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Note

Separation of the enkephalins from proteins in an aqueous medium by chromatofocusing

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The principle of chromatofocusing was developed by Sluyterman and co-workers in 1978[1,2] to concentrate and separate peptides and proteins on the basis of their isoelectric point. The application of this technique to concentrate and possibly separate Leu- and Met-enkephalin has been investigated during which information regarding the p*I* values of these two peptides has been obtained. On a theoretical basis, calculating the p*I* from the pK_a values of the amino- and carboxy-terminal amino acids, it was thought that a separation of these two peptides might be effected by chromatofocusing.

Santagostino *et al.*[3] have recently published the isoelectric point of β-endorphin and other related peptides including the enkephalins using isoelectrofocusing on thin polyacrylamide gel slab and found that p*I* values for Leu- and Met-enkephalin were 5.5 and 5.45 respectively.

EXPERIMENTAL

Columns were prepared and calibrated according to the Pharmacia manual on chromatofocusing. The columns were eluted at a flow-rate of 27 cm h^{-1} using a peristaltic pump and the eluate monitored at 280 nm using an LKB Uvicords detector. Samples of Leu- and Met-enkephalin, insulin, albumin and β-lactoglobulin were applied to the columns which had been pre-equilibrated with imidazol buffer to pH 7 and eluted with Polybuffer (74). All experiments were carried out at 21–22°C.

Polybuffer exchanger (PBE 94), Polybuffer (74), $C_{10/40}$ columns were purchased from Pharmacia (Hounslow, U.K.), Horse heart cytochrome *c*, Leu-enkephalin, Met-enkephalin both acetate salt, albumin (bovine) fraction 5, insulin (bovine) from Sigma (Poole, U.K.), β-lactoglobulin from Miles Labs., U.S.A. and imidazol hydrochloride from BDH (Poole, U.K.).

RESULTS

Assuming that the pK_a values of the amino group of tyrosine and the carboxy group of Leu- or Met-enkephalin are not altered by the inclusion of the amino acids in peptides then calculations of the p*I* of the pentapeptides from published values of their pK_a values[4–8] provides a range of p*I* values the extremes of which are listed in

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TABLE I

p*I* FOR LEU- AND MET-ENKEPHALIN CALCULATED FROM pK_a VALUES

Peptide	*pK_a*		*pI*	*Mean pI*
	Tyr	*Leu or Met*		
Leu-enkephalin	9.11	2.125	5.617	5.656
	9.11	2.28	5.695	
Met-enkephalin	9.11	2.320	5.715	5.725
	9.11	2.36	5.735	

Table I. However, the inclusion of an amino acid in a peptide is known to alter the pK_a of both the amino and carboxy groups[4].

Results from the chromatofocusing of Leu- and Met-enkephalin separately yielded p*I* values of 5.61 ± 0.120 and 5.58 ± 0.168 (Table II, Figs. 1 and 2) respectively. The technique failed however to separate a mixture of equal amounts of these peptides (Fig. 3). A single-peak band was observed on each occasion showing a p*I* of 5.56 ± 0.276 (Table II). The values for p*I* obtained experimentally are of the same order as those calculated on the basis of the pK_a values of the free amino acids.

To test the validity of the technique in separating molecule species according to their p*I* values, three protein markers, insulin, albumin and β-lactoglobulin were analysed separately and their p*I* values were determined. The p*I* values for insulin and albumin were found to be 4.88 ± 0.068 and 4.69 ± 0.117 respectively. β-Lactoglobulin in agreement with the reported data[9] was separated as two major components; the first with the p*I* of 4.48 ± 0.091 and the second with the p*I* of 4.34 ± 0.093 (Figs. 4–6).

TABLE II

p*I* VALUES FOR LEU AND MET-ENKEPHALIN, INSULIN, ALBUMIN AND β-LACTOGLOBULIN DETERMINED BY CHROMATOFOCUSING AND COMPARED WITH VALUES QUOTED IN THE LITERATURE DETERMINED BY ISOELECTRIC FOCUSING

Figures in parenthesis are the number of determinations.

Peptide/protein	*pI*	
	Chromatofocusing ± S.D.	*Isoelectric focusing*
Leu-enkephalin	5.61 ± 0.120 (5)	5.50*
Met-enkephalin	5.58 ± 0.168 (7)	5.40*
Leu + Met-enkephalin	5.56 ± 0.276 (5)	–
Insulin (bovine)	4.88 ± 0.086 (6)	5.72**
Albumin (bovine) fraction 5	4.69 ± 0.117 (6)	4.90**
β-Lactoglobulin B	4.48 ± 0.091 (6)	5.31**
β-Lactoglobulin A	4.34 ± 0.093 (6)	5.14**

* Santagostino *et al.*[3].

** Righetti and Caravaggio[9].

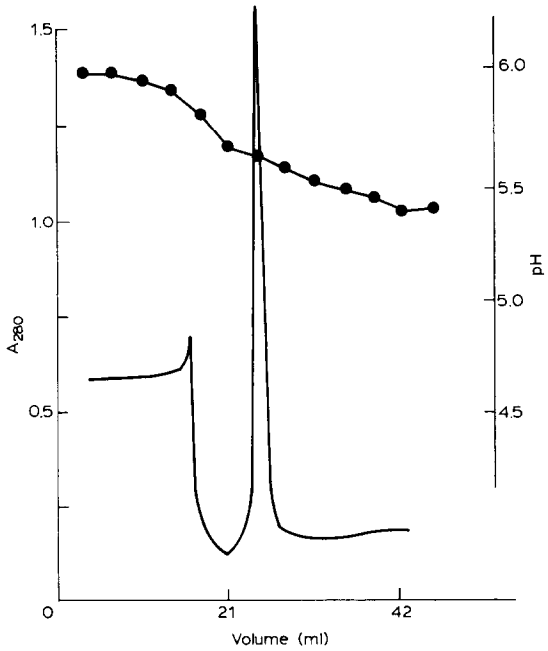

Fig. 1. Chromatofocusing of Leu-enkephalin on a column of PBE 94. Column: $C_{10/40}$. Bed height: 40 cm. Sample: 0.1 ml Leu-enkephalin (10 m*M*). Elution conditions: start buffer 0.025 *M* imidazole–HCl, pH 7; elution buffer, Polybuffer 74–water (1:8) pH 4.2. Flow-rate: 27 cm h^{-1}.

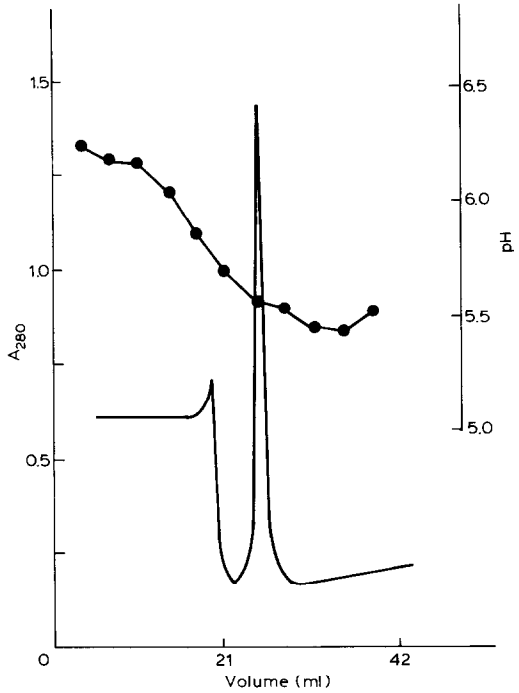

Fig. 2. Chromatofocusing of Met-enkephalin on a column of PBE 94. Column: $C_{10/40}$. Bed height: 40 cm. Sample: 0.1 ml of Met-enkephalin (10 m*M*). Elution conditions: start buffer 0.025 *M* imidazole–HCl, pH 7; elution buffer, Polybuffer 74–water (1:8) pH 4.2. Flow-rate: 27 cm h^{-1}.

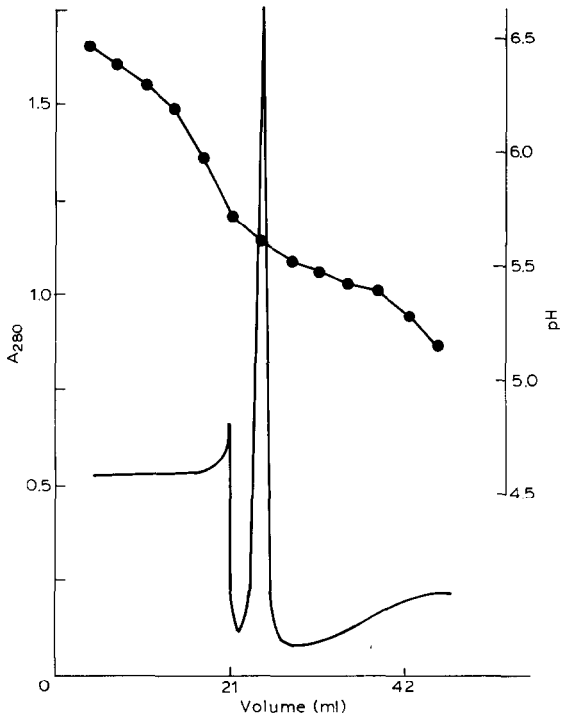

Fig. 3. Chromatofocusing of a mixture of Leu- and Met-enkephalin on a column of PBE 94. Column: $C_{10/40}$. Bed height: 40 cm. Sample: 0.1 ml of Leu-enkephalin (10 m*M*) + 0.1 ml Met-enkephalin (10 m*M*). Elution conditions: Start buffer 0.025 *M* imidazole–HCl, pH 7; elution buffer, Polybuffer 74–water (1:8) pH 4.2. Flow-rate: 27 cm h^{-1}.

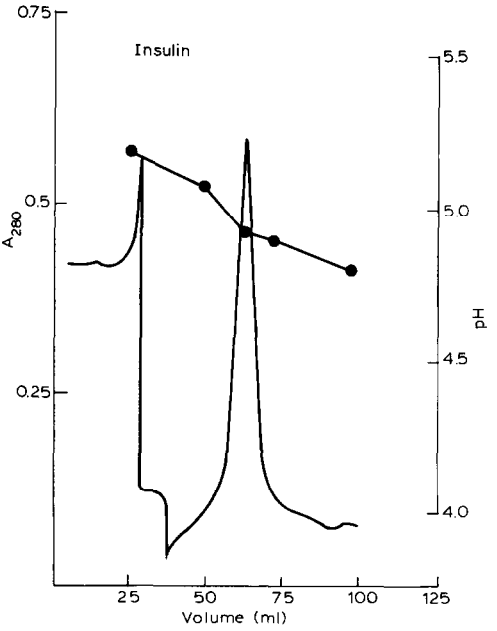

Fig. 4. Chromatofocusing of insulin on a column of PBE 94. The sample (1 ml) containing 1 mg of insulin. Column: $C_{10/40}$. Bed height: 40 cm. Elution condition: start buffer 0.025 *M* imidazole–HCl, pH 7; elution buffer, Polybuffer 74–water (1:8) pH 4.2. Flow-rate 27 cm h^{-1}.

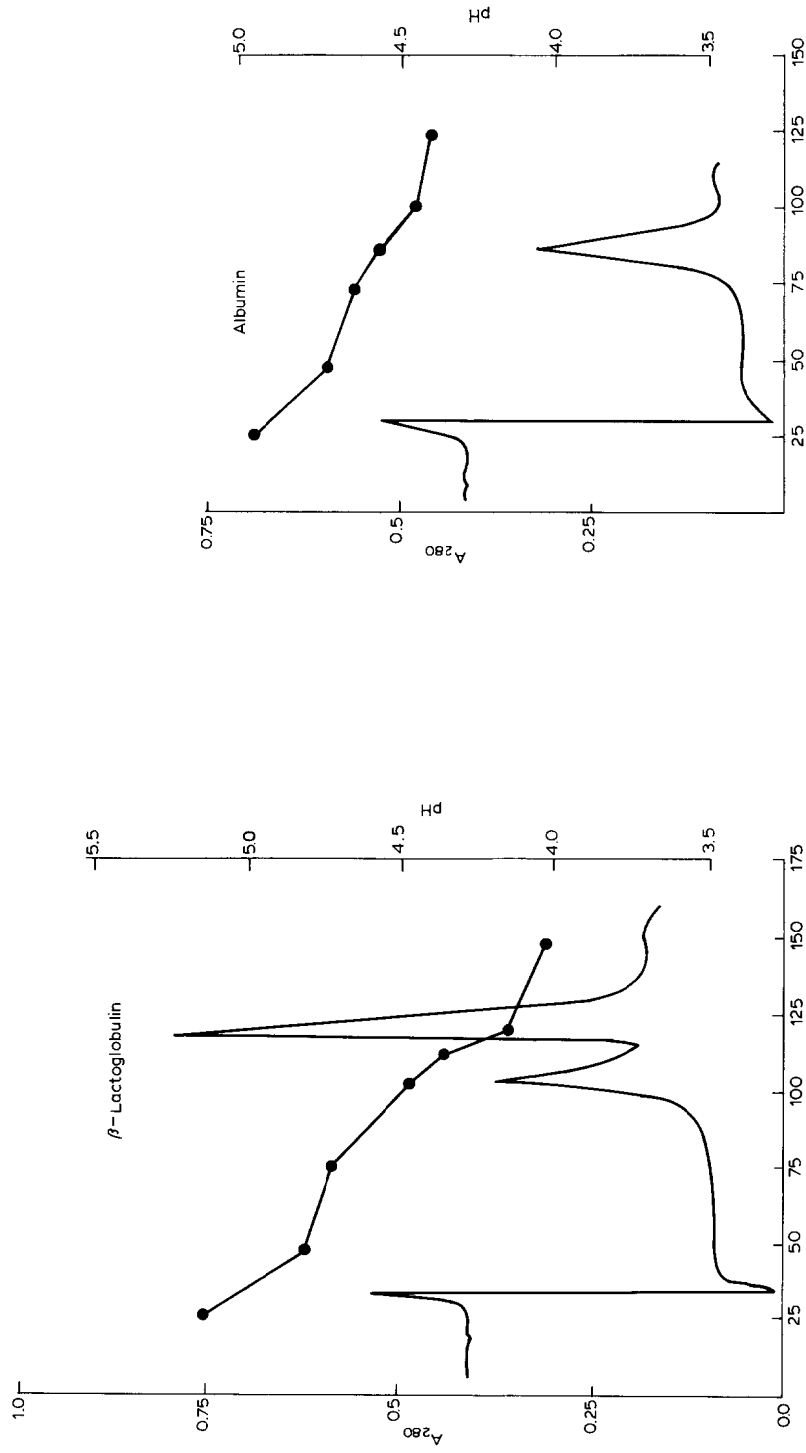

Fig. 5. Chromatofocusing of albumin on a column of PBE 94. The sample (1 ml) containing 1 mg of albumin. Column: $C_{10/40}$. Bed height: 40 cm. Elution condition: start buffer 0.025 *M* imidazole–HCl, pH 7; elution buffer, Polybuffer 74–water (1:8) pH 4.2. Flow-rate 27 cm h^{-1}.

Fig. 6. Chromatofocusing of β-lactoglobulin on a column of PBE 94. The sample (1 ml) containing 1 mg of β-lactoglobulin. Column: $C_{10/40}$. Bed height: 40 cm. Elution condition: start buffer 0.025 *M* imidazole–HCl, pH 7; elution buffer, Polybuffer 74–water (1:8), pH 4.2. Flow-rate 27 cm h^{-1}.

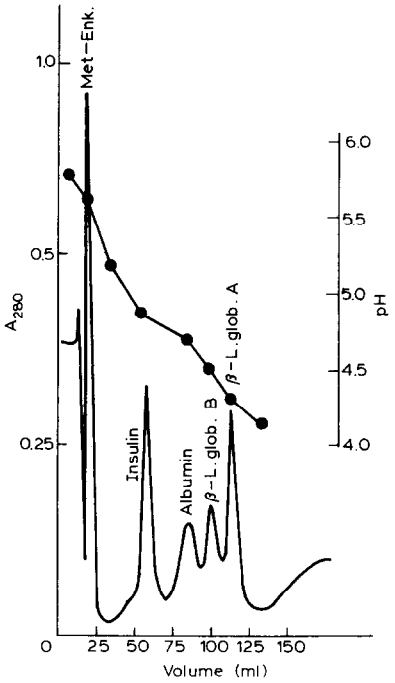

Fig. 7. Separation of a protein mixture containing insulin, albumin, lactoglobulin and Met-enkephalin by chromatofocusing. The sample (1.6 ml) containing 0.5 mg of each protein (1 mg/ml) plus 0.63 mg of Met-enkephalin (10 m*M*). Bed height: 40 cm. Elution condition: start buffer 0.025 *M* imidazole–HCl, pH 7; elution buffer, Polybuffer 74–water (1:8) pH 4.2. Flow-rate 27 cm h^{-1}.

A mixture of the same proteins, to which Met-enkephalin was added, was also analysed and a clear separation of Met-enkephalin from other proteins was observed (Fig. 7). Met-enkephalin was eluted first, followed by insulin, albumin and then the two components of β-lactoglobulin.

DISCUSSION

Santagostino *et al.*[3] have reported that p*I* values for Leu- and Met-enkephalin using isoelectric focusing as 5.5 and 5.45 respectively. The results in this study using chromatofocusing have shown that the p*I* values for both peptides were slightly higher than those reported by the above authors but nevertheless are in close agreement with their values and with the theoretically predicted values. However the technique (or the difference in the p*I* values of Leu- and Met-enkephalin) does not permit these peptides to be separated.

The p*I* values of other proteins, estimated by chromatofocusing, appear to differ quite markedly from the p*I* values estimated by isoelectric focusing. This difference could arise from the distribution of charges in the more complex proteins analysed, relative to the simple pentapeptides which have little secondary or tertiary structure. Alternatively the difference in the estimated p*I* values of the proteins could be due to the non-specific adsorption of the protein to the column since the proteins

elute at a lower pH than expected. Since Leu- and Met-enkephalin are eluted so close to their estimated p*I* they do not adsorb to the matrix of the chromatofocusing column.

Whilst this work serves to demonstrate sthe ability to concentrate and separate the enkephalins from a protein mixture, the recent work of Cooper *et al.*[10] would support the contention that none of the proteins found in plasma and cerebrospinal fluid are eluted in the region pH 5.6 with the possible exception of a small proportion of immunoglobulin G.

Chromatofocusing does therefore provide a very useful means of concentrating the enkephalins from an aqueous protein mixture which would then be amenable to quantitation by radioimmunoassay[11].

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