ISOLATION AND PARTIAL CHARACTERIZATION OF A PEPTIDASE WITH TRYPSIN-LIKE ACTIVITY FROM BOVINE ADENOHYPOPHYSIS

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A reproducible method has been developed for the isolation of the adenohypophyseal enzyme with a trypsin-like activity. The enzyme is able to hydrolyze N^{α}-benzoyl-L-arginine-*p*-nitroanilide, a fluorogenic substrate CBzl-Arg-Arg-β-naphthyl amide and some peptides with one or two accumulated basic amino acids in the chain. The optimum pH for hydrolysis of the chromogenic substrate was within the range 6·0—7·0 (K_m = 0·66 mmol l^{-1}), in the case of the fluorogenic substrate the range was between 7·0 and 7·5 (K_m = 1·2 μmol l^{-1}). The enzyme is activated by cysteine and dithiothreitol and inhibited by SH-poisons. The molecular weight of the enzyme, determined by means of two independent methods, was approximately 25 kDA.

The releasing of biologically active peptides from non-active higher-molecular forms is connected with existence of recognition signals in form of pairs of basic amino acids in a non-active precursor, which determine the sequence of the biologically-active peptide[1-10]. Enzymes recognizing this signal are involved in the post-translational proteolytic cleavage of the protein or peptide precursors. In the case of enzymes splitting preferentially the sequence at the site of localization of accumulated pairs of basic amino acids, the analogy with enzymes with trypsin-like activity is often examined[11-19]. The specificity of these enzymes varies. For example, Cathepsin B isolated from calves brains[18] splits the peptide bond following a pair of basic amino acids, as well as that following a single one. On the other hand, yeast protease requires for its action presence of an accumulated pair of basic amino acids[20]. The coexistence of [8-arginine]vasopressin and its des-glycine amide derivative in the brains[21-23] raises a question on the enzyme system involved in the creation of vasopressin and its des-glycine derivative respectively.

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The isolation of an enzyme system from bovine adenohypophysis, with the ability to cleave the Arg-GlyNH_2 bond in vasopressin and with the retained affinity to peptides with a pair of basic amino acids, is described in this paper.

EXPERIMENTAL

Frozen bovine hypophyses were purchased from the Prague Meat Industry (Slaughter-houses in Písnice and Čakovice). AH-Sepharose 4B, Sephadex G-75 from Pharmacia (Uppsala, Sweden), TES from Sigma (U.S.A.). TLC plates Silufol were produced by Kavalier (Czechoslovakia), dithiothreitol, 1-ethyl-3-diaminopropyl carbodiimide HCl and TEMED (N,N,N',N-tetramethylene diamine) by SERVA (Heidelberg, F.R.G.). DSS was purchased from BioRad (Richmond, U.S.A.), Triton X-100 from Fluka (Basel, Switzerland), trypsin from Worthington (Freehold, U.S.A.). Other chemicals were produced in Czechoslovakia and were of analytical grade.

N^{α}-Benzoyl-L-arginine-*p*-nitroanilide (BANA) was synthesized by one of us (E.K.), the octapeptide Tyr-Gly-Gly-Phe-Leu-Lys-Lys-Leu[24,25] was given to us kindly by Dr J. Vičar of the Institute of Chemistry, Faculty of Medicine, Palacky University, Olomouc, [8-L-Arg]vasopressin[26], by Dr M. Flegel of the Institute of Pharmacology, Czechoslovak Academy of Sciences. [8-Lys]vasopressin, [8-L-Arg]deamino-6-carba vasopressin sulfoxide[27] and [8-L-ornithine]deamino-6-carba vasopressin[28] were obtained from Dr K. Jošt of the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, [8-L-homolysine]vasopressin[29] from Dr G. Lindeberg of Pentapharm (Basel, Switzerland) and the lysozyme preparation from Dr M. Tichá from the Department of Biochemistry, Faculty of Natural Sciences, Charles University, Prague. CBzl-Arg-Arg-β-naphthyl amide was purchased from Bachem (Bubendorf, Switzerland).

Methods

Homogenization and acetone fractionation of bovine hypophyses. Frozen hypophyses (1 kg) were left overnight at 4°C. Then adenohypophases were separated (300 g) and homogenized successively in 0·1% NaCl containing 2% 1-butanol and 1 mmol l^{-1} EDTA (w : v = 1 : 2), in a high-speed homogenizer (6 000 rpm, 2 × 1 min). The homogenate was filtered through double gauze, 20% Triton X-100 was added to the filtrate up to a final concentration of 0·2% and the pH was adjusted to 6·1 with H_3PO_4. The homogenate was stirred for 1 h at 4°C, the precipitate formed was separated by centrifugation (Beckman JM-6, 4 000 rpm, 30 min, 4°C) and the pH of the supernatant was adjusted with H_3PO_4 (5 mol l^{-1}) to a value of 4·5. The supernatant was left overnight at 37°C, then cooled to 4°C and the precipitate was separated by centrifugation (4 000 rpm, 30 min, 4°C). The temperature of the supernatant was lowered to 0°C and cooled acetone (−5°C) was added gradually up to a final concentration of 45%. The mixture was stirred for 30 min at 0°C and the precipitate was separated by centrifugation (3 000 rpm, 40 min, 0°C). Cooled acetone was added again to the supernatant up to a 65% final concentration, the precipitate was collected by centrifugation (3 000 rpm, 20 min, 0°C), suspended in 100 ml of water with EDTA (5 mmol l^{-1}). The solution was dialyzed 16 h at 4°C against Na-phosphate buffer (10 mmol l^{-1}), pH 6·8, containing EDTA (1 mmol l^{-1}). Precipitated proteins were separated by centrifugation (3 000 rpm, 15 min, 4°C) and the supernatant was kept at 4°C before further processing (not longer than 16 h).

Chromatography of the enzyme on DEAE-Sephadex A-50. The supernatant obtained by the preceding operations (total amount of proteins 480 mg) was applied to a column of DEAE-

-Sephadex A-50 (3·5 × 12 cm) in equilibrium with Na-phosphate buffer (10 mmol l^{-1}), pH 6·8, containing EDTA (1 mmol l^{-1}). The column was rinsed with 70 ml of the equilibration buffer. The elution was carried out with NaCl (step-wise, first with 100 mmol l^{-1}, then with 150 mmol . l^{-1} in the equilibration buffer). Enzymatically active fractions (see further) were collected, dialyzed against water with EDTA (1 mmol l^{-1}) and lyophilized.

Gel filtration on Sephadex G-75. The fraction obtained by means of ion-exchange chromatography (100 mg) was dissolved in 5 ml of Na-acetate buffer (50 mmol l^{-1}), pH 5·5, with EDTA (1 mmol l^{-1}) and adsorbed on a column of Sephadex G-75 (117 × 3·2 cm) equilibrated in the same buffer. The elution was carried out at a rate of 4·2 ml/15 min. Active fractions were collected, dialyzed against water containing EDTA (1 mmol l^{-1}) and lyophilized.

Covalent chromatography on chlormercuribenzoate-AH-Sepharose 4B. Preparation of modified Sepharose 4B — 1 g of AH-Sepharose 4B was swelled in NaCl solution (0·5 mol l^{-1}) and rinsed with water. Then the gel was suspended in 4 ml of 40% N,N'-dimethylformamide and 125 mg of sodium *p*-chlormercuribenzoate were added during stirring. The pH of the suspension was adjusted to 4·8 with HCl (6 mol l^{-1}). Then, 154 mg of 1-ethyl-3-dimethylaminopropyl carbodiimide were added, the pH being maintained at 4·8 by means of 2M-HCl. The modified Sepharose was washed after 16 h successively witz 40% N,N'-dimethylformamide, water and Na-acetate buffer (25 mmol l^{-1}), pH 5·0, containing NaCl (0·2 mol l^{-1}).

Chromatography on modified Sepharose. The enzyme fraction obtained after the gel filtration (5 mg) was dissolved in 5 ml of Na-acetate buffer (25 mmol l^{-1}), pH 5·0, containing NaCl (0·2 mol l^{-1}), and adsorbed on a column of the modified Sepharose (0·8 × 6 cm) in equilibrium with the same buffer. After rinsing of the column, the elution was carried out with a solution of 1 mmol l^{-1} dithiothreitol in the equilibration buffer and the active fractions were collected.

Determination of the enzyme activity by means of N^{α}*-benzoyl-*L*-arginine-p-nitroanilide.* To 0·5 ml of Na-phosphate buffer (0·2 mol l^{-1}), pH 6·0, containing EDTA (2 mmol l^{-1}) and dithiothreitol (2 mmol l^{-1}), 20—250 μl of the enzyme fraction were added and the volume was adjusted to 0·75 ml. Enzyme activation proceeded at laboratory temperature for 15 minutes. Then 250 μl of N^{α}-benzoyl-L-Arg-*p*-nitroanilide solution (5 mmol l^{-1} in 10% dimethylformamide or dimethylsulfoxide) were added to the reaction mixture and the incubation was carried out for 1—2 h at 37°C. The reaction was stopped by the addition of 0·1 ml concentrated acetic acid. The concentration of the released *p*-nitroaniline was determined spectrophotometrically at 405 nm; the enzyme activity is expressed in pKat.

Determination of the pH *dependence of* K_m *and* V_{max} *of the enzymic hydrolysis of* N^{α}*-benzoyl-*L*-Arg-p-nitroanilide.* For the measurement of the pH dependence of kinetics of the enzymic hydrolysis (pH range 6·0—8·0), Na-phosphate buffers (0·2 mol l^{-1}) with EDTA (2 mmol l^{-1}) and dithiothreitol (2 mmol l^{-1}) were utilized.

Determination of the effect of activators (dithiothreitol, cysteine) and inhibitors on enzyme activity. The reaction mixture consisted of 0·5 ml of the appropriate activator, 0·02—4·0 mmol . l^{-1} of inhibitor (iodacetamide 0·2—2·0 mmol l^{-1}, *p*-Cl-mercuribenzoate and $HgCl_2$ in a concentration range of 0·02—0·2 mmol l^{-1}), 0·25 ml of the enzyme fraction and 0·25 ml of 5 mmol . l^{-1} solution of N^{α}-benzoyl-L-Arg-*p*-nitroanilide. The substrate was added after preincubation of the mixture at laboratory temperature, and the reaction proceeded further as described in the paragraph concerning the determination of the enzyme activity.

Fluorescence measurement. The measurement of the enzymic degradation of the fluorogenic substrate with adenohypophyseal enzyme was carried out with the aid of a microprocessor-controlled fluorimeter Spex (U.S.A.). Excitation and emission maxima, 335 and 410 nm respecti-

vely, were determined from uncorrected excitation and emission spectra of the substrate (CBzl--Arg-Arg-β-naphthyl amide) and the product of the enzymic hydrolysis (β-naphthyl amide). Uncorrected emission spectra for β-naphthyl amide at concentrations of 1, 0·5, 0·2, 0·1, 0·07 and 0·05 μmol l^{-1} respectively, served for the construction of a calibration curve for determination of the initial rates of the hydrolysis.

The measurement of the kinetics of the hydrolysis of CBzl-Arg-Arg-β-naphthyl amide with the adenohypophyseal enzyme was carried out at 35°C and pH range 5·0—8·5. The solution containing 300 μg of the enzyme, 1 ml of TES buffer (0·05 mol l^{-1}) of the appropriate pH and 1 ml H_2O was left for 15 min at laboratory temperature for enzyme activation (the concentration of dimethylformamide utilized for substrate solubilization did not exceed 0·5%). The solution was transferred into the thermostated fluorimeter cuvette and after temperature equilibration, the reaction was started by the addition of the substrate (0·3—1·5 μmol l^{-1}; the volume was adjusted to 2·7 ml with water) and then followed for 20 minutes.

Determination of relative molecular weight of the enzyme. The determination was carried out electrophoretically in polyacrylamide gel in the presence of 0·1% sodium dodecylsulfate according to Weber and Osborn[30], and by means of sedimentation centrifugation[31].

Hydrolysis of a model peptide — enkephalin analogue — with a pair of basic amino acid residues at the carboxyterminal part of the chain with the adenohypophyseal enzyme (or trypsin). The reaction mixture consisting of 50 μl of 10 mmol l^{-1} octapeptide Tyr-Gly-Gly-Phe-Leu-Lys-Lys--Leu, 25 μl of adenohypophyseal enzyme solution (1 mg ml^{-1}) obtained by means of covalent chromatography or of bovine trypsin solution (2 mg ml^{-1} in 1 mmol l^{-1} $CaCl_2$) and 25 μl buffer (in the case of trypsin 50 mmol l^{-1} Na-phosphate buffer, pH 7·6, for adenohypophyseal enzyme 20 mmol l^{-1} Na-acetate buffer, pH 5·1), was incubated for 1 h at 37°C. Analyses of the products were carried out by means of TLC in two systems: 1-butanol–acetic acid–water (2 : 1 : 1) and 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6). Detection was carried out with ninhydrin.

Hydrolysis of vasopressin and its synthetic analogues with the adenohypophyseal enzyme. Peptides were dissolved in 1 mmol l^{-1} HCl to a final concentration of 1 mmol l^{-1}, the enzyme was dissolved in Na-phosphate buffer (0·1 mol l^{-1}), pH 6·0, containing dithiothreitol (4 mmol . l^{-1}) and EDTA (4 mmol l^{-1}) to a concentration of 0·6 mg ml^{-1}. 10 μl of the peptide solution was diluted with 0·05 mol l^{-1} Na-phosphate buffer to 0·5 ml, 0·5 ml of enzyme solution was added and the mixture was incubated for 2 h at 37°C. The reaction was terminated by the addition of 0·5 ml of acetic acid (1 mol l^{-1}). The concentration of pressorically active peptides was determined on despinalized rats[32], after neutralization of the solution. A sample incubated without the enzyme served as a control sample.

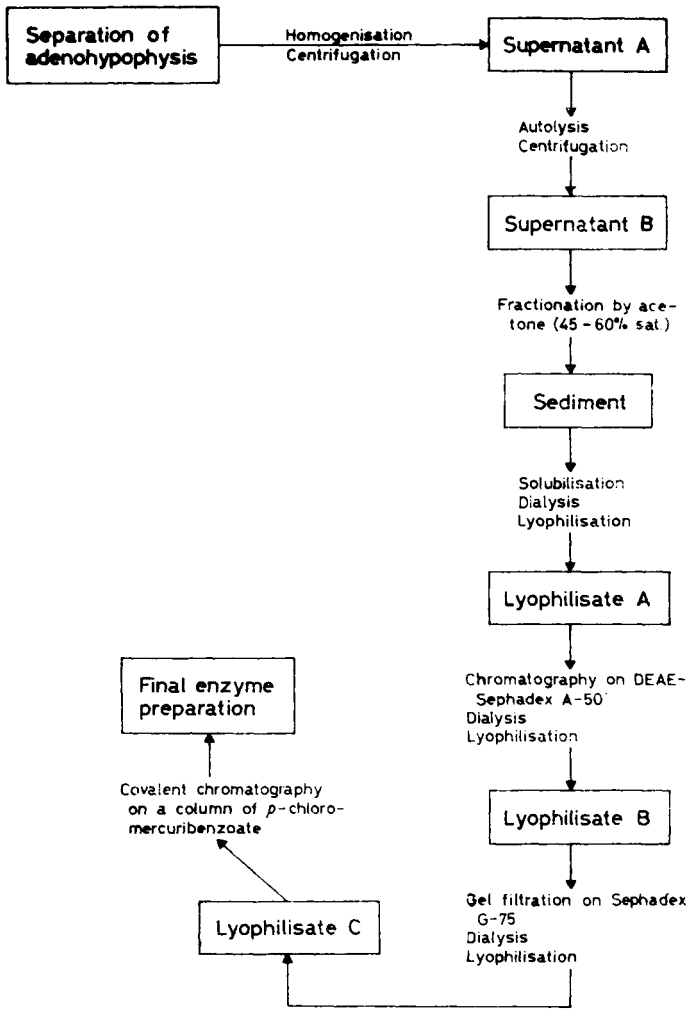
The concentration of proteins in the samples was determined according to Lowry et al.[33] and by means of polyacrylamide electrophoresis according to Maurer[34].

RESULTS

The balance of the process of isolation of the adenohypophyseal enzyme (Scheme 1) is shown in Table I*. Autolysis caried out after the homogenization represents a crucial operation which results in an almost 30 times higher specific activity of the enzyme in comparison with that of the homogenate. Acetone fractionation, together with

* A preliminary report was presented at the Meeting Molecular and Cellular Regulation of the Enzyme Activity, Halle/Saale, August 17th, 1986.

ion-exchange and permeation chromatography, increased further the specific activity (both these steps are illustrated in Figs 1 and 2). Covalent chromatography on



SCHEME 1

Procedure for the isolation of bovine adenohypophyseal enzyme

modified AH-Sepharose represented the final step of the purification process (Table II). The electrophoretically homogeneous enzyme preparation obtained by elution of the column with dithiothreitol was employed for the characterization of its properties.

The relative molecular weight of the adenohypophyseal enzyme was determined by means of two independent methods. SDS electrophoresis in polyacrylamide gel revealed a value of approximately 25 000 daltons (Fig. 3), the value determined by means of the sedimentation analysis (300 000 rpm, 17 h) was 24 500 ± 3% DA.

TABLE I

Balance of the isolation of the adenohypophyseal enzyme

Isolation step	Amount of proteins mg	Total activity pKat	Total activity %	Specific activity pKat/mg	Degree of purification
Homogenate	72 790	7 280	100	0·10	1·0
Extract	32 490	5 950	81·7	0·18	1·8
Autolysate	6 690	18 280	251	2·73	27·3
Acetone fraction 45—65%	482	15 270	210	31·67	317·0
DEAE-Sephadex A-50	80	6 420	88·2	80·20	802·0
Sephadex G-75	26	4 720	64·8	181·38	1 814·0

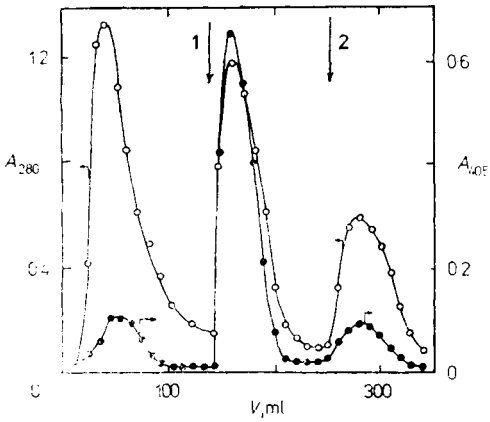


FIG. 1

Chromatography of the adenohypophyseal enzyme on a column of DEAE-Sephadex A-50. ○ Absorbance at 280 nm, ● absorbance at 405 nm; *V* eluate volume, 1 0·1 mol l^{-1} 2 0·15 mol l^{-1}

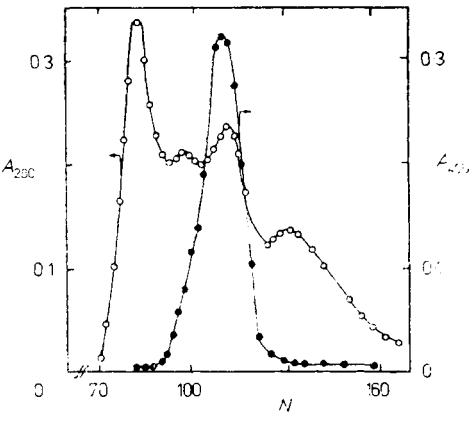


FIG. 2

Gel filtration of the adenohypophyseal enzyme on Sephadex G-75. ○ Absorbance at 280 nm, ● absorbance at 405 nm; *N* number of fractions

N^{α}-benzoyl-L-arginine-*p*-nitroanilide was employed as a basic substrate for the adenohypophyseal enzyme. The highest affinity of the enzyme of this substrate was

TABLE II

Balance of the covalent chromatography of adenohypophyseal enzyme with the use of two different elution solutions

Isolation	1 mmol l^{-1} DTT				0·5 mmol . l^{-1} $HgCl_2$			
	Proteins		Activity		Proteins		Activity	
	mg	%	pKat	%	mg	%	pKat	%
Adsorbed on column	2·14	100	450	100	2·14	100	450	100
Passed through	1·50	53	124	27	1·15	53	124	27
Eluted from column	0·79	37	311	69	0·87	40	270	60
	Degree of purification 2				Degree of purification 1·5			

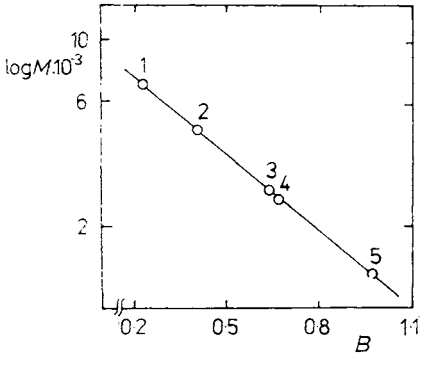


FIG. 3

Determination of the relative molecular weight of the adenohypophyseal enzyme by means of SDS-polyacrylamide gel electrophoresis. 1 Bovine albumin, 2 ovalbumin, 3 lysozyme dimer, 4 adenohypophyseal enzyme, 5 lysozyme monomer; log *M* logarithm of the relative molecular weights, *B* electrophoretic mobility

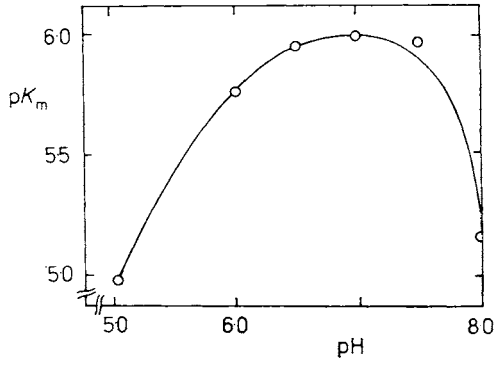


FIG. 4

Dependence of pK_m ($-\log K_m$) on pH for the hydrolysis of CBzl-Arg-Arg-β-naphthyl amide

found at pH 6·0 (Table II), the rate of the complex enzyme–product breakdown was greatest at pH 7·0. The pH value of 6·5 was shown to represent the optimum for the binding and hydrolysis of this chromogenic substrate. The enzyme activity is increased in the presence of SH-compounds, e.g. cysteine or dithiothreitol. Cysteine increased the activity (in a concentration range of 0·05 – 3·0 mmol l^{-1}) by 28 – 61%, dithiothreitol (at a concentration of 0·01 mmol l^{-1}) by 23 – 96%. Iodacetamide (at concentrations from 0·1 to 1·0 mmol l^{-1}) and *p*-chlormercuribenzoate or $HgCl_2$ in a concentration range of 0·01 – 0·1 mmol l^{-1} caused a substantial decrease in the enzyme activity or complete inhibition.

TABLE III

pH-Dependent changes of K_m and V_{max} of hydrolysis of N^{α}-benzoyl-L-arginine-*p*-nitroanilide (*A*) and CBzl-Arg-Arg-β-naphthyl amide (*B*) with adenohypophyseal enzyme

pH	*A*		*B*	
	$K_m \cdot 10^4$ mol l^{-1}	V_{max} nmol min^{-1}	$K_m \cdot 10^6$ mol l^{-1}	V_{max} nmol min^{-1}
5·0	—	—	10·0	7·0
6·0	5·0	200	1·9	7·0
6·5	6·6	238	1·4	10·0
7·0	3·3	250	1·0	18·0
7·5	1·0	50	1·2	14·0
8·0	1·0	50	6·7	6·0

TABLE IV

Inactivation of vasopressin and its analogs with adenohypophyseal enzyme

Peptide	Residual amount of peptide, after incubation with enzyme, % of the initial amount
[8-L-Arginine]vasopressin	20
[8-L-Arginine]deamino-6-carba vasopressin sulfoxide	60
[8-L-Arginine]deaminovasopressin	35
[8-L-Lysine]vasopressin	50
[8-L-Homolysine]vasopressin	10
[8-L-Ornithine]deamino-6-carba vasopressin	10

Optimum pH for hydrolysis of the fluorogenic substrate CBzl-Arg-Arg-β-naphthyl amide was within the range 7·0–7·5. Table III summarizes K_m and V_{max}determined for pH values between 5·0 and 8·0, in comparison with those obtained with the chromogenic substrate. Fig. 4 demonstrates the course of the pH dependence of pK_m for the fluorogenic substrate.

The specificity of the adenohypophyseal enzyme was studied in relation to peptides containing either a pair of accumulated basic amino acids, or, at least one such amino acid in their chain. In the first case, we employed an analogue of Leu^5-enkephalin*, the carboxyterminal part of which was extended with a tripeptide Lys-Lys-Leu. In the second case we used several analogues of [8-arginine]vasopressin.

A comparative study of the octapeptide hydrolysis with trypsin and the isolated adenohypohyseal enzyme revealed different specificity of both the enzymes*. Trypsin hydrolyzed the peptide bond between amino acid residue 6 and 7, i.e. splits off the terminal dipeptide Lys-Leu, meanwhile the adenohypophyseal enzyme hydrolyzed the peptide bond between Lys in position 7 and the terminal Leu.

Results of the study of the hydrolysis of vasopressin and its analogs with the adenohypophyseal enzyme are summarized in Table IV. The reaction was carried out with low substrate concentrations in order to prevent interaction between disulfidic bridges in the peptides and enzyme SH-group(s). The decrease in the biological activity (pressoric test) of the carba analogues of vasopressin containing disulfide bridges shows, that at least a part of the decrease can be attributed to hydrolysis of the peptide bond between the basic aminoacid and terminal glycine amide. Biological activity of peptides with lysine, homolysine or ornithine in position 8 was also decreased after the incubation with enzyme.

DISCUSSION

Our procedure for the isolation of the adenohypophyseal enzyme employs an autolytic step, during which the specific activity of the enzyme increases by one order and the overall activity 2·5 times. The degree of purification of the enzyme obtained after acetone fractionation, ion-exchange and permeation chromatography was almost 2 000, yield 65%). Covalent chromatography using two different elution solutions resulted in a further 1·5–2·0 degree of purification, with regard to the solution employed (yield approximately 60–70%).

The bovine adenohypophyseal enzyme prepared as described in the Experimental was electrophoretically homogeneous. Its relative molecular weight was determined by two independent metods and the obtained results (25 and 24·5 – kDA, respectively) were in good agreement. The autolytic step in the isolation procedure compli-

* A preliminary report was presented on the occasion of the 18th EPS, Djuronäset, June 1984 (ref.[24]).

cates correlation with molecular weights found in literature. The enzyme, characterized as Cathepsin B (ref.[18]), isolated from whole calve brains by a procedure which involved a 2 h autolysis at 37°C had a molecular weight of 27 kDA. Enzymes specific to a pair of basic amino acids, isolated from rat brain cortex[11], using a procedure without the autolytic step exhibited molecular weights 90 kDA and 60 kDA respectively. Kinin-generating proteinases were released from their latent forms in pork adenohypophysis only after certain processes (autolysis, dialysis, enzyme degradation[35]), unfortunately the changes in molecular weights of the enzyme were not followed.

The adenohypophyseal enzyme was characterized by means of two substrates which exhibited affinities to the enzyme differing by 4 orders. Characteristics of the enzyme obtained with N^{α}-benzoyl-L-arginine-*p*-nitroanilide as a substrate reflects its sensitivity to SH-poisons, as well as the dependence of the rate of substrate hydrolysis and affinity on pH. The optimum pH for the hydrolysis was within the range 6·0 – 7·0.

The K_m value determined for the hydrolysis of the fluorogenic substrate CBzl-Arg--Arg-β-naphthyl amide with the adenohypophyseal enzyme ($1·0 - 1·2 \cdot 10^{-6}$ mol l^{-1} at optimum pH) demonstrates a high affinity of this substrate, which thus is very advantageous for the determining of low levels of the enzyme. Moreover, at substrate concentrations in the region of the K_m, the concentration of the organic solvent used for its dissolving is lower than 0·5% and, thus, below the limit affecting the enzyme activity adversely. The observed significantly higher affinity of the enzyme to a substrate with two accumulated basic amino acids proves that peptides with such molecular arrangement will represent more natural substrates than those with one isolated basic amino acid in the chain. Therefore, it is possible to assume that this type of enzyme will participate during the generation of biologically active peptides under physiological conditions. The enzyme is able to cleave the peptide bond following either one or two basic amino acids, but not between them, and thus exhibits the same specificity as the esteropeptidase isolated from rat brain cortex[11].

The study of SH-enzyme interactions with peptides containing a disulfidic bridge in their chain can be complicated by the reaction of SH-groups with the disulfide (at higher concentrations of both components), resulting in subsequent inactivation of the enzyme[36] and elimination of the substrate from the solution, which remains bound in the precipitate with the enzyme. The introduction of peptides with a monocarba bridge into the study eliminated this problem and showed that the adenohypophyseal enzyme cleaves the bond formed by the basic amino acid and glycine amide in these compounds.

Inactivation of carba vasopressin with ornithine in position 8 by the adenohypophyseal enzyme is also of great interest, as lower lysine homologues in position 8 protect reliably the bond between the basic amino acid and glycine amide from splitting with trypsin[37].

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