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Purification and Properties of an Arylaminopeptidase of Rat Wound Tissue, Acting Chiefly on the 2-Naphthylamides of L-Methionine and L-Valine

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The purification and properties of an arylaminopeptidase of rat wound tissue acting chiefly on the 2-naphthylamides of L-methionine and L-valine have been studied. The purification was accomplished by CM-Sephadex, DEAE-cellulose, and CM-cellulose. The purity of the final preparation was studied with disc electrophoresis. Only one protein band was observed which coincided with the enzyme activity in the gel. The preparation was shown to hydrolyse various amino acid 2-naphthylamides in the following order and ratios: L-methionyl-, L-valyl-, L-isoleucyl-, L-leucyl-, L-phenylananyl-, and L-alanyl-2-naphthylamide (8:4:2:2:2:1). The enzyme required SH-compounds to maintain its full activity. It was inhibited by divalent metal cations and activated by EDTA. *p*-Chloromercuribenzoate, *N*-ethylmaleimide, NaCl, and KBr inhibited the enzyme reactions. The most rapid hydrolysis of L-methionyl-2-naphthylamide by the enzyme occurred at pH 6.5. The molecular weight of the enzyme, determined by gel filtration, was found to be approximately 190 000.

In our earlier papers [1,2] enzymic hydrolysis of 2-naphthylamides of L-methionine and L-valine by rat wound tissue enzymes was described. The enzyme preparations, hydrolysing very rapidly these amides, possessed also a considerable activity on the 2-naphthylamides of L-phenylalanine and L-isoleucine and it seems that in the wound tissue there is an enzyme which is capable of hydrolysing all of the mentioned substrates. This kind of enzyme activity was found only in the wound tissue, the normal skin preparation displaying no or only a low activity when the hydrolysis of L-valyl- and L-isoleucyl-2-naphthylamides was studied. It is noteworthy that there seems to be no report in the literature on arylaminopeptidases, acting specifically or even rapidly on L-valyl-2-naphthylamide. Many enzyme preparations, possessing high activity against other amino acid 2-naphthylamides, have been described and some of the corresponding aminopeptidases have been purified, for example, aminopeptidase B from rat liver acting specifically on L-arginyl- and L-lysyl-2-naphthylamides,[3,4] an aminopeptidase of the ocular lens acting

on L-leucyl-2-naphthylamide,[5] pituitary arylamidases and peptidases acting on various amino acid 2-naphthylamides,[6] arylamidases of rat liver and kidney acting on L-leucyl-2-naphthylamide,[7] arylamidase of *Neisseria catarrhalis* hydrolysing most rapidly L-alanyl-2-naphthylamide,[8] *etc.*

The present paper will provide a method for partial purification of an arylaminopeptidase from rat wound tissue, capable of hydrolysing at a considerably high rate also the 2-naphthylamides of L-valine and L-isoleucine. In addition, attention has been paid to the differences between the arylaminopeptidase described here and those demonstrated in the normal skin preparations.[1,2,9]

MATERIALS AND METHODS

Reagents. The 2-naphthylamides of amino acids, *N*-ethylmaleimide (NEM) and human hemoglobin were purchased from Mann Research Laboratories Inc. (New York, USA). Diethyl-*p*-nitrophenyl phosphate (E-600) was purchased from Fluka AG (Buchs, Switzerland), dithiothreitol (DTT) and sodium *p*-chloromercuribenzoate (pCMB) from Calbiochem (Los Angeles, USA). Bovine γ-globulin (fraction II) was obtained from Nutritional Biochemical Corporation (Cleveland, Ohio, USA). All other reagents were obtained from E. Merck AG (Darmstadt, Germany).

Chemical determinations. The determination of enzyme activity and of protein concentration was performed as described earlier.[1]

Column chromatography. In the preparation of the ion exchange columns and in conducting the fractionation, the instructions of Peterson and Sober [10] have been followed with the exceptions mentioned earlier.[1] The gel filtration was conducted on Sephadex G-200 and CM-Sephadex C-50 columns, following the instructions of the manufacturer of the gel (Pharmacia, Uppsala, Sweden). For details, see the legends to the figures.

Analytical disc electrophoresis. Disc electrophoresis was conducted with a Canalco Model 6 electrophoresis apparatus following the method of Ornstein and Davis. The concentration and desalting of the sodium chloride containing samples is described in our preceding paper.[2]

Preparative disc electrophoresis. The preparative disc electrophoresis was conducted with Shandon Preparative Acrylamide Electrophoresis Apparatus. The instructions of the manufacturer (Shandon Scientific Co, London, England) were followed, with the following exceptions: In the polymerization of the gel, riboflavin (B_2-vitamin) was used instead of ammonium persulphate. The gels in this study did not contain urea and the molarity of the electrolyte buffer was diminished to one tenth. Approximately the same original salt concentration in the buffer was then ensured by adding enough of sodium chloride.

RESULTS

The starting material in the purification of the wound tissue arylaminopeptidases was the supernatant fraction obtained after centrifugation (23 500 *g*, 15 min) of a wound tissue homogenate. The preparation of the homogenate is described earlier.[1] The first step in the purification procedure was the fractionation of the supernatant fluid on CM-Sephadex C-50 gel. In this way about three quarters of foreign proteins of the preparation were removed and, thus, the enzyme fraction (CMS—II) under investigation contained only a quarter of the total protein amount, applied on the column. The active fractions were pooled as indicated in Fig. 1. This pooled CMS—II was still considered to contain a considerable amount of foreign proteins but was not obviously contaminated by other enzymes hydrolysing the 2-naphthylamides of L-valine

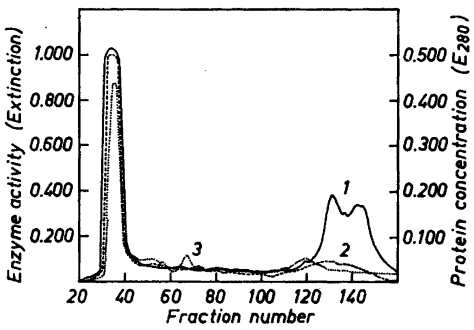

Fig. 1. Fractionation of one day old wound tissue and normal skin arylaminopeptidases on CM-Sephadex C-50. Column: 10 × 850 mm; Elution: 0.05 M tris-HCl, pH 7.15; Salt gradient: linear sodium chloride gradient from 0 to 1 M (mixing volume 150 + 150 ml); Fraction volume: 1.5 ml; Temperature: 4°C; Sample: supernatant fluid of the homogenate of wound tissue or normal skin. Total protein amount of each sample was about 50 mg; Substrate: L-methionyl-2-naphthylamide. 1 = wound tissue (enzyme activity), 2 = normal skin (enzyme activity), 3 = wound tissue (protein concentration).

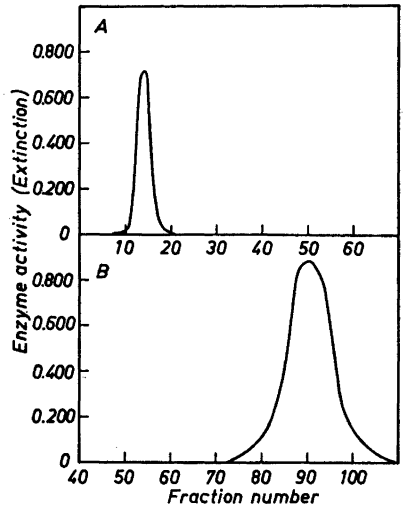

Fig. 2. A. Fractionation of the CMS—II pool (from the experiment given in Fig. 1) on DEAE-cellulose, 200—230 mesh (Schleicher & Schüll). Column: 15 × 200 mm; Elution: 0.01 M tris-HCl, pH 7.15; Salt gradient: linear sodium chloride gradient from 0 to 1 M (mixing volume 150 + 150 ml); Fraction volume: 2 ml; Temperature: 4°C; Sample: pooled CMS—II peak, concentrated and desalted as described in the text; Substrate: L-methionyl-2-naphthylamide. *B.* Fractionation of the pooled active fractions from the DEAE-cellulose fractionation (Fig. 2 A) on CM-cellulose, 140—200 mesh (Schleicher & Schüll). Column: 15 × 950 mm; Elution and salt gradient were similar to 2 A; Sample: pooled fractions from the above mentioned DEAE-cellulose fractionation (Fig. 2 A); Substrate: L-methionyl-2-naphthylamide.

and L-methionine. The arylaminopeptidase of the CMS—II pool was not at all adsorbed on DEAE-cellulose and advantage was taken of this observation in the further purification of the enzyme.

Before applying the pooled fractions from the CM-Sephadex fractionation on DEAE-cellulose columns, the sodium chloride present in the active fractions, due to the use of a salt gradient elution, had to be removed. For this purpose the proteins of the pooled fractions were precipitated with ammonium sulphate (40—60 %), the solution was allowed to stand for 60 min at +4°C, followed by centrifugation (12000 *g*, 15 min). Desalting of the dissolved precipitate was performed on columns, packed with Sephadex G-25 gel. The resultant desalted solution was then applied on the DEAE-cellulose column. Using DEAE-cellulose fraction an about 30 fold purification could be achieved,

as compared to the starting material. In these DEAE-cellulose fractionations the arylaminopeptidase constantly behaved as a homogeneous peak (Fig. 2 A).

The active fractions from the DEAE-cellulose fractionation were pooled and applied on a CM-cellulose column. The arylaminopeptidase preparation appeared homogeneous (Fig. 2 B), displaying about 60 fold higher activity than the starting material.

Testing of the purity of the arylaminopeptidase. To test the purity of the obtained arylaminopeptidase preparation, a disc electrophoretic investigation was performed. For this purpose the active fractions from the CM-cellulose fractionation were pooled, precipitated with ammonium sulphate (80 %), desalted with Sephadex G-25 as earlier, and the sample was freeze-dried. The freeze-dried powder was dissolved in 100 μl of cold water. Fig. 3 shows the results. Only one protein band and only one arylaminopeptidase peak can be seen in the disc electrophoresis slab using L-methionyl-2-naphthylamide as substrate.

As shown in Fig. 3, the enzyme studied moved only a very short distance in polyacrylamide gel under the standard conditions. On the other hand, its molecular weight (about 190 000, see Fig. 7) as determined by gel filtration, suggested that its mobility in the gel would be higher than that observed. Therefore, the following two reasons could be given to explain the immobility of the enzyme. 1) The enzyme protein is adsorbed to, for example, γ-globulin, the mobility of which was, in separate experiments, also shown to be very low. 2) The net charge or the charge density of the enzyme molecule favours

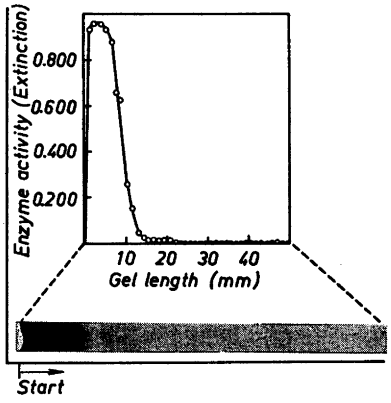

Fig. 3. Disc electrophoresis experiment on the freeze-dried enzyme preparation obtained by pooling the active fractions from CM-cellulose fractionation (Fig. 2 B). For details see section Methods.

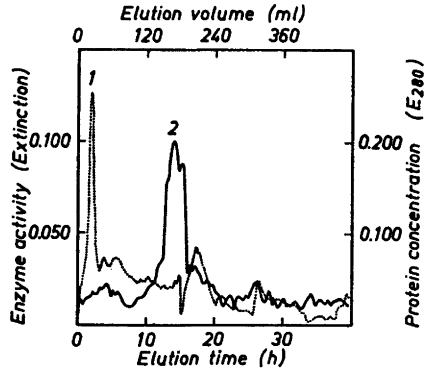

Fig. 4. Preparative disc electrophoresis experiment on the freeze-dried enzyme preparation obtained by pooling the active fractions from CM-cellulose fractionation (Fig. 2 B). Pre-elution: 6 h at 30 mA; Elution: 0.15 M phosphate buffer, pH 6.5, containing 10^{-4} M dithiothreitol; Flow rate: 12 ml/h; Electrophoresis current: 70 mA; Sample: 1 ml of the enzyme solution involved: 1=protein concentration, 2= enzyme activity.

immobility. To test these two possibilities, the enzyme and γ-globulin were separately applied on urea-containing gels in order to release the enzyme from the possible combination. 5 and 10 M urea caused a movement of about 5—10 mm of γ-globulin. When the enzyme preparation was analyzed in the presence of the same urea concentrations, the enzyme activity was found in the borderline of the stacking gel and the separating gel as in the gels containing no urea. This result suggested that the enzyme molecules had not been adsorbed to γ-globulin. Hence it seems more probable that the net charge or the charge distribution of the enzyme molecule had been the principal reason for the observed immobility.

The specific activity of the final enzyme preparation, estimated with the 2-naphthylamides of L-methionine and L-valine, was 7.0×10^{-2} μmole 2-naphthylamine mg^{-1} min^{-1} and 3.2×10^{-2} μmole 2-naphthylamine mg^{-1} min^{-1}, respectively. Any exact purification factor cannot be given because the crude starting material contained several enzymes capable of hydrolysing the named substrates, especially L-methionyl-2-naphthylamide.

Preparative disc electrophoresis. Further purification of the enzyme preparations from the CM-cellulose fractionations by preparative disc electrophoresis was tried (Fig. 4). Unfortunately, however, the enzyme activity, tested with L-methionyl-2-naphthylamide as the substrate, was almost constantly rather low after the procedure. The lowering of the enzyme activity during the preparative disc electrophoresis suggested further investigation, to find the most proper conditions for the electrophoresis. It was also found that when the preparative electrophoresis was conducted, following strictly the instructions of Shandon Scientific Co., the arylaminopeptidase studied moved very slowly only, if at all, in the gel column. The modifications presented in the section "Methods" for the conducting of the electrophoresis, increased the movement of the protein in the gel.

Substrate specificity studies. The substrate specificity of the enzyme preparation obtained from CM-cellulose fractionations was studied using 2-naphthylamides of eight different amino acids. The most rapidly hydrolysed substrate seemed constantly to be L-methionyl-2-naphthylamide. The rate of the hydrolysis of the amino acid 2-naphthylamides was found to occur in the following approximate order and ratio: L-methionyl-, L-valyl-, L-isoleucyl-, L-leucyl-, L-phenylalanyl-, and L-alanyl-2-naphthylamide (8:4:2:2:2:1). The 2-naphthylamides of the basic amino acids, L-arginine and L-lysine, were not hydrolysed by the wound tissue arylaminopeptidase studied.

Effect of pH. The effect of pH on the hydrolysis of L-methionyl-2-naphthylamide by the enzyme preparation obtained from CM-cellulose fractionation (Fig. 2 B) is shown in Fig. 5. The observed pH optimum was found to be considerably lower than those found for arylaminopeptidases of rat normal skin.[9]

Effect of some chemical compounds. When studying the effect of some SH-compounds, *i.e.* L-cysteine, reduced glutathione, and dithiothreitol, on the wound tissue arylaminopeptidase, it was found that maximum activity was attained in the presence of a thiol, the best one of the above mentioned compounds being dithiothreitol. In addition, it was found that the activation, expressed in percent, seemed to be subject to some variations, evidently due

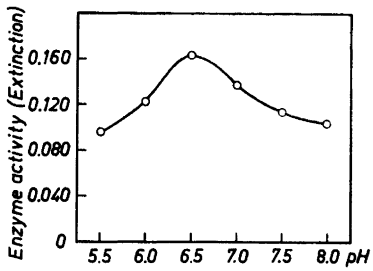

Fig. 5. Effect of pH on the hydrolysis of L-methionyl-2-naphthylamide by the purified wound tissue arylaminopeptidase.

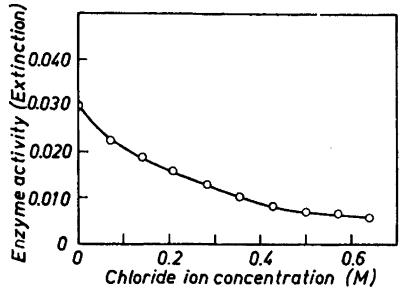

Fig. 6. Effect of sodium chloride on the hydrolysis of L-methionyl-2-naphthylamide by the purified wound tissue arylaminopeptidase.

to the different stage of oxidation of the enzyme molecule. It was possible to reactivate a fully inactivated enzyme preparation by adding dithiothreitol to the reaction mixture.

Because hydrolyses, catalysed by several arylaminopeptidases, have been found to reach the maximum velocity in the presence of certain monovalent anions only, in particular chloride ions,[11,13] the effect of sodium chloride and potassium bromide on the hydrolysis of L-methionyl-2-naphthylamide was studied. Results are given in Fig. 6 for NaCl. Essentially similar results were obtained also with KBr. According to them, addition of the salts to the reaction mixtures caused an inhibition.

Table 1 shows the effect of several chemical compounds on the enzyme reaction. The most noticeable results were the inhibitory effect of the tested metal cations as well as the inhibition caused by *p*-chloromercuribenzoate and

Table 1. Effect of certain chemical compounds on the hydrolysis of L-methionyl-2-naphthylamide by the purified arylaminopeptidase. The results are given in relative ratio, the value of 100 refering to conditions without any added affector.

Modifier	Modifier concentration (M)			
	1.43×10^{-6}	1.43×10^{-5}	1.43×10^{-4}	1.43×10^{-3}
KCN	111	118	123	129
EDTA	140	154	152	152
NEM	89	82	62	29
E-600	100	100	86	70
pCMB	88	80	75	13
$MnCl_2$	83	62	7	7
$MgCl_2$	100	100	100	80
$CaCl_2$	100	100	90	61
$CoCl_2$	94	83	56	33
DTT	100	265	500	452

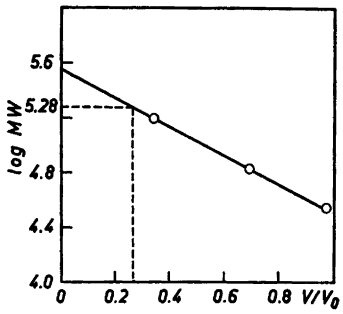

Fig. 7. Determination of the molecular weight of the purified wound tissue arylaminopeptidase. Column: Sephadex G-200, 12×970 mm; Elution: 0.05 M tris-HCl, pH 7.15; hydrostatic pressure: 10 cm H_2O; Fraction volume: 0.5 ml; Temperature: 4°C; Void volume: 64 ml; Protein standards: γ-globulin (MW 160 000), hemoglobin (MW 68 000) and pepsin (MW 35 000); Sample: partly purified wound tissue arylaminopeptidase; The molecular weight obtained: 186 000.

N-ethylmaleimide. The inhibition by the metal cations seemed to be consistent with the observed activation by EDTA. It is to be noted that when studying the influence of the given compounds on the hydrolysis of L-methionyl-2-naphthylamide, the reaction mixtures were 10^{-4} M with respect to dithiothreitol.

Determination of the molecular weight. Fig. 7 shows the determination of the molecular weight of the enzyme by gel filtration on Sephadex G-200. The calculated value of the molecular weight was 186 000.

DISCUSSION

The enzyme described in this paper was shown to be rather unspecific as to the nature of the amino acid side chain of the substrate. This suggestion is based on the evidently high purity of the enzyme preparation (see, for example, the results of the disc electrophoresis experiments). Consequently, also the results with various chemical compounds, used to characterize the enzyme, may be claimed to describe properties of just the enzyme studied, and not those of contaminating enzymes. Therefore it may be well founded to compare the properties of the present wound tissue enzyme to those of normal skin.[9] Some of the most important properties of such enzymes are given in Table 2.

As is seen the wound tissue enzyme differs with regards to many important properties from normal skin enzymes. One interesting characteristic is also the observed inhibition by NaCl. Hence it is necessary to compare this finding with the proposed role of aminopeptidase B in tissue injury,[14] the latter enzyme being strongly activated by NaCl, and supposed to perform its function after being liberated from tissue cells to spaces with high NaCl concentration. The suggestion which could be done on the basis of the presented differences is that the arylaminopeptidase described in this paper participates, more or less directly, in reactions leading to synthesis of some cell components of the regenerating tissue. These reactions, for instance protein synthesis, occur in the cell itself where the NaCl concentration is low. On the contrary, aminopeptidase B, evidently another wound tissue enzyme, performs its function in the extracellular space, where the NaCl concentration is high. All this is supported by the observed short period of time during which the present

Table 2. Properties of enzymes hydrolysing various amino acid 2-naphthylamides from rat normal skin and wound tissue. In this table the normal skin arylaminopeptidases (described elsewhere [9]) are called I, II, III, and IV, according to the order in which they are eluted out of a Sephadex G-200 column. The substrates most rapidly hydrolysed by these enzymes are L-methionyl- (I and II), L-alanyl-, and L-arginyl-2-naphthylamide, respectively.

Property	I	II	III	IV	The present enzyme
pH optimum	7.5—8.0	7.5—8.0	7.0—7.5	7.0—7.5	6.5
Requirement for SH-compounds	Not necessary	Not necessary	Activates, but not necessary	Activates, but not necessary	Absolute requirement [a]
Adsorption to CM-cellulose at pH 7	Not adsorbed	Not adsorbed	Not adsorbed	Not adsorbed	Strong adsorption
Adsorption to DEAE-cellulose at pH 7	Adsorbed	Adsorbed	Adsorbed	Adsorbed	Not adsorbed
Effect of EDTA	Inhibition	Inhibition	Inhibition	Inhibition	Activation
Effect of pCMB and NEM	No effect	No effect	Inhibition	Inhibition	Inhibition
Effect of Co^{2+}-ions	Activation	Activation	Activation	No clear effect	Inhibition
Effect of alkali metal salts (NaCl,KBr)	Activation	Activation	Strong activation	Strong activation	Inhibition

[a] This absolute requirement implies probably the maintaining of the structure of the enzyme.

arylaminopeptidase is demonstrable in the wound tissue.[1] The highest activity is usually seen after 24 h after making the wound.

It has been shown earlier [15] that human and bovine γ-globulin possess proteolytic activity, due to enzyme molecules adsorbed to the globulin. As the molecular weight of the present arylaminopeptidase was found to be about 186 000, it was possible to think that even it could exist as a complex with γ-globulin. The disc electrophoresis experiments with urea, however, did not reveal any such property of the arylaminopeptidase. Consequently, the relatively high molecular weight of the enzyme shows that it differs also from cathepsins, other intracellular proteolytic enzymes, whose molecular weight is considerably lower.

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