activity found in the sediment and in the fluid = 100%

Reagent	Time	pH	Temperature	Yield
	h		°C	%
1% Deoxycholate	1	8	0	10
1% Saponin	2	6	0	2
10% Butanol	1	8	0	5
1% Triton X-100	1	8	0	70
1% Nonidet P40	1	8	0	70
Autolysis	24	7	30	3
Phospholipase	24	7	30	5

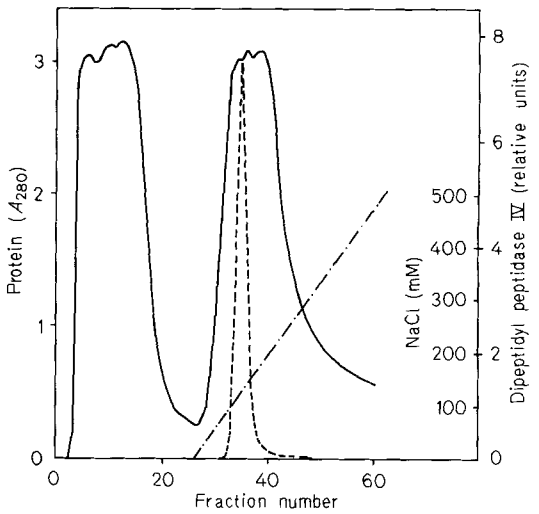

Fig. 1. *Affinity chromatography on glycylprolyl-AH-Sepharose.* (———) Absorbance at 280 nm; (– – – –) dipeptidyl peptidase IV activity; (– . – . –) NaCl concentration

Table 2. *Purification of dipeptidyl peptidase IV from human placenta*
The specific activity was determined by the standard assay with 0.2 mM Gly-Pro-β-naphthylamide at pH 8/37 °C. The assays of the ammonium sulfate fraction were performed after dialysis and the CL-Sepharose fraction was assayed after readjustment to pH 7

Fraction	Total protein	Specific activity	Purification	Yield
	mg	U/mg		%
Microsomes	8100	0.015	1	100
Butanol treatment	5690	0.023	1.5	105
Solubilizate after NP40	1260	0.124	8.2	127
DEAE-Sephacel	94.8	0.987	65.2	96
80% Ammonium sulfate	73.8	1.20	79	72
Affinity chromatography	13.3	5.63	372	61
CL-Sepharose 4B	3.15	16.4	1082	42
CM-Sephadex	0.75	38.0	2510	23
DEAE-Sephacel	0.53	46.3	3055	20

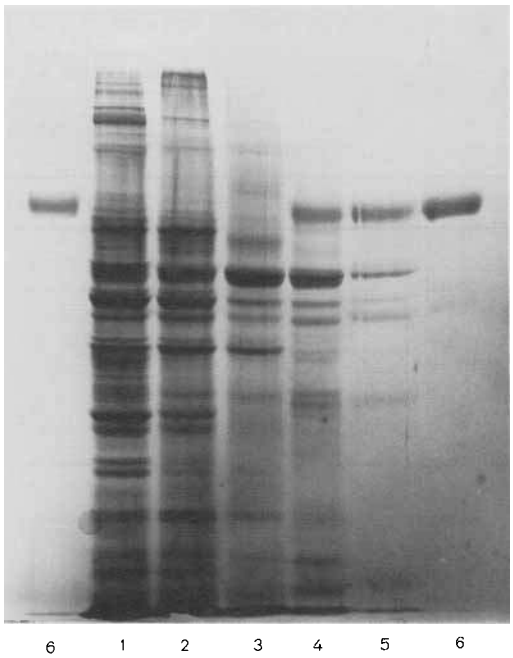

Fig. 2. *Dodecyl sulfate electrophoresis after various steps of purification.* 10% Polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate. (1) Placenta microsomes; (2) raw extract; (3–6) pooled fractions after chromatography on (3) DEAE-Sephacel, (4) glycylprolyl-AH-Sepharose, (5) CM-Sephadex, (6) rechromatography on DEAE-Sephacel. Staining with Coomassie brilliant blue

Table 2 summarizes the purification procedure (data from a single, representative experiment). Starting with microsomes from four placentas we achieved an approximately 3000-fold purification with a final yield of 0.5 mg purified enzyme.

PHYSICAL PROPERTIES

Using RNA polymerase (three chains), monomeric and dimeric bovine serum albumin as standards, we estimated a M_r of 120000 for the center of the broad peptidase band after dissociation in dodecyl sulfate and mercaptoethanol. The molecular size of the native enzyme was estimated by gel filtration on CL-Sepharose 4B with bovine serum albumin, rat liver esterase pI 6.0 [17], catalase, and ferritin as standards. The gel was equilibrated with 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. Under these conditions the elution volume of purified dipeptidyl peptidase IV (< 0.1 mg/ml) was slightly smaller than that of catalase and corresponded to a M_r of about 250000.

Analytical isoelectric focussing of placenta dipeptidyl peptidase IV revealed heterogeneity of the enzyme both in raw placenta extracts and in the highly purified state. The isoelectric points of the multiple forms were in the range of pH 3–4 (Fig. 3A). After treatment with sialidase the range of the isoelectric points shifted to pH 5–5.5, but the enzyme remained heterogenous (Fig. 3B). We concluded that the enzyme was a neuraminic-acid-containing glycoprotein. The purified enzyme had a carbohydrate content of 28.8%. This value was calculated from an estimation of total hexose with the phenol method and a Lowry protein estimation.

The purified peptidase did not lose activity if it was stored at 25 °C (1 h) in Hepes buffers, pH 6–12. Under these conditions the enzyme lost 4% activity in buffer pH 5 and 10% in buffer pH 4. In Hepes buffer, pH 3.0, the peptidase was completely inactivated in 1 h at 25 °C.

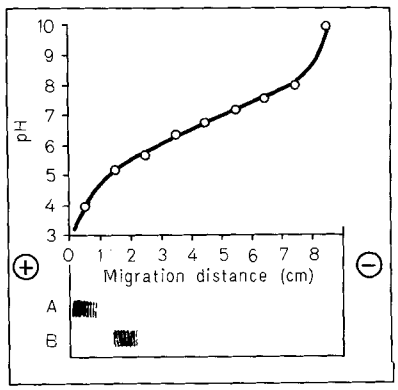

Fig. 3. *Isoelectric focussing of purified dipeptidyl peptidase IV.* Polyacrylamide flat gel containing ampholyte pH 3–10. (A) Native peptidase; (B) peptidase pretreated with neuraminidase. Enzyme stained with glycylprolyl-4-methoxynaphthylamide [23]

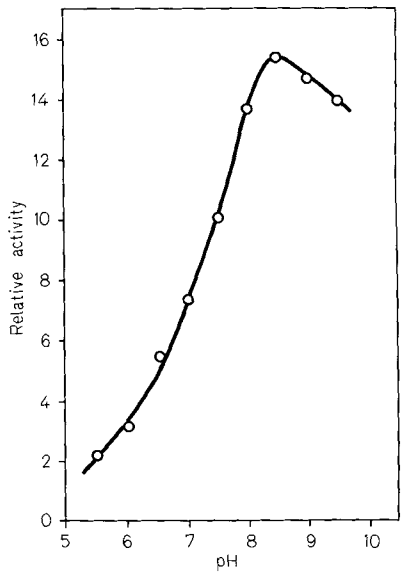

Fig. 4. *Influence of the pH on the hydrolysis of glycylprolyl-β-naphthylamide by purified dipeptidyl peptidase IV.* 0.25 M Tris/citrate buffer

HYDROLYSIS OF DIPEPTIDYLNAPHTHYLAMIDES

With glycylprolyl-β-naphthylamide the pH optimum of the purified dipeptidyl peptidase IV was at pH 8.5 (Fig. 4), which was normal for this type of enzyme. The values of Fig. 4 were corrected for the relative fluorescence of β-naphthylamine at varying pH values in Tris/citrate buffer. In spite of this result we undertook all kinetic and specificity studies in Tris/HCl buffer, pH 8.0, which seemed to be a compromise between physiological conditions and optimal pH.

We investigated the hydrolysis of a series of β-naphthylamides and 4-methoxy-β-naphthylamides by the purified placenta enzyme (Tables 3 and 4). The 4-methoxy-β-naphthylamide of glycylproline had a K_m value tenfold lower than that of the corresponding unsubstituted naphthylamide. In this context it was remarkable that an addition of ethanol reduced the K_m found with glycylprolyl-β-naphthylamide although ethanol generally inhibited the enzyme (Table 4). This might be an effect of the dielectric constant in the vicinity of the reaction site.

Table 3. *Release of naphthylamine from naphthylamides by purified dipeptidylpeptidase IV of human placenta*
Incubation in 50 mM Tris/HCl buffer, pH 8, 37 °C. Abbreviations: 2-NNap, β-naphthylamide; Cbz, carbobenzoxy; n. d., not determined. Activity with 0.2 mM Gly-Pro-2-NNap = 100

Substrate	Relative activity at a substrate concentration of	
	0.2 mM	0.05 mM
Gly-Pro-2-NNap	100	39
Phe-Pro-2-NNap	243	193
Ala-Ala-2-NNap	5	n. d.
Leu-Ala-2-NNap	4	n. d.
Asp-Ala-2-NNap	0	n. d.
Gly-Arg-2-NNap	0	n. d.
Phe-Arg-2-NNap	0	n. d.
Gly-Pro-4-methoxy-2-NNap	54	82
Lys-Pro-4-methoxy-2-NNap	53	97
His-Ser-4-methoxy-2-NNap	0	0
Pro-2-NNap	0	n. d.
Cbz-Ala-2-NNap	0	n. d.

Table 4. *Kinetic data for the hydrolysis of peptidylnaphthylamides*
All values have been obtained with highly purified placenta enzyme in 50 mM Tris buffer pH 8.0/37 °C. Abbreviation: 2-NNap, β-naphthylamide. N_t, maximal turnover number (s^{-1})

Substrate	K_m	V	N_t/K_m
	mM	U/mg	s^{-1} mM^{-1}
Gly-Pro-2-NNap	0.20	69	690
Gly-Pro-2-NNap with 2% ethanol	0.1	16	320
Gly-Pro-4-methoxy-2-NNap	0.018	40[a]	4440
Ala-Ala-2-NNap	1.0	28	56
Phe-Pro-2-NNap	0.019	92	9680
Lys-Pro-4-methoxy-2-NNap	0.016	43[a]	5380

[a] Substrate inhibition above 0.1 mM

A free amino-terminal NH_2 group of the first amino acid and a penultimate proline residue are the essential features of good substrates for placenta dipeptidyl peptidase IV. Of several substrates with amino acids other than proline in position two only those with alanine (Tables 3 and 4) and hydroxyproline (Table 5) were cleaved at low rates. Generally, the results of Tables 3 and 4 are similar to those reported for purified dipeptidyl peptidase IV from other sources [3–10].

HYDROLYSIS OF PEPTIDES

Part A of Table 5 lists several peptides that proved to be good substrates for the placenta peptidase. It was not surprising that two N-terminal dipeptides were cleaved from substance P, this had already been demonstrated with dipeptidyl peptidase IV from other sources [11, 12]. The procedure for the identification of the released peptides by thin-layer chromatography and by identification of the dansylated amino termini was the same as in our earlier report [11]. β-Casomorphin-5 was an even better substrate. The pentapeptide was completely cleaved into three components, two of which could be identified as phenylalanylproline and the carboxy-terminal glycine. Dansylphenylalanine and dansylglycine could be identified in addition to the N-terminal tyrosine if the mixture was dansylated after incubation with dipeptidyl peptidase IV. Recently Barth et al. [14] reported that casomorphin was hydrolyzed by dipeptidyl peptidase IV of pig kidney.

The renin inhibitor mentioned in Table 5A was a synthetic angiotensin analog [28]. This octapeptide was a good substrate for dipeptidyl peptidase IV (Fig. 5), as well as the corresponding tripeptide histidylprolylphenylalanine. Phenylalanine was the only newly produced N-terminal amino acid during the degradation of these peptides.

The degradation rates of casomorphin-5, histidylprolylphenylalanine and prolylprolylglycine were similarly high.

The rates of the other peptides of Table 5A decreased in the order casomorphin > renin inhibitor > substance P > kentsin.

Table 5. *Cleavage of peptides by dipeptidylpeptidase IV of human placenta*
(A) Good substrates. Purified enzyme (0.6 μg/ml) cleaves the bulk of a 2 mM peptide solution in 1 h at 37 °C/pH 8. The fragments are identified by cochromatography with reference substances (detection with ninhydrin) and by identification of the newly appearing N-terminal amino acids. (B) Moderate substrates. (Fragments can be demonstrated with ninhydrin after 4 h at 37 °C; other conditions as in A.) (C) Peptides not cleaved. (No fragments detectable with ninhydrin after 4 h at 37 °C; other conditions as in A.)

Category	Peptide	Identified fragments	Identified additional amino termini
A. Good substrates	substance P	Arg-Pro, Lys-Pro	Lys, Glu
	casomorphin-5(Tyr-Pro-Phe-Pro-Gly)	Phe-Pro, Gly	Phe, Gly
	kentsin (Thr-Pro-Arg-Lys)	Thr-Pro	Arg
	renin inhibitor (His-Pro-Phe-His-Leu-D-Leu-Val-Tyr)	His-Pro	Phe
	His-Pro-Phe	His-Pro, Phe	Phe
	Pro-Pro-Gly	Gly	Gly
B. Moderate substrates	Gly-Hyp-Ala	Ala	Ala
	Tyr-D-Ala-Gly-Phe-Met-NH_2	–	Gly
C. Peptides not cleaved	Arg-Pro-Pro		
	Bradykinin (Arg-Pro-Pro-Gly ...)		
	Glu-Val-Phe		
	Gly-Phe-Phe		
	Gly-Gly-Gly-Gly-Gly		
	Leu-Gly-Leu		
	Pro-Phe-Asp		
	Pro-Pro-Pro		
	Tuftsin (Thr-Lys-Pro-Arg)		

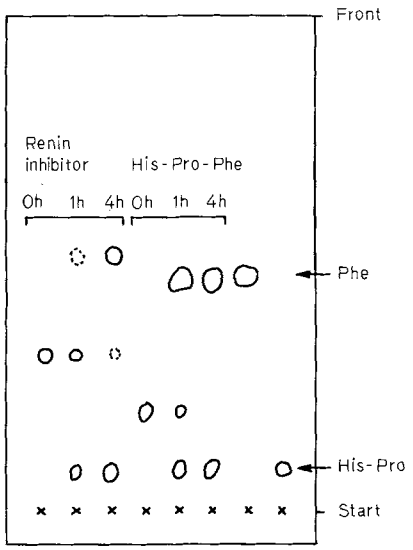

Fig. 5. *Hydrolysis of histidylprolylphenylalanine and a synthetic renin inhibitor by purified dipeptidyl peptidase IV.* Thin-layer chromatography on kieselgel in butanol/pyridine/acetic acid/water (150:100:3:100). Staining with ninhydrin. The digestion mixtures contained 0.1 μmol of each peptide and 1 mU peptidase in 50 μl 10 mM Tris/HCl buffer, pH 8.0. The last two lanes contain phenylalanine and histidylproline as references

Part B of Table 5 lists further peptide substrates which showed ninhydrin-positive fragments upon incubation with dipeptidyl peptidase IV and thin-layer chromatography. However, in these cases the release of peptides was not complete within a reasonable time. We cannot exclude the possibility that the last peptide of Table 5B contained some of the L-alanine-containing isomer. The last part of Table 5 lists peptides that were not attacked by dipeptidyl peptidase IV. We could confirm that a proline in position three of the sequence or an amino acid other than proline, hydroxyproline or alanine in position two were absolutely inhibitory.

Table 6. *Effect of inhibitors on dipeptidylpeptidase IV activity*
Standard assay with 0.2 mM Gly-Pro-2-NNap. (A) Organophosphates: enzyme (about 1 μg/ml) was preincubated in 50 mM Tris pH 8 for 30 min at 30 °C. (B) Inorganic ions

Inhibitor	Concentration	Remaining activity
	mM	%
A. Organophosphates		
Diisopropylfluorophosphate	1	0
	0.5	0
	0.1	54
	0.05	76
	0.01	100
Diethyl-4-nitrophenyl-phosphate (Paraoxon)	1	16
	0.1	74
	0.01	100
Bis-(4-nitrophenyl)phosphate	1	0
	0.1	36
	0.01	85
Bis-(4-cyanophenyl)phosphate	1	89
B. Inorganic ions		
NaF	1	68
Na_2SO_4	250	100
$ZnCl_2$	1	0
	0.1	12
	0.01	42
	0.001	89
$CdCl_2$	0.1	45
	0.01	88
	0.001	100
$HgCl_2$	1	2
4-Hydroxymercuribenzoate	1	120
$CuSO_4$	1	41
$Pb(CH_3COO)_2$	1	0

INHIBITORS AND ACTIVATORS

The organophosphorus triesters diisopropylfluorophosphate and paraoxon irreversibly inhibited placenta dipeptidyl peptidase IV (Table 6A). Surprisingly the diester bis-(4-nitrophenyl)phosphate was a more effective inhibitor. The corresponding peptidase of rat liver was far less sensitive to inhibition by this diester [11].

After incubation with bis-(4-nitro[^{14}C]phenyl)phosphate (30 min at 30 °C, pH 8.0) and subsequent dodecyl sulfate electrophoresis a substantial amount of radioactivity remained bound to the placenta peptidase. Thus, the enzyme obviously is organophosphorylated by this inhibitor, as has been reported for the similar reaction with liver carboxylesterase [29].

We assayed the possible inhibitory action of some amino acids and peptides. The dipeptides glycylproline and phenylalanylproline had significant effects, but only at relatively high concentrations: the remaining activities at 1 mM (0.1 mM) peptide concentration were 51% (90%) with phenylalanylproline and 76% (100%) with glycylproline. We thought that tuftsin might act as a competitive inhibitor because of the ε-amino group of lysine in the neighborhood of the proline residue, but it did not. Proline, glycine, and arginylprolylproline also were not inhibitory at 0.1 mM.

Zinc was a remarkably strong inhibitor of dipeptidyl peptidase IV (Table 6B). It was more effective than cadmium or mercury. Mercury or lead ions seemed not to act on free SH groups of the enzyme because 4-hydroxymercuribenzoate was not inhibitory. The activating effect of 4-hydroxymercuribenzoate might be explained as a detergent-like action similar to that of Triton X-100 and Nonidet P40, both of which activated the purified enzyme by about 30% at 0.1% detergent concentration. Mercaptoethanol (1 mM), EDTA (1 mM), wheat germ lectin (0.25 mg/ml), acetonitrile (0.1%), and several inhibitors of lysosomal proteases (1 mM), namely leupeptin, chymostatin and antipain, did not influence dipeptidyl peptidase IV. 1,10-Phenanthrolin inhibited 72% at 1 mM and 20% at 0.1 mM inhibitor concentration.

DISCUSSION

Dipeptidyl peptidase IV of human placenta is a membrane-bound neuraminic-acid-containing glycoprotein. The enzyme probably is a dimer composed of two identical subunits of M_r 120000 as has been proposed for dipeptidyl peptidases IV from other sources [5–10]. However, these data have to be confirmed by other methods, because dodecyl sulfate electrophoresis often gives false results with glycoproteins [27]. Both in raw extracts and in the purified state the enzyme exists in multiple forms with respect to the isoelectric points. This heterogeneity is not caused by the neuraminic acid moiety of the molecules (Fig. 5). The range of the isoelectric points of the

placenta enzyme is significantly lower than those reported for other dipeptidyl peptidase IV preparations. In the placenta the enzyme seems to be localized on the mesodermal chorion cells, and in placental villi on myofibroblasts and the capillary endothel, especially of the arteries [30]. Recently we could show that the enzyme interferes with blood clotting in that it cleaves a dipeptide from monomeric fibrin [16]. However, this is probably not the only natural role of dipeptidyl peptidase IV in the placenta. It has been reported that human cord blood contains considerable amounts of substance P produced by the fetus [15]. Dipeptidyl peptidase IV probably increases the vasoactive effect of substance P by removing two N-terminal dipeptides [11]. Thus, the enzyme may be involved in the regulation of contractile processes in the placenta. In this context it seems to be biologically significant that bradykinin and many active analogs with X-Pro-Pro-... structure [31] are not cleaved by dipeptidyl peptidase IV and remain stable in the circulation. Peptides of the bradykinin type exert an unrestricted effect, e.g. on smooth muscles, whereas the similar action of substance P is correlated with the presence of the activating dipeptidyl peptidase.

It is questionable whether the other natural peptides mentioned in Table 5 are important as physiological substrates of the placenta peptidase. Kentsin that has been isolated from hamster embryos [32] may be an exception. This peptide exhibits complex biological actions on cultured fibroblasts [33]. Since the specialized, myosin-containing fibroblasts of the placenta (M. R. Parwaresch and A. Feller, personal communication) contain the bulk of dipeptidyl peptidase IV [30], kentsin or related peptides might transmit signals from the fetus.

Casomorphin is a partial sequence of casein with morphin-like biological activity [34]. It is not cleaved by proteinases and aminopeptidases [34] and could, therefore, escape intestinal degradation. The primary structure of casein [31] contains several partial sequences with alternating proline residues, i.e. positions 109–113, 149–153, 171–175, 178–182 and 203–207, in addition to the casomorphin-containing part (position 60–68). Dipeptidyl peptidase IV would digest such peptides with biological activity. However, it is unknown whether such peptides occur in the blood.

The synthetic renin inhibitor mentioned in Table 5 is an analog of angiotensin. Inhibitors of this type are frequently used in pharmacological experiments. It should be kept in mind that a degradation by dipeptidyl peptidase IV could alter the pharmacodynamic properties of such peptides.

The remarkable inhibitory effect of zinc may also be biologically important, because the normal zinc concentration in human plasma is of an order of magnitude [35] that inhibits about 50% of the dipeptidyl peptidase IV activity. Thus, any regulation of the plasma or tissue zinc concentration will exert a regulatory effect on the peptidase activity.

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