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ISOELECTRIC FOCUSING OF PEPTIDES

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SUMMARY

Analytical isoelectric focusing of peptides in 0.7-mm thick gel slabs, in presence of 8 *M* urea, is made possible by staining the gel directly in Coomassie Brilliant Blue G-250 dissolved in 1 *N* H_2SO_4–12% trichloroacetic acid solution. The carrier ampholytes are soluble in this solvent whereas the peptides form a macromolecular aggregate with the dye, thus being trapped and precipitated within the gel matrix. The minimum critical peptide length for precipitation and staining is of the order of 15 amino acids. Below this length, only some basic, lysine-containing peptides are able to form faint, stained precipitates. About 20 μg peptide per an average band contained in a 3.5-mm^3 gel volume can be detected by this technique. It is hypothesized that the dye cross-links different peptide chains by binding to basic residues *via* its SO_3^- groups.

INTRODUCTION

In theory, isoelectric focusing (IEF) should be adaptable to the separation of any amphoteric compound, including substances of low molecular weight, such as peptides, and even free amino acids. In practice, this is often a difficult proposition as the carrier ampholytes used in IEF, being themselves polyamino-polycarboxylic acids, exhibit many of the properties typical of proteins and peptides, including similar behaviour on ion-exchange chromatography and on molecular sieves and similar reactivities with common protein stains. Thus, it is often difficult not only to detect the focused peptides, but also to separate them, after focusing, from contaminant carrier ampholytes. In the case of free amino acids, the situation is even worse as all mono-amino-monocarboxylamino acids are practically isoelectric over the pH range 4–8 (ref. 1), and are thus found as several peaks spread over most of the separation column[2,3]. Only recently, by performing IEF in presence of 0.1-*M* NaCl, has it been possible to condense them at their theoretical p*I*'s, in single peaks of rather broad distribution[4].

Examples of the separation of peptides are very scarce in the literature. This

is because only peptides containing aromatic residues can be easily distinguished from ampholytes by UV absorbancy. Thus Catsimpoolas[5], by *in situ* analytical scanning IEF, has been able to show separations of chromophoric (UV absorbing) amino acid derivatives and dipeptides, such as L-lysyl-L-tyrosine and L-hystidyl-L-tyrosine. Kopwillem *et al.*[6] used gel IEF and analytical isotachophoresis to analyse peptides in the amino acid sequence 125–156 from human growth hormone (HGH)[7], synthesized by the Merrifield technique[8]. In order to detect the various bands, the peptide was synthesized with a dinitrophenyl-His residue, so that the focused bands were visible as bright yellow zones under UV light. In the case of peptide antibiotics of low solubility in water, the separation has been performed by IEF in gels containing 50% dimethyl sulphoxide (DMSO)[9]. After focusing, the gel is extruded into distilled water: the DMSO is rapidly leached out and the focused antibiotic zones precipitate at their p*I* values as opalescent bands that can be quantitated by a scan at 600 nm and recovered fully active. Clearly, these examples are only of limited applicability to very particular cases. Recently, Noble *et al.*[10] have applied IEF to the analysis and purification of the amino terminal 54-residue fragment of HGH; however, this instance too is not representative for the separation of peptides as the long fragment behaves as a protein and can thus be fixed in trichloroacetic acid (TCA) and stained by the usual techniques[11].

In the present report, we describe a new method for the separation and detection of peptides with lengths ranging from 8 to 54 amino acids.

MATERIALS AND METHODS

Acrylamide, N,N'-methylenebisacrylamide (Bis), ammonium persulphate and tetramethylethylenediamine (TEMED) were from Bio-Rad Labs., Richmond, Calif., U.S.A. Coomassie Brilliant Blue G-250 (xylene brilliant cyanin G) was purchased from Serva, Heidelberg, G.F.R. The peptides used were mostly sequences of the HGH molecule, or of the ovine growth hormone (OGH), synthesized by the Merrifield technique[8] or by conventional methods. Their lengths, molecular weights (MW) and amino acid sequences are listed in Table I. The peptides were dissolved in 8 *M* urea (ultrapure, from Mann Labs., New York, N.Y., U.S.A.) and 10% 2-mercaptoethanol (Serva) at a concentration of 10 mg/ml.

Isoelectric focusing

IEF was performed in gel slabs, in the LKB Multiphor 2117 cell, using an LKB constant wattage power supply. However, instead of using the usual 2-mm thick slab, we cast a 0.7-mm thick gel slab by using a home-made rubber gasket cut out of a 0.7-mm rubber sheet. The gel contained 7% acrylamide (the ratio of acrylamide to Bis being 25:1), 2% Ampholine (pH 3.5–10) and 8 *M* urea. 1 *M* NaOH and 1 *M* H_3PO_4 were used as catholyte and anolyte, respectively. The gel was cooled at 10° with a Lauda thermostat. After pre-focusing for 1 h at 10 W, the samples (10–15 μl) were loaded soaked in filter paper strips at the anodic side. After focusing for 1 h, all the filter paper strips were removed from the gel surface and focusing continued for an additional 2 h (total running time: 4 h). The voltage, at equilibrium, was usually 1000 V. pH measurements were made as previously described[16]. No p*I* corrections were made for the presence of urea, as suggested by Ui[17].

TABLE I

PEPTIDES USED IN THIS STUDY

*Peptides**	*Number of residues*	*Sequence*	*MW*
HGH 139–146[12].**	8	Phe-Lys-Gln-Thr-Tyr-Ser-Lys-Phe	1048
OGH 125–133[13].**	9	Arg-Glu-Leu-Glu-Asp-Val-Thr-Pro-Arg	1114
HGH 35– 44**.***	10	Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe	1302
HGH 33– 44**.***	12	Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe	1502
HGH 31– 44**.***	14	Phe-Glu-Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr--Ser-Phe	1778
		Dnp Dnp	
HGH 15– 36§.§§	22	Leu-Arg-Ala-His-Arg-Leu-His-Gln-Leu-Ala-Phe--Asp-Thr-Tyr-Gln-Glu-Phe-Glu-Glu-Ala-Tyr-Ile	3082
HGH 1– 24[14].§.§§§	24	Phe-Pro-Thr-Ile-Pro-Leu-Ser-Arg-Leu-Phe-Asp--Asn-Ala-Met-Leu-Arg-Ile-Ser-Leu-Leu-Leu-Ile--Gln-Ser	2759
HGH 111–134§.§§	24	Tyr-Asp-Leu-Leu-Lys-Asp-Leu-Glu-Glu-Gly-Ile--Gln-Thr-Leu-Met-Gly-Arg-Leu-Glu-Asp-Gly--Ser-Pro-Arg	2749
HGH 166–191[15].§ (oxidized with O_2)	26	Phe-Arg-Lys-Asp-Met-Asp-Lys-Val-Glu-Thr-Phe--Leu-Arg-Ile-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly--Ser-Cys-Gly-Phe	3049
HGH 25– 51[14].§.§§§	27	Trp-Leu-Glu-Pro-Val-Glu-Phe-Ala-His-Arg-Leu--His-Gln-Leu-Ala-Phe-Asp-Thr-Tyr-Gln-Glu--Phe-Glu-Glu-Ala-Tyr-Ile	3382
HGH 157–191[14].§ (tris-*S*-carbamido-methylated)	35	Leu-Lys-Ans-Tyr-Gly-Leu-Leu-Tyr-Cys-Phe-Arg--Lys-Asp-Met-Asp-Lys-Val-Glu-Thr-Phe-Leu--Arg-Ile-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser--Cys-Gly-Phe	4287
HGH 1– 36[15].§	36	Phe-Pro-Thr-Ile-Pro-Leu-Ser-Arg-Leu-Phe-Asp--Asn-Ala-Met-Leu-Arg-Ala-His-Arg-Leu-His-Gln--Leu-Ala-Phe-Asp-Thr-Tyr-Gln-Glu-Phe-Glu--Glu-Ala-Tyr-Ile	4354
HGH 96–134[15].§	39	Val-Phe-Ala-Asn-Ser-Leu-Val-Tyr-Gly-Ala-Ser--Asn-Ser-Asp-Val-Tyr-Asp-Leu-Leu-Lys-Asp-Leu--Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met-Gly-Arg--Leu-Glu-Asp-Gly-Ser-Pro-Arg	4273
HGH 115–156§.§§	42	Lys-Asp-Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met--Gly-Arg-Leu-Glu-Asp-Gly-Ser-Pro-Arg-Thr-Gly--Gln-Ile-Phe-Lys-Gln-Thr-Tyr-Ser-Lys-Phe-Asp--Thr-Asn-Ser-His-Asn-Asp-Asp-Ala-Leu	4757
HGH 103–156§.§§	54	Tyr-Gly-Ala-Ser-Asn-Ser-Asp-Val-Tyr-Asp-Leu--Leu-Lys-Asp-Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu--Met-Gly-Arg-Leu-Glu-Asp-Gly-Ser-Pro-Arg--Thr-Gly-Gln-Ile-Phe-Lys-Gln-Thr-Tyr-Ser-Lys--Phe-Asp-Thr-Asn-Ser-His-Asn-Asp-Asp-Ala-Leu	6055

* All peptides are crude products of reaction.
** Synthetized by conventional methods.
*** Manuscript in preparation.
§ Synthetized by solid-phase method.
§§ Unpublished results.
§§§ Synthetized before the HGH primary structure revision.

Staining

We used the method described by Blakesley and Boezi[18] for disc gel electrophoresis. To a 0.2% (w/v) aqueous solution of Coomassie B.B. G-250 an equal volume of 2 *N* H_2SO_4 is added. After stirring for 3 h, the precipitate is filtered through a Whatman No. 1 paper. To the clear brown filtrate 1/9 volume of 10 *N* KOH is added, producing a purple solution into which 100% (w/v) TCA is poured to give a final concentration of 12% (w/v). The resulting clear greenish light-blue solution is then ready for use. After focusing, the gel is bathed directly in this staining solution, and shaken for at least 3 h. It is then transferred into distilled water, where the blue colour of the bands is intensified. No background staining is observed in the gel, as the carrier ampholytes are soluble in this solution, and only the precipitated peptides slowly absorb the dye from the solution. One litre of this staining mixture can be used at least ten times with our thin gels (30 ml of gel solution are used to polymerize a gel slab).

RESULTS

Fig. 1 shows the focusing pattern, in the pH range 3.5–10, of the 15 peptides investigated. These peptides are representative of practically any amino acid composition, as there are acidic (samples 1, 3 and 6), close to neutral (sample 2) and basic (samples 4, 5 and 7) components, covering essentially any possible p*I* range. The first four peptides used (octa-, nona-, deca- and dodeca-peptides) are not precipitated in the H_2SO_4–TCA mixture, nor can they fix the stain, except for the octapeptide HGH 139–146, which is seen as a very faint band against the cathodic filter paper strip (pH *ca.* 12; not shown). This peptide contains two Lys and no acidic residues (except for the carboxyl terminus) and it ceases migrating at this very high pH because of partial deprotonation of the two Lys groups. Oddly, the nonapeptide, which also contains two basic residues (Arg, but also three acidic amino acids, two Glu and one Asp) does not precipitate nor pick up any stain. Starting from the tetradecapeptide, all the peptides analysed are fixed and stained in this stain mixture. The tetradecapeptide exhibits a single, homogeneous band with an uncorrected p*I* of 4.7. This p*I*, upon correction for the urea presence, as recommended by Ui[17], is in excellent agreement with the theoretical p*I* calculated with the Linderstrøm–Lang[19] equation, on the basis of its known amino acid composition (one carboxyl terminus, three Glu, two Lys and one amino terminus). The same applies to all other peptides investigated, which rules out any spurious band formation due to carrier ampholyte–peptide interaction. This has also been confirmed by excising and rerunning single, homogeneous bands in the focusing pattern. They run through to their original p*I* without splitting into several bands (not shown). This is a clear indication of absence of artefacts[20].

Fig. 2A shows the pattern obtained by focusing the same peptides and then fixing the gel in 10% TCA. It can be seen that only the major bands can be distinguished and much of the fine heterogeneity is lost. Moreover, all peptides shorter than 24 amino acids are lost, as they are soluble in 10% TCA. It is customary, in IEF, to fix first the protein pattern in 10% TCA, in order to leach out the carrier ampholytes, then to remove the excess of TCA by washing the gel three times in water and then to stain the protein bands in Coomassie B.B. R-250 dissolved in alcohol–acetic acid[11]. When this is done, quite a few of the peptide bands, already precipitated

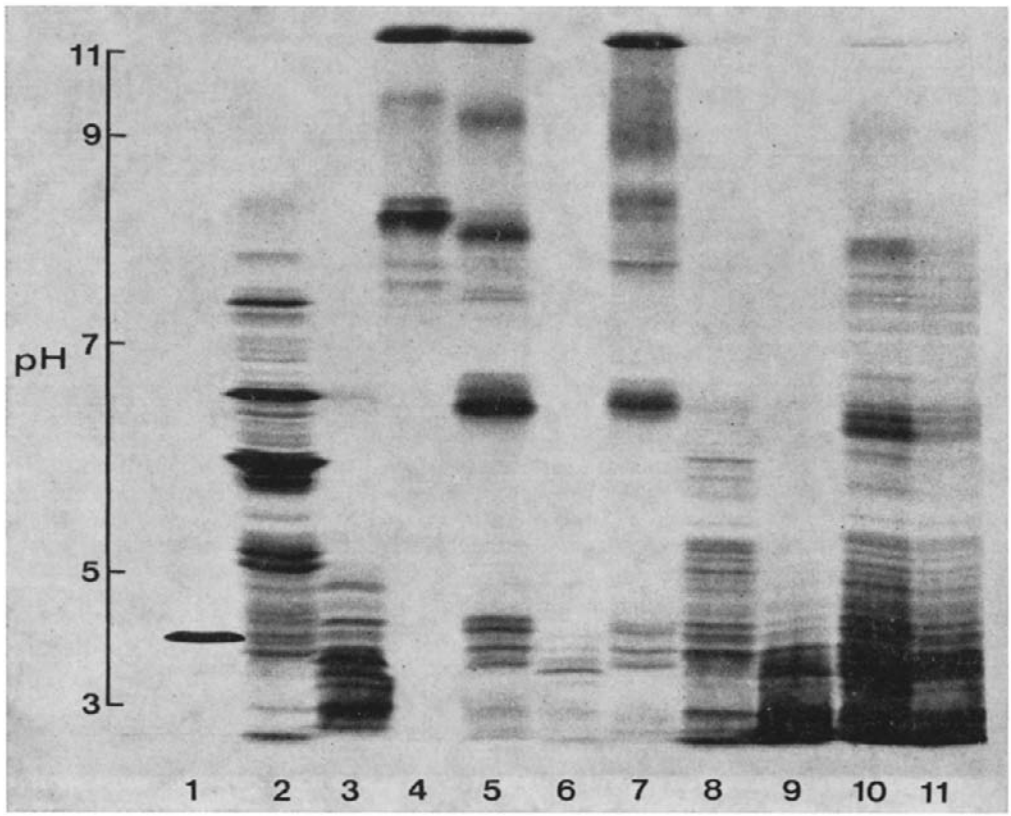

Fig. 1. IEF of peptides in the length range 8–54 amino acids. The gel slab was 0.7 mm thick and contained 7% acrylamide, 2% Ampholine pH 3.5–10 and 8 *M* urea. 10–15 μl of sample (10 mg/ml) were applied in filter paper strips at the anode after 1 h prefocusing. Total running time: 4 h at 10 W (1000 V at equilibrium). The gel was then dipped in the staining solution of Blakesley and Boezi[18]. Samples: 1 = HGH 31–44; 2 = HGH 15–36; 3 = HGH 111–134; 4 = HGH 1–24; 5 = HGH 166-191; 6 = HGH 25–51; 7 = HGH 157–191; 8 = HGH 1–36; 9 = HGH 96–134; 10 = HGH 115–156 and 11 = HGH 103–156. The octa, nona-, deca- and dodeca-peptides were neither fixed in the gel nor stained.

in 10% TCA, are lost in subsequent washings, as shown in Fig. 2B, so that the peptide patterns thus obtained are totally unreliable.

By running a series of standard dilutions of the tetradecapeptide, we have seen that by this method *ca.* 20 μg peptide per band (in *ca.* 3.5 mm^3 of gel volume, assuming an average band 10 mm long, 0.7 mm thick and 0.5 mm wide) can be detected (Fig. 3). This detection limit is much lower than the 0.2 μg detectable protein limit in a gel when stained with Coomassie B.B. R-250 in solution[21]. On the other hand, it must be emphasized that in the present method there is no need for background destaining and also that, in the latter method, because the gel is often destained at high temperatures, there is a great risk of the protein zones losing their stain even before the background is destained[22]. Moreover, this detection limit applies to the most difficult cases, *i.e.* to short peptides, which have a greater tendency to be solubilized and a lower dye affinity.

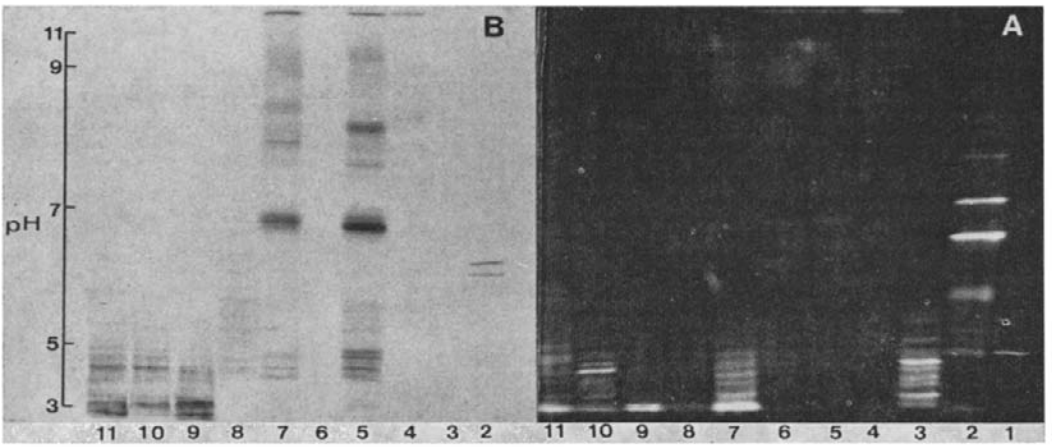

Fig. 2. IEF of peptides followed by conventional staining. IEF conditions and samples as in Fig. 1. At the end of focusing, the gel was bathed in 10% TCA and photographed (side A). After fixing the peptides, the excess TCA was washed with three rinses in water and the gel was then stained by conventional methods (Coomassie B.B. R-250 dissolved in alcohol–acid, side B). Note the total loss of samples 1, 3 and 6 and the heavy loss of material in all other samples, including high MW peptides. Since the same amount of material has been applied, the stain intensity here should be compared with the stained pattern in Fig. 1.

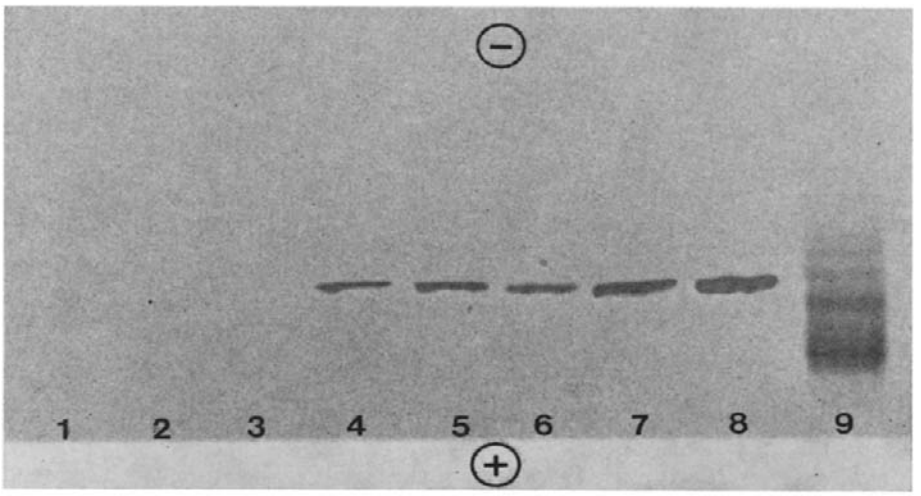

Fig. 3. Sensitivity of the staining method. Samples 1–8 refer to the tetradecapeptide HGH 31–44, in different loads. 1 = 1 μg; 2 = 5 μg; 3 = 10 μg; 4 = 20 μg; 5 = 30 μg; 6 = 40 μg; 7 = 70 μg; 8 = 100 μg; 9 = 100 μg of HGH 157–191. In the case of HGH 31–44, the minimum critical concentration for staining appears to be of the order of 20 μg.

DISCUSSION

For a successful fractionation of peptides by IEF, the following general experimental rules apply.

(1) Focusing should always be performed in the presence of urea or other appropriate dissociating agents (such as DMSO, dimethylformamide or tetramethyl-

urea[23]) in order to prevent aggregation of peptides and also any possible carrier ampholyte–peptide interaction, which would then generate an artefactual heterogeneity, because the peptide, existing in solution mostly as a random coil, has all its possible binding sites exposed to the solvent.

(2) IEF should be performed in thin gel layers. We have found a 0.7-mm thickness highly satisfactory because it is thin enough to allow for rapid fixing in the H_2SO_4–TCA solution, quick dye absorption and rapid leaching out of urea and carrier ampholytes, thus preventing diffusion of small peptides, and thick enough to allow for proper gel handling. Moreover, there is a considerable saving of expensive Ampholine and acrylamide and a much easier drying of the gel slab for permanent storage.

(3) The sample, when soaked in filter paper, should always be applied at the anodic side. As paper always contains a few carboxyl groups, they are fully ionized at basic pH but completely protonated at this low pH, thus preventing any absorption of the samples, especially if basic, on the filter paper. When applying the sample at the cathode, we have noticed strong absorption of basic peptides (samples 4, 5 and 6 in Fig. 1) with consequent loss of several bands from the stained pattern, which could only be abolished when the sample was applied in a liquid form in pockets pre-cast in 2-mm thick gels (not shown). In addition, the low pH at the anode will prevent possible carbamylation of the protein basic groups, in case traces of cyanate are present in the sample. The 1-h pre-run of the gel, prior to sample application, will also discharge at the anode any harmful persulphate and traces of cyanate in the gel.

(4) One drawback of the present method is that not all peptides are solubilized in the 8 *M* urea–10% 2-ME mixture. Two of the 15 peptides are poorly soluble in this solvent, so that only a few components (the most acidic, see samples 6 and 9, in Fig. 1) out of a very heterogeneous mixture can be focused and detected. Unfortunately, neutral detergents, which would often help solubilizing hydrophobic compounds, cannot be used (see below).

(5) Detergents (such as Nonidet P-40 or Triton X-100) should not be used in the present method. They bind to carrier ampholytes, forming complexes that are insoluble in 10% TCA (the gel becomes milky) but soluble in hot (60–70°) water. However, with the present staining technique, a tertiary complex detergent–dye–carrier ampholyte is formed, which cannot be solubilized without disaggregating too the dye–peptide complex.

From a theoretical point of view, we should like to stress the following points.

(1) The minimum critical peptide length for fixation in the gel matrix and dye binding appears to be around 15 amino acids. Insolubilization of peptide by dye binding is a function of both size and amino acid composition. Above this length, the size plays the most important role, whereas below it the peptide composition is critical.

(2) Dye binding is favoured by the presence of basic amino acids (Arg, Lys and His) and by a relatively high content of hydrophobic amino acids on the peptide. Besides binding to basic groups, the dye can interact with hydrophobic residues, particularly if they are close together. As a corollary, a relatively high content of hydrophilic (especially acidic) amino acids does not favor dye binding.

(3) Our data suggest that dye binding is stronger with Lys than with Arg (possibly for steric reasons). The octapeptide, which contains two Lys residues, binds

the dye (even if very poorly) and forms a faint precipitate. The nonapeptide, which contains two Arg residues instead of Lys, does not seem to bind the dye or at least forms soluble complexes, which are eluted from the gel.

(4) We hypothesize that the peptide is insolubilized because the dye binds two chains simultaneously (via its SO_3^- groups), thus acting as a bridge or a cross-link between chains and forming a macromolecular aggregate that is trapped within the random meshwork of fibres of the gel (for the dye formula, see Diezel *et al.*[24]). If this is so, a peptide has to have at least one basic residue, so that the dye can act as a cross-link over several chains, utilizing the amino terminus plus the basic residue. A chain with no basic residues would only have the amino terminus as a binding site, so that the dye could only link two chains at a time, thus forming a soluble complex that would escape detection (see the model in Fig. 4). It must also be emphasized that multiple binding sites on the polypeptide will also increase the stability of the complex through stacking of the aromatic rings of the dye molecules.

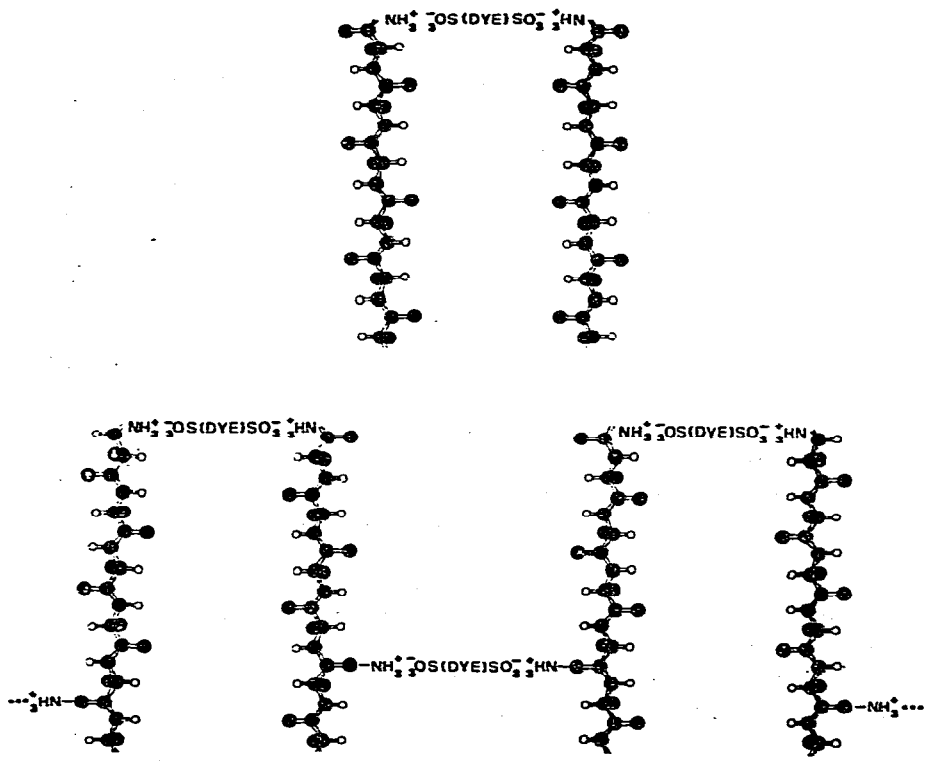

Fig. 4. Proposed dye–peptide binding model. A peptide with no basic residues (upper part), will only bind the dye via its NH_2 terminus, thus forming a dimeric complex that readily splits because it is extremely unstable in H_2O. The lower part shows a peptide with an additional binding site in the chain (an ε-NH_2 group of Lys). In this case, a macromolecular aggregate is formed, that is trapped in the gel matrix and is insoluble in water. This model should be seen not as linear, as depicted, but as a three-dimensional array of chains. The aggregate is further stabilized by stacking among aromatic rings of different dye molecules, made possible by the ionic interactions of the two sulphate groups of the dye with the peptide amino groups.

We feel that, with the present method, it is possible to analyse by IEF peptides in the length range 15–50 amino acids, the range in which most of the biologically active peptides are located.

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