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ANALYTICAL SEPARATION OF AMINO ACIDS ON A CATION-EXCHANGE RESIN CROSS-LINKED WITH *m*-DIVINYLBENZENE

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SUMMARY

The elution bands of acidic and neutral amino acids of protein hydrolysates, emerging from the column of a cation-exchange resin cross-linked with pure *m*-divinylbenzene, are narrower than those from a resin prepared from styrene and technical divinylbenzene. As a result of these narrower bands, a more complete resolution of the critical pairs threonine–serine, glycine–alanine and tyrosine–phenylalanine is obtained. The most probable reason for the narrower elution peaks is the more rapid diffusion of the exchanged components through the bulk of the resin as a result of a more regular arrangement of cross-linkages in the cation-exchange resin prepared from *m*-divinylbenzene.

INTRODUCTION

The matrix of the cation-exchange resins that are used for the separation of amino acids according to the Stein and Moore method is formed from a styrene–divinylbenzene (DVB) copolymer. The cross-linking comonomer (technical-grade DVB), as is well known, is not a single compound but contains, in addition to *m*- and *p*-divinylbenzene, other monomers (*e.g.*, ethylvinylbenzenes) and also components (mainly diethylbenzenes) that do not polymerize but that affect the properties of the copolymer.

Of *m*- and *p*-divinylbenzene, the former isomer is considered to be a more suitable cross-linking agent[1–3] because, owing to its lower reactivity, which is similar to that of styrene[4], it creates conditions for the formation of a copolymer with a more regular arrangement of cross-linkages. Nevertheless, even with the copolymer of styrene and *m*-DVB, structural inhomogeneities have been reported[5], resulting from the dependence of the reactivity of the second double bond of divinylbenzene (the so-called "pendant" vinyl) upon conversion during the polymerization. From this point of view, the marked differences in the structure that it is sometimes suggested exist between the two copolymers[6] seem not to be sufficiently established.

Several workers[7–9] have mentioned the possible consequences that the composition of divinylbenzene might have upon the resolving ability of the cation-exchange resin during the separation of amino acids. However, no experimental

evidence was given. Zuev *et al.*[10] synthesized resins based on styrene–*m*-DVB and styrene–*p*-DVB copolymers but they did not compare them with the standard resin in the chromatography of amino acids. This encouraged us to attempt to solve the problem by comparing the performance of two resins, cross-linked by the same amounts of technical-grade DVB and *m*-DVB, in the separation of acidic and neutral amino acids present in protein hydrolysates.

MATERIALS AND METHODS

Resins

The resins for comparison were prepared by sulphonation of a suspension of a copolymer of styrene and divinylbenzene (8.5%, w/w) under identical conditions[11].

The crosslinking agents were as follows. Technical-grade divinylbenzene (Dow Chem., Midland, Mich., U.S.A.) contained *m*-divinylbenzene (45.0%), *p*-divinylbenzene (16.4%), *m*-ethylvinylbenzene (27.9%) and *p*-ethylvinylbenzene (5.1%), the balance being diethylbenzenes, styrene and xylenes. *m*-Divinylbenzene (K & K Labs., Plainview, N.Y., and Hollywood, Calif., U.S.A.) contained *m*-divinylbenzene (94.1%) and *p*-divinylbenzene (3.3%), the balance being ethylvinylbenzenes, styrene, diethylbenzenes and xylenes.

The cation-exchange resins were graded by hydrodynamic classification and their basic properties were virtually identical as can be seen from Table I. Lord and Student tests proved that the differences between the individual mean values were not statistically significant. The exchange capacity and water regain (relative to the H^+ forms) of the resins was determined according to Czechoslovak Standards[12]. Mean particle sizes and their standard deviations were determined by a microscopic method by classifying a minimum of 300 particles into size groups of range 1 μm.

Conditions of separation

An automatic amino acid analyzer, Type AAA 881 (Mikrotechna Co., Prague-Modřany, Czechoslovakia) was used with 60.0 × 0.8 cm columns. Sodium citrate

TABLE I

BASIC PROPERTIES OF TWO CATION-EXCHANGE RESINS

Property	*Component copolymerized with styrene in resin*		
	Technical-grade DVB	*m-DVB*	*u(t)**
Strong acid capacity (mequiv./g)	5.10 ± 0.03	5.06 ± 0.05	0.443
Weak acid capacity (mequiv./g)	0.09 ± 0.01	0.08 ± 0.01	0.443
Total exchange capacity (mequiv./g)	5.19 ± 0.03	5.14 ± 0.05	0.554
Sulphur/carbon ratio	0.313 ± 0.006	0.310 ± 0.004	0.266
Water regain (g/g)	0.98 ± 0.04	1.10 ± 0.06	1.063
Mean particle size (μm)	17.3 ± 1.3	17.2 ± 1.4	0.178

* The critical values of the Lord and Student's *t* test are $u_{0.05} = 1.714$ and $t_{0.05} = 1.960$, respectively.

lution buffers of pH 3.25, 4.25 and 5.28 were used at a flow-rate of 70 ml/h, and the ninhydrin flow-rate was 35 ml/h. The temperature was 53°. Amounts of 100 nmoles of each amino acid were applied, and the standard series of acidic and neutral amino acids studied consisted of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine.

RESULTS AND DISCUSSION

As criteria for the resolving efficiency of the resins prepared from technical DVB and from *m*-DVB, the following parameters were employed: elution peak widths; differences in peak elution volumes and therefrom the resulting degree of resolution of pairs of amino acids that are difficult to resolve, *i.e.*, threonine–serine, glycine–alanine and tyrosine–phenylalanine; analysis time expressed by the elution time of the last amino acid of the standard series (phenylalanine); and the pressure in the chromatographic column. These criteria are affected by numerous factors, some of which have already been described, others being considered for the first time. It is known that the resolution of amino acids is affected by the properties of the cation-exchange resin, such as mean particle diameter and its variance[13], the degree of cross-linking of the matrix[7,13], the reaction conditions under which the exchange group was introduced into the matrix[11], the degree of extraction of non-cross-linked polymer from the resin, and the concentration of functional groups[14]. Also, long-term experiments revealed statistically significant effects of the condition of the apparatus, of the fluctuation of the separation conditions and, in some instances, of the packing of the resin bed (arrangement of particles) prevailing in the particular working cycle.

Under these circumstances, in order to be able to distinguish exclusively the effect of the structure of the resin matrix, determined by the character of the cross-linking monomer, upon the separation criteria selected, comparative experiments were arranged in such a manner that cation-exchange resins of identical properties, prepared under identical conditions, were used. These resins were filled into both of the long columns of the analyzer, the same standard amino acid mixture was loaded on both columns and the same buffers and regenerant were introduced alternately. At the half-point of the series of experiments, the resins in both columns were mutually exchanged. In this way, all of the factors that affect the resolution were maintained the same and the effect of the column packing conditions was also reduced. The parameters of resolution obtained are given in Table II. The significance of the differences in the mean criterial values obtained with the resins being compared was evaluated by the Student's t test at $p = 0.01$.

From the comparison with the critical value of t, it follows that all of the differences between the resolution criteria investigated, with exception of the differences in the elution volumes of glycine and alanine and the analysis time, are statistically significant. The following conclusions on the behaviour of the two cation-exchange resins in the separation of amino acids can therefore be drawn:

(1) Bands of amino acids eluted from the column packed with the *m*-DVB resin are narrower that those of the resin cross-linked with technical-grade DVB.

(2) With the *m*-DVB resin, difference in the peak elution volume of threonine and serine is less than, that of glycine and alanine is equal to and that of tyrosine and

TABLE II

CRITERIA FOR THE RESOLUTION OF AMINO ACIDS WITH TWO RESINS

Amino acid pair	*Criteria for resolution**	*Component copolymerized with styrene in resin*		$\bar{x}_B - \bar{x}_A$	t^{**}
		Technical-grade DVB ($\bar{x}_A$)	*m-DVB* ($\bar{x}_B$)		
Threonine–serine	$V_{Ser} - V_{Thr}$ (cm³)	1.798 ± 0.075	1.698 ± 0.055	− 0.100	3.630
	σ_{Thr} (cm³)	0.428 ± 0.018	0.387 ± 0.011	− 0.041	6.634
	σ_{Ser} (cm³)	0.409 ± 0.024	0.363 ± 0.010	− 0.046	5.935
	$R_{Thr,Ser}$	2.148 ± 0.065	2.264 ± 0.076	+ 0.116	3.942
Glycine–alanine	$V_{Ala} - V_{Gly}$ (cm³)	3.954 ± 0.273	3.931 ± 0.633	− 0.023	0.111
	σ_{Gly} (cm³)	0.597 ± 0.038	0.500 ± 0.019	− 0.097	7.918
	σ_{Ala} (cm³)	0.687 ± 0.023	0.572 ± 0.041	− 0.115	8.345
	$R_{Gly,Ala}$	3.079 ± 0.209	3.667 ± 0.456	+ 0.588	3.917
Tyrosine–phenyl-alanine	$V_{Phe} - V_{Tyr}$ (cm³)	2.677 ± 0.074	3.513 ± 0.164	+ 0.836	15.499
	σ_{Tyr} (cm³)	0.580 ± 0.083	0.487 ± 0.042	− 0.093	3.443
	σ_{Phe} (cm³)	0.641 ± 0.044	0.556 ± 0.048	− 0.085	4.434
	$R_{Tyr,Phe}$	2.192 ± 0.244	3.368 ± 0.113	+ 1.176	15.033
	t_{Phe} (sec)	8631 ± 133	8710 ± 106	+79	1.565
	Pressure in column (kg/cm²)	15.2 ± 1.0	16.1 ± 1.3	+ 0.9	1.870

* V_a is the elution volume of component a; σ_a is a peak half-width at the point of inflection; $R_{a,b}$ is the resolution of components a and b; $R_{a,b} = V_a - V_b/\sigma_a + \sigma_b$; t_a is the elution time of component a.

** The critical values of the Student's t test are $t_{0.10} = 1.721$ and $t_{0.01} = 2.831$.

phenylalanine is greater than the corresponding values with the resin prepared from technical-grade DVB.

(3) The narrower elution peaks obtained with the *m*-DVB resin have a positive effect on the resolution. This effect is partially impaired by the smaller differences in the peak elution volumes of threonine and serine. Nevertheless, the R values of all three critical amino acid pairs found with the *m*-DVB resin are markedly greater than those with the standard product. The pairs glycine–alanine and tyrosine–phenylalanine are completely resolved by the *m*-DVB resin.

(4) The analysis time of the standard amino acid mixture is the same with both resins.

(5) The hydrodynamic resistance of the *m*-DVB resin is slightly higher than that of the standard resin. The difference found is, however, statistically less significant than with the other criteria of separation.

In order to illustrate the resolution obtained with the *m*-DVB resin, a typical chromatogram of the standard amino acid series is given in Fig. 1.

According to Hamilton[13], the elution peak width, characterized by the variance, σ^2, depends upon the cross-sectional area of the chromatographic column (A), its length (Z), a dimensionless number that characterizes eddy diffusion[15] (λ), the linear flow-rate of the elution buffers (U_0), the distribution coefficient of amino acids between the stationary and mobile phases (K_d), the mean particle size of the resin (d_p), the void volume (F_I) and the diffusion coefficient of the components being exchanged across the stationary phase (D_s).

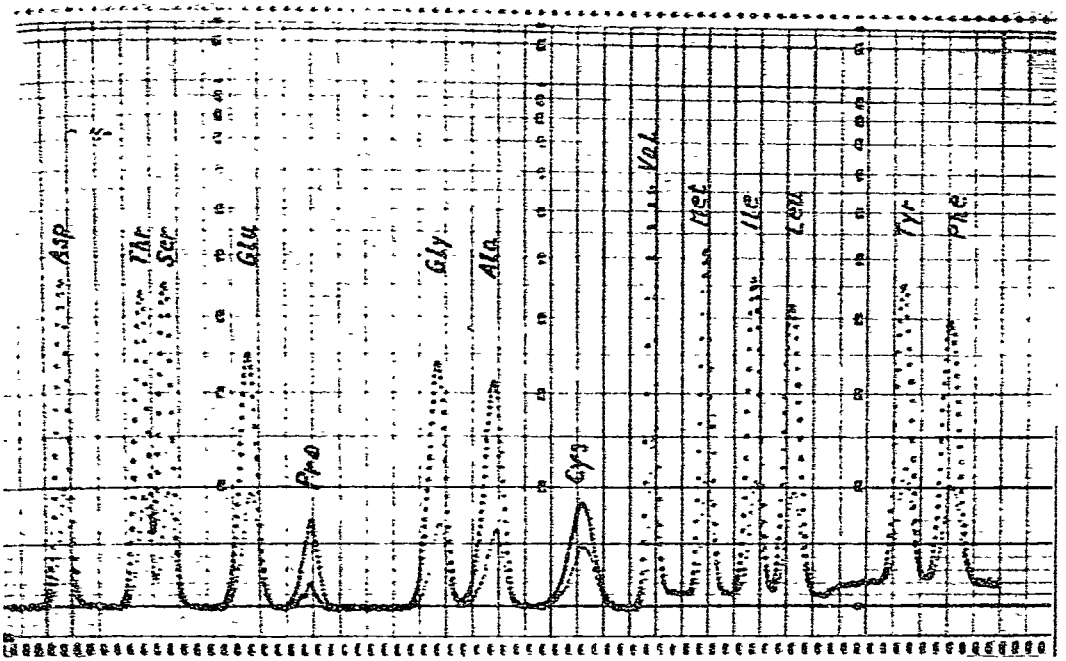

Fig. 1. Resolution of a standard series of acidic and neutral amino acids on the *m*-DVB resin.

As identical columns, identical flow-rates of eluents and resins of the same particle size distribution were used in this comparison, the factors A, Z, λ, U_0, d_p and F_I must be constant in both experimental runs. The differences in the elution band widths found with the two resins must therefore be attributed to the distribution coefficient, K_d, and to the solid-phase diffusion coefficient of the components, D_s.

Although the distribution coefficients of some pairs of amino acids apparently differ, the narrowing of the elution bands of all amino acids of the standard series found with the *m*-DVB resin cannot be explained by this difference. The narrowing of peaks must therefore be caused by higher values of the diffusion coefficients in the resin phase. The accelerated transport of the components that take part in the exchange is in agreement with the more regular arrangement of cross-linkages in the matrix of the resin prepared from pure *m*-DVB.

The cause of the differences in the distance between the elution bands if the column sizes are identical is, according to the interpretation of Hamilton[13], the different affinities of the two resins for the amino acids tested. In view of a recent paper by Wiley[16], who reported on the different selectivities of cation-exchange resins based on styrene–*m*-DVB and styrene–*p*-DVB copolymers, this fact is not very surprising.

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