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THE USE OF QUATERNARY AMMONIUM ETHYL CELLULOSE IN THE FRACTIONATION OF TYROSINE-RICH PROTEINS FROM WOOL

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SUMMARY

A group of tyrosine-rich proteins from wool which are only soluble to any extent above pH 10.0, have been chromatographed on quaternary ammonium ethyl cellulose at pH 10.5. The elution profile consisted of two major and nine minor peaks. This fractionation was based largely on differences in the tyrosine content of the proteins which covered the range from 7 to 20 residues %. A combination of chromatography at pH 10.5 followed by gel electrophoresis at pH 8.5 revealed the presence of at least thirty components in this protein preparation, the great majority of which contained 27–33 residues % of glycine, 8–12 residues % of phenylalanine, 9–16 residues % of serine and 3–7 residues % of carboxymethylcysteine.

INTRODUCTION

Small proteins, which contain relatively large proportions of glycine and the aromatic amino acids, particularly tyrosine, appear to be a common constituent of mammalian keratins, occurring in amounts up to an apparent maximum of 30% in *Echidna* quill[1,2]. In spite of the major role which they can play in some keratins, they have received only scant attention, and little is known about them, particularly the extent of their heterogeneity and the limits of their amino acid compositions. One reason for this is that certain components, particularly those low in sulphur, in the reduced and S-carboxymethylated form, are difficult to handle by many conventional techniques, being only slightly soluble below pH 10.0. The anion exchangers based on cellulose, commonly used in protein fractionation are uncharged at these pH values. Although these proteins are soluble in concentrated formic acid and 6 *M* urea solutions, fractionation at acid pH values has had only limited success because they contain few basic groups[3,4], whilst in urea at neutrality, and at slightly alkaline pH values, it has so far proved impossible to prevent carbamylation reactions taking place. This is an important consideration, for the cystine-poor components at least, appear unique amongst keratin proteins in having non-acetylated end groups.

The availability of quaternary ammonium ethyl (QAE) cellulose, which contains anion-exchange groups still ionized at pH values above 10 where the proteins

are soluble, has made it possible to explore the nature and extent of the heterogeneity of these proteins and to isolate one component in a state approaching purity. This paper describes a study made of the group of cystine-poor, high-tyrosine proteins, which have been termed Type I components[1].

EXPERIMENTAL

Preparation of high-tyrosine proteins

Wool was solubilized by alkaline reduction in 6 *M* urea, alkylated with iodoacetate, and the solution dialysed[5]. Both the high-tyrosine and low-sulphur proteins were precipitated from this solution by addition of zinc acetate to 0.02 *M* (pH 6.0). The precipitate was collected by centrifugation, stirred with 0.02 *M* sodium citrate solution for 2 h, and the turbid solution was then dialysed against running deionized water for 2 days.

This heavily turbid solution of zinc-precipitable proteins was centrifuged at 40,000 *g* for 1 h to sediment those high-tyrosine proteins (Type I) which are essentially insoluble below about pH 10. The precipitate was dissolved in 0.1 *M* ammonium hydroxide and the high-tyrosine proteins reprecipitated by dialysing the solution against 0.1 *M* ammonium bicarbonate solution, leaving any contaminating low-sulphur proteins in solution. The precipitate was dissolved in 0.1 *M* ammonium hydroxide and the solution freeze-dried[1].

Chromatography

QAE-cellulose (Schleicher and Schüll, Lot 2282, 0.84 mequiv./g) (20 g) was suspended in 2 l 0.5 *M* NaOH, stirred for 1 h, collected by filtration on a Büchner funnel, and then washed on the funnel with several litres of deionized water. The cellulose was then suspended in 1 l of starting buffer (0.05 *M* β-alanine–0.05 *M* NaOH, pH 10.5) with the aid of a Waring Blendor, stirred for 1 h, collected by filtration, resuspended in 500 ml of starting buffer, deaerated, and used to pack a column immediately. If the suspension stood for more than a few hours before use, the cellulose packed as sheets with pronounced layering. The column dimensions were 50 × 2 cm, and a packing pressure of 5 lbs/in.^{2} was used.

300 mg of protein was dissolved in 20 ml of starting buffer, the pH adjusted to 10.5 by the dropwise addition of ammonia, centrifuged to remove any insoluble material, and applied to the top of the column. Proteins were eluted by a linear gradient of sodium chloride in the starting buffer to a limiting concentration of 0.5 *M*. The total volume of eluant was 1200 ml with a pumping rate of 1.6 ml/min and a fraction size of about 10 ml. The column was regenerated between runs by passing through 2 l of the starting buffer, and periodically the cellulose was washed with 0.5 *M* NaOH and the equilibration and packing procedure repeated.

Amino acid analysis

The samples of protein were hydrolysed for 22 h *in vacuo* at 108° with 6 *M* HCl and 2 m*M* phenol. Each hydrolysate was freeze-dried, and the amino acid composition estimated with a Beckman-Spinco 120C amino acid analyzer.

Electrophoresis in starch gels

The gel was composed of 13.0 g hydrolysed starch (Connaught), 40 g urea, and 50 ml buffer (pH 8.46), containing citric acid 8.5 m*M*, Tris 75 m*M* and sodium tetraborate 3.7 m*M*. The bridge buffer (pH 8.35) contained boric acid (0.3 *M*) and NaOH (0.06 *M*). The gels were run in a cooled plate apparatus at a voltage gradient of 15 *V*/cm for about 4 h. At the completion of electrophoresis the gels were sliced and stained with nigrosine (0.01%) in water–ethanol–acetic acid (45:45:10)[1].

RESULTS AND DISCUSSION

Type I high-tyrosine proteins are almost completely eluted from QAE-cellulose at pH 10.5 with a salt gradient with a limiting value of 0.5 *M* (Fig. 1). The elution profile consists of two major peaks (C and F) and at least nine minor ones. Ten fractions were prepared for further examination by pooling appropriate fractions as shown on the profile. They were then dialysed against deionized water and freeze-dried. The yields of fractions after dialysis and freeze-drying are shown on Table I. The yield is about 87% which is remarkably good considering the possibility of losses during handling.

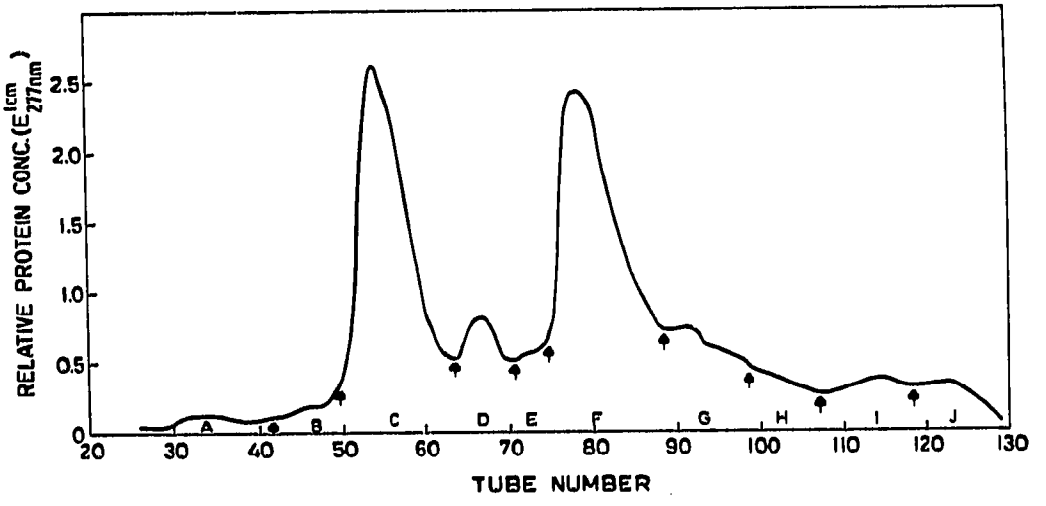

Fig. 1. The chromatography of Type I high-tyrosine proteins on QAE-cellulose at pH 10.5 in β-alanine–NaOH buffer, 0.05 *I*. A linear gradient of sodium chloride rose from 0 to 0.5 *M*. Arrows indicate the pools made for the recovery of the ten fractions referred to in the text, in Fig. 2, and in Table I.

These ten fractions, together with the unfractionated protein, were compared by starch gel electrophoresis. It can be seen (Fig. 2) that there are large differences between the fractions, both in the number and the type of components they contain. The fractionation appears to have been relatively sharp as there is surprisingly little overlap between successive fractions. In the gel patterns of the parent protein a maximum of ten bands can be counted but some of these single bands now appear to contain a number of proteins with very similar mobility but with substantially different chromatographic properties. It is difficult to make an accurate estimate of the total number of components but it is not less than thirty.

TABLE I

PROTEIN YIELDS (mg) AND AMINO ACID COMPOSITIONS (RESIDUES %) OF FRACTIONS OBTAINED FROM TYPE I HIGH-TYROSINE PROTEINS BY CHROMATOGRAPHY ON QAE-CELLULOSE AT pH 10.5

For fraction identification see Fig. 1.

Amino acid	*Amino acid composition*									
	Fraction									
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Yield of protein (mg)	*2*	*5*	*100*	*25*	*5*	*70*	*10*	*15*	*25*	*3*
Lys	0.29	0.25	0.17	0.21	0.33	tr[b]	0.21	0.71	0.27	0.27
His	1.76	2.89	1.53	1.43	1.00	0.52	0.85	0.90	0.34	0.36
Arg	7.64	7.72	5.66	7.01	5.58	4.04	4.18	4.72	5.08	4.99
CmCys[a]	5.71	7.27	5.69	6.01	6.25	5.72	4.42	3.68	3.94	3.71
Asp	2.77	3.18	4.40	3.36	3.12	2.57	2.23	4.13	4.35	4.33
Thr	5.95	1.74	4.71	2.07	2.21	2.94	2.23	3.23	3.07	3.17
Ser	15.80	12.40	13.10	12.90	11.90	13.10	12.10	10.90	9.14	9.52
Glu	3.02	0.65	0.44	0.72	0.88	0.74	0.41	1.10	1.60	1.72
Pro	9.87	3.78	6.10	2.15	2.96	5.20	4.08	5.94	6.00	6.08
Gly	15.70	30.20	26.50	33.00	32.90	28.10	31.10	28.00	26.60	27.30
Ala	1.87	0.85	1.16	0.50	0.83	1.89	1.47	1.03	0.60	0.63
Val	3.51	0.90	1.53	tr	1.00	3.15	2.09	2.45	2.95	2.90
Met	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ile	1.14	0.25	tr	tr	tr	tr	tr	0.71	1.12	1.09
Leu	7.83	6.47	6.10	5.08	6.09	4.99	6.10	6.65	4.56	4.35
Tyr	7.75	10.00	12.10	13.20	16.20	17.60	19.70	17.50	19.80	20.20
Phe	9.22	11.60	10.70	12.40	8.67	9.34	8.58	8.33	9.25	9.34

[a] CmCys = S-carboxymethylcysteine.
[b] tr = trace.

Fraction F appears to be substantially (>90%) a single component and this material, after further purification, is being used for physico-chemical and amino acid sequence studies. The amino acid compositions of the ten fractions are shown in Table I. All except fraction A fall into the class of Type I high-tyrosine proteins as they contain between 27 and 33 residues % of glycine, between 20 and 30 residues % of the aromatic amino acids, between 9 and 13 residues % of serine, and no more than 7 residues % of CmCys*[2]. There are other familial resemblances between the fractions, notably their comparatively small content of lysine, histidine, glutamic acid, and isoleucine, and their moderate content of arginine, proline, and leucine. In spite of these similarities there are substantial differences between them. Tyrosine occurs over an almost 2 to 1 range and there are quite significant differences between fractions in their contents of CmCys, arginine, serine, proline, phenylalanine, and leucine. It is clear therefore, that Type I high-tyrosine proteins consist of a group of related proteins which cover quite a range of amino acid compositions.

Methionine, isoleucine, lysine, histidine, valine, glutamic acid, and alanine may be absent or present to less than 1 residue per 100 residues in a number of fractions. Most components contain 90 residues or less and therefore must be deficient in some or all of these amino acid residues[1]. For example, the major component of fraction F

* CmCys = S-carboxymethylcysteine.

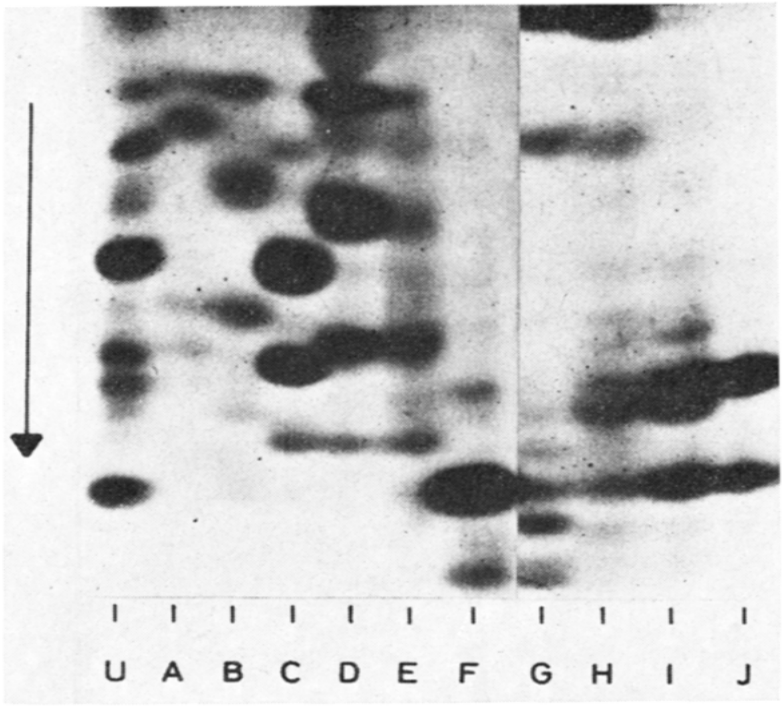

Fig. 2. Starch gel electrophoresis patterns of Type I high-tyrosine fractions isolated by chromatography at pH 10.5. Fraction numbering corresponds to that used in Fig. 1. U is the gel pattern of unfractionated Type I material.

after complete purification by further chromatography was found to be devoid of lysine, histidine, glutamic acid, methionine, and isoleucine.

The actual position in which each fraction elutes is almost certainly dictated by the contribution made to the net charge by ionized tyrosine residues. The relatively minor contribution from other charged groups can be illustrated by reference to fractions C and I which have a large difference in net charge at pH 10.5. However, if their net charge is calculated, excluding tyrosine, it is found to be the same if all carboxyls are free, and to differ by a maximum of two negative charges per mole in the extreme situation that all carboxyls in C are amidated but none of those in I.

Some separations appear to be unrelated to tyrosine content. For example, fractions G, I and J have substantially different elution positions although their tyrosine contents are similar. Other charged residues may in this case provide a secondary separation, although the possibility cannot be excluded that the relative proportions of tyrosine residues which are ionized may differ in the three fractions. Fraction A contains somewhat less tyrosine and glycine than the other fractions but significantly more threonine, serine, proline, leucine, and glutamic acid. Its higher glutamic acid content suggests that it may contain the glutamic acid-rich cell membrane proteins which are only moderately rich in glycine and the aromatic amino acids[6].

QAE-cellulose chromatography has thus proved a useful fractionation procedure for a protein system which is insoluble at pH values below 10 and where contact with urea has to be avoided. No evidence was found for protein damage at pH 10.5, for when a sample of high-tyrosine protein was chromatographed and then wholly recovered, without collecting separate fractions, the starch gel pattern obtained had the same number of bands with the same relative intensities. With more easily damag-

ed proteins it might be necessary to lower the temperature and to place a neutralizing buffer in each collector tube to minimize the time the proteins are at the high pH.

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