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GEL ELECTROPHORETIC SEPARATION OF ANGIOTENSINS I, II and III.

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SUMMARY

The hepta-, octa- and decapeptides, angiotensins III, II, and I with molecular weights 930, 1,045, and 1,295, were separated by polycrylamide gel electrophoresis at pH 6.41, positive polarity, using gel concentrations ranging from 24 to 40 %T, 2 $\%C_{Bis}$. The peptides were detected by a conventional protein staining method using Coomassie Brilliant Blue R-250 modified by the addition of 1.25% glutaraldehyde, thus crosslinking the peptides to "proteins". Separation between the 3 peptides is due nearly entirely to net charge rather than to size. Resolution is therefore optimal in charge fractionation methods such as electrofocusing, chromatofocusing, ion exchange chromatography and liquid-liquid partition chromatography.

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INTRODUCTION

The need for a simultaneous detection and quantitation of the hepto-, octa- and decapeptides, angiotensins III, II and I arose in the analysis of a perfused kidney system[1]. Although every major separation tool has previously been applied to the individual angiotensins and their radioactive derivatives[2], their joint analysis presented a new problem. "Quantitative" polyacrylamide gel electrophoresis (PAGE) was applied to this problem in order to find the optimally resolving pore size [3] for the separation of the 3 peptides, and determine any multiplicity of aggregation states for each. Discontinuous buffer systems were applied to render this separation independent of the degree of sample dilution (in the physiological application) and to allow for the optimization of the pH of separation by a convenient procedure, using stacking gels[4]. To solve the problem conveniently, it was attempted to find a simple procedure for the fixation and staining of the angiotensins.

While this investigation proceded, two reports [5,6] appeared on the "charge separation" [4] of the 3 angiotensins by high performance liquid chromatography. The first [5] used reverse phase partition chromatography which may be considered a "charge fractionation" method since the partition coefficient between immiscible phases is largely a function of molecular net charge (neglecting adsorption onto the column matrix). The second [6] used chromatofocusing on the iodinated angiotensins, but had to be

conducted in 1 M NaCl on the native peptides to overcome adsorption onto the column matrix. Both of these methods excel from the viewpoint of rapid quantitation at a minimal workload, thus pre-empting the need for zone quantitation by densitometry in this electrophoretic study.

MATERIALS AND METHODS

1) Peptides: Angiotensins I, II and III were obtained from Sigma (Cat. No. A 9402, A 9525 and A 9514) and used without further purification.

2) Polyacrylamide gel electrophoresis (PAGE):

A) Buffer system: The pH was optimized, and independence of sample volume was secured by conducting PAGE in discontinuous buffer systems, using stacking gels only[4]. Since the angiotensins were known to be basic peptides, buffer systems of the positive polarity [7] were used. The temperature of PAGE was kept at 0-2°C to secure chemical stability and to guard against enzymatic hydrolysis. Several buffer systems of an operative pH of 3.50 to 6.41 with trailing ion net mobility (TINM) values ranging from 0.050 to 0.100 were tested with regard to their capacity to stack angiotensins I, II and III in gels of 5 %T, 15 $\%C_{DATD}$[8], using methyl green to mark to stack. The highest pH capable of stacking the 3 angiotensins was 6.41 (TINM = 0.095). The composition of the buffer system is given in Table I.

B) PAGE procedure: The angiotensins I, II and III were subjected separately, and in a mixture, to polyacrylamide gel

electrophoresis at gel concentration ranging from 20 to 40 %T, 2 $\%C_{Bis}$, 0°C, using cylindrical gels of 1.2 ml volume and 6 mm diameter [9] in the buffer system specified in section A. Stacking gel volume was 0.5 ml. Tracking dye was methyl green.

TABLE I

Prepared Buffer				Operative Buffer	
			$pH_{25°}$	$pH_{0°}$	TINM
Anolyte (Upper Buffer)	pyridine 1 N HCl	1.58g per 1; 10.0 ml	3.59		
4 x concentrated Stacking Gel Buffer	imidazole ACES	4.73g per 100 ml; 4.45g	7.40	6.70	0.052
4 x concentrated[b] Resolving Gel Buffer	imidazole ACES	2.58g per 100 ml; 7.24g	6.87	6.41	0.095
Catholyte (Lower Buffer)	1 N KOH ACES	10.0ml per 1; 3.64g	6.80		

[a]"Operative" conditions are those in the gel after passage of the imidazolium/pyridinium boundary.

[b]The 4-fold concentration allows for polymerization of a gel by a 7:2.5:0.5 volume ratio of acrylamide stock solution:buffer:catalyst mixture.

Stacking of the 3 peptides was tested in stacking gels (Table I) of 1.7 ml and 5 %T, 15 $\%C_{DATD}$, using as a criterion for stacking the coincidence of the stained peptide bands and the hypersharp zone of methyl green which does not broaden with increasing migration distance.

Staining was carried out by a modification of the procedure of Axelsen et al[10]. Gels were suspended in 40 ml of fixative (20% acetic acid, 1.4% picric acid and, 1.25% glutaraldehyde) for 30 min. Then 2 ml 0.25% Coomassie Brilliant Blue R-250 was added. After 1 h, gels were destained in 20% acetic acid, using the apparatus described previously [11].

Resolving gels were prepared from a stock solution of 60 %T, 2 $\%C_{Bis}$, stored at R.T. for a period not exceeding 1 week. Polymerization mixtures were prepared [9] by mixing 7 ml of an appropriate dilution of this stock solution with 2.5 ml resolving gel buffer (Table I) and 0.5 ml of 0.3% K-persulfate, 5×10^{-3} % riboflavin. After a 5 min evacuation by oil pump at 0°C (ice-water bath), 24, 28, 30, 32, 35, 38 and 40 %T gels were photopolymerized by addition of 6, 5, 10, 4, 5, 2 and 2 µl TEMED/ml gel respectively.

RESULTS

Fig. 1 depicts the Ferguson plots at pH 6.41, 0°C, obtained from 50 µg each of angiotensins I, II and III. The 3 peptides are most conveniently resolved on the same gel at 24 %T. However,

since the Ferguson plots are nearly parallel, the theoretical optimum [3] for their resolution is at 0 %T, i.e, by charge separation methods [4] such as electrofocusing, chromatofocusing, ion exchange or partition chromatography.

DISCUSSION

It is widely held that polyacrylamide gel electrophoresis is inapplicable to oligopeptides with molecular weights of the order of 1,000 for 2 reasons: Inability to prepare a gel matrix capable of exerting molecular sieving effects on molecular species of such small molecular size, and second, inability to fix such species, with consequent transient character of staining.

This study shows clearly that both of these arguments against the feasibility of oligopeptide separation are untenable. Polyacrylamide gels in the ranges of 24 to 40 %T can be prepared at 0°C within the optimal polymerization time of about 10 min by conventional photopolymerization procedures [9] and catalyst concentrations. Within that concentration range, polyacrylamide is clearly restrictive to the peptides as demonstrated by their Ferguson plots (Fig. 1). It should be noted that R_f values refer to the migration distance of the peptides divided by the migration distance of the imidazolium-pyridinium moving boundary front. The Ferguson plot of methyl green (Fig. 2) provides the positions of this front (see Legends to Figs. 1 and 2).

The problem of peptide fixation prior to staining has been solved by crosslinking in a generally applicable fashion. Cross-

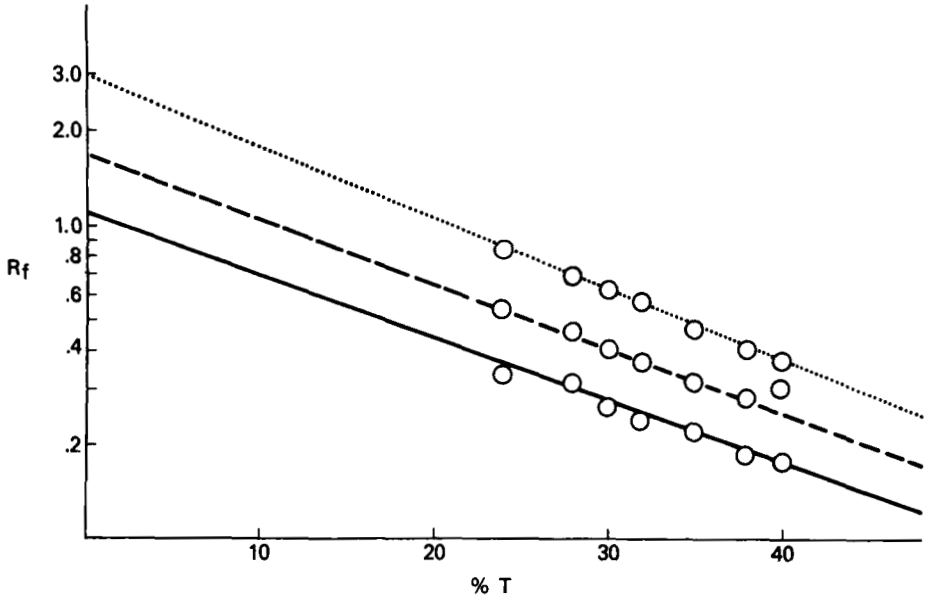

FIGURE 1

Ferguson plots of angiotensins I (dashed line), II (solid line) and III (dotted line). pH 6.41, TINM 0.095, 0°C, positive polarity. The tracking dye, methyl green, marks the imidazolium/pyridinium moving boundary up to 29 %T. At larger gel concentrations, the migration distance of methyl green divided by its R_f (Fig. 2) provides the position of the moving boundary front.

linked oligopeptides can be visualized by the conventional protein stain, Coomassie Blue R-250. It seems obvious that peptide staining with prior fixation has advantages of reproducibility and reproducible color yield compared to time-dependent staining procedures either without fixation, or under fixation conditions of questionable efficacy such as alcohol-acetic acid solutions[12]. Oligopeptide fixation in a nitrocellulose matrix has been reported previously[13], but it seems not clear at this time to what degree

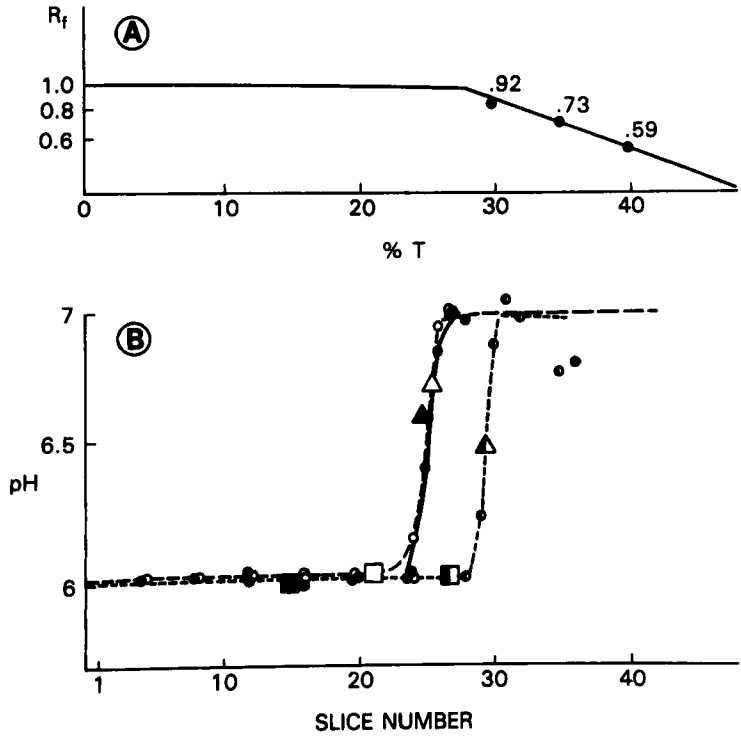

FIGURE 2

Panel A: Ferguson plot of methyl green under the conditions of Fig. 1. Panel B: The imidazolium/pyridinium boundary was located by slicing the gels into 1 mm slices, using the electro-vibrator slicer (Hoefer), suspending the slices in 0.025 M KCl and pH analysis on the slice suspensions. Half-closed symbols: 30 %T; open symbols: 35 %T; closed symbols: 40 %T. Squares designate the position of methyl green, triangles those of the moving boundary.

such adsorption onto a matrix is related (inversely) to peptide chain length. A similar argument holds with regard to isoelectric precipitation may provide a pseudo-fixation[14]. But again the degree of isoelectric precipitation, and the relative insolubi-

lity of the precipitate, must vary greatly among peptides, compared to the general effectiveness of glutaraldehyde crosslinking.

For the purposes of quantitation of the 3 angiotensins in the mixture, densitometry of the stained bands appears feasible but has not been carried out in view of the fact that separation of the 3 angiotensins and quantitation by HPLC (partition chromarography and chromatofocusing) has become available while this study was in progress[5,6]. It seems obvious that for purposes of quantitation, HPLC is the less laborious and faster method. Interestingly, the partition column at pH 3 eluted the angiotensins in the order III, II and I [15] while the elution from the chromatofocusing column as well as the electrophoretic mobility at pH 6.41 is in order of isoelectric points (7.11, 7.05 and 6.56 for iodinated angiotensins III, I and II respectively [6]). This suggests that the partition column, just like the chromatofocusing column applied to the native peptides, specifically adsorbs angiotensin II. This adsorption may be related to the fact that angiotensin II possesses a conformation, probably as an antiparallel beta-pleated sheet [p. 155 of [16]].

The nearly parallel Ferguson plots of angiotensins I, II and III show that the optimal gel concentration for their resolution occurs at 0 %T. Thus, isoelectric focusing, chromatofocusing, isotachophoresis and ion exchange chromatography are the preferred methods for their separation[3,9]. However, isoelectric focusing cannot employ peptide detection by staining after glutaraldehyde fixation since carrier ampholytes - both of the synthetic mixture (e.g., Ampholine, LKB) or the simple buffer [17] type - crosslink

with glutaraldehyde. Chromatofocusing in 1 M NaCl containing eluent appears suitable for both separation and quantitation [6]. Isotachophoresis in the capillary apparatus in conjunction with either thermal or UV-detection [18], ion exchange chromatography on either 1 or 2% crosslinked Dowex-50 or on HPLC columns remain to be applied to the angiotensins.

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