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PURIFICATION AND SUBSTRATE CHARACTERIZATION OF A HUMAN ENKEPHALIN-DEGRADING AMINOPEPTIDASE

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Summary

A 5000-fold purification of the enzyme responsible for the rapid inactivation of enkephalin in human blood has been achieved: this enzyme cleaves the N-terminal tyrosine from enkephalin and from short peptides provided their first amino acid is aromatic. The enzyme, an enkephalin-degrading aminopeptidase (α-aminoacyl-peptide hydrolase, EC 3.4.11.11), requires a free amino group on the substrate and has a maximum activity around pH 8. Its apparent molecular weight is in the range of 80 000—90 000 and an apparent Michaelis constant of 0.4 mM was determined.

Enkephalins (Tyr-Gly-Gly-Phe-Leu or -Met) are opiate peptides, which may act as neurotransmitters or neuromodulators [1—4]. It is well known that the physiological activity of enkephalins is rather transitory, due to their rapid degradation by blood and brain proteolytic enzymes [5,6]: the potent analgesic and behavioural action of these peptides has led to considerable interest in the nature of the proteolytic enzymes involved in their activation and destruction.

The nature of the brain inactivating system is rather controversial [7—9], whilst a general agreement exists about the enkephalin-inactivating enzyme(s) present in serum, which cleaves the peptide bond of tyrosine in position 1.

We wish to report here on the purification and partial characterization of a human serum aminopeptidase, which preferentially hydrolyses enkephalins. Although the existence of other serum aminopeptidases has been reported the enzyme described here was not isolated previously.

Materials and Methods

Enkephalins and other peptides were purchased from Bachem AG, β-casomorphin from Peninsula Lab., other chemicals were the purest commercially available products. Amino acid analyses were performed with a Beckman Analyser Model 118C (operating parameters as recommended for 3 h single column, 6 × 510 mm, analysis). Protein concentrations were determined from the 280 nm absorbance with a Beckman DB and/or by the method of Lowry et al. [20]

Assay of enkephalin degradation. Enzyme activity was routinely measured by the appearance of free tyrosine from Leu-enkephalin, in an incubation mixture consisting of 2 mM solution of the substrate in sodium phosphate buffer (pH 7.8) and a suitable amount of the enzyme-containing solution. The incubations were carried out at 37°C for time intervals ranging from 10 to 30 min, depending upon the expected activity of the extract; reactions were terminated by acidification. When plasma or crude $(NH_4)_2SO_4$ precipitate were concerned, this step was performed by acidic acetone precipitation, the sample was centrifuged and the deproteinized supernatant analysed. Semi-quantitative analysis of the free N-terminal amino acid was performed by TLC (solvent system was 2-butanol/acetic acid 1% in water, 10 : 3, v/v) and quantitative determination by automatic amino acid analysis (Fig. 1). Total enzyme activity was expressed as I. munits (International milliunits) (nmol of tyrosine liberated from Leu-enkephalin/min at 37°C). Appropriate 0-time blanks, control incubations and amino acids and/or peptide standards were included in each experiment.

Purification of enkephalin-degrading aminopeptidase. All steps in the purification were carried out at room temperature. Blood was collected in heparinized tubes, centrifuged and the resulting plasma dialyzed overnight, before $(NH_4)_2SO_4$ fractionation.

Chromatography on a DEAE-Sephadex column. A column (1.6 × 50 cm) of DEAE-Sephadex A-50 was equilibrated with 5 mM phosphate buffer, pH 7.8. The dialyzed material from 30—40% $(NH_4)_2SO_4$ fractionation was applied to this column followed by 50 ml of 0.1 M phosphate buffer pH 7.8 (Fig. 2). Following the wash a 400 ml linear gradient up to 0.3 M NaCl was started and fractions (4-ml) containing enkephalin-degrading activity pooled, dialyzed and lyophilized.

Gel filtration. A Sephadex G-100 column (1.6 × 135 cm) was equilibrated with phosphate buffer pH 7.8. The enkephalin-degrading aminopeptidase activity from DEAE-Sephadex was applied to the column and fractions (4-ml) collected at a flow rate of 8—10 ml/h. The enzyme was eluted from the column as shown in Fig. 3, activity-containing fractions were pooled, dialyzed against dilute buffer and lyophilized.

Chromatography on Sepharose-Blue Dextran. A column (1.8 × 30 cm) of Blue Sepharose CL-6B was equilibrated with 2 mM phosphate buffer, pH 7.0. The enkephalin-degrading aminopeptidase activity from Sephadex G-100 was applied to the column and 2-ml fractions were collected using the same buffer as eluent. The enzyme eluted just after the void volume, impurities were either retarded or eluted with 0.5 M NaCl buffer.

Product analysis of substrates degradation. Besides the N-terminal amino

acid, essentially two peptides were obtained from the experiments reported in Table II. They were identified by TLC and quantified by automatic amino acid analysis; Gly-Gly-Phe-Leu from enkephalin (R_F on TLC = 0.24, 58 ml on the autoanalyser) and Gly-Gly from X-Gly-Gly, where X is an aromatic amino acid (R_F on TLC = 0.05, 47 ml on the autoanalyser).

pH optimum and kinetic parameters of enkephalin-degrading aminopeptidase. The purified enzyme was incubated at appropriate pH values as described for routine assays and at the optimum (pH 7.8) with suitable substrate concentrations. The kinetic parameters were calculated by Lineweaver-Burk plots (Fig. 4).

Results and Discussion

Human plasma degrades and inactivates enkephalin, the enzyme responsible for this degradation was purified as summarized in Table I. The procedure involved $(NH_4)_2SO_4$ fractionation, chromatography on DEAE-Sephadex A-50 (Fig. 2), gel filtration on Sephadex G-100 (Fig. 3) and chromatography on Blue-Sepharose. All parasite proteolytic activity from plasma disappeared after chromatography on DEAE-Sephadex and the favourable chromatographic behaviour of the substrate and of the products on automatic amino acid analyser (Fig. 1), allowed us to simplify the overall procedure, which yielded about 5000-fold enrichment of enzyme activity over the starting material. Polyacrylamide gel electrophoresis [10] showed less than 10—15% impurities. After staining with Coomassie blue the active band migrated with a relative velocity of 75%, if compared with Bromophenol blue standard migration at pH 8.9.

As previously postulated by other research groups, the enzyme was shown to catalyse only the cleavage of the N-terminal amino acid acid thus confirming its affinity to the aminopeptidase family. Moreover, Tyr-β-naphthylamide, Gly-Gly-Phe-Leu and insulin B chain were not degraded, allowing us to rule out the presence in the preparation of other proteolytic enzymes, possessing chymotrypsin-like or dipeptidyl-carboxypeptidase activity.

The enzyme exhibits a good stability at room temperature, and remains active after lyophilisation and dialysis, it can therefore be stocked as lyophilized powder at −18° C for months. The only denaturing conditions noticed are pH values below 5.5. The apparent molecular weight, as inferred by the gel-filtration elution volume is around 80 000—90 000, but this result should be inter-

TABLE I

PURIFICATION OF HUMAN PLASMA ENKEPHALIN-DEGRADING AMINOPEPTIDASE

Starting material 90 ml human plasma.

Step	mg protein	Total activity (I. munits)	Specific activity (I. minits)	Yield (%)
Dialyzed plasma	5850	6500	1.11	
$(NH_4)_2SO_4$	1600	6400	4.0	98.4
DEAE-Sephadex	297	4270	14.4	69.2
Sephadex G-100	33	2778	84.2	43.7
Blue Sepharose	0.21	1022	4870	15.7

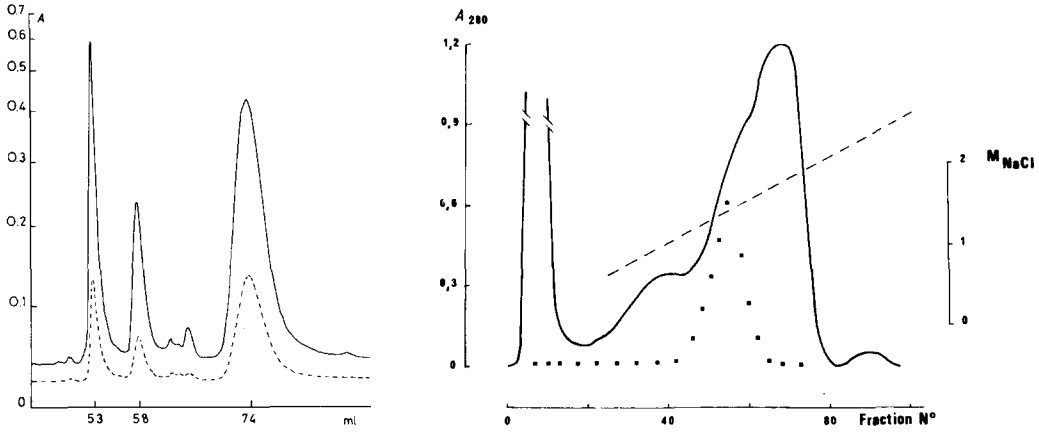

Fig. 1. Product analysis on amino acid analyser of enkephalin-degrading aminopeptidase-treated Leu-enkephalin. 52 ml peak = Tyrosine; 58 ml peak = Gly-Gly-Phe-Leu and 74 ml peak = Leu-enkephalin.

Fig. 2. Chromatography of 30—40% $(NH_4)_2SO_4$ fraction on DEAE-Sephadex G-50. The enzyme activity (■) was measured with Leu-enkephalin as substrate and is expressed in arbitrary units.

preted with some caution since gel-filtration anomalous behaviour was reported for many serum proteins [11]. The enzyme exhibits a maximum activity between pH 7.5 and 8.3; the Lineweaver-Burk plot of enzyme activity using enkephalin as substrate is reported in Fig. 4 and an apparent Michaelis constant of 0.4 mM was calculated. A slight inhibition was observed at high substrate concentrations.

Other peptides behave as substrates for the enkephalin-degrading aminopeptidase (Table II): Tyr-OEt, Tyr-NH_2, Tyr-β-naphthylamide, Tyr-Gly, Leu-Gly-

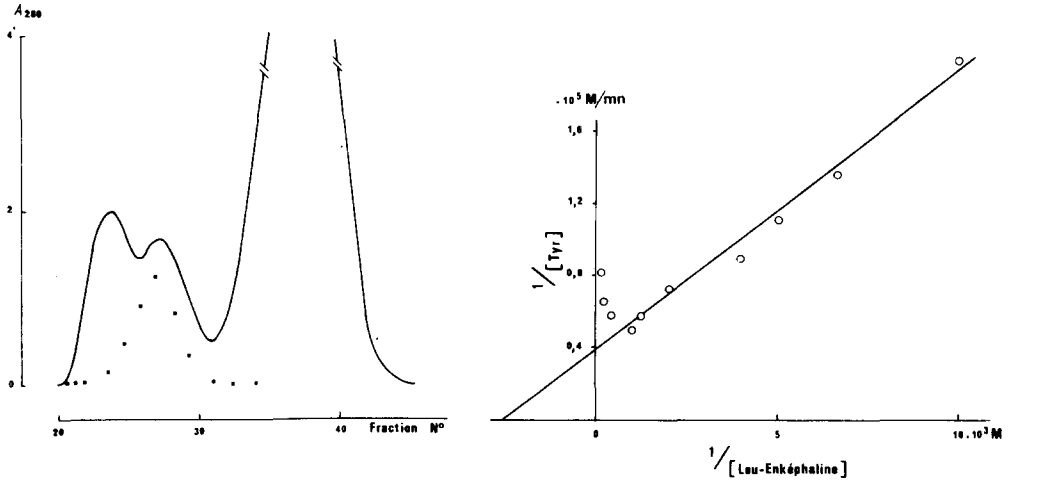

Fig. 3. Chromatography of DEAE-Sephadex active fractions on Sephadex G-100. The enzyme activity (■) was measured with Leu-enkephalin as substrate and is expressed in arbitrary units.

Fig. 4. Lineweaver-Burk plot of enkephalin-degrading aminopeptidase activity.

TABLE II

SUBSTRATE SPECIFICITY OF HUMAN PLASMA ENKEPHALIN-DEGRADING AMINOPEPTIDASE

Activities are expressed as % of Leu-enkephalin.

Peptide	Activity (%)	K_m	Products
Tyr-Gly-Gly	78.8	$3.5 \cdot 10^{-3}$ M	Tyr and Gly-Gly
Phe-Gly-Gly	79.5	$3.5 \cdot 10^{-3}$ M	Phe and Gly-Gly
Trp-Gly-Gly	46.5	$3.5 \cdot 10^{-3}$ M	Trp and Gly-Gly
Tyr-Gly-Gly-Phe-Leu (enkephalin)	100	$4 \cdot 10^{-4}$ M	Tyr and Gly-Gly-Phe-Leu

Gly, Val-Gly-Gly, β-casomorphin, insulin B chain, β-endorphin and desamino-enkephalin are not cleaved by the enzyme. Two features seem essential for an appreciable susceptibility to enzyme action, a minimum of length of three amino acids and an aromatic amino acid as N-terminal of the peptide. Tyr-Gly-Gly and Phe-Gly-Gly show similar susceptibility to the enzyme action, while Trp-Gly-Gly is a poorer substrate. A special comment is occasioned by β-casomorphine (Tyr-Pro-Phe-Pro-Gly-Pro-Ile): this opiate peptide resulting from the digestion of casein [12] has been recently characterized [13], no liberation of tyrosine could be observed even at high concentrations of the enzyme, thus showing the resistance of the Tyr-Pro bond to the aminopeptidase activity.

The enkephalin-degrading aminopeptidase specificity differs from brain [14] and serum [15] aminopeptidases so far characterized, in respect to both substrate size and amino acid sequence, in fact, the enzyme does not appreciably hydrolyse naphthylamines or dipeptides and is not inactivated by EDTA, it displays its activity only against relatively short peptides, larger ones like insulin B chain (N-terminal phenylalanine) not being degraded. Analogous results were recently reported with aminopeptidase M on chemically prolonged enkephalins [16]. Similarly endorphin is a very poor substrate for the aminopeptidase, this is presumably due to its size as well as to the steric situtation of the N-terminal tyrosine, which is believed to be hindered by an intramolecular bonding with the C-terminal part of the molecule [17]. The plasma aminopeptidase shows, on the basis of the kinetic parameters, a marked preference for enkephalins, when compared with other similar substrates. The actual efficiency of the enzyme could be questioned if the K_m of the enzyme (0.4 mM) is compared to the low concentration of the substrate actually circulating in vivo. This low substrate concentration (approx. 1 nM) is in fact partly compensated for by a high circulating enzyme activity (cf. Table I, $1.11 \times 70\,000 \approx 77\,000$ I. munits/l plasma for an average of 65 g/l of circulating proteins). In these conditions (at pH 7.3 and 37°C) the Michaelis-Menten equation predicts an enzyme activity of $77\,000 \cdot 10^{-9}/(4 \cdot 10^{-4} + 10^{-9}) \approx 0.2$ nmol · min^{-1} for a postulated substrate concentration of 1 nM. If pseudo-first-order kinetics are postulated for the degradation, a half-life of $0.693/0.2 \approx 3$ min is to be expected at such a concentration. This value is in good agreement with the half-life of a few minutes, reported for enkephalins in vivo [18]. It thus appears that, by itself, the activity of the enzyme we have purified could account for most of the enkephalin-inactivating activity of human plasma. With caution and awaiting deeper

characterization, this enzyme could therefore be considered as the human blood 'enkephalinase'.

It can be legitimately asked, though probably not yet answered at this point, whether the presence of such a potent enkephalin-degrading activity in human blood finds its physiological significance in the regulation of the peptide concentration before reaching the receptor. The fact that endorphin is resistant to this inactivating action and the absence of correlation between analgesic potency and biodegradation of enkephalin analogues, recently pointed out [19], seems to indicate that generally speaking the significance of this proteolytic action has been somewhat over-estimated. Notwithstanding the importance of enkephalin inactivation by aminopeptidase, the liberation of free aromatic amino acids and/or of the residual peptide by this enzyme in the presence of the appropriate substrates might also be of physiological interest.

However, if a more general pharmacological use of these peptides develops, it will be a considerable advantage to have a better understanding of how blood enzymes alter the level of enkephalins and moreover, proper use of knowledge about aminopeptidase may allow a better definition of long-lasting enkephalin analogues of biological and/or pharmacological interest.

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