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CYSTINE-RICH PROTEINS OF HUMAN HAIR: CHARACTERIZATION AND STRUCTURE AS REVEALED BY REVERSED-PHASE AND SIZE-EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HAYEL M. SAID*, A.E. NEWSOM, ALAN J. FEIGE, SCOTT L LAUREN and ROGER A. MATHEWS

Redken Laboratories, Inc., Biochemistry Department, 6625 Variel Avenue, Canoga Park, CA 91303 (U.S.A)

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SUMMARY

The human hair cystine-rich proteins have been separated through the combined use of reversed-phase and size-exclusion chromatography into more than fifty components. These have been grouped, based on molecular weight, into six families of closely related members. The families range in molecular weight from less than 6500 for the low-molecular-weight components to more than 67 000 for the high-molecular-weight components, with average intermediate values for the other families of 8000, 11 500, 15 500 and 19 000. The results also suggest an organized structure of the hair matrix proteins. The combined use of reversed-phase and size-exclusion high-performance liquid chromatography in these studies presents an example where the quaternary structure of a multi-component protein can be largely deduced from its chromatographic behaviour.

INTRODUCTION

Human hair, which belongs to the large family of epidermal derivatives known as hard α-keratins, is its least studied member. Much of our knowledge about these proteins has been gathered from a wealth of research on wool [1—3], in which three groups of structural proteins have been identified. The first, which makes up the organized fibrillar components of the hair cortex, has a relatively low content of cystine [4]. The second group is rich in cystine residues and forms the amorphous matrix cementing the microfibrils [5]. The third and minor group, also a constituent of the matrix, consists of the high

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tyrosine—glycine proteins. These have not been detected in human hair, and their function has not been fully established [6].

The difficulty in studying human hair and other keratin proteins stems from two aspects: first, the large insolubility of these proteins in normal protein solvents due to their elaborately cross-linked structures mainly by disulfide linkages [7]; second, the lack of a reliable and effective procedure for their separation. Despite the tremendous and skillful efforts of several workers in the field, conventional methods of chromatography and electrophoresis have yielded modest results so far, specifically with human hair where none of its components has ever been purified [8]. In addition, almost nothing is known about the quaternary structure of these matrix proteins and very little is understood about their arrangement in situ.

We have recently reported a procedure for the fractionation of human hair cystine-rich proteins (HHCRPs) by reversed-phase high-performance liquid chromatography (RP-HPLC) [9]. We present here the detailed studies which led to the further purification and molecular-weight characterization of those proteins by the combined use of reversed-phase and size-exclusion (SE) HPLC.

EXPERIMENTAL

Keratin protein extraction

Human hair cystine-rich proteins were extracted and isolated from their cystine-poor counterparts and insoluble material as described in detail elsewhere [9]. All solutions containing urea were freshly prepared to minimize the generation of cyanate and possible carbamylation of lysyl residues. Under these conditions no homocitrulline was detected by amino acid analysis.

Reversed-phase high-performance liquid chromatography

All RP-HPLC studies described here were performed on a Waters Assoc. (Milford, MA, U.S.A.) system [9], equipped with a Hi-Pore RP-304 reversed-phase C_4 column (250 × 4.6 mm) (Bio-Rad Labs., Richmond, CA, U.S.A.) and an LKB 7000 UltroRac fraction collector (LKB, Bromma, Sweden). The system was interfaced to an HP9836 computer (Hewlett-Packard, Fullerton, CA, U.S.A.) utilizing Nelson Analytical chromatography software (Nelson Analytical, Cupertino, CA, U.S.A.). Conditions for the separations are described in the legends to the figures.

Size-exclusion high-performance liquid chromatography

A Bio-Rad HPLC gel chromatography column (Bio-Sil TSK 125, 30 cm × 7.5 mm I.D.) was employed for molecular weight (MW) analysis. The following reference proteins were used for calibration: bovine serum albumin (MW 67 000) and ovalbumin (MW 43 000) were purchased from Pharmacia (Piscataway, NJ, U.S.A.); carbonic anhydrase (bovine erythrocytes, MW 29 000), cytochrome c (horse heart, MW 12 500), insulin (bovine, MW 5700), somatostatin (SRIF, MW 1640) and isotocin (MW 966) were from Sigma (St. Louis, MO, U.S.A.); lysozyme (hen egg white, MW 14 300) was from Calbiochem (La Jolla, CA, U.S.A.). Prior to use, all reference proteins were heated in a reducing buffer (0.2 *M* Tris, pH 9.5—8 *M* urea—0.03 *M* dithio-

threitol) for 10 min at 90°C and carboxymethylated with iodoacetic acid as described for the hair proteins [9]. Myoglobin (horse heart type III, MW 17 000, Sigma) which is void of cystine was treated similarly and included as an internal reference in all preparations. Component SCMK-B2A from wool cystine-rich proteins was generously provided by Drs. J.M. Gillespie and R.C. Marshall (CSIRO, Division of Protein Chemistry, Parkville, Australia) and used untreated in some comparative studies. The column void and total volumes were determined from the elution profiles of blue dextran and phenylalanine, respectively. Unless otherwise stated, the eluting solvent was made up of 5 *M* urea—25% acetonitrile—0.03 *M* Na_2SO_4—0.2% trifluoroacetic acid (TFA).

Amino acid analysis

Proteins were hydrolyzed in 6 *M* hydrochloric acid and analyzed as previously noted [9]. Values reported are amino acid mole percent, and no corrections were made for serine and threonine losses during hydrolysis.

RESULTS

RP-HPLC of the cystine-rich proteins

The HHCRPs were resolved by RP-HPLC into 27 fractions (Fig. 1). The elution conditions adopted here are slightly modified from those published earlier [9]. The TFA concentration was raised to maintain a pH below 2.3 in solvent B, and the gradient slope was adjusted to enhance the separation of the middle peaks. As a result, the relative amounts of the peaks differ significantly from previous data [9]. However, under either set of conditions the

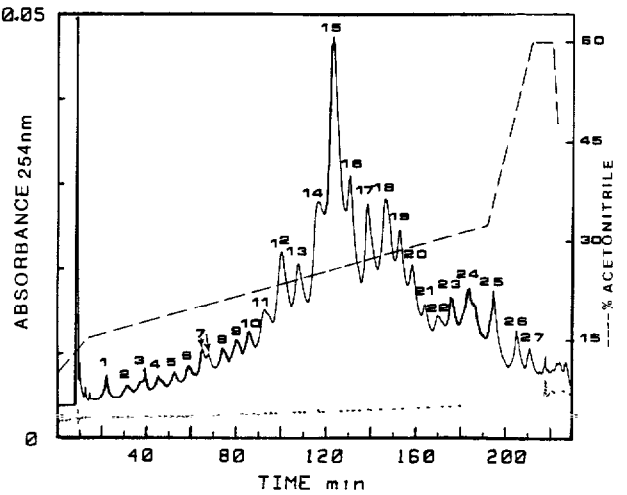


Fig. 1. RP-HPLC profile of the human hair cystine-rich proteins. Samples dissolved in solvent A (0.025% TFA) were injected (0.3 mg) onto a Bio-Rad Hi-Pore RP-304 column. Initial mobile phase composition: A—B (85:15) (B = 60% acetonitrile—0.025% TFA). The gradient (dashed line) was linearly increased from 15% to 25% B in 15 min, to 55% B in 178 min and to 100% B in 20 min. The flow-rate was 0.4 ml/min and detection was achieved at 254 nm. Dotted line is a tracing from a blank run. Numericals refer to the fractions in their order of elution.

reproducibility of the separations was consistently high, and the new retention times were only slightly affected.

Because heterogeneity is suspected in several fractions of Fig. 1, each peak was rechromatographed separately on the same reversed-phase column. When the original conditions were employed (see legend of Fig. 1), the early-eluting fractions (1—9) could not be detected and the late-eluting fractions (14—27) revealed column-adsorption patterns. However, when the conditions were slightly modified to decrease interaction time of the proteins with the hydrophobic matrix, the chromatograms shown in Fig. 2 were obtained. At this point the reader is referred to Fig. 3 for a flow chart of the various separation steps as well as the terminology adopted for subfraction identification. In this chart three RP-HPLC and two SE-HPLC steps are shown. The first RP separation leading to the 27 fractions is termed R_1P (e.g. R_1P15 is the same as fraction No. 15). Subfractions resulting from the second and third RP steps are identified together with the parent fractions and the separation step (e.g. R_2P15-1, R_3P15-1.1). Similarly, R_1S_1E and R_2S_2E refer to the two SE-HPLC steps (see below).

Each chromatogram in Fig. 2 has a broad peak (arrow) whose retention time increased in parallel to the fraction number. This peak is the major protein subfraction in each fraction based on amino acid analysis, and is designated as peak 1. Another large peak, always eluting in the time interval 87—89 min (peak X),

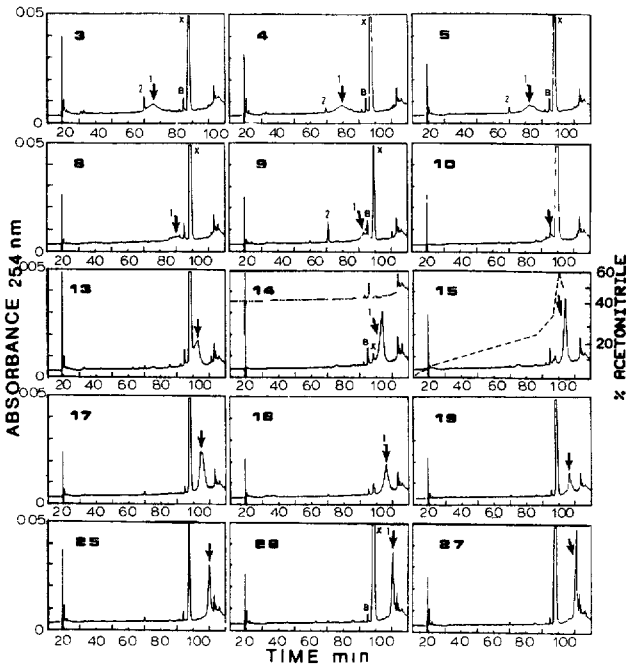


Fig. 2. Rechromatography of some of the fractions of Fig. 1 on Bio-Rad Hi-Pore RP-304 column. Solutions of 1 mg/ml of some of the lyophylized fractions (identified by the numbers at upper left-hand corners) were prepared in 4 *M* urea—0.2% TFA, and 50-μl samples were chromatographed. Initial, mobile phase conditions: 100% A. The gradient (dotted line in chromatogram 15) was linearly increased to 15% B in 15 min, to 40% B in 60 min, to 60% B in 10 min and to 100% B in 5 min. Solvents A and B, flow-rate and detection are the same as in Fig. 1. Upper tracing in chromatogram No. 14 is that of a blank run. Peak B is a background peak; peak X is to be identified; arrows point to the major protein peaks.

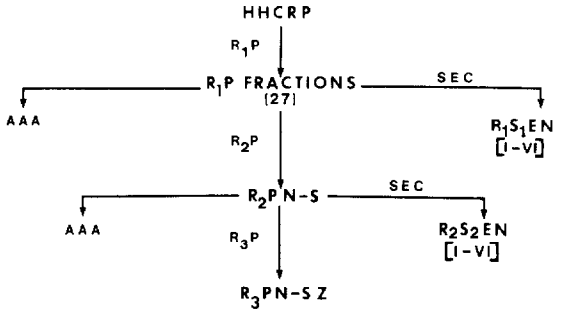


Fig. 3. Flow chart for the further purification, labeling and analysis of the various components resulting from RP- and SE-rechromatography of the fractionated human hair cystine-rich proteins (HHCRP). R_1P, R_2P, and R_3P are first, second and third RP-HPLC steps, respectively. R_1S_1E and R_2S_2E are first and second SE-HPLC steps. N identifies the original fraction number: S and Z identify the subfractions of the second and third RP-HPLC steps, respectively. AAA = amino acid analysis, and I—VI refer to the six molecular weight families of Table I.

is evident in all of the chromatograms albeit to varying extents. Amino acid analysis of this peak reveals a very low protein content and typical aromatic amino acid composition. Its high absorption at 254 nm is still under investigation. Other peaks observed in some profiles are numbered in their order of elution. One such component apparently of constant elution time (peak 2), is well resolved in fractions 1—9 at the 60-min mark, and appears in trace amounts in the remaining fractions.

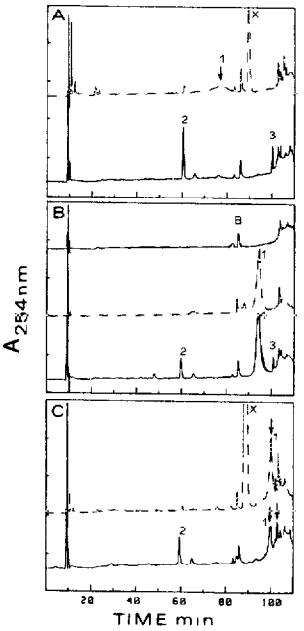


Fig. 4. Third reversed-phase chromatography step of the major protein peaks of some R_2P chromatograms (dashed lines). R_2P6-1 (A), R_2P15-1 (B) and R_2P24-1 (C) were separately collected, lyophylized, dissolved in 4 *M* urea, and injected onto the C_4 column. Conditions are identical to those of Fig. 2. Uppermost tracing in B is that of a blank. Peak B is a background peak; peak X is unknown; arrows point to the major protein peaks.

When subfractions R_2P6-1, R_2P15-1 and R_2P24-1 were collected and separately rechromatographed (R_3P) under the same conditions, some minor but interesting differences in their patterns were observed (Fig. 4). For R_2P6-1, the third RP-HPLC step (Fig. 4A) resulted in the virtual disappearance of the component and the appearance of component R_3P6-1.2, with the same retention time of 60 min as R_2P6-2. Also, a more hydrophobic component, R_3P6-1.3, at 100 min was observed. On the other hand, R_2P15-1 (Fig. 4B) showed little or no change except for the emergence of the minor components: R_3P15-1.2 at 60 min and R_3P15-1.3 at 101 min. Rechromatography of R_2P24-1 (Fig. 4C), again produced the 60-min component (R_3P24-1.2) in addition to the main subfraction. These results, together with those of Fig. 2, establish component 2 as a common constituent of all fractions.

Molecular weight analysis

The behaviour of some S-carboxymethylated reference proteins and peptides is shown in Fig. 5 which was used for the estimation of the molecular weights of the various HHCRP components. The SE-HPLC profiles of the R_1P fractions are presented in Fig. 6. The predominant feature of these chromatograms is the similarity observed among the early eluting as well as among the middle and late-eluting fractions, and the broad distribution of molecular sizes around the detected components (indicated by arrowheads). Molecular weight determination for some species therefore, may not be accurate. Six molecular weight ranges of the HHCRPs are identified (Table I), and arranged in ascending order of their size into six families: family I (fractions 1—8) includes the low-molecular-weight components (LMWCs) which elute in two peaks: one, Ia, is close to the total permeation peak and consists of very short peptides (MW < 1000), and the other (Ib) is made up of peptides of molecular weights increasing up to about 6500. Family II (MW 7000—8600, fractions 9—12) is distinguished from family I on the basis of simultaneous resolution in the chro-

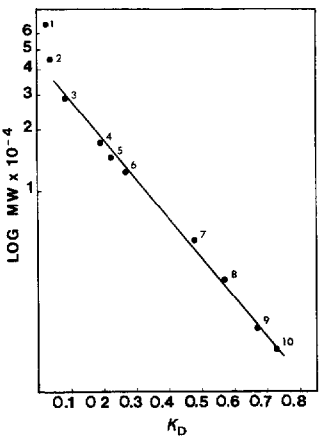


Fig. 5. Semi-logarithmic plot of the molecular weights of some S-carboxymethylated reference proteins and peptides versus their distribution coefficients (K_D). 1 = Bovine serum albumin; 2 = ovalbumin; 3 = carbonic anhydrase; 4 = myoglobin; 5 = lysozyme; 6 = cytochrome c; 7 = insulin (partially carboxymethylated); 8 = insulin B-chain; 9 = insulin A-chain; 10 = somatostatin. See legend of Fig. 6 for chromatographic conditions.

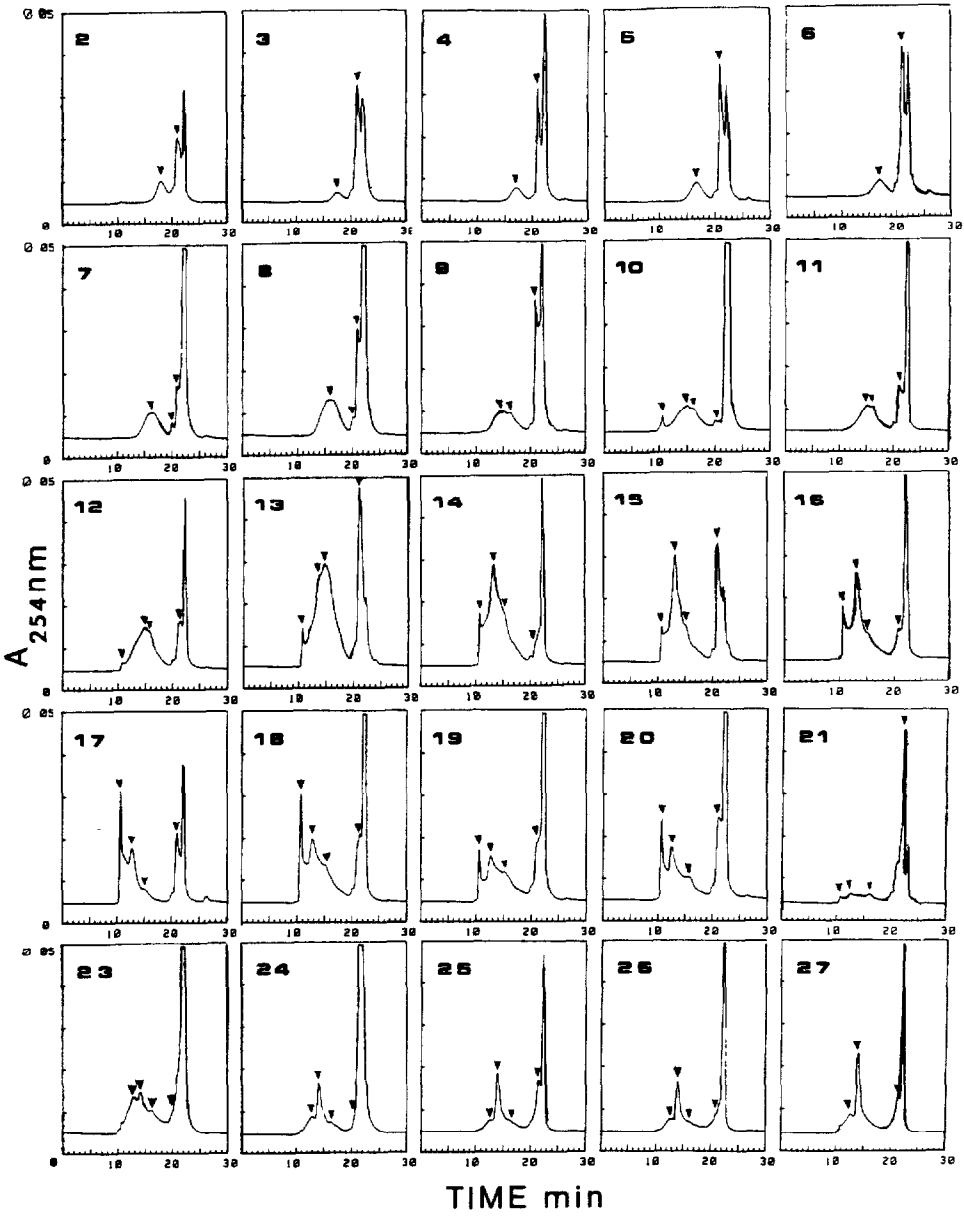


Fig. 6. SE-HPLC of the fractions of Fig. 1 on a Bio-Rad Bio-Sil TSK 125 gel chromatography column. The fractions were dissolved in 4 *M* urea containing 0.2% TFA, injected (50 μg per injection) onto, and isocratically eluted from the column with a solution of 5 *M* urea—30% acetonitrile—0 03 *M* Na_2SO_4—0.2 % TFA at a flow-rate of 0.5 ml/min. Exclusion and total permeation times were 10.7 and 22.4 min, respectively. Detection was at 254 nm. Arrows indicate detected components (see Table I).

matograms, and family III (MW 11 500, fractions 23—27) is characterized by a constant molecular weight. Family IV (MW 15 000—15 500, fractions 13—16) comprises members whose profiles did not change significantly upon RP re-

TABLE I

MOLECULAR WEIGHTS OF THE COMPONENTS OF THE HUMAN HAIR CYSTINE-RICH PROTEINS

(*) The major component in each fraction; (++) large component; (+) component is detected; (—) component is absent; SCMK-B2A: wool cystine-rich protein component. Family I includes the low-molecular-weight components which elute in two peaks (Ia and Ib); family VI includes the high-molecular-weight components.

Fraction No.	Molecular weight family						
	Ia	Ib	II	III	IV	V	VI
1	++	2600*					—
2	++	2600*					—
3	++	2800*					—
4	++	3300*					—
5	++	3800*					—
6	++	3700*					—
7	++	4400*					—
8	++	4600*					—
9	++	4600*	8200*				—
10	+	4800*	8000*				+
11	+	5600*	8000*				—
12	+	6400*	8200*				+
13	++		8600*		12 500*		+
14	+		7600		15 000*		+
15	++		8000		15 500*		+
16	+		8000		15 500*		+
17	+		8000			18 000*	++
18	+		8000			18 000*	++
19	+		7200			19 000*	++
20	+		7000			20 000*	++
21	++	5000		11 500		19 000*	+
22	++	5000		11 500		19 000	+
23	+	5000		11 500*		18 000	—
24	+	5300		11 500*		19 000	—
25	+	5300		11 500*		19 500	—
26	+	5300		11 500*		20 000	—
27	+			11 500*		20 000	—
R_2P15-1	+		7600		15 000*		—
R_2P19-1	+		7200			19 000*	—
R_2P24-1	+	5000		11 500*			—
SCMK-B2A				10 500		19 500*	31 000

chromatography. Families V and VI (MW 18 000—20 000 and > 67 000, respectively) coelute in fractions 17—20, and preliminary data (not shown) indicate that the high-molecular-weight components (HMWCs) of family VI may consist of aggregates of lower molecular weight resulting from uncleaved cross-links.

In an effort to correlate the R_1S_1E-peaks with those of R_2P, subfractions R_2P15-1, R_2P19-1 and R_2P24-1 representing families IV, V and III, respectively, were chromatographed by SE-HPLC under the original conditions. The results shown in Fig. 7 reveal that each R_2P subfraction is made up of at least two components whose molecular weights agree very well with the

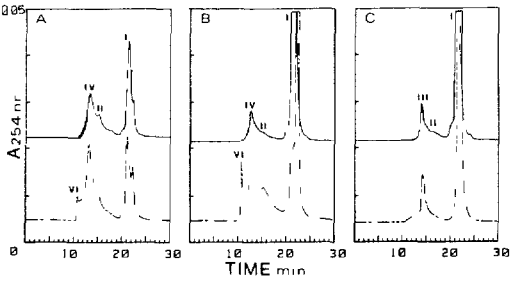
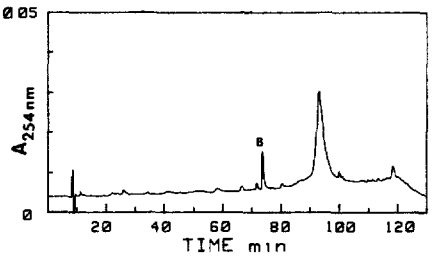


Fig. 7. SE-HPLC of subfractions R_2P15-1 (A), R_2P19-1 (B) and R_2P24-1 (C). Lower tracing in each panel is that from the corresponding parent fraction. Peaks (in panel A): I = R_2S_2E15-I; II = R_2S_2E15-II; IV = R_2S_2E15-IV; VI = R_1S_1E15-VI (parent fractions as given in Table I). Conditions as in Fig. 6.

corresponding components on the parent fractions (Table I). However, the HMWCs seen in the R_1S_1E chromatograms (lower tracings of Fig. 7) are not detected in the R_2S_2E panels and they are also absent from the R_2P chromatograms (where subfractions 1 are the only major detectable protein peaks). This absence may be accounted for by two alternatives: either that the HMWCs were not eluted from the column during the second RP-HPLC separation, or that the extended exposure to the separation environment may have induced their breakdown into smaller components (see Discussion).

Chromatography of a wool cystine-rich protein

SCMK-B2A is a well characterized member of the wool cystine-rich proteins whose sequence has been determined [10], so it was of interest to compare its chromatographic behaviour with that of HHCRPs. The RP-HPLC of this protein (Fig. 8) shows one large peak which is flanked by minute shoulders.



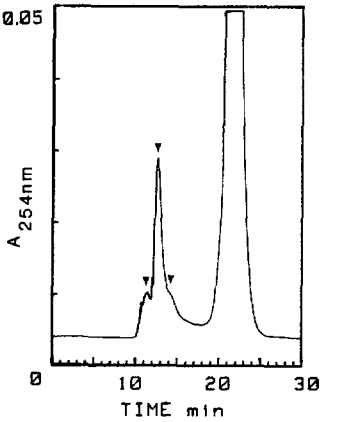


Fig. 8. RP-HPLC of the wool cystine-rich protein SCMK-B2A The protein was dissolved in solvent A and a 100-μg sample was chromatographed. Conditions are the same as in Fig. 3 except that the gradient was stepped up from 40% to 100% B in 60 min Peak B is a background peak.

Fig. 9. SE-HPLC of the wool cystine-rich protein SCMK-B2A. Conditions are those of Fig. 7. The solvent peak is greatly enhanced in this case due to the presence of UV-absorbing additives (dithiothreitol, iodoacetic acid, Tris) in the sample solution. Arrows indicate determined molecular weight components.

This profile is reproduced by SE-HPLC (Fig. 9) where the MW of 19 500 determined for the major component is in good agreement with that of approximately 20 000 determined elsewhere [10]. The two minor components in Fig. 9 (MW 31 000 and 10 500) may be due either to changes in the protein structure induced by prolonged storage, or to some minor impurities in the sample.

Effect of sodium dodecyl sulfate on keratin proteins

Normally, sodium dodecyl sulfate (SDS) is added to buffers when molecular weight determination of multi-component proteins is intended. In some pilot experiments this was the case when the R_1P fractions and SCMK-B2A were chromatographed by SE-HPLC. Under such conditions, the early fractions 1—8) showed no discrete peaks, but rather a continuous distribution of molecular sizes ranging between the lower limit of the LMWCs (MW < 1000) and 8000 (unpublished data). The remaining fractions exhibited patterns similar to those of Fig. 6 except for the appearance of additional components in the molecular weight range of 40 000—45 000. The problem encountered with SDS in these studies, however, was the increased adsorption of the proteins onto the column as evident by the elution of components after the solvent peak. SCMK-B2A showed two major peaks (data not shown) of MW 20 000 and 8500. Whether these additional peaks are real or mere SDS-related artifacts, remains to be determined (see Discussion). It is worth mentioning here that under both sets of SE-HPLC conditions, the remaining components in each fraction were very similar although the molecular weights determined in the presence of SDS were slightly but consistently higher.

DISCUSSION

The HHCRPs consist of a large number of components which have been characterized and classified in the present studies, based on molecular weight distribution, into six families.

Very few studies in the literature have dealt in great detail with hair proteins. Pollitt and Stonier [11] reported limited fractionation of these keratins by the combined and repeated use of isoelectric precipitation, gel chromatography, and DEAE-cellulose chromatography. Gillespie and Marshall [12] obtained five fractions of the HHCRPs by gel chromatography on Sephadex G-200, and they identified in their fractions components with molecular weights of 72 000, 37 000—38 000, 21 000—23 000, 17 000, 11 000 and 6000—7000. As mentioned earlier, a 40 000-dalton component was detected in some studies where SDS was employed: but the confirmation of its existence under other conditions awaits further experiments due to the artifacts that SDS confers on these proteins. In this regard, Marshall [13] has reported the anomalous behaviour of the cystine-rich proteins in SDS polyacrylamide gel electrophoresis (pH 8.9) where as much as 150% overestimation in molecular weights was obtained. We have recently documented [9] the effect of SDS on the hydrophobic separation of these proteins and noted the increment in their hydrophobic character on reversed-phase columns. It is very likely that such an effect, whether in hydrophobic or electrophoretic separations, is brought about

by SDS-induced conformational changes in the carboxymethylated molecules, that would shield their large anionic load from the polyanionic reagent. In such instances, SDS would serve a purpose opposite to the one intended, by causing aggregation of these proteins.

The similarity of the SE-HPLC profiles of Fig. 6 and the data of Table I strongly allude to an organized quaternary structure for the cystine-rich proteins. Such a structure conceivably can be made up of four to six subunits each of which may possess constant as well as variable domains. The assumption of different domains is supported by sequence analysis data of some wool proteins which reveal a disproportionate concentration of cystine in these molecules, with the first half containing much more than the second [14, 15]. Similarly, several studies (reviewed in ref. 7) have established the presence of peptide repeats in wool proteins which, if present in hair, would constitute the constant domains. In this aspect, the relationship of subfraction 2 (the 60-min component, Figs. 2 and 4) to the proposed subunits seems of particular interest to investigate. The variable domains of the subunits may explain the heterogeneity of these proteins and prevent their polymerization into any super-structure identifiable by electron microscopy or X-ray diffraction.

Based on the order of elution of the fractions (Fig. 1) and the data of Table I, some light can be shed on the interactions among the different subunits. It appears that the 7000—8600 and 11 500 dalton proteins maintain only weak hydrophobic interactions after the fission of whatever inter-chain disulfide bonds were originally present. Similarly do the 11 500 and the 15 500 dalton subunits, as well as the 15 500 and the 18 000—20 000 dalton subunits. On the other hand, the 11 500 and the 18 000—20 000 dalton subunits, for example, are possibly still held together by such bonds, as reflected by their coelution and close association (see Table I). Although the relative number of the subunits is not determined, the 15 500 dalton protein is the most abundant (Fig. 1). Unrefined estimates point to a possible ratio of 2:1:1:1 for subunits 15 500, 7000—8000, 11 500 and 18 000—20 000 dalton, respectively.

The superiority of RP-HPLC over other forms of fractionation of keratin proteins may reside in its ability to induce conformational changes [16, 17] in these protein molecules that would expose inaccessible inter-chain disulfide linkages to more polar media and render them susceptible to cleavage. As much as 25% of these disulfides may escape oxidation or reduction of wool [18] due to differences in their reactivity [19]. Lindley and Cranston [20] found these reactivity variations to be a function of the polarity of the environment surrounding these bonds, so that those involved in short intra-chain links or located in hydrophobic crevices away from charged carboxyl groups, are the least reactive.

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