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ISOTACHOPHORETIC ANALYSIS OF PEPTIDES

SELECTION OF ELECTROLYTE SYSTEMS AND DETERMINATION OF PURITY

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SUMMARY

Capillary isotachophoresis (ITP) was applied to the qualitative and quantitative analysis of both natural and synthetic oligo- and polypeptides. Based on the mathematical model of acid–base equilibria for a general ampholyte, a procedure and a computer program for the calculation of the pH dependence of the effective and specific charge and effective mobility of peptides with known amino acid sequence were developed which allow the selection of electrolyte systems for peptide isotachophoretic analysis to be rationalized.

Basic peptides (bovine pancreatic trypsin inhibitor, bull seminal isoinhibitors of trypsin, arginine vasopressin and adamantylamide–alanylisoglutamine) were analysed with a cationic ITP system at acidic pH. Neutral and acidic peptides (insulin, proinsulin, bull seminal isoinhibitors of trypsin, cow colostrum isoinhibitors of trypsin) were analysed with an anionic ITP system, mostly at alkaline pH.

Peptide purity (electrophoretic homogeneity) was determined from the ITP degree of purity defined by a peptide itself and the zone length ratio of its admixtures. Enrichment of peptide in the sample during the purification procedure was measured by its zone length relative to unit mass of the amount of sample analysed.

INTRODUCTION

In the chemistry of peptides, capillary isotachophoresis (CITP) is most frequently used as a method to control the purity of both natural and synthetic peptide preparations. Utilization of CITP for this purpose was introduced by Kopwillem *et al.*[1,2] in the analysis of the fragments of human growth hormone. Following this work, CITP has been used for the analysis of many other naturally occurring and/or synthetically prepared biologically active peptides, *e.g.*, hormones [oxytocin, vasopressin, adrenocorticotropic hormone (ACTH)[3] and insulin[4,5], drugs[6,7], nutritional additives[8,9] and others[10–18]. CITP was used not only as a method to control the purity of the final peptide preparation but also for monitoring the peptide purity after individual steps in a purification procedure, thus providing information on the

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efficiency of the preparative separation method used. In addition, CITP has also been used for the determination of low-molecular-weight ionic admixtures in peptide preparations[19,20].

The aim of this work was to apply CITP to the determination of the purity of several peptide preparations either isolated from a natural material or synthesized, *e.g.*, enzyme inhibitors, hormones, protein fragments and immunomodulators. In order to rationalize the selection of the conditions for ITP analyses of peptides, a procedure and a computer program for the calculation of the pH dependence of the effective and specific charges of peptides was developed and rules for the selection of the pH of the leading electrolyte were established.

THEORETICAL

For the rational selection of conditions for peptide ITP analysis, it is advantageous to know the dependence of their effective or specific charge on pH. Therefore, we have developed a procedure and a computer program that make it possible to calculate the pH dependence of the effective and specific charges of any peptide the amino acid sequence of which is known. The procedure is based on a mathematical model of the acid–base equilibria of a general ampholyte.

Let X(M) and X(N) be ionic forms of peptide X with a maximum charge M and a minimum charge N. The magnitude of the charge is considered in elementary units, including the sign, *i.e.*, maximum and minimum are meant in the mathematical sense. If $K(J)$ is the apparent dissociation constant of the equilibria between the components with charge $(J+1)$ and J, then

$$\mathrm{X}(J+1) \rightleftharpoons \mathrm{X}(J) + \mathrm{H}^+ \tag{1}$$

$$K(J) = c_{\mathrm{X}(J)} \cdot H/c_{\mathrm{X}(J+1)} \tag{2}$$

where $c_{\mathrm{X}(J)}$ and $c_{\mathrm{X}(J+1)}$ are the equilibrium concentrations of the corresponding ionic forms of peptide X and H is the equilibrium concentration of hydrogen ions. Further, the molar fraction, $D_{\mathrm{X}(J)}$, of the component X(J) is introduced, referred to the total concentration c_{X} of peptide X:

$$D_{\mathrm{X}(J)} = c_{\mathrm{X}(J)}/c_{\mathrm{X}} \tag{3}$$

This formulation[21] permits the derivation of the following relationship for the molar fraction $D_{\mathrm{X}(J)}$:

$$D_{\mathrm{X}(J)} = \frac{\left[\prod_{l=0}^{J-1} K(l)/H^{|J|}\right]^{-1} \cdot (J>0) + \left[\prod_{l=J}^{-1} K(l)/H^{|J|}\right] \cdot (J<0)}{1 + \sum_{i=N}^{-1} \prod_{l=i}^{-1} K(l)/H^{|i|} + \sum_{i=1}^{M} \left[\prod_{l=0}^{i-1} K(l)/H^{|i|}\right]^{-1}} \tag{4}$$

$J \in \langle N,M \rangle$, $J \neq 0$

where $K(l)$ are dissociation constants of the acid–base equilibria between the components $X(l+1)$ and $X(l)$, and i and l are auxiliary variables.

The effective charge, z_X, of compound X at a given pH is then given by the equation

$$z_X = \sum_{J=N}^{M} J \cdot D_{X(J)} \tag{5}$$

where $D_{X(J)}$ is given by eqn. 4 in which $H = 10^{-pH}$. From the effective charge, z_X, the specific charge, $z_{X,s}$, may be calculated, *i.e.*, the charge referred to unit relative molecular mass, M_X, of the peptide X:

$$z_{X,s} = z_X/M_X \tag{6}$$

In addition to the effective or specific charge, we can also calculate the effective mobilities, m_X, of peptide X as a function of pH, if known or estimated values of the actual mobilities, $m_{X(J)}$, of individual ionic forms $X(J)$ are available:

$$m_X = \sum_{J=N}^{M} \text{sign}(J) \cdot m_{X(J)} \cdot D_{X(J)} \tag{7}$$

where $D_{X(J)}$ is given by eqn. 4 in which $H = 10^{-pH}$.

From the obtained dependences of effective mobility, effective charge and specific charge on pH, important conclusions can be inferred for the selection of conditions for the ITP separation of peptides and proteins. For the ITP separation the only pH that is suitable is that at which the effective mobilities are sufficiently high, *i.e.*, if their absolute values are approximately higher than $1 \cdot 10^{-9}$–$2 \cdot 10^{-9}$ $m^2\ V^{-1}\ s^{-1}$ (ref. 22), having the same sign, *i.e.*, moving in the same direction (either cationic or anionic), and the differences in their mobilities, Δm, are sufficient for their separation, *i.e.*, $\Delta m \approx 1 \cdot 10^{-9}$ $m^2\ V^{-1}\ s^{-1}$ (ref. 23).

If only the dependence of specific charge on pH is known, then a pH for separation is selected such that the absolute value of the specific charge of the peptide to be separated is greater than $2 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ e (ref. 24).

From the course of the calculated dependence of effective and/or specific charge on pH, the regions of minimum and maximum charge, the regions important for influencing the charge and the isoionic (isoelectric) point can be determined.

Fig. 1 shows the calculated pH dependence of the effective charge of pig insulin. Based on its amino acid sequence[25], the ionogenic groups and the values of the minimum and maximum charge of fully ionized forms of insulin ($N = -10$, $M = +6$) were determined. The average values from the pK ranges of amino acid residues in polypeptide chains[26] were used as values of the dissociation constants of ionogenic groups present in insulin. This leads to a certain inaccuracy because the pK values of individual ionogenic groups can be different not only in different peptides but also in the molecule of the same peptide, as a consequence of the electrostatic and configurational effects of their environment. This inaccuracy and the fact that we consider only the charges formed by dissociation or association of hydrogen ions and

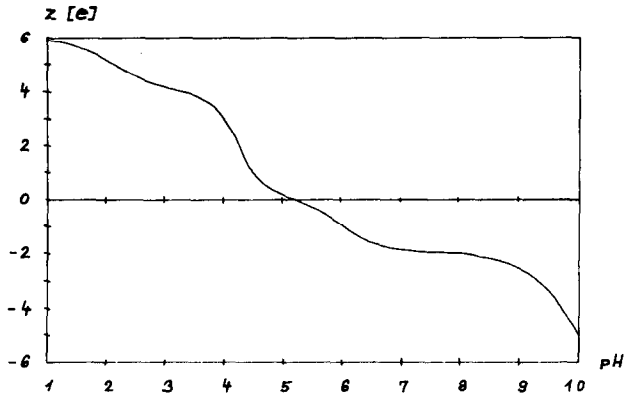

Fig. 1. Calculated pH dependence of effective charge z of insulin (for more details, see text).

not the charges formed by interaction of the peptide with other ions present in solution accounts for the difference between the calculated isoelectric (isoionic) point (5.2) and the experimentally determined values 5.3–6.15[27].

From the course of the pH dependence of the specific charge, z_s, of insulin (see Fig. 2), the pH region of sufficient charge density, *i.e.*, $z_s > 2 \cdot 10^{-4}$–$5 \cdot 10^{-4}\,e$ can be determined. Comparison of the specific charges of insulin and glycine in Fig. 2 allows the ability of glycine to serve as a terminating ion to be judged. Glycine can be considered as a candidate for a terminating ion in the region where its specific charge is lower than that of insulin but greater than the minimum specific charge required for sufficient electrophoretic mobility.

In spite of the inaccuracy of the model, the calculation affords useful information; the calculated isoionic point and the dependences of both the effective and specific charges of insulin of pH were important characteristics that were taken into account in the selection of conditions for its ITP analysis (see Experimental).

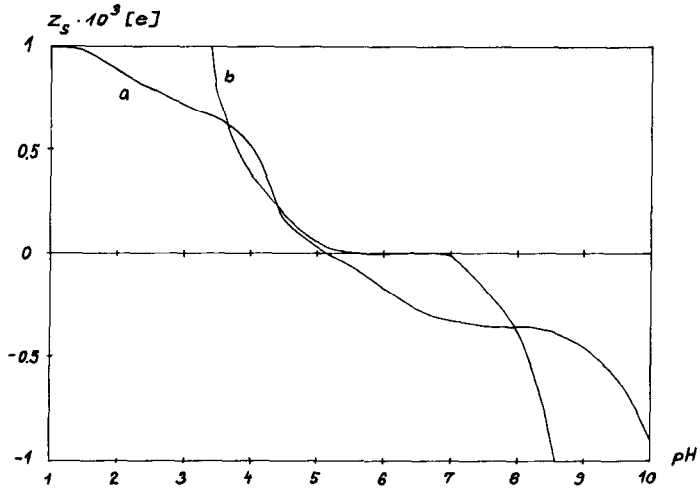

Fig. 2. Calculated pH dependence of specific charge (z_s) of (a) insulin and (b) glycine.

The inaccuracy (given by the inaccuracy of the chosen pK values) and the complexity of the calculation increase with increasing number of ionogenic groups in the molecule. Therefore, the calculation could afford more reliable data for oligopeptides than for polypeptides and proteins. With polypeptides and proteins it is more advantageous to use the electrophoretic titration curves[28], which, however, cannot be obtained for small peptides (up to 20–30 amino acids) as a consequence of the difficulties with their fixation and staining in the gel. Hence both methods, *i.e.*, based on theoretical calculation and experimental titration curves, are complementary, covering a wide range of relative molecular masses of peptides and proteins.

The separation conditions and the pH of the leading electrolyte must be selected not only according to the effective or specific charge of the peptides to be separated, but also with respect to their solubility, stability and biological activity. For the correct choice of the pH of leading electrolyte, the following rules should be adopted: let pH_1 be the pH range compatible with the ITP separation principle (*ca.* 2–11); let pH_2 be the pH range of sufficient solubility of the given peptides (at least about 1 mmol/l); let pH_3, be the pH range in which the given peptides are chemically stable; let pH_4 be the pH range in which the biological activity of the given peptides is preserved; let pH_5 be the pH range in which the given peptides possess sufficient effective mobility or sufficient specific charge of the same sign, *i.e.*, $|m_{ef}| > 1 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} or $|z_s| > 2 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ e; and let pH_6 be the pH range in which the relative differences in the effective mobilities of the peptides (Δm_{ef}) are sufficient for their separation ($\Delta m_{ef} \geqslant$ 2–3%). Then, the pH range suitable for the ITP separation of these peptides pH (ITP, PEP) is given by the multiplication in the algebraic sense of classes (logical product) of the above ranges:

$$\text{pH (ITP, PEP)} = \bigcap_{i=1}^{6} (\text{pH}_i) \tag{8}$$

If the conservation of biological activity for a given ITP peptide separation is not required (*e.g.*, for amino acid and sequence analyses even denaturated polypeptides suffice), then the pH_4 interval is not included in the logical product[8].

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade. Sodium hydroxide, potassium hydroxide, acetic acid and glycine were obtained from Lachema (Brno, Czechoslovakia), N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), tris-(hydroxymethylaminomethane) (Tris) from Serva (Heidelberg, F.R.G.), β-alanine (BALA) from Koch-Light (Colnbrook, U.K.), histidine (His) from Pierce (Rockford, IL, U.S.A.), poly(vinyl) alcohol (PVA) (Mowiol) from Hoechst (Frankfurt, F.R.G.) and barium hydroxide from Merck (Darmstadt, F.R.G.).

Bovine pancreatic trypsin inhibitor (BPTI) (Trasylol) was purchased from Bayer (Leverkusen, F.R.G.), pig proinsulin from Novo (Bagsvaerd, Denmark) and insulin and adiuretin ([8-D-Arg]deaminovasopressin) from Léčiva Pharmaceuticals (Prague, Czechoslovakia).

The other samples were obtained from laboratories where they were isolated

from natural material or synthesized. Bull seminal isoinhibitors of trypsin BUSI II[29], BUSI IIb[30], BUSI IA, IB1, IB2[31], cow colostral isoinhibitors of trypsin CTI A, B, C[32] and bovine basic pancreatic inhibitor of trypsin BPTI[33] were obtained from Dr. D. Čechová and Dr. V. Jonáková (Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). Tryptic and cyanogen bromide fragments of human haemopexin[34,35] were obtained from Dr. B. Meloun and Dr. L. Morávek and muramine dipeptide (Mur–Ala–Gln)[36] from Dr. J. Ježek (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). Adamantylamide-L-alanyl-D-isoglutamine[37] was provided by Dr. M. Flégl (Pharmacological Institute, Czechoslovak Academy of Sciences, Prague, Czechoslovakia).

Apparatus

All ITP analyses were performed in an apparatus of our own construction[38]. It is an all-PTFE capillary-type ITP analyser equipped with two universal potential gradient detectors and a specific UV photometric detector operated at 254 nm. The separation capillary (23 cm × 0.45 mm I.D. × 0.7 mm O.D.) is placed in a thermostated bath filled with Savant EC 123 electrophoresis coolant. Its temperature is controlled over the range 7–25°C by Peltier thermocouples. Samples were introduced by means of a dosing valve (constant volume 2 μl) or with a Hamilton microsyringe (0.5–10.0 μl).

RESULTS AND DISCUSSION

CITP was used for both qualitative and quantitative analysis of selected oligo- and polypeptides. CITP was utilized not only for determination of purity (electrophoretic homogeneity) of peptides both isolated from natural material and synthesized, but also for the analysis of complex polypeptide mixtures of protein fragments resulting from enzymatic and chemical protein cleavage. Further, CITP was applied to monitoring the efficiency of peptide purification procedures.

A survey of peptides analysed in the cationic mode is shown in Table I and in the anionic mode in Table II; qualitative and quantitative characteristics of the peptides are also given. As a qualitative index the relative step height (*RSH*)[39] of the analysed peptide was used, defined by

$$RSH = [(h_P - h_L)/(h_T - h_L)] \cdot 100 \quad (9)$$

where h_L, h_P and h_T are the step heights (voltage of the potential gradient detector) of the leading electrolyte, peptide sample and terminating electrolyte, respectively.

As a quantitative index of the purity (electrophoretic homogeneity) of peptides, the so-called ITP degree of purity was adopted[3]. Let l_S represent the total zone length of all zones having absorption at a given wavelength of the UV detector on an isotachophoregram for the analysis of peptide sample S, and l_A the length of the zone of peptide A, representing the main component of the sample S (see Fig. 3). Then the ITP degree of purity of peptide A, p_A, is given by the ratio of the lengths l_A and l_S:

$$p_A = (l_A/l_S) \cdot 100 \quad (10)$$

TABLE I

SURVEY OF PEPTIDES ANALYSED IN THE CATIONIC ITP MODE

ES No. = electrolyte system number; *RSH* = relative step height; *p* = ITP degree of purity (*RSH* and *p* are average values of two experiments). HOAc = acetic acid.

ES No.	*Leading electrolyte*				*Terminating electrolyte*				*Sample*	*RSH (%)*	*p (%)*
	L:	*leading ion constituent (mol/l)*		*pH*	*T:*	*terminating ion constituent (mol/l)*		*pH*			
	C:	*counter ion constituent (mol/l)*			*P:*	*pH-adjusting constituent (mol/l)*					
	A:	*additive (%, w/v)*									
1	L:	NaOH	0.01	4.8	T:	BALA	0.01	4.5	Bovine pancreatic trypsin		
	C:	HOAc			P:	HOAc			inhibitor (BPTI)	33.3	73.9
	A:	PVA	0.02						Bull seminal trypsin inhibitors:		
									BUSI II	42.9	72.7
									BUSI IIb	31.4	69.4
2	L:	NaOH	0.01	5.0	T:	BALA	0.01	4.7	Adiuretin ([8-D-Arg]deamino-		
	C:	HOAc			P:	HOAc			vasopressin)	81.7	90.4
3	L:	KOH	0.005	5.0	T:	BALA		5.0	Adamantylamide–L-alanyl-D-		
	C:	HOAc			P:	HOAc			isoglutamine	82.5	88.2

TABLE II

SURVEY OF PEPTIDES ANALYSED IN THE ANIONIC ITP MODE

ES No. = electrolyte system number; *RSH* = relative step height; *p* = ITP degree of purity (*RSH* and *p* are average values of two experiments).

ES No.	*Leading electrolyte*				*Terminating electrolyte*				*Sample*	*RSH (%)*	*p (%)*
	L:	*leading ion constituent (mol/l)*		*pH*	*T:*	*terminating ion constituent (mol/l)*		*pH*			
	C:	*counter ion constituent (mol/l)*			*P:*	*pH-adjusting constituent (mol/l)*					
	A:	*additive (%, w/v)*									
4	L:	HCl	0.005	6.1	T:	HEPES	0.01	8.1	Muramine dipeptide		
	C:	His	0.01		P:	$Ba(OH)_2$			(Mur–Ala–Gln)	32.8	88.0
	A:	PVA	0.2								
5	L:	HCl	0.01	6.1	T:	HEPES	0.01	8.1	Tryptic fragments of human		
	C:	His	0.02		P:	$Ba(OH)_2$			haemopexin	–	–
6	L:	HCl	0.005	8.1	T:	Gly	0.01	10.5	Bull seminal trypsin inhibitors:		
	C:	Tris	0.01		P:	$Ba(OH)_2$			BUSI IA	12.3	76.4
	A:	PVA	0.02						BUSI IB1	12.3	61.3
									BUSI IB2	12.2	83.0
									Cow colostral trypsin inhibitors:		
									CTI A	33.3	34.9
									CTI B	44.1	51.1
									CTI C	54.7	46.8
									Pig insulin	33.3	79.5
									CNBr fragments of human		
									haemopexin	–	–
7	L:	HCl	0.005	8.1	T:	Gly	0.01	10.2	Pig insulin	15.2	69.2
	C:	Tris	0.01		P:	$Ba(OH)_2$			Pig proinsulin	50.0	87.3
	A:	2-propanol 10% (v/v)									

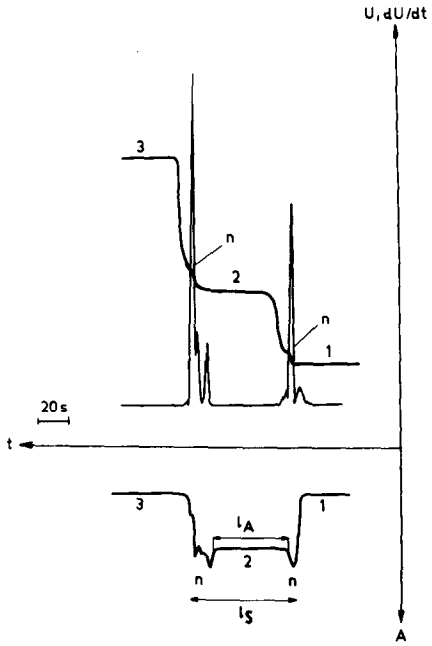

Fig. 3. Determination of ITP degree of purity of bull seminal trypsin isoinhibitor (BUSI II). l_A = Zone length of pure BUSI II; l_S = total length of UV-positive zones. 1 = Na^+; 2 = BUSI II; 3 = BALA; n = unidentified sample admixtures. A = Absorbance at 254 nm; U = voltage of PG detector; t = time; dU/dt = differentiation of PG detector signal. Analysis in ES No. 1 (see Table I); 9 μg of BUSI II in 2 μl of leading electrolyte were applied.

However, the zone lengths of possible impurities from the electrolyte system must not be included in the total length l_S. The lengths of these impurity zones can be determined during the blank run of the ITP analysis without the application of a sample. The degree of purity defined in this manner can be approximately identified with the molar fraction of the given peptide in the analysed mixture. This approximation is the more precise the closer are the charges and the effective mobilities of the components present and the higher is the proportion of the main component in the sample. This requirement is fulfilled to a considerable extent with peptide preparations purified to a relatively high degree. The error caused by this approximation is in the range of units of percent and in some instances [with equal values of the charge, close effective mobilities (deviations up to several percent) and at a relatively low mobility of the counter ion of the leading electrolyte] it can be almost negligible[40,41].

The degree of purity or the ratio of concentrations of individual components can therefore be approximately determined even without standards of individual compounds, merely on the basis of the ratio of zone lengths. This is an advantage of ITP over high-performance liquid chromatography, because in chromatographic analysis with photometric detection at one constant wavelength (254 or 280 nm) the concentration ratios of individual components cannot be determined without a knowledge of their molar absorption coefficients.

Some examples of peptide ITP analyses are given. Fig. 4 shows the ITP analysis

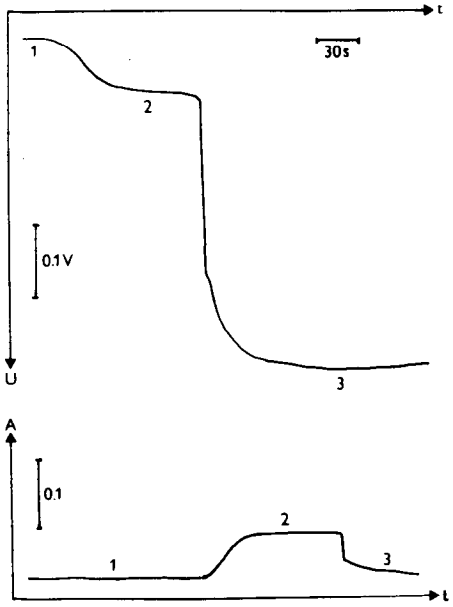

Fig. 4. ITP analysis of pig insulin. 1 = Carbonates; 2 = pig insulin; 3 = glycine. A = Absorbance (at 254 nm); U = voltage of PG detector; t = time. Analysis in ES No. 6 (see Table II); 10.2 μg of pig insulin in 3 μl of leading electrolyte were applied. Separation current, 50 μA; detection current, 20 μA; time of analysis, 30 min.

of pig insulin, the conditions of which and the pH of the leading electrolyte (pH_L) were selected according to the following parameters described and calculated under Theoretical:

(1) Isoelectric (isoionic) point pI: 5.2–6.15 $\Rightarrow$ pI − 1.5 > pH_L > pI + 1.5, *i.e.*, 3.7 > pH_L > 7.65.

(2) Specific charge z_s: $|z_s| > 2 \cdot 10^{-4}\ e \Rightarrow 4.5 > pH_L > 6.2$ (see Fig. 2).

(3) Effective charge z: weakly dependent on pH in the range 6.5–8.5 (see Fig. 1).

(4) Solubility: bad solubility near pI $\Rightarrow$ pH_L should not be in the range pI ± 1, *i.e.*, 4.2 > pH_L > 7.15.

(5) Chemical stability and biological activity: trans-sulphidation can occur under higher alkaline conditions, pH $\Rightarrow$ pH_L < 8.5.

Based on these data, electrolyte system ES 6 (see Table II) was chosen for the ITP analysis of insulin. A relatively high degree of purity of the given preparation was confirmed (see Table II).

One of immunologically most dangerous admixtures in insulin preparations is proinsulin, the prohormone form of insulin. A very good separation of these two polypeptides (see Fig. 5) was achieved in a mixed-solvent ITP electrolyte system (ES 7 in Table II) using water–2-propanol (90:10, v/v) as the solvent of the leading electrolyte.

Determination of peptide purity is important not only with regard to the final products. Using CITP as a control technique for purity determination after different steps in a purification procedure, we can obtain important information about the

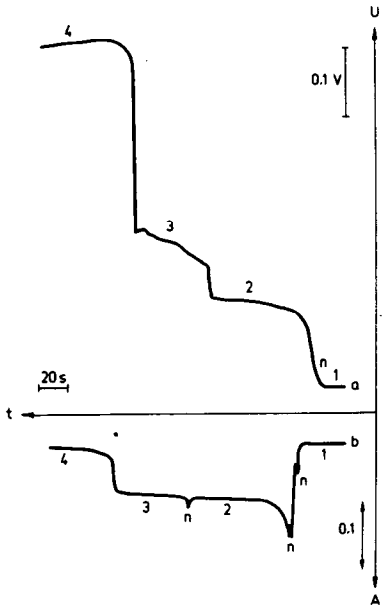

Fig. 5. ITP separation of pig insulin and pig proinsulin. 1 = Carbonates; 2 = insulin; 3 = proinsulin; 4 = glycine; n = unidentified sample components. (a) Signal of PG detector; (b) signal of photometric detector. A = Absorbance at 254 nm; U = voltage of PG detector; t = time. Analysis in ES No. 7 (see Table II); sample, 8.8 μg of lyophilized pig insulin and 8.5 μg of lyophilized pig proinsulin in 5 μl of leading electrolyte were applied. Separation current, 30 μA; detection current, 20 μA; time of analysis, 20 min.

efficiency of different purification methods, from which the suitability of applied methods can be judged.

Using CITP, the efficiency of the purification procedure for the preparation of pure basic bovine pancreatic trypsin inhibitor (BPTI) was monitored. Fig. 6a shows the ITP analysis of the crude BPTI product which was obtained by magnesium sulphate precipitation[33] from the waste solution after trypsin isolation. In this record the zone of BPTI forms a relatively small part of the total length of UV-positive zones, whereas in Fig. 6b, which shows the ITP analysis of BPTI after ion-exchange chromatography on CM-Sephadex, the zone of BPTI already prevails. The ITP degree of purity was further increased after rechromatography on CM-Sephadex. The determined degrees of purity of different BPTI preparations (including a commercial one) are given in Table III.

The degree of enrichment of BPTI was also monitored by another quantitative parameter, the relative zone length, *i.e.*, the zone length relative to unit mass of the applied amount of sample:

$$p_{A,m} = l_A/m_S \tag{11}$$

The calculated values of $p_{A,m}$ are given in Table III. These values are useful especially for monitoring the first steps in a purification procedure, because in their values content of non-ionogenic admixtures and salts is also reflected. In the analysis shown

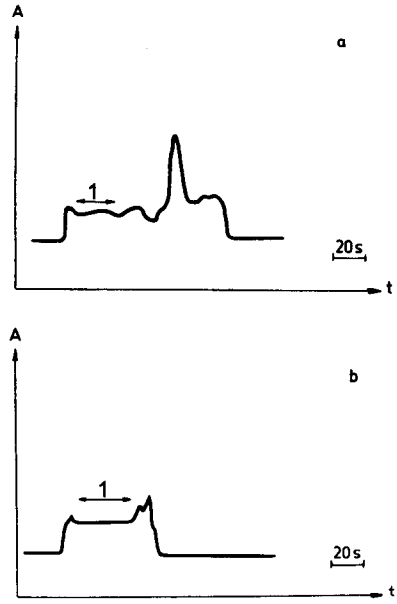

Fig. 6. ITP analysis of bovine pancreatic trypsin inhibitor (BPTI) in ES No. 1 (see Table I) (a) 200 μg of crude BPTI product (precipitated by $MgSO_4$); (b) 6 μg of BPTI purified by ion-exchange chromatography. A = Absorbance at 254 nm; t = time. 1 = BPTI zone.

in Fig. 6a this relative zone length is 0.05 mm/μg (the amount of sample applied was 200 μg) and in Fig. 6b the relative zone length is 2.3 mm/μg (the amount of sample applied was 6 μg). The ratio of these relative zone lengths gives the degree of enrichment after the purification step. In the given example this means that sample analysed in Fig. 6b contains 46 times more BPTI per unit mass than that analysed in Fig. 6a. This high degree of enrichment (in comparison with only a 2.2 times higher ITP degree of purity derived from the UV zone-length ratio) is caused by the high salt content in the crude BPTI product.

TABLE III

ITP DEGREE OF PURITY OF DIFFERENT PREPARATIONS OF BOVINE PANCREATIC TRYPSIN INHIBITOR (BPTI)

p = Degree of purity defined on the basis of zone-length ratio (see eqn. 10); p_m = degree of purity defined on the basis of zone length relative to unit mass (see eqn. 11); IEC = ion-exchange chromatography.

BPTI preparation	*Degree of purity*	
	p (%)	*p_m (mm/μg)*
Crude	20.3	0.05
Purified by IEC	43.1	2.3
Repurified by IEC	73.2	4.1
Commercial (Trasylol)	50.0	3.0

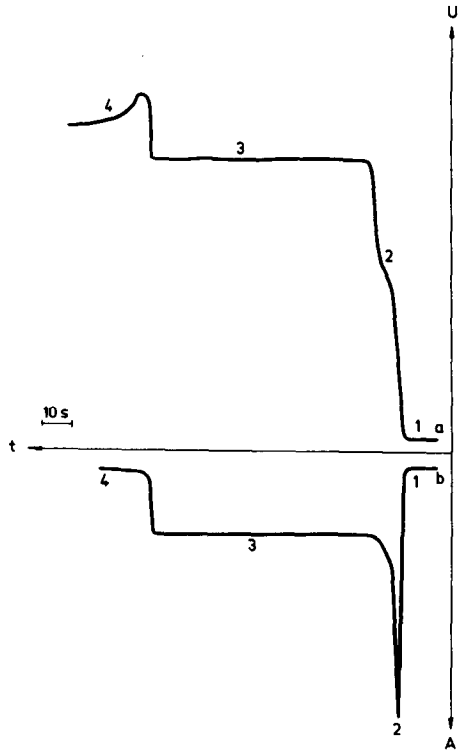

Fig. 7. ITP analysis of adiuretin, synthetic ([8-D-Arg]deaminovasopressin), in the cationic mode, in ES No. 2 (see Table I). 2 μl of an aqueous solution of 8 μg of adiuretin were applied. Current during separation, 50 μA; during detection, 35 μA. (a) Signal of PG detector; (b) signal of UV detector. A = Absorbance at 254 nm; U = voltage of PG detector; t = time. 1 = Na^+; 2 = unidentified sample component; 3 = adiuretin; 4 = BALA.

The zone lengths were measured manually and in some instances, *e.g.*, in Fig. 6, only approximately from the UV record of the ITP analysis as only a contactless UV detector affords a reproducible signal in ITP analyses of polypeptides and proteins. The potential gradient (PG) detector does not provide completely reliable and reproducible data owing to protein adsorption on the electrodes of the detector and to electrode polarization. For this reason, the PG detector signal is of minor importance in polypeptide ITP analysis and its differentiation was not used.

Determination of peptide purity is important especially with peptides used as drugs and in biological tests, where admixtures may cause unwanted additional side effects. An example of the ITP analysis of adiuretin ([8-D-Arg]deaminovasopressin), which is used as a drug against diabetes insipidus, is shown in Fig. 7. A relatively high degree of purity was confirmed (see Table I).

CONCLUSION

CITP has been demonstrated to be a fast, high-performance, sensitive method of peptide microanalysis on the nanomole and subnanomole scale, giving both qualitative and quantitative information on peptide purity.

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