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A NEUTRAL SOLUBLE HIGH MOLECULAR WEIGHT KERATIN FROM EPIDERMIS

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SUMMARY: Previously described keratins, the structural proteins of epidermis, are soluble at pH values of less than 2.5, or greater than 10. At intermediate pH values, denaturants such as urea or SDS must be added in order to dissolve them, and in addition, with some types of keratin, disulfide reducing agents are also required to effect solution. These proteins are composed of several polypeptide chains in the molecular weight range of 45,000 to 70,000 daltons. This report describes the isolation and partial characterization of a new form of keratin, called neutral soluble keratin, which is freely soluble in low ionic strength buffers at neutral pH. The molecular weights of the polypeptides comprising neutral soluble keratin are in the range of 79,000 to 90,000 daltons.

Epidermis contains several layers of cells and as one progresses from the lower, viable layers to the outer, anucleate layer known as stratum corneum, each layer is mitotically less active and has a greater accumulation of intercellular structural proteins or keratins. Keratins were first identified as the principal structural proteins of epidermis on the basis of their α fibrous x-ray diffraction pattern (3,4). Studies by Rudall (5), Matoltsy (6), and Mercer (7) showed that one type of keratin, termed prekeratin, could be isolated from the lower viable layers of epidermis by extraction with citrate buffer at pH = 2.65. A second type of keratin was extracted from the outer cornified stratum corneum layers with alkaline buffers containing urea. A third keratin which contains inter and/or intra chain disulphide bonds can be isolated from epidermis by extraction with alkaline buffers containing urea and a disulphide reducing agent. These three forms of epidermal α fibrous proteins have been isolated and partially characterized from various sources and have been shown

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to be similar with respect to polypeptide composition, immunological reactivity, and amino acid composition and to differ principally in their solubility properties and the presence or absence of disulphide bonds (8,9,10,11,12,13,14,15). This report describes the isolation and characterization of a new form of keratin which is strikingly different in molecular weight and solubility from these previously described keratins.

Materials and Methods

Preparation of Epidermal Keratins

Neutral soluble protein: Epidermis was removed from the snouts of freshly slaughtered cows by slicing with a razor blade, and only tissue which did not include dermis was used. The resulting slices were placed in 0.25M sucrose (300 ml per 36 gm of wet tissue), ground in a Virtis homogenizer for 4 min at 4°C and the resulting suspension centrifuged at 35,000 g for 20 min. The supernatant was adjusted to pH = 4.5 and the floculent precipitate allowed to stand overnight at 4°C after which the supernatant was decanted and the precipitate dissolved in 40 ml of 0.01M Tris pH = 8.3. The volume was reduced to 3 mls by Amicon ultrafiltration and applied to a Sepharose 4B (0.9 x 46 cm) column packed and run in 0.01M Tris, pH = 8.3 at a flow rate of 5 ml/hr. All tubes were tested for reactivity toward a keratin specific and a BSA specific antibody. The excluded peak from this column which was keratin positive and BSA negative was pooled, concentrated, made 8M in urea, 0.1M in NaCl, 0.01M in DTT and 1% in SDS and the pH was adjusted to 8.3. The sample was then heated at 100°C for 1 min followed by 50°C for 1 hr prior to application to G200 column for final purification. A typical column and details of its operation are shown in Fig. 1.

Prekeratin protein: The pellet from the above centrifugation was suspended in 0.1M citrate-citric acid buffer pH = 2.65 (150 ml per 18 gm of wet tissue) and ground in the Virtis homogenizer for an additional 4 min and the resulting mixture stirred at 4°C for 1 hr. After centrifugation at 35,000 g for 20 min for a second time, the prekeratin in the supernatant was purified by a series of precipitations at pH's = 7.0, 6.0, 5.0, and 4.5.

Stratum corneum protein: The insoluble pellet from the second centrifugation was rehomogenized in a Virtis homogenizer in 6M urea containing 0.1M Tris, pH 9.0, stirred under nitrogen at room temperature for 18 hr, centrifuged for a third time at 35,000 x g. Two additional Tris-urea washes were done in order to insure complete removal of this protein.

Stratum corneum SH protein: The final pellet from the above preparation of stratum corneum protein was extracted in 6M urea containing 0.1M Tris, pH 9.0 and 0.1M mercaptoethanol (Tris-urea-mercaptoethanol) for 25 hr at room temperature under nitrogen and the suspension centrifuged at 35,000 g for 20 min. The resulting supernatant contained the stratum corneum SH protein.

Electrophoresis

Polyacylamide gel electrophoresis in the presence of SDS was

done using the concentrating gel system described by Neville (1) and stained with Coomassie Brilliant Blue.

Amino Acid Analysis

Samples for amino acid analysis were hydrolyzed in 6M hydrochloric acid for 24 hrs under vacuum at 110°C and run in duplicate on a Beckman 116 amino acid analyzer.

Immunodiffusion

Gel diffusion experiments were done using modifications of classical Ouchterlony techniques previously described (2).

Results and Discussion

The initial step in the isolation of previously described epidermal keratins involves grinding the tissue in sucrose to remove soluble proteins. Such sucrose extracts were shown to contain a protein that was immunologically identical to the epidermal keratins (16). The antibody that was used to detect and monitor the purification of this new form of keratin, designated neutral soluble keratin, has been previously described (2,17) in detail. It was shown that this antibody is specific for the polypeptide chains of prekeratin in the molecular weight range of 67,000 to 45,000 daltons and that it does not cross react with sucrose extracts of any other cow organs or appendages including hair and nail. The prekeratin antigen used to ellicit this antibody had no high molecular weight (>67,000 daltons) species present. As an initial purification step neutral soluble keratin was precipitated from sucrose extracts by adjusting the pH to 4.5 and could be redissolved at 50 to 75 times its initial sucrose concentration in the low ionic strength buffers shown in Table I. The solubility properties of the other forms of keratins are also shown in this table. SDS gel electrophoresis showed that these preparations of neutral soluble keratin contained a large number of polypeptide chains with widely ranging molecular weights.

One major contaminant was shown immunologically to be BSA which was removed on a Sepharose 4B column run in 0.01M Tris at pH 8.3, as described under methods. The excluded peak from the Sepharose

TABLE I

Type of Keratin	Polypeptide Composition and Molecular Weight	Solubility Properties
NEUTRAL SOLUBLE KERATIN (sucrose extractable)	Undetermined number of chains with MW > 79,000 < 90,000	0.01M PO_4 pH = 7.0 0.01M Tris pH = 8.0 0.01M Tris pH = 9.0 0.25M Sucrose
PREKERATIN (citric acid ectractable)	Four principal chains with MW's ranging from 67,000 to 45,000	pH > 10.5 < 2.5 8M urea 6M GuHCl or dilute buffer with 0.1% SDS
STRATUM CORNEUM PROTEIN (urea extractable)	Chains similar, but not identical to prekeratin with MW's in the same range	8M urea 6M GuHCl or dilute buffer with 0.1% SDS
STRATUM CORNEUM - SH PROTEIN (urea - SH extractable)	Chains similar but not identical to prekeratin with MW's in the same range	Same as the urea soluble protein but requires presence of reducing agent

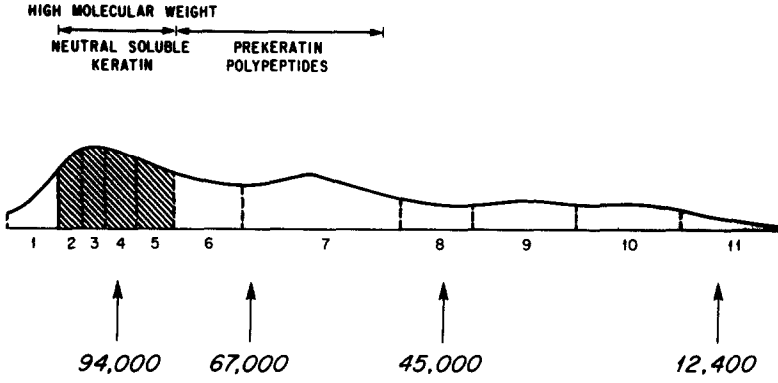

Figure 1. Elution profile of Sephadex G200 (0.9 x 190 cm) run in 8M urea, 0.1M NaCl, 0.1% SDS and 0.01M Tris, pH = 8.3. The sample applied to the column was obtained from Peak 1 of Sepharose 4B as described in the text. The column was run at 4.4 ml/hr and tubes were collected at 7.5 min intervals and combined into 11 pools as shown in the figure. The column was calibrated with the following standard proteins Phosphorylase A (94,000), BSA (67,000), Ovalbumin (45,000) and Cytotochrome C (12,400).

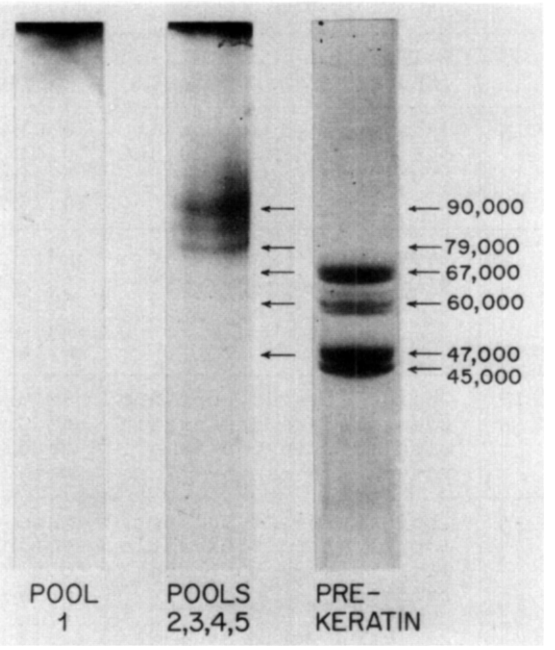

Figure 2. SDS-polyacrylamide electrophoresis of pools from Sephadex G200 column shown in Fig. 1. The gels are 7% acrylamide 3% bisacrylamide and run at pH 9.5 using the discontinuous gel system described by Neville (17). Gels were run at 2 ma per gel for approximately 1 hr at 25°C and fixed and stained with Coomaissie blue in 7.5% acetic acid (v/v) and 5% methanol (v/v/). The direction of migration is downward. Samples were heated in 8M urea, 1% SDS and 1% mercaptoethanol for 1 min at 100°C followed by an hour at 50°C and dialyzed against 1,000 volumes of upper gel buffer before loading on gels. Standard protein markers used for molecular weight determinations were phosphorylase (94,000), BSA (67,000), heavy chain of globulin 50,000 and creatinine phosphokinase (40,000).

4B contained the neutral soluble keratin plus a number of proteins whose molecular weight in 0.01M Tris, pH 8.3 was greater than 67,000; but which subsequent gel electrophoresis in the presence of SDS showed were aggregates of lower molecular weight species. Therefore, the excluded peak was concentrated and subjected to gel filtration on a Sephadex G200 (see Fig. 1) run under denaturing conditions which included 1% SDS, 8M urea, 0.1M DTT and 0.1M NaCl. Fractions were pooled as indicated in the figure. Double diffusion studies showed that pool 1, containing the highest molecular weight material,

was not immunoreactive but that pools 2, 3, 4, and 5 were immunologically identical to other previously defined forms of keratin as were pools 6 and 7. Calibration of the Sephadex G200 with standard proteins showed the material in pool 1 had a molecular weight greater than 100,000 daltons while that in pools 2, 3, 4, and 5 was seen to be between 83,000 and 100,000 daltons. Pools 6 and 7 contained material in the molecular weight range of the keratin polypeptide chains (i.e. 45,000 to 67,000 daltons). These pools were run on SDS electrophoresis (1) along with standard proteins as markers and some of the results are shown in Fig. 2. Pool 1 contained high molecular weight material which did not enter the gel. Pools 2, 3, 4, and 5 also contained some of this high molecular weight material as well as three polypeptide chains with molecular weights calculated to be between 79,000 and 90,000 daltons. It is important to note that material from this region of the chromatogram contained no keratin polypeptide chains. Even when material from these pools was run at ten times the concentration shown here, there were still no traces of the usual keratin chains (which are also shown in Fig. 2 for comparison). We have previously shown that the sensitivity of the antibody was such that between 0.3 and 1.0 ug of keratin was required for an optimum precipitin line (17). This is also the same range of protein required to give a visible band on a SDS gel. Therefore, we can assume that the precipitin line on the Ochterlony plate is formed by one of the visible bands on the gel and not some visually undetectable amount of keratin with molecular weights 45,000 to 67,000 daltons. The aggregated material at the top of the gel was preparatively eluted and shown not to react with the antikeratin antibody.

Keratins are distinguished by a high content of glycine, glutamic, and serine and much lower values for proline and no hydroxyproline.

TABLE II
(Expressed as Res/1000 Res)

	PREKERATIN (Citric Acid) (Extractable)	STRATUM CORNEUM - SH PROTEIN (Urea SH Extractable)	NEUTRAL SOLUBLE KERATIN (Sucrose) (Extractable)
Serine	111.0	113	113
Glutamic	141.0	135	187
Glycine	164.0	176	158
Proline	14.0	22	28
Hydroxyproline	0	0	0

The amino acid composition of neutral soluble keratin is compared to that of previously reported keratins in Table 2 and it is apparent that these proteins are closely related. We have shown that a high molecular weight neutral soluble keratin similar to the one described in this report is also present in pig and human epidermis.

We hypothesize that neutral soluble keratin is either 1) a chemically modified form of a previously described keratin, 2) a structural protein which is immunologically related to the keratins but whose role in the epidermis is as yet undefined or, 3) a soluble precursor of all the more fully differentiated forms of keratins found in the epidermis. Although the modifications that occur when prekeratin protein is converted to stratum corneum protein have not as yet been fully detailed, it is felt that such changes occur concomitant with the migration of cells from the lower living malpighian layers to the outer cornified layers. It seems reasonable that the process of keratinization consists of an early step involving elaboration from the ribosomes of a highly soluble form of keratin (neutral soluble keratin) which is subsequently converted to a more specialized protein (prekeratin) and then finally to the fully differentiated forms (stratum corneum proteins) and stratum corneum SH.

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