HUMAN PREGNANCY OXYTOCINASE ISOLATION AND SUBSTRATE CHARACTERISTICS

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Ethanol fractionation and chromatography on DEAE-cellulose and on Sephadex G-200 were used for preparing an electrophoretically homogeneous oxytocinase (cystinaminopeptidase) from human pregnancy serum. The enzyme hydrolyzes a number of aminopeptidase and aminoacylarylamidase substrates. An excess of arylamides of aromatic amino acids inhibits the hydrolytic activity of the enzyme. The hydrolytic activity of oxytocinase is also suppressed in the presence of the tetrapeptide H-Cys(Bzl)-Pro-Leu-GlyNH_2. The isolation method appears to be suitable for large-scale preparations.

In a previous paper[1] we described the separation of some aminopeptidase components of pregnancy serum using ion-exchange chromatography and gel filtration. In the present paper we describe a modification of the method which makes it possible on a large scale to isolate one of the aminopeptidases, oxytocinase, which during pregnancy represents a substantial component of the total aminopeptidase activity of serum. Using this electrophoretically homogeneous oxytocinase we studied its substrate specificity toward aminoacyl-β-naphthylamides and some peptides.

EXPERIMENTAL

Material. Leucine *p*-nitranilide and S-benzylcysteine *p*-nitranilide were obtained from Dr E. Kasafírek, Research Institute of Pharmacy and Biochemistry, Prague; leucine β-naphthylamide was synthesized according to Hanson and coworkers[2,3], cysteine di-β-naphthylamide and S-benzylcysteine-β-naphthylamide were prepared at the Institute of Physiological Chemistry (Halle), β-naphthylamides of Ala, Arg, Asn, Gln, His, Ile, Lys, Leu, Phe, Pro, Tre, Trp, Tyr and Val were kindly provided by Dr H. Nesvadba (Vienna)[4]. The peptides Leu-GlyNH_2, Pro-Leu-GlyNH_2, Cys(Bzl)-Pro-Leu-GlyNH_2, Asn-Cys(Bzl)-Pro-Leu-GlyNH_2, Gln-Asn-Cys(Bzl)-Pro-Leu-GlyNH_2 and Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-GlyNH_2 were prepared in the Department of organic syntheses of this Institute[5–9] and we are indebted in this connection to Dr K. Jošt and Prof. J. Rudinger.

Polyacrylamide gel electrophoresis. The gels were prepared according to Davis[10] with the slight modification in that Temed was replaced with EDTA and the concentration gel was omitted[11]. The amidopeptidase activity was estimated by incubating the gel in a solution of leucine β-naphthylamide in the presence of Echtblausalz BB at pH 7·0. The proteins were detected with 0·5% amido-black in 1M acetic acid. Agar electrophoresis was done according to Wieme[12].

Estimation of enzyme activites. Aminoacylarylamidase activity was expressed by the rate of hydrolysis of the chromogenic substrate leucine *p*-nitranilide, either by direct spectrophotometry of *p*-nitraniline or by diazotization of *p*-nitraniline and its coupling with N-1-naphthylethylenediamine[13–14]. The degree of hydrolysis of aminoacyl-β-naphthylamides was determined as follows. The incubation mixture consisted of 0·25 ml 10 mM solution of S-benzylcysteine β-naphthylamide in 15% dimethylformamide (0·5 ml 100 mM phosphate buffer of pH 6·85). The enzyme was added in such an amount as to permit during incubation at most a 20% substrate hydrolysis. The volume was made to 1 ml with water. The enzyme reaction was stopped by adding 1 ml 10% trichloroacetic acid. 0·5 ml of an acidified reaction mixture was taken into the reaction for determination of free β-naphthylamine.

Hydrolysis of peptide fragments of oxytocin. The peptide (2 mM solution) was incubated with oxytocinase (1·5 mU) in 40 mM sodium phosphate buffer of pH 7·0. The reaction was stopped by acidification and the free amino acids were separated electrophoretically. The amount of split amino acid was determined after detection with ninhydrin and fixation with cupric nitrate colorimetrically.

Paper electrophoresis. Electrophoretic separation of amino acids and peptides on Whatman No 3 paper was carried out according to Durrum[15] in a pyridine–acetate buffer of pH 5·5 or in 1M acetic acid for 60 min at a potential gradient of 26 V/cm. Proteins were determined according to Lowry and coworkers[16].

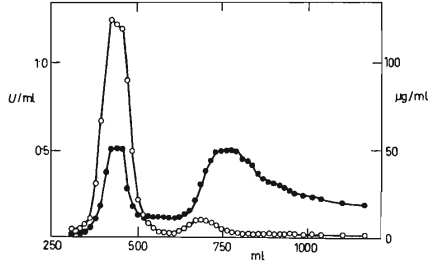

FIG. 1

Gel Filtration on a Sephadex G-200 Column

12 ml fraction obtained from a DEAE-cellulose column (2·0—2·5) mg protein/ml) was separated chromatographically on a column of Sephadex G-200 (4·5 × 75 cm) which was in equilibrium with 0·01M phosphate buffer of pH 7·0. The column was eluted at a rate of 10 ml/h. The aminopeptidase activity is expressed in U/ml eluate (○), the protein concentration is given in μg/ml (●). The eluate volume is given in ml.

Preparation of enzyme. The ethanol fractionation of retroplacental serum was done as described before[1]. The enzymically active fraction separated during alcohol precipitation was stirred in 50—70 ml 0·05M sodium phosphate buffer of pH 5·5 and centrifuged. The precipitate was repeatedly washed with 50 ml 0·05M buffer, centrifuged, the supernatant was combined with the previous one and the precipitate discarded. A column of DEAE-cellulose (0·7—0·8 m-

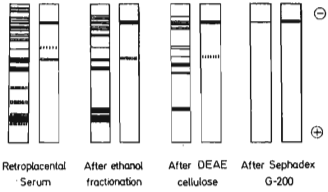

FIG. 2

Polyacrylamide Gel Electrophoresis

Samples of protein (5—50 µl) were placed in 27% sucrose. Electrophoresis was carried out at 2—3°C, for 3 h at 200 V and 4—5 mA per gel tube. The gels were stained for proteins and for aminopeptidase activity.

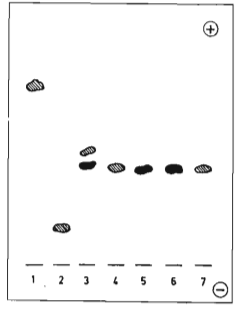

FIG. 3

Agar-Gel Electrophoresis

For comparative samples we used aminopeptidases from bovine lens (*1*), particle-bound aminopeptidases from pig kidney (*2*), eluate from DEAE-cellulose containing oxytocinase and aminopeptidase from retroplacental serum (*3*) and the peak fractions of oxytocinase obtained by gel filtration (*4—7*). Proteins and aminopeptidases were detected as described in the text.

equiv/g dry weight) 3 × 10 cm was equilibrated with a 0·05M phosphate buffer of pH 5·5 and the above supernatant was applied to it. After adsorption, the column was washed with 100 ml of the same buffer and eluted with 0·2M-NaCl in the same buffer. The active fractions leave the column together with the front of eluted proteins. Oxytocinase kept at the pH of the elution buffer and at 2–4°C is stable for several weeks.

Gel filtration on Sephadex G-200. 10–15 ml eluate from the DEAE-column was chromatographed on a column of Sephadex G-200 (4·5 × 75 cm) which was equilibrated with 0·01M sodium phosphate buffer of pH 7·0 at an elution rate of 10 ml/h. The distribution of proteins and of activity toward leucine *p*-nitranilide is shown in Fig. 1, an electrophoretic pattern from polyacrylamide and from agar indicates the purity of the isolated enzyme (Fig. 2 and 3). Table I presents the average balance sheet of the isolation procedure for oxytocinase as found on using leucine *p*-nitranilide.

TABLE I

Isolation of Oxytocinase

Enzyme activity and protein concentration were determined as described in the text.

Fraction	Specific activity U/mg protein	Yield %
Retroplacental serum	0·0024	100
Ethanol fraction	0·024–0·030	50–60
Ion-exchange chromatography	0·2–0·6	40–45
Gel chromatography on Sephadex G-200	8–12	25–38

RESULTS AND DISCUSSION

The modification of the original isolation method concerning the chromatographic procedures affected positively both the purity of the final preparation and its yield. At pH values lower than 6 most of the ballast proteins are not adsorbed to DEAE-cellulose, the remainder, mostly proteins of the size of albumins, are separated during gel filtration. During this operation, the electrophoretically most mobile component with aminopeptidase activity is separated. It was shown in preliminary experiments that this component shows a substrate specificity different from that of oxytocinase, *e.g.* it hydrolyzes most rapidly Ala-β-naphthylamide and Met-β-naphthylamide. It will also split faster the peptide bonds in the studied fragments of the oxytocin molecule. Its electrophoretic mobility, size and specificity suggest the identity of this enzyme with the aminopeptidase isolated recently and studied in detail by Kulling and Yman[17].

Like some other aminopeptidases, oxytocinase will hydrolyze a number of substrates, whether peptides or aminoacylarylamides. The study of Tuppy and coworkers[18]

TABLE II

Hydrolysis of Aminoacyl β-Naphthylamides

Hydrolytic activity is expressed in μmol split substrate per 1 mg protein nitrogen per hour. The value for Leu-β-naphthylamide is set equal to 100%.

Naphthylamide	Relative rate of hydrolysis %	Naphthylamide	Relative rate of hydrolysis %
Leu	100·0	Asp (β)	1·5
Lys	72·5	Glu (α)	16·5
Ala	50·4	Glu (γ)	7·0
Ser	15·2	Tre	12·5
His	8·0	Met	62·5
Cys-Cys	1·2	Arg	18·5
Pro	8·0	Phe[a]	11·0 — 65·0
Val	4·5	Tyr[a]	15·5 — 35·0
Ile	3·2	Trp[a]	2·6 — 12·0
Hypro	2·3	H-Cys(Bzl)[a]	32·0 — 120·0
Asp (α)	3·5		

[a] Substrates which showed excess-substrate inhibition of enzyme activity.

TABLE III

Rate of Splitting of the N-Terminal Amino Acid from Peptide Fragments of the Oxytocin Molecule

The rate of hydrolysis of the N-terminal peptide bond in the peptide was determined as shown in the experimental section and was expressed as a first-order rate constant k_1 in min^{-1}. For comparison, the rate of splitting of the N-terminal amino acid is referred to the rate of hydrolysis of leucine *p*-nitranilide.

Peptide	Hydrolysis k_1	Peptide	Hydrolysis k_1
Leu-$GlyNH_2$	2·760	Asn-Cys(Bzl)-Pro-Leu-$GlyNH_2$	0·062
Gln-Asn-Cys(Bzl)-Pro-Leu-$GlyNH_2$	0·083	Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-$GlyNH_2$	0·820

demonstrated the ability of the enzyme to split off the acid residue from aminoacyl β-naphthylamides. Beránková and coworkers [19] studied the properties of this enzyme in a mixture with other aminopeptidases isolated from pregnancy serum using

a number of peptides derived from oxytocin. The hydrolysis of oxytocin, vasopressin and angiotensinamide was taken up in detail by Sjöholm and Yman[20]. The determination of the hydrolytic properties expressed as the rate of hydrolysis of aminoacyl β-naphthylamides showed (Table II), in addition to confirming earlier results[18], that this enzyme can be inhibited by excess substrate, as observed under our conditions first of all with β-naphthylamides of aromatic amino acids and S-benzylcysteine.

Fig. 4 shows schematically the splitting of some peptides and Table III contains the rate constants k_1 for the rate of splitting of the N-terminal amino acid from the individual peptides. (The value of k_1 refers to oxytocinase activity toward leucine *p*-nitranilide). It may be seen that peptides with Leu and Ile at the N-terminus of the chain are split most rapidly, followed by peptides with Gln and Asn. The participation of proline in the peptide bond is demonstrated by the considerable resistance toward aminopeptidase splitting with oxytocinase as was observed in the case of H-Cys(Bzl)-Pro-Leu-GlyNH_2 and Pro-Leu-GlyNH_2. All the peptides shown, with the exception of the tripeptide, inhibit hydrolysis of leucine-*p*-nitranilide with oxytocinase but only one of them (H-Cys(Bzl)-Pro-Leu-GlyNH_2) may be considered as a true inhibitor while the others are merely competing substrates.

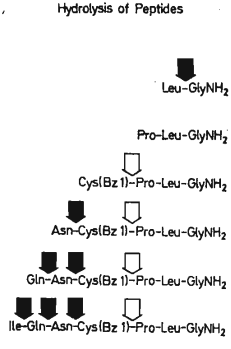

FIG. 4

Oxytocinase, regarded from the point of view of its catalytic properties, has the properties of most of the hitherto described aminopeptidases or aminoacylarylamidases, *i.e.* a broad substrate specificity extended by the ability to split peptide bonds in the cyclic peptides oxytocin and vasopressin.

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