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CHANGES IN THE α-FIBROUS PROTEIN DURING EPIDERMAL KERATINIZATION

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Abstract. The epidermal proteins of cow snout have been extracted and fractionated. Proteins extracted with citrate buffer pH 2.65 (pre-keratin) formed filaments with an α-helical pattern and contained 0.58% sulfur. S-carboxymethyl derivatives of this protein had more than one component by disc gel electrophoresis. The protein soluble in 6 M guanidine had a fibrous structure and an amino acid composition similar to pre-keratin. The microsomal fraction of epidermis contained small amounts of a protein with the characteristics of pre-keratin. Neutral buffer soluble precursors of keratin were not found. Protein which required 6 M guanidine and sulfhydryl reducing agents for extraction had chemical features similar to those of pre-keratin suggesting that it is a more highly cross-linked or aggregated form of the original protein.

The fibrous proteins of epidermis, myosin and fibrinogen, have been shown to share a common type of helical structure, as determined by X-ray diffraction analysis (13). However, the epidermal protein, unlike the muscle and blood proteins, is insoluble in neutral buffers (1, 6, 11, 14, 15). Recently, Matoltsy (12) described the extraction of an α-fibrous protein (pre-keratin) from cow snout using citrate acid buffer, pH 2.65, while Baden (2) has reported similar results when using mammalian epidermis. The fibrous proteins isolated in this way represent a small percentage of the total fibrous protein present in the tissue. The reduction of disulfide bonds is necessary to solubilize the remaining fibrous protein and still have it maintain a helical structure (1).

Biochemical studies of hair protein have shown that the structural proteins became insoluble as a result of cystine cross-linkages developing between the helical and matrix components (5). Although it has been suggested that similar changes occur with epidermal proteins, the various stages in the process have not been clearly elucidated. One of the major difficulties in studying this problem has been the limited amount of epidermal tissue available from most laboratory animals. The purpose of this report is to describe our studies with cow snout epidermis. In these studies it has been possible to describe the various stages of the α-protein during keratinization.

MATERIALS AND METHODS

Guanidine and mercaptoethanol were purchased from Eastman Organic Chemicals; DEAE cellulose from Merck Chemicals; Sephadex and Sepharose from Pharmacia Fine Chemicals, dithiothreitol from Nutritional Biochemicals; tris (hydroxymethyl) aminomethane and iodoacetic acid from Sigma Chemicals. The chemicals were reagent grade and used as supplied except iodoacetic acid which was crystallized from water.

Fresh cow snouts were obtained from a local slaughterhouse and packed in ice until used (2–3 hours). The epidermis was removed by free-hand slicing using razor blades and then rinsed with distilled water at 4°C. Histologic studies showed that the skin was sliced through the tops of the dermal papillae. The washed tissue was minced with scissors and then homogenized in buffer (Table I) for 5 min at 4°C. The volume of the particular buffer used was calculated to give a 20% homogenate. The homogenate was then centrifuged according to the scheme in Table 1. The various pellets obtained from the sucrose experiments were re-extracted with citrate buffer, pH 2.65, as above and centrifuged at 40 000 *g* for 1 hour to obtain clear supernatants.

The 20 000 *g* pellet obtained from the citrate-extracted tissue (Table 1) was then stirred in 6 M guanidine in 0.1 M tris at pH 9.0 at room temperature for 24 hours. Following centrifugation at 40 000 *g* for 1 hour the supernatant was dialysed repeatedly against 0.1 M citric acid at pH 2.65 and clarified by centrifugation. The guanidine-washed pellet was re-extracted with the 6 M guanidine in 0.1 M tris at pH 9.0 with 0.1 M mercaptoethanol. An aliquot was treated with iodoacetic acid to obtain the S-carobxymethyl (SCM) derivative; the alkylated

Table I. *Fractionation scheme of tissue homogenates*

Buffer		Centrifuged at *g*	Min
0.1 M sodium citrate buffer at pH 2.65	(1)	20 000	10
	(2)	40 000	60
0.1 M sodium phosphate buffer at pH 7.0	(1)	40 000	60
0.25 M sucrose	(1)	1 000	15
	(2)	15 000	15
	(3)	40 000	15
	(4)	100 000	60

and remaining untreated extracts were then dialysed against distilled water and lyophilized.

The α-protein (pre-keratin) was isolated from the solution of citrate-soluble protein by successive precipitations at pH 7.0, 6.0, 5.0 and 4.5 as described by Matoltsy (12). The final solution was centrifuged at 40 000 *g* for 1 hour. The SCM derivative was prepared as described above folowing dialysis against 6 M urea in 0.1 M tris, pH 9.0, with 0.1 M mercapthethanol.

The solution of phosphate-soluble protein was dialysed repeatedly against 0.5 M acetic acid at 4°C and centrifuged at 1 000 *g*. The precipitate was redissolved in phosphate buffer and the procedure repeated two more times. The final precipitate was lyophilized. The solution of acetic acid soluble protein was made 0.7 M with respect to potassium chloride by the slow addition of salt with constant stirring at 4°C. The suspension was clarified by centrifugation and the supernatant dialysed against distilled water and lyophilized. The precipitate was redissolved in 0.5 M acetic acid and the procedure repeated twice. The final pellet was dissolved in acetic acid, dialysed against distilled water, and lyophilized.

Fractionation of the proteins was attempted with ascending column gel filtration on Sephadex G-200 and Sepharose 4B with 0.1 M citrate at pH 2.65 or 6 M urea in 0.1 M tris at pH 9.0. Exchange chromatography was also done using DEAE cellulose equilibrated with 6 M urea in 0.01 M tris at pH 8.0. A sodium chloride gradient was then used to elute the protein. All the columns were run at 4°C and/or 25°C. The eluates from the columns were monitored at 280 nm and selected fractions were dialysed and lyophilized.

Table II. *Yield of protein by each of the successive extraction procedures*

Aliquots of the initial homogenate and various extracts were dialysed, lyophilized, extracted with ether, dried and weighed

Extraction medium	Percent yield of total tissue protein
0.1 M citric acid, pH 2.65	5–10
6 M guanidine in 0.1 M tris, pH 9.0	15
6 M guanidine in 0.1 M tris, pH 9.0, with 0.1 M mercapto-ethanol	50–60

Table III. *Amino acid composition of purified acid-soluble α-fibrous proteins*

Data are expressed as residues/100 residues

	Protein isolated from whole epidermal tissue	Protein isolated from 100 000 *g* pellet
Aspartic acid	9.1	9.1
Threonine	4.0	4.1
Serine	11.1	10.8
Glutamic acid	14.1	13.4
Proline	1.4	1.9
Glycine	16.4	15.9
Alanine	6.7	7.0
Cystine ($\frac{1}{2}$)[a]	0.6	0.6
Valine	4.0	3.8
Methionine	1.3	2.1
Isoleucine	3.5	3.1
Leucine	9.2	9.1
Tyrosine	2.8	2.9
Phenylalanine	3.6	3.8
Lysine	5.1	5.1
Histidine	1.0	1.2
Arginine	6.1	6.1

[a] As determined by S-carboxymethyl cysteine

Disc electrophoresis was done at pH 8.3 using a 7% acrylamide gel with and without 6 M urea (7).

Samples for amino acid analysis were hydrolysed at 110°C for 24 hours and run in duplicate on a Beckman Model 116 amino acid analyzer.

Total sulfur was determined gravimetrically following its oxidation to sulfate and the addition of barium (Belmont Analytical Laboratories).

X-ray diffraction analysis was done on stretch-oriented specimens using nickel-filtered copper Kα-radiation (λ = 1.54 Å) at 40 kV at a specimen-to-film distance of 1.54 cm.

RESULTS

Acid-soluble protein

Treatment of cow snout epidermis with citrate buffer, pH 2.65, extracted 5–10% of the total tissue protein from which the α-protein (pre-keratin) could be purified with a yield of 1% of the total tissue protein (Table II). X-ray diffraction analysis of oriented filaments prepared from the purified protein showed an α-pattern with 9.8 Å equatorial and 5.14 Å meridional reflections. The total sulfur content of the fibrous protein was 0.58% and the amino acid analysis is shown in Table III.

Fractionation of the protein on Sephadex G-200

using 0.1 M citrate buffer, pH 2.65, yielded a single peak at the end of the void volume. The α-fibrous protein was dissolved in 6 M urea with 0.1 M tris buffer, pH 9.0, and fractionated on Sepharose 4B using the same buffer. A single peak was observed as shown in Fig. 1. Disc electrophoresis of this protein on acrylamide gel in a urea buffer showed the protein was held up by the spacer gel and no distinct bands could be seen in the running gel.

Conversion of the α-protein to the S-carboxymethyl derivative alters markedly the solubility characteristics of the protein, but X-ray diffraction patterns of oriented fibres still show an α-pattern. The converted protein can be dissolved in neutral buffers, unlike the native α-protein which is insoluble. Disc electrophoresis with 6 M urea shows a number of distinct components (Fig. 2). Band *a* is the most prominent and consistently present in all the preparations which have been made. Bands *b* and *c*, although almost always present, varied in intensity in different preparations and different electrophoretic runs of the same material. A disc electrophoretic pattern similar to this can be demonstrated with the native protein if it is treated with 6 M urea and 0.1 M mercaptoethanol at pH 9.0 and run in the urea system with the addition of 0.1 M mercaptoethanol.

Gel filtration of the SCM in protein on Sepharose 4B in 6 M urea with 0.1 M tris, pH 9.0, showed a single peak (Fig. 1). The eluting volume of SCM protein was greater than the untreated α-protein, suggesting the former has a smaller molecular size. It was not possible to separate the SCM protein into several components by using 8 M urea or 6 M guanidine. When the SCM protein was chromatographed in DEAE cellulose in a urea buffer, 60–70% of the protein was irreversibly bound to the column. The remainder could be eluted with a sodium chloride gradient (0 to 0.2 M) and one major peak and several minor ones were observed. Protein isolated from the major peak was identical with the starting material as judged by electrophoretic patterns and amino acid analyses and no apparent separation could be achieved.

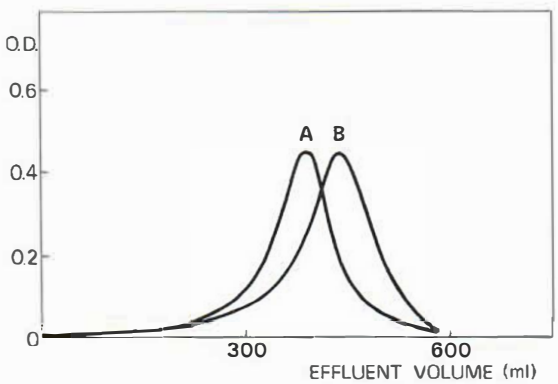

Fig. 1. Column chromatography of pre-keratin on Sepharose 4B using 6 M urea in 0.1 M tris pH 9.0 as the eluant. *A* is the untreated protein and *B* is the SCM derivative.

Fig. 2. Acrylamide gel electrophoresis in 6 M urea of S-carboxymethyl pre-keratin.

Neutral-soluble proteins

The proteins extracted from cow snout with 0.1 M phosphate buffer, pH 7.4, showed a number of components by disc electrophoresis. This extract was fractionated by dialysis against 0.5 M acetic acid and then 0.5 M acetic acid with KCl. This procedure is similar to that used for collagen (9) which does share certain common features with pre-keratin. Amino acid analysis of these fractions showed a number of differences from the α-protein particularly with respect to proline content (Tables III and IV). X-ray diffraction patterns obtained from these various neutral-soluble components showed no evidence of an α-pattern.

Guanidine-soluble protein

Treatment of the tissue with a 6 M guanidine buffer extracted an additional 5% more protein

Table IV. *Amino acid analysis of neutral-soluble epidermal proteins*

Data are expressed as residues/100 residues

	Insoluble in 0.5 M acetic acid	Insoluble in 0.5 M acetic acid with 0.7 M KCl	Soluble in 0.5 M acetic acid with 0.7 M KCl
Aspartic acid	10.9	8.7	9.5
Threonine	5.3	6.8	6.2
Serine	7.3	7.3	7.2
Glutamic acid	13.0	14.4	14.0
Proline	5.4	7.8	6.2
Glycine	9.9	9.2	9.5
Alanine	8.0	6.3	7.5
Cystine (½)	1.5	3.6	1.1
Valine	5.0	6.5	6.5
Methionine	2.3	1.5	1.2
Isoleucine	3.6	3.0	3.7
Leucine	8.4	7.6	8.0
Tyrosine	2.9	1.7	2.4
Phenylalanine	3.9	2.4	3.4
Lysine	6.1	8.0	7.6
Histidine	2.0	2.1	2.0
Arginine	4.5	3.1	4.0

(Table II). The clear acidic supernatant obtained from the guanidine extract was precipitated successively at pH 7.0, 6.0, 5.0 and 4.5 to obtain a protein insoluble at neutral pH which amounted to about 0.5 % of the total tissue protein. X-ray diffraction analysis of oriented filaments prepared from the protein showed an α-pattern while the amino acid composition is similar to the acid-soluble α-protein.

Fig. 3. Acrylamide gel electrophoresis of the SCM derivative of α-fibrous protein insoluble at pH 7.8. The α-fibrous protein was extracted with guanidine and 0.1 M mercaptoethanol.

Table V. *Amino acid composition of proteins dissolved with guanidine buffer and mercaptoethanol*

Data are expressed as residues/100 residues

	Unfractionated protein	Protein insoluble at pH 7.8	Protein insoluble at pH 4.5	Protein soluble at pH 4.5
Aspartic acid	8.5	8.6	8.6	7.4
Threonine	3.9	3.8	4.0	4.2
Serine	11.3	11.0	11.6	10.0
Glutamic acid	13.5	13.8	14.1	12.5
Proline	2.2	1.5	1.9	8.6
Glycine	17.6	18.0	17.4	20.7
Alanine	6.8	6.5	6.9	7.1
Cystine (½)[a]	0.9	0.6	0.7	1.7
Valine	4.2	4.3	4.1	4.0
Methionine	1.6	1.2	1.2	1.3
Isoleucine	3.3	3.8	3.2	2.7
Leucine	8.7	9.1	8.8	5.9
Tyrosine	2.8	2.7	2.8	1.8
Phenylalanine	3.5	3.5	3.5	2.5
Lysine	4.8	5.0	4.8	4.5
Histidine	1.0	0.7	0.8	1.3
Arginine	5.4	5.9	5.6	3.8

[a] As determined by S-carboxymethyl cysteine.

Protein extractable with reducing agents

Treatment of the tissue with a guanidine buffer containing mercaptoethanol extracted about 50–60 % of the total protein leaving about 20 % which could not be solubilized (Table II).

The sulfur content of the protein dissolved with reducing agents was 0.64 % and the amino acid composition is shown in Table V. The SCM derivative was dissolved in 6 M urea with 0.1 M tris, pH 8.5, and dialysed against 0.1 M ammonium bicarbonate. The suspension was clarified by centrifugation at 40 000 *g* and then the supernatant adjusted to pH 4.5 with 1 N sodium hydroxide. Protein precipitated out of solution continuously during this procedure but there was no additional precipitation below pH 4.5. The precipitate was collected by centrifugation and then the two precipitates and clear supernatant dialysed free of salt and lyophilized. The ratio of pH 7.8 insoluble protein to pH 4.5 insoluble protein was 2 to 1, while only about 5 % of the total protein remained soluble at pH 4.5. Disc electrophoresis patterns of

the two principal components were identical and showed two distinct bands corresponding very closely in migration to *a* and *c* of the SCM derivative of pre-keratin (Fig. 3). X-ray diffraction patterns of oriented fibres prepared from these proteins showed an α-pattern, while the protein soluble at pH 4.5 showed only unoriented halos. Amino acid analysis of the various components (Table V) suggests that the pH 7.8 insoluble and pH 4.5 insoluble fractions are identical and different from that remaining in solution.

Microsomal fraction

The yield of citrate-soluble protein extracted from the microsomal fraction (100 000 *g* pellet) at pH 2.65 was quite low and the final protein purified by repeated precipitation was less than 1 % of total amount of pre-keratin extracted from the whole tissue. X-ray diffraction patterns of oriented filaments prepared from the protein purified from the citrate extract of the microsomes had an α-pattern, and the amino acid analysis (Table III) indicates the similarity to citrate-soluble pre-keratin. Furthermore, disc electrophoresis of the SCM derivative showed a pattern identical with SCM pre-keratin.

DISCUSSION

The neutral-soluble character of collagen, myosin, and fibrinogen suggested that a similar situation might exist for the fibrous proteins of epidermis. Although the studies of Matoltsy (12) on pre-keratin pointed to an acid-soluble protein as the precursor of the highly insoluble fibrous proteins of the stratum corneum, the results were not entirely conclusive. The data indicate that a neutral soluble α-protein cannot be identified but a fibrous protein can be isolated from the microsomal fraction which has the same solubility characteristics as pre-keratin. Previous studies (3) using labelled amino acids have indicated that this α-fibrous protein from the microsomal fraction is indeed a precursor pool of pre-keratin. This suggests that pre-keratin is released from ribosomal aggregates in a completed form similar to what has been described for collagen biosynthesis (8). It appears likely, therefore, that a neutral-soluble precursor of the α-fibrous protein does not occur.

Chromatographic and electrophoretic studies of acid and urea solutions of non-reduced pre-keratin indicated that it was a large single molecular species. Conversion of the protein to the S-carboxymethyl derivative, however, produced significant changes in its physical properties. The change in solubility could not be explained by an increase in polar groups since the number of 1/2-cystine residues, particularly with respect to the number of polar amino acids, was quite small. This suggests that the pre-keratin was disassociated into smaller components. Chromatographic studies of the SCM protein in urea did indicate that it had a smaller size than the untreated protein and this was confirmed by electrophoretic studies which showed that the SCM protein was able to enter the running gel and consisted of several components. The fastest moving component appeared to be the most consistent in different preparations whereas the two others observed showed considerable variation in the amount present. This suggested that there was only one component but in various stages of aggregation. These aggregates could have been present in the initial preparation or formed during the electrophoretic run. However, it is possible that different components do occur but they were not separable by the techniques used.

The α-protein purified from the guanidine extract seemed identical with citrate-extracted material as judged by amino acid composition. This protein was apparently aggregated in a way which required treatment with a denaturing agent before it could be solubilized.

The bulk of the fibrous protein was extracted with epidermis was treated with urea buffers containing a cystine reducing agent. The SCM derivatives which were isolated at pH 7.8 and pH 4.5 appeared to be identical by electrophoresis and amino acid analysis. Furthermore, they were similar in amino acid composition and total sulfur content to pre-keratin. This suggests that they represent a cross-linked form of pre-keratin rather than a new complex containing fibrous protein plus some additional component. The protein soluble at pH 4.5 was clearly different and could be considered as a matrix component equivalent to what has been found in hair.

These studies have clearly identified the various forms of the α-fibrous protein, but have demonstrated only small amounts of a possible matrix protein. These and other biochemical studies have failed to confirm electron microscopic observations

(4) which have suggested that equal amounts of filamentous and matrix-protein occur in stratum corneum. Lavker (10) showed that in ruminal epithelium the matrix appears to be a mixture of endoplasmic reticulum and kerato-hyaline proteins. The same situation may occur in epidermis and the matrix could consist of a number of different components with different solubility characteristics including lipids. Whether any of these are linked to the fibrous protein through disulfide bonds remains to be demonstrated.

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