THE ADSORPTION OF ENKEPHALIN TO POROUS POLYSTYRENE BEADS: A SIMPLE ASSAY FOR ENKEPHALIN HYDROLYSIS

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1. Introduction

Two pentapeptides with opioid activity, H–Tyr–Gly–Gly–Phe–Met–OH (Met-enkephalin) and H–Tyr–Gly–Gly–Phe–Leu–OH (Leu-enkephalin) have been recently detected in the brain [1,2]. It was shown that the biological activity of the enkephalins disappears upon incubation with rat plasma, or brain homogenate, or after injection into the animal [3–5].

Hambrook et al. have shown that rat plasma and rat brain homogenates contain an enzymatic activity which rapidly cleaves the enkephalin molecules preferentially at the tyrosyl–glycine peptide bond [4]. This enzymatic activity might have an important physiological role in regulating the function of enkephalins in the nervous system.

A rapid quantitative method for determinining the hydrolysis of enkephalin is described. It is based on the observation that in aqueous solutions the enkephalins are quantitatively adsorbed onto beads of the porous copolymer Porapak Q.

2. Materials and methods

Leu–enkephalin, Met–enkephalin and phenylalanyl–methionine were kindly donated by Miles Yeda Inc., Rehovot, Israel. Tyrosyl-[^{3}H]Leu–enkephalin and [^{3}H]tyrosine were obtained from Amersham Radiochemical Center, other amino acids and peptides from the Sigma Chemical Company. Fluorescamine was obtained from Hoffmann-La-Roche Inc., Nutley, N.J. and Porapak Q (100–120 mesh) from Waters Associates, Milford, Mass. Leucine aminopeptidase (EC 3.4.1.1, 101 units/mg) was obtained from Worthington Biochemical Corp. and was activated as described in the Worthington Manual.

2.1. *Preparation of crude brain homogenate*

Male Sprague–Dawley adult rats were decapitated and the brains without the cerebellum were homogenized in 10 volumes of cold 0.3 M sucrose in 10 mM Tris–HCl (pH 7.5). The homogenate was centrifuged for 10 min at 1000 × *g* and the supernatant stored in small aliquots in liquid nitrogen. Protein was determined by the method of Lowry et al. [6].

2.2. *Porapak Q columns*

Constant volumes of dry Porapak Q beads (80 mg in weight) were packed into a series of small columns and the beads were equilibrated with absolute ethanol for at least 1 h. Prior to use, columns were washed with 4 ml aliquots of: absolute ethanol, 50% ethanol, and twice with either 10 mM Tris–HCl, or with 10 mM sodium phosphate buffer, both at pH 7.5. After use, the columns were washed with ethanol and stored in a beaker with ethanol. The same columns were reused many times.

2.3. *Column assay for enzymatic hydrolysis*

Mixtures of labeled (approximately 40 000 cpm per assay) and unlabeled Leu–enkephalin were incubated at 30°C in an Eppendorf centrifuge tube in a final volume of 100 μl, containing the enzymatic activity and buffer as specified in the Legends. After 10 min of incubation, unless otherwise indicated, the reaction was stopped by immersing the tubes in a boiling water bath for an additional 10 min. The tubes were spun for 2 min in an Eppendorf tabletop centrifuge. Eighty μl of the supernatants were applied

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to the columns, which were then washed with 4 ml of 10 mM Tris–HCl (pH 7.5). The effluents were directly collected into scintillation vials and counted.

2.4. *Thin-layer chromatography*

Samples were chromatographed on silica plates in chloroform–methanol–acetic acid–water (45:30:6:9). The locations of amino acids or peptides were determined by spraying the dry chromatogram with a solution of 0.01% fluorescamine in acetone followed by immediate spray with 0.5% pyridine in acetone. The fluorescent spots were visualized with a long wave ultraviolet light. Strips were then cut into 1/2 cm sections and counted for radioactivity.

3. Results

3.1. *Adsorption of enkephalin to Porapak Q beads*

The ability of Porapak Q, a porous copolymer of styrene and ethylvinylbenzene crosslinked with divinylbenzene, to discriminate between Leu–enkephalin and tyrosine is illustrated in fig.1. Tyrosyl-[^{3}H] Leu-enkephalin was quantitatively adsorbed to the beads in 10 mM Tris–HCl (pH 7.5), and was readily eluted with 50% ethanol (fig.1A). Upon storage of the enkephalin solutions, there was an increase in the radioactivity not adsorbed to the Porapak beads. This minor component was shown by thin layer chromatography to migrate differently from enkephalin and is probably a decomposition product. [^{3}H]Tyrosine, which was used as a control in this experiment, was not adsorbed to the column under these conditions (fig.1B).

Table 1 shows the elution characteristics of various unlabeled amino acids and short peptides which are part of the enkephalin molecule. The results indicate that in 10 mM phosphate buffer tyrosine, glycine, tyrosyl–glycine and tyrosyl–glycyl–glycine did not adsorb to the column, while phenylalanine was partially retained by the beads. The dipeptides phenylalanyl–leucine and phenylalanyl–methionine and the two enkephalins were readily adsorbed to the beads and could be eluted with 50 and 100% ethanol. It is, therefore, the carboxyterminal dipeptide containing the phenylalanine residue which is important for the quantitative adsorption of the enkephalins to the Porapak.

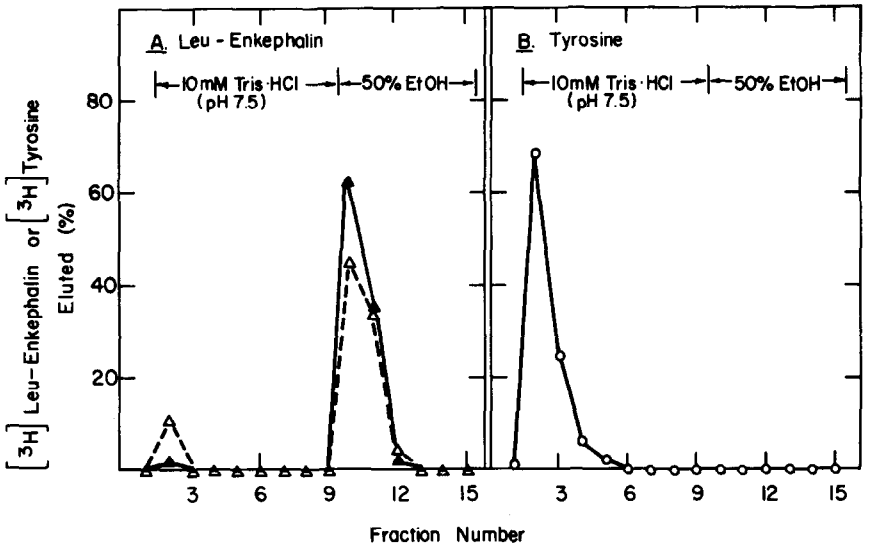

Fig.1. Elution pattern of Leu–enkephalin and of tyrosine from Porapak Q columns. Two hundred μl of (A) 7.5 × 10^{-6} M tyrosyl-[^{3}H]Leu–enkephalin (30 000 cpm) or (B) 6.25 × 10^{-5} M [^{3}H]tyrosine (15 000 cpm) in 10 mM Tris–HCl (pH 7.5), were applied to the columns. The columns were washed with 10 mM Tris–HCl (pH 7.5), and then with 50% ethanol. Fractions of 0.5 ml were collected, and counted. (▲—▲) Fresh solution of enkephalin; (△- - -△) 4-months-old solution of enkephalin.

Table 1
Stepwise elution of amino acids and peptides from Porapak Q columns

Amino acid or peptide assayed	Yield of stepwise elution (%)			
	Phosphate buffer	50% Ethanol	100% Ethanol	Total eluted
Tyr	95	0	0	95
Gly	87	4	3	94
Phe	62	34	1	97
Tyr–Gly	100	0	0	100
Tyr–Gly–Gly	88	5	6	99
Phe–Leu	12	80	3	95
Phe–Met	6	76	11	93
Leu–enkephalin	9	75	6	90
Met–enkephalin	6	81	7	94

The indicated amino acids or peptides were applied to the columns (100 μl of 4 × 10^{-5} M). The columns were sequentially washed with: 5 ml of 10 mM sodium phosphate buffer (pH 7.5); 3 ml of 50% ethanol and 2 ml of 100% ethanol. Fractions of 1 ml were collected, ethanol fractions were evaporated and redissolved in 1 ml of the phosphate buffer. Amounts of amino acids and peptides were determined by their fluorescence with fluorescamine [7]. Results presented are the average of duplicate columns.

3.2. *Hydrolysis of Leu–enkephalin with leucine aminopeptidase*

The formation of free tyrosine upon hydrolysis of tyrosyl-[^{3}H]Leu–enkephalin by this enzyme was monitored by the use of Porapak columns. Figure 2 illustrates the change in the adsorption of the labeled material as the result of partial and complete hydrolysis. Under conditions of complete hydrolysis only a minor amount of the labeled material was adsorbed

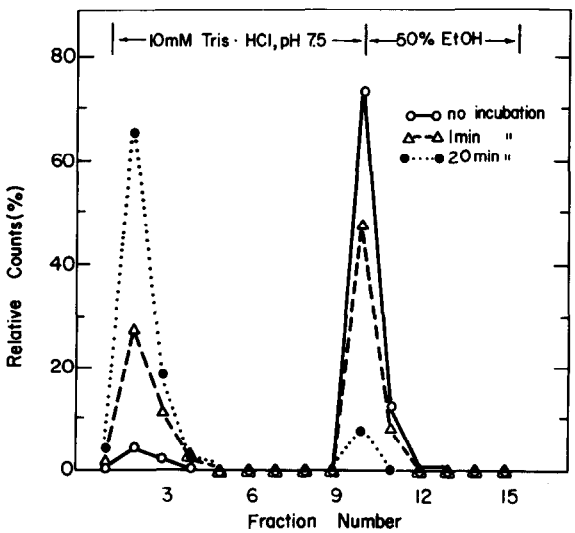

Fig.2. Hydrolysis of Leu–enkephalin by leucine aminopeptidase. Each 100 μl incubation mixture contained: 1.6 μg leucine aminopeptidase; 1 × 10^{-4} M tyrosyl-[^{3}H]Leu-enkephalin, 2 mM $MgCl_2$ and 10 mM Tris–HCl (pH 8.5). Hydrolysis was stopped as described in Materials and methods, columns were washed and eluted as described in fig.1.

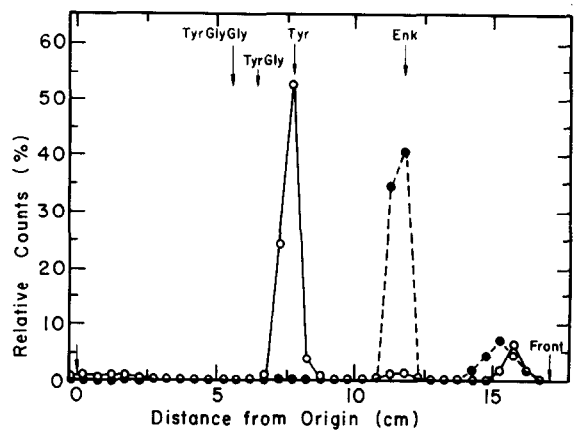

Fig.3. Thin-layer chromatography of Leu–enkephalin hydrolyzed by rat brain homogenate. Mixtures containing 3 × 10^{-7} M tyrosyl-[^{3}H]Leu–enkephalin were incubated for 60 min at 37°C in 0.15 M sucrose and 10 mM Tris–HCl (pH 7.5). Samples of 3 μl were analyzed. No homogenate present (●- - -●); 10 μg homogenate present (○——○). Arrows show location of markers.

to the column. It was shown by thin-layer chromatography that the residual material is a contaminant present in the commercial labeled enkephalin (see also fig.3). The initial rate of hydrolysis found in the presence of 1×10^{-4} M Leu–enkephalin was approximately 3100 nmol/min/mg of enzyme (experiments not shown).

3.3. *Hydrolysis of Leu-enkephalin by rat brain homogenates and by rat serum*

Figures 3 and 4 demonstrate that brain homogenate contains an enzymatic activity (or activities) which hydrolyses Leu–enkephalin. The hydrolysis products of tyrosyl-[^{3}H]Leu–enkephalin were analyzed by thin-layer chromatography using several solvents. It was revealed that the labeled product formed comigrated with the tyrosine marker (fig.3). Under conditions of limited hydrolysis, the same product co-migrating with tyrosine was formed (data not shown). It is therefore the peptide bond between the tyrosine and the glycine which is first cleaved.

The rate of hydrolysis of Leu–enkephalin as a function of the amount of homogenate is shown in fig.4. With 1×10^{-4} M Leu–enkephalin, the rate was proportional to the amount of homogenate present; 40 nmol/min of Leu–enkephalin were hydrolyzed at 30°C by 1 mg protein of crude brain homogenate. With 1×10^{-7} M Leu–enkephalin, the initial rate of hydrolysis was about 100 times lower than with 1×10^{-4} M Leu–enkephalin. Preliminary experiments have shown that the Michaelis–Menten constant for this hydrolytic reaction is around 2×10^{-5} M.

Rat serum also contains a potent enzymatic activity; 70 pmol/min of Leu-enkephalin were hydrolyzed by 1 μl of serum, at 30°C, in the presence of 1×10^{-4} M substrate.

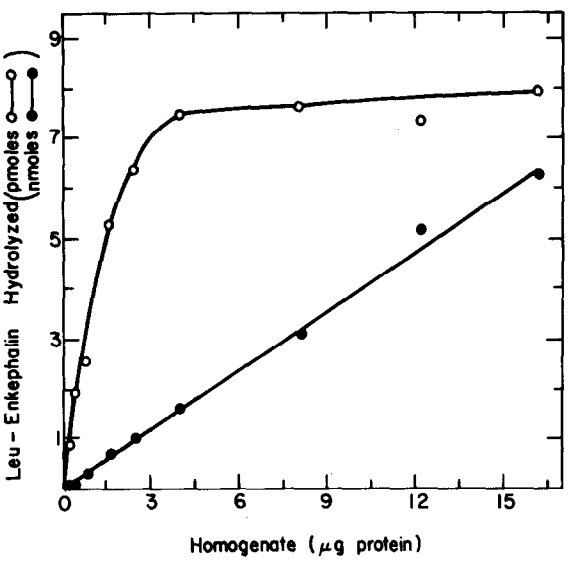

Fig.4. Effect of brain homogenate concentration on the rate of enkephalin hydrolysis. Reaction mixtures contained 1×10^{-4} M (●——●), or 1×10^{-7} (○——○) of tyrosyl-[^{3}H] Leu–enkephalin, 100 μg bovine serum albumin, 10 mM Tris–HCl (pH 7.5) and the indicated amounts of homogenate. Radioactivity eluted in the absence of homogenate was subtracted.

4. Discussion

The two pentapeptides Leu–enkephalin and Met–enkephalin are quantitatively adsorbed to Porapak Q. The adsorption is reversible and the enkephalins are readily eluted with ethanol. The adsorption capacity is very high; one gram of beads adsorbs 100 mg or more of enkephalin (Dr M. Oreg, Miles Yeda Inc., personal communication). Porapak Q beads are now being utilized for the purification and for the concentration of the enkephalins and might be used as a tool in the purification of substituted enkephalins as well. Porous polystyrene beads have been utilized for the adsorption of various materials; for example Porapak Q was used to adsorb dinitrophenyl derivatives of amino acids [8] and a similar copolymer was used to adsorb Triton X-100 from protein solutions [9].

It was shown by Niederwieser [8] that phenylalanine is partially adsorbed to Porapak Q (20–60%, depending on conditions), while all the other amino acids are not adsorbed. Our results agree with this observation, and also indicate that the assayed dipeptides and pentapeptides containing a phenylalanine residue, are more strongly adsorbed than free phenylalanine. Porapak Q beads might therefore find use in the separation of small phenylalanine containing peptides from other peptides.

The physiological activity of enkephalin is rapidly lost upon contact with various animal tissues [3–5]. We have confirmed the observation of Hambrook et al. [4] that rat brain homogenate contains an enzymatic activity which hydrolyses enkephalin and that

the tyrosyl–glycine peptide bond is the most susceptible bond to the cleavage.

We have described a simple method for the quantitative analysis of the enzymatic hydrolysis of enkephalin. The method demonstrated here with tyrosyl-[^{3}H]Leu–enkephalin should be applicable as well for tyrosyl-[^{3}H]Met–enkephalin, which is also commercially available. The method is reproducible (in the range of ±3%) and is highly sensitive; with specific activity of 50 Ci/mmol it was possible to determine the hydrolysis of 5 fmol of the enkephalin. The assay is rapid; more than a hundred samples were processed within 1 h.

Acknowledgement

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