Characterization of the Keratin Filament Subunits Unique to Bovine Snout Epidermis

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Steinert [*Biochem. J.* (1975) **149**, 39–48] reported that the α -keratin polypeptides (the subunits of the intracellular keratin filaments) of bovine hoof and snout epidermis are the same. We now demonstrate that this is not so: in addition to the seven polypeptides previously identified in hoof epidermis, snout epidermis also contains at least three other polypeptides of higher molecular weight. These unique polypeptides were isolated, purified and characterized. They are chemically and structurally very similar to the other polypeptides of bovine epidermis and readily polymerize *in vitro* with them to form native-type epidermal keratin filaments.

Keratin filaments are the principal differentiation product of mammalian epidermis. They contain a heterogeneous group of α -helix-rich polypeptide subunits of very similar properties and structure. The subunits have been isolated from bovine (Lee *et al.*, 1975; Steinert & Idler, 1975), rat (Shimizu *et al.*, 1974; Huang *et al.*, 1975), mouse (Steinert *et al.*, 1979) and human (Fuchs & Green, 1978; Steinert *et al.*, 1980) epidermis and partially characterized. Perhaps their most interesting property is their facility for polymerization *in vitro* into native-type epidermal keratin filaments (Steinert *et al.*, 1976).

We (Steinert, 1975; Steinert & Idler, 1975) initially studied the polypeptides of bovine epidermis, and reported that the polypeptides extracted from different animals and from different regions of the same animal were invariant. More recently, however, Drochmans *et al.* (1978) and Lee *et al.* (1979) have convincingly demonstrated the presence in bovine snout epidermis of a prominent α -keratin polypeptide component that was absent from the epidermis proximal to the hoof. The purpose of the present paper is twofold. Firstly, our new results described here fully concur with those of Drochmans *et al.* (1978) and Lee *et al.* (1979), in contradiction to our earlier finding (Steinert, 1975). Secondly, we report the isolation and characterization of the polypeptides unique to the snout epidermis, which, because of incomplete separation of the components, were not reported by Lee *et al.* (1975). We also demonstrate that these unique polypeptides assemble *in vitro* into keratin filaments that are ultra-structurally identical with all other keratin filaments so far studied.

Experimental

Materials

The snout and skin adjacent to the hooves were obtained from several steers and transported to the laboratory within 2 h of slaughter.

Methods

Extraction and separation of the α -keratin polypeptides. The epidermis was dissected from the tissue and then extracted with a urea buffer as described previously (Steinert, 1975; Steinert & Idler, 1975). The polypeptides unique to the snout were partially separated into an enriched fraction by chromatography on DEAE-cellulose (peak A, at the break-through peak), and then separated by preparative polyacrylamide-gel electrophoresis (Steinert & Idler, 1975). Polypeptide 5 was prepared similarly.

Analytical procedures. Polyacrylamide-gel electrophoresis with 0.1% (w/v) sodium dodecyl sulphate was done with polyacrylamide gradient (10–18% acrylamide) gels by the method of Laemmli (1970). Other techniques for the characterization of the polypeptides were as described previously (Steinert & Idler, 1975). Filaments were reconstituted from separated polypeptides (Steinert *et al.*, 1976). Fully reduced polypeptides were treated with 4-vinylpyridine to convert the cysteine residues into their *S*- β -pyridylethyl derivatives (Cavins & Friedman, 1970) and then hydrolysed with 3 M-methanesulphonic acid at 110°C for 22 h *in vacuo*. This permitted simultaneous analysis of cysteine and tryptophan with the other amino acids

(Simpson *et al.*, 1976). Hydrolysates were analysed in a Beckman 119CL analyser equipped with a model 126 data-reduction system. Polypeptides were dissolved in aq. 70% formic acid (2mg/ml) and treated with CNBr (equal weight of protein to CNBr) for 2 days at 23°C. The resulting peptides were examined by isoelectric focusing on polyacrylamide (7.5% acrylamide) gels (Hearing *et al.*, 1978).

Results and Discussion

Nomenclature of α-keratin polypeptides

Polyacrylamide-gel electrophoretograms of the polypeptides extracted from bovine snout and hoof epidermis are shown in Fig. 1 and clearly demonstrate different band patterns. In addition to the seven polypeptides of hoof epidermis, snout epidermis also contained at least three other polypeptides, of higher molecular weight, that were absent from hoof epidermis. The polypeptides unique to snout epidermis were name 1c and 1d (major components) and 1e (minor component of highest molecular weight), to be consistent with our earlier nomenclature system (Steinert & Idler, 1975). Marked quantitative differences in the relative molar amounts of the polypeptides were also evident in Fig. 1: in snout there was relatively less polypeptides 1a, 1b, 3 and 5, but relatively more polypeptide 4, in comparison with hoof epidermis. Although similar gel systems were used, Fig. 1 shows the presence of more bands than were seen by Lee *et al.* (1975,

1979). They described the presence of only four bands of polypeptides, enumerated B (which corresponds to our polypeptides 1c, 1d and 1e), B' (polypeptides 1a, 1b, 2 and 3), A (polypeptide 4) and A' (poly-

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the α-keratin polypeptides extracted from bovine hoof and snout epidermis

The samples were prepared and electrophoresed as described in the Experimental section and by Steinert & Idler (1975). The nomenclature system and *M_r* values of the different polypeptides are indicated. The gels are of the polypeptides extracted from hoof (H) and snout (S) epidermis from the same animal.

Table 1. Amino acid compositions of α-keratin polypeptides from bovine snout epidermis

All of our data are the averages of at least three different preparations of polypeptides and were corrected for hydrolytic losses. Cysteine was measured in this study as *S*-β-pyridylethylcysteine. The values for polypeptides 1a, 1b and 5 are from Steinert & Idler (1975). The values for fractions B and B + B' are from Lee *et al.* (1975). N.D., Not determined.

Amino acid	Polypeptide ...	Amino acid composition (residues/100 residues)							
		1e	1d	1c	B	1b	1a	B + B'	5
Aspartic acid		8.80	8.65	8.58	9.00	8.80	8.58	8.35	9.65
Threonine		3.75	3.70	3.38	4.45	4.45	4.40	3.35	3.85
Serine		12.15	11.75	12.05	11.51	13.75	13.90	11.76	10.50
Glutamic acid		14.10	13.90	14.05	13.69	16.00	16.10	13.69	14.75
Proline		1.05	1.10	1.20	1.86	0.60	0.65	1.98	1.25
Glycine		19.25	19.05	18.65	19.21	15.20	16.75	21.03	12.85
Alanine		6.00	6.10	5.85	6.41	4.40	3.75	5.99	6.55
Valine		4.50	4.35	4.45	4.45	4.85	4.65	4.44	5.65
Methionine		1.20	1.20	1.25	1.52	2.15	2.00	1.10	2.00
Isoleucine		3.30	3.40	3.35	3.48	3.70	3.90	3.56	3.75
Leucine		8.65	8.80	8.75	8.89	7.85	7.70	8.47	10.15
Tyrosine		2.05	1.65	1.30	1.86	2.10	2.60	1.31	3.65
Phenylalanine		3.35	3.70	4.00	3.83	2.85	2.20	3.41	2.40
Histidine		0.95	1.00	1.05	1.06	1.35	1.25	1.23	1.15
Lysine		4.75	4.50	4.65	4.79	5.05	5.10	4.68	4.60
Cysteine		1.30	1.35	1.35	N.D.	1.49	1.55	N.D.	0.91
Tryptophan		0.60	0.65	0.65	N.D.	0.37	0.38	N.D.	0.44
Arginine		5.15	5.05	5.15	5.06	5.70	5.40	5.14	6.45

peptides 5 and 6). Possible reasons for the different observations have been discussed (Steinert & Idler, 1975). The different components that we detect on gels are distinct polypeptide species that are unrelated to one another by degradation or other post-translational events (Steinert & Idler, 1975; see below).

Characterization of polypeptides 1c, 1d and 1e

After chromatography on DEAE-cellulose, an enriched fraction of polypeptides 1c, 1d and 1e was obtained as peak A (Steinert & Idler, 1975), which was then used to purify the three polypeptides to electrophoretic homogeneity by preparative polyacrylamide-gel electrophoresis.

The molecular weights of polypeptides 1c, 1d and 1e were determined on polyacrylamide gels by construction of Ferguson plots (Steinert & Idler, 1975) and were respectively 64 000, 66 000 and 68 000. These values agree with those obtained by Lee *et al.* (1975, 1979). The α -helix contents of polypeptides 1c, 1d and 1e were estimated by spectropolarimetric measurements to be (means $\pm$ S.D.) $38 \pm 4\%$, $36 \pm 6\%$ and $35 \pm 6\%$ respectively.

Fig. 2. Isoelectric focusing of the peptides derived on cleavage of the purified α -keratin polypeptides with CNBr. For experimental details see the text. The polypeptide numbers and approximate pH values along the focused gels are indicated.

Each polypeptide possessed *N*-acetylserine at its *N*-terminus, like all of the other bovine epidermal polypeptides (Steinert & Idler, 1975). The amino acid contents of polypeptides 1c, 1d and 1e were virtually identical with one another and with that of fraction B obtained by Lee *et al.* (1975), and very similar to those of polypeptides 1a and 1b and fraction B + B' (Table 1). Polypeptides 1c, 1d and 1e, like polypeptides 1a and 1b, contained about 8 mol of cysteine/mol, 2–3 mol of tryptophan/mol and about 84 mol of glutamic acid/mol. Their amino acid contents were clearly different from that of polypeptide 5, a major component common to both snout and hoof epidermis (Table 1). The peptides resulting on cleavage of polypeptides 1c, 1d and 1e at their methionine residues with CNBr were clearly different from one another, and from those from polypeptide 1a (Fig. 2), as well as from those from all of the other bovine epidermal polypeptides (results not shown). These results indicate that polypeptides 1c, 1d and 1e are distinct species, with major differences in amino acid sequences, and thus are unrelated to one another or other subunits by degradation or precursor-product events.

Fig. 3. Stoichiometry of filaments polymerized *in vitro* from mixtures of polypeptides 1c and 5.

The experimental details are as given by Steinert *et al.* (1976). Samples of solutions of purified polypeptides 1c and 5 were combined to give 12 mixtures containing the polypeptides in amounts varying between about 0.2 and 0.8 mg (total of about 1 mg). The detergent was removed by adsorption on ion-exchange resin. Filaments were assembled by standard procedures and then pelleted from suspension. Samples of the mixtures before filament assembly and the pellets of filaments were electrophoresed on gels for determination of the relative molar amounts of the two polypeptides. ●, Relative molar amount of polypeptide 5; ○, relative molar amount of polypeptide 1c.

Assembly and structure of filaments containing polypeptides 1c, 1d and 1e

Purified polypeptides 1c, 1d and 1e separately or together did not form filaments *in vitro* under the standard conditions used. Most combinations of polypeptides 1c, 1d or 1e with another purified bovine epidermal polypeptide (except with polypeptides 1a or 1d) readily assembled into filaments *in vitro*. Filaments were examined in an electron microscope after negative staining and in all cases were 7–8 nm wide and 1–20 μ m long. The polypeptide mixture 1c and 5, which formed long filaments in high yield, was examined further to investigate stoichiometric properties (Fig. 3). Irrespective of the relative molar amounts of polypeptides 1c and 5 in the mixtures, the resulting filaments contained the two polypeptides in the molar ratio of 1:2, which is indicative of a three-chain unit structure of the composition 1c+5+5 (Steinert *et al.*, 1976). In similar experiments, polypeptides 1c and 1d always assembled with other subunits in ratios of 1:2, as is characteristic of polypeptides 1a and 1b (Steinert *et al.*, 1976). Thus it seems likely that polypeptides 1c, 1d and 1e in snout epidermis replace the functions of polypeptides 1a and 1b of hoof epidermis.

The structure of a three-chain unit containing polypeptide 1c was investigated by isolation of two α -helix-enriched fragments, particle 1 and particle 2, that were released on limited tryptic digestion of 1c+5+5 filaments. These two particles were physicochemically very similar to the comparable particles previously isolated from bovine epidermal keratin filaments of other subunit compositions (Steinert, 1978). From this, we conclude that the structure of the three-chain unit of the 1c+5+5 filaments is also the same as that of all other possible three-chain units of bovine epidermal keratin filaments (Steinert *et al.*, 1976; Steinert, 1978).

The present results demonstrate that polypeptides 1c, 1d and 1e are chemically, structurally and

functionally most similar to polypeptides 1a and 1b, but also very similar to the other smaller subunits of bovine epidermal keratin filaments. Further studies are required to understand the molecular mechanisms underlying the variable expression of the keratin-filament-subunit genes in different regions of the epidermis of the same animal.

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