

LIVER DIPEPTIDYL AMINOPEPTIDASE IV HYDROLYZES SUBSTANCE P

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1. Introduction

Dipeptidyl aminopeptidase IV (EC 3.4.14.-) has been isolated from the microsomal fractions of various tissues [1], including rat liver [2]. The activity of this enzyme has normally been determined with Gly-Pro- β -naphthylamide or similar synthetic substrates with a proline-residue next to the N-terminal amino acid. It has been assumed, that this enzyme might be involved in the turnover of collagen [1], but this is not very likely for a membrane-bound intracellular enzyme. So far, no physiological substrate has been found for dipeptidyl aminopeptidase IV.

The hormone-like undecapeptide, substance P, produces a variety of physiological effects which are not yet fully understood [3,4]. It is mainly found in the nervous system and in the intestine. Besides its distinct effects on the smooth muscles of intestine and of blood vessels, substance P is also discussed as a neurotransmitter. The amino acid sequence of substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) suggests that it might be a good substrate for dipeptidyl aminopeptidase IV. Since it has recently been reported [5] that liver has a high capacity to inactivate substance P, we wondered whether this peptidase might be responsible for this effect.

2. Materials and methods

2.1. Reagents

Substance P, Gly-Pro- β -naphthylamide, dansyl-

Abbreviation: dansyl-, 5-dimethylaminonaphthyl-1-sulfonyl-

chloride, dansyl-amino acids, diethyl-4-nitrophenylphosphate, and diisopropyl fluorophosphate were purchased from Serva, Heidelberg, Germany. Saponium album, sodium dodecyl sulfate, bis-(4-nitrophenyl)phosphate and thinlayer plates with silica gel were from Merck (Darmstadt, Germany). Polyamide thinlayer plates were from Cheng Chin, Taipeh, Taiwan. Bis-(4-nitro [¹⁴C]phenyl)phosphate was from Höchst AG, Frankfurt, Germany.

2.2. Preparation of rat liver dipeptidyl aminopeptidase IV

We did not follow the known procedures involving an extensive autolysis step for the isolation of the rat liver enzyme [2] or similar enzymes from other origins [1], because we intended to isolate various other serine hydrolases from the same material. Therefore, the procedure described here is similar to our method for the isolation of liver and kidney esterases [6]. Apart from an enrichment by a single ion-exchange chromatography or alternatively by gel filtration [2] a purification of rat liver diaminopeptidase IV has not yet been described.

Rat liver microsomes were prepared by ultracentrifugation [6] starting with 500 g of fresh livers from male Wistar rats. The 105 000 g_{max} sediment was resuspended with 250 ml 0.1 M Tris buffer, pH 8.5 and kept under refrigeration. After thawing, 500 ml 0.1 M Tris, pH 8.5 and 7.5 g saponin (1%) were added to 250 ml of the microsomal suspension, and the mixture was stirred at 0°C for 60 min. After centrifugation (105 000 g_{max}, 2 h) the supernatant contained 25% of the Gly-Pro- β -naphthylamide hydrolyzing activity. (This activity was measured fluorometrically according to McDonald [7].) The peptidase activity was

precipitated completely with $(\text{NH}_4)_2\text{SO}_4$ in the fraction from 40–70% saturation together with various esterases. The sediment after 70% $(\text{NH}_4)_2\text{SO}_4$ -saturation was redissolved in 75 ml 5 mM phosphate buffer, pH 8 and applied to a gel filtration column equilibrated with the same buffer (column 90×2.5 cm filled with Sephadex G-150, Pharmacia, Freiburg, Germany). The Gly-Pro- β -naphthylamide hydrolyzing activity coincided with the first protein-containing fractions of this column (peak after 245 ml). This peak, however, was combined with the overlapping esterase peak (259 ml). The separation from most of the esterase activity was achieved by chromatography on DEAE-Sephadex A50 (2.5×25 cm, eluted with a linear salt gradient, fig.1). The combined fractions (59–87, fig.1) were dialyzed against 5 mM phosphate buffer, pH 8 (2×12 h at 4°C) and mixed into the sucrose gradient (440 ml) of an isoelectric focussing column (LKB, no. 8100, Gräefelfing, Germany). A mixture of 2.5 ml Ampholine (LKB) pH 3.5–10, 2.5 ml Ampholine, pH 5–7, and 5 ml Ampholine,

pH 4–6 served as ampholyte. After 2 days at an increasing voltage (from 300 to 700 V) the dipeptidyl aminopeptidase IV was found as a sharp peak at pI 4.76, completely separated from acetanilide and butyric acid-methyl ester hydrolyzing activities. The peptidase was freed from ampholytes by chromatography on Sephadex G-200 (column 2.5×90 cm, 5 mM phosphate buffer, pH 8). The resulting peptidase solution was enriched 480-fold as compared to the microsomes, and gave a single band in the analytical dodecyl sulfate electrophoresis (fig.3). Protein was estimated with the biuret procedure [8].

2.3. Dodecyl sulfate electrophoresis

7.5% polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate were used for the separation of denatured proteins. The protein samples were heated (2 min, 95°C) in 62 mM Tris buffer, pH 6.8 containing 2.5% dodecyl sulfate and 5% mercapto-ethanol. Gel and electrode buffers were prepared according to Karam and Bowles [9]. Purified

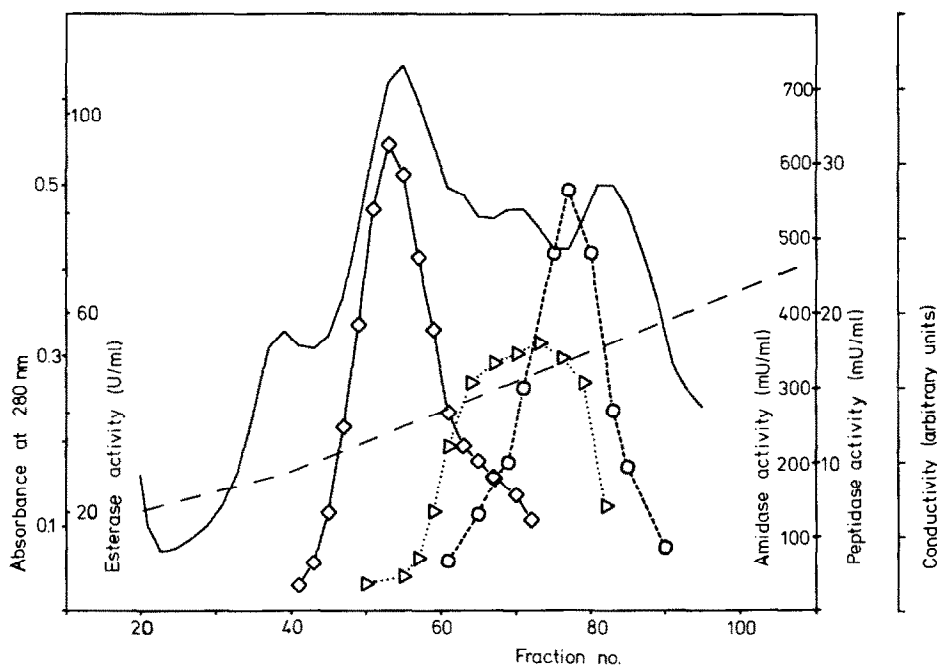


Fig.1. Purification of dipeptidyl aminopeptidase IV by ion exchange chromatography (DEAE-Sephadex A50 column eluted with 5 mM phosphate buffer pH 8 containing a sodium chloride gradient from 0.04 M to 0.5 M). Fractions of 10 ml were collected and tested for absorbance at 280 nm (—), activity against *n*-butyric acid-methylester (◇—◇), activity against acetanilide (▷...▷), activity against Gly-Pro- β -naphthylamide (○—○), and for conductivity (---).

dipeptidyl aminopeptidase IV was labeled with 1 mM bis-(4-nitro[14 C]phenyl)-phosphate (16 h at 3°C in 5 mM phosphate buffer, pH 8) before the denaturing treatment. The gels were stained with 0.1% Coomassie brilliant blue R 250 in 50% trichloroacetic acid/water and destained with 7% acetic acid. For autoradiography the gels were dried on filter paper in vacuo and pressed on X-ray films (Agfa-Gevaert Osray T 4) for 3 weeks.

2.4. Determination of *N*-terminal amino acids

Samples of the incubation mixture of substance P and the peptidase were dansylated according to Gray [10] 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 thinlayer sheets. All dansyl-amino acids in question could be identified by one-dimensional chromatography either in water/formic acid (200:3, by vol.) or in 1M NH₃ ethanol (1:1, by vol.) with dansyl-Arg, -Glu, and didansyl-Lys as references. The NH₃-containing solvent served for the identification of ϵ -dansyl-Lys in addition to dansyl-Arg.

3. Results and discussion

3.1. Degradation of substance P

100 μ g of substance P in 20 μ l water were mixed with 24 μ g of purified dipeptidyl aminopeptidase IV in 40 μ l 5 mM phosphate buffer, pH 8 and heated to 37°C for 3 h. Samples (2 μ l) of the reaction mixture were applied to a silica gel thinlayer plate at intervals of 15, 30 or 60 min (fig.2). After chromatography in phenol/water (3:1, by weight) and detection with ninhydrin (0.2% in 2,4,6-collidine/acetic acid/ethanol 1:5:25, by vol.) the time-dependent production of two new peptides with R_F -values of 0.056 and 0.019 becomes clearly visible, while the original spot of substance P disappears. Since the spots R_F 0.056 are the first to appear, it is probable that these spots correspond to the N-terminal dipeptide Arg-Pro of substance P. This observation is confirmed by spraying a corresponding thinlayer plate with the phenanthroquinone spray [11] for arginine derivatives: besides substance P the spot R_F 0.056 is the only fluorescent spot. Consequently, the spots R_F 0.019 should corre-

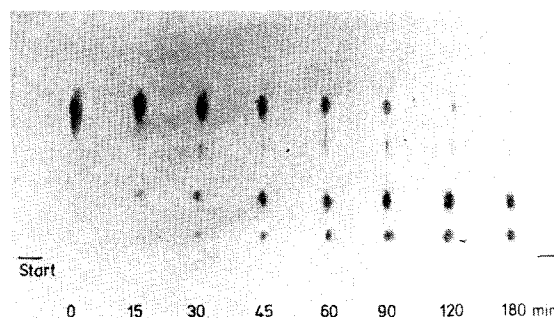


Fig.2. Thinlayer chromatography of the degradation peptides from substance P after different times of incubation with purified dipeptidyl aminopeptidase IV (see text).

spond to Lys-Pro, which occupy position 3-4 in the sequence of substance P. An additional peptide spot (R_F 0.119), which gives a much weaker colour with ninhydrin, might correspond to the heptapeptide with N-terminal Gln, which remains after the removal of 4 amino acids from the N-terminus of substance P.

The production of the two dipeptides and the heptapeptide mentioned above is proven by a dansylation experiment. 15 μ l of the above-mentioned reaction mixture (\approx 25 μ g of substance P) after 3 h of incubation with the peptidase were dansylated and hydrolyzed in order to determine the N-terminal amino acids (see methods). Both didansyl-Lys and dansyl-Glu were found in addition to dansyl-Arg and traces of ϵ -dansyl-Lys. If substance P was incubated (3 h at 37°C) in 5 mM phosphate buffer without enzyme, only dansyl-Arg and ϵ -dansyl-Lys were visible; the corresponding blank with the peptidase - without substance P - gave no dansyl-amino acid. The identity of the dansyl-amino acids was proven both by comparison to the mobility of the corresponding reference substances and by cochromatography with these standards. No other fluorescent spots besides dansyl-OH, dansyl-NH₂ and the above-mentioned dansyl-amino acids were visible on the thinlayer plates.

We see only one possibility to explain the results described here: dipeptidyl aminopeptidase IV subsequently splits the two dipeptides Arg-Pro and Lys-Pro from the N-terminal end of substance P. The possibility that a tripeptide Lys-Pro-Gln is split off besides Arg-Pro would also be in accordance with the experimental results, since a further Gln-residue is

in position 6 of substance P. However, this is extremely unlikely in the light of the known behaviour of the peptidase towards small synthetic peptide derivatives [1]. Though we did not prove that the hydrolysis of substance P reported here destroys its biological activity, it is likely that the degradation of substance P by liver dipeptidyl amino peptidase IV leaves the residual heptapeptide ready for further degradation by amino-peptidases and therefore corresponds to the reported [5] capacity of the liver to inactivate the biological activity of this peptide. It might well be that the main task of this peptidase is to eliminate circulating substance P, which otherwise would strongly influence the permeability of the blood vessels and the blood pressure [4].

On the other hand, it has been reported that the C-terminal hexapeptide of substance P is up to 12 times more biologically active than the undecapeptide itself [12]. If this is so, it might also be possible that substance P is only a precursor of the active hormone (or transmitter or whatever it is), which is activated by the action of dipeptidyl amino-peptidase IV.

3.2. Some properties of rat liver dipeptidyl amino-peptidase IV

Hopsu-Havu and Sarimo [2] reported dipeptidyl aminopeptidase IV of rat liver not to be a serine enzyme, because they failed to get a complete inhibition with diethyl-4-nitrophenylphosphate (paraoxon)

Table 1
Inhibition of purified rat liver dipeptidyl aminopeptidase IV by organophosphates

Inhibitor	Remaining activity ^a (%), inhibitor concentration	
	10 ⁻³ M	10 ⁻⁴ M
Bis-(4-nitrophenyl)-phosphate	67	87
Diethyl-(4-nitrophenyl)-phosphate (paraoxon)	34	84
Diisopropyl-fluoro-phosphate	0	0

^a Enzyme was preincubated with inhibitor for 60 min (30°C, pH 8.0), and assayed with Gly-Pro-β-naphthylamide

or diisopropyl fluorophosphate. However, this was doubted by McDonald and Schwabe [1]. Our preparation of the peptidase was completely inhibited by diisopropylfluorophosphate, and also reacted slowly with bis-(4-nitrophenyl)phosphate, a specific inhibitor [13] or liver serine esterases (table 1). These organophosphates are active-site-directed inhibitors which phosphorylate a single serine residue in various proteases or esterases [14,15]. The autoradiogram of the gel shown in fig.3 demonstrates that some of the

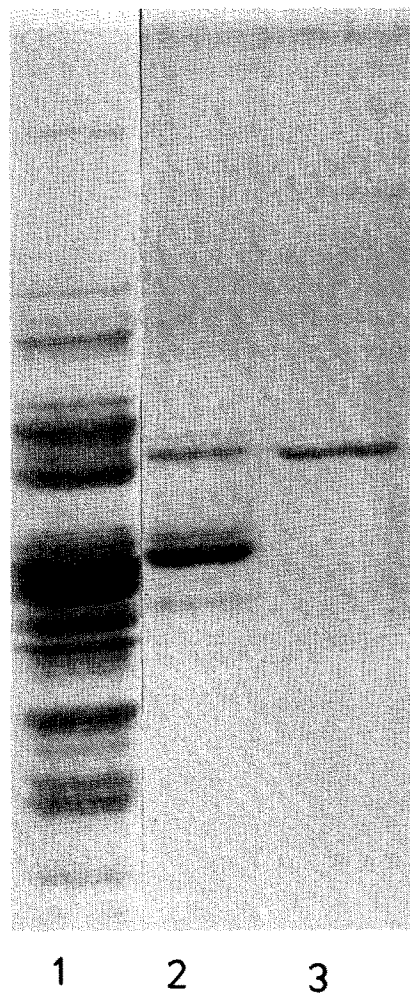


Fig.3. Dodecyl sulfate electrophoresis after various steps in the purification of dipeptidyl aminopeptidase IV. (1) After gel chromatography; (2) after ion-exchange chromatography; (3) after preparative isoelectric focussing. Stained with Coomassie brilliant blue R 250.

radioactivity of bis-(4-nitro [^{14}C]phenyl)phosphate is bound to the peptidase subunit, which behaves like a liver carboxylesterase (EC 3.1.1.1) in this respect [14], though the phosphorylating reaction is much slower. We conclude that rat liver dipeptidyl aminopeptidase IV clearly is a serine enzyme as has already been shown for the corresponding enzymes from other animals and tissues [1,16].

We also checked whether substrates of the microsomal carboxylesterases EC 3.1.1.1 [17] are hydrolyzed by this peptidase. However, we found no activity with butyric acid-methyl ester [6], acetanilide [18], *N*-butylglycyl-(2-chloro-6-methyl-anilide)HCl [19] procain [18], or α -naphthyl acetate [20].

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