ISOLATION OF SUBSTRATES OF EPIDERMAL TRANSGLUTAMINASE FROM BOVINE EPIDERMIS[1]

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SUMMARY: Substrates of the transglutaminase specific to epidermis were identified by fluorescent labeling of bovine epidermal homogenates with dansyl cadaverine. This lysine analog was preferentially incorporated into a soluble protein of 150,000 MW. A highly insoluble protein was also labeled; this protein was solubilized and extracted following chemical cleavage with cyanogen bromide. The soluble and insoluble substrates of epidermal transglutaminase were immunochemically related, as shown by precipitation in agar or by chromatography on antibody affinity columns. They were distinguished from the fibrous, α-helical and sulfur-rich matrix proteins of skin as well as from fibrinogen and cold insoluble globulin of plasma.

Identification of the ε-(γ-glutamyl)lysine dipeptide in enzymatic digests of wool (1) or human callus (2) suggested that isopeptide crosslinks may serve to stabilize structural proteins in keratinizing epithelia or epithelial appendages. An enzyme which catalyzes ε-(γ-glutamyl)lysine bonds was highly purified from guinea pig hair follicles (3) and from bovine (4) and human epithelium (5). Transglutaminases purified from these sources differ from those purified from liver (6,7) and plasma (8).

Preliminary evidence has been presented that a citrulline-rich protein of the inner root sheath and medulla of hair may be cross-bonded by the hair follicle transglutaminase (9). Substrates for epidermal transglutaminase have not heretofore been isolated, although we have demonstrated an acceptor (glutamine-bearing) protein in keratinizing zones of human or bovine epidermis and hair follicle inner root sheath by a fluorescent histochemical method (10).

We now report the isolation and purification of soluble and insoluble substrates of epidermal transglutaminase which were preferentially labeled by dansyl

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cadaverine in epidermal homogenates. The labeling was specific in that maneuvers which inactivate the transglutaminase (e.g., calcium deprivation or sulfhydryl blocking) abrogate the reaction. Further, we wish to report that the soluble and insoluble substrates of epidermal transglutaminase bear common antigenic markers and thus may represent soluble precursor and insoluble crosslinked structural proteins.

MATERIALS AND METHODS

Labeling of epidermal homogenates with dansyl cadaverine: Quantities of 0.5 gm (wet weight) of frozen pulverized bovine epidermis removed at 0.2 mm by Storz keratotome were homogenized in 3 ml of .025 M tris-acetate pH 7.5 containing .15 M NaCl and .001 M EDTA for three minutes in a Polytron (Brinkman Instruments). An equal volume of 2 mM dansyl cadaverine-10 mM $CaCl_2$ was added at time 0, and the homogenates were agitated at 37°C for times from 0 to 5 hours. The reaction was terminated with .1 ml 400 mM disodium EDTA. Controls contained excess EDTA through out incubation. All studies were performed in triplicate.

Following centrifugation of homogenates at 20,000 rpm and 4°C for 60 minutes, the supernatants were removed and dialyzed against the tris-acetate buffer to remove unreacted dansyl cadaverine. Fluorescence was read in an Aminco-Bowman spectrophotofluorometer with excitation = 355 and emission = 525 nm.

Pellets remaining after tris buffer removal were serially extracted at room temperature for 24 hours, with agitation, with 5 ml volumes of the following solvents: 0.1 M citric acid-sodium citrate, pH 2.5; .5% SDS-8 M urea-.002 M dithiotreitol; and 10% cyanogen bromide in .1 M HCl. The pellets were then re-extracted several times with SDS-urea-dithiothreitol, and these extracts were added to the cyanogen bromide extracts. Finally, the pellets were digested with .2 N NaOH.

Tris buffer and cyanogen bromide extracts were further analyzed.

Affinity chromatography: Rabbit anti-human fibrinogen (Behring Diagnostics) was coupled to AH-Sepharose 4B (Pharmacia) by a standard procedure (11). As a control, nonimmune rabbit serum was similarly coupled to Sepharose 4B.

To remove possible traces of contaminating fibrinogen, maximally fluorescent buffer and cyanogen bromide extracts were dialyzed against .025 M tris-.15 M NaCl, pH 7.5 and separately passed over a .5 x 6 cm bed of Sepharose-anti-fibrinogen equilibrated with the same buffer. The column was thoroughly washed with tris buffer, then with .5 M NaCl in the tris buffer, and finally with 3 M sodium thiocyanate to clear strongly adsorbed proteins. For comparison, 1 ml of .2% fibrinogen, 95% clottable (Kabi Products) or 1 ml of .2% bovine fibrinogen, 65% clottable (Miles-Yeda), was passed over the column. All experiments were repeated using the Sepharose-nonimmune rabbit serum as affinity medium.

Proteins eluting from the affinity column were quantitated by the Lowry method (12). They were dialyzed against tris buffer and studied by immunodiffusion in agar plates against rabbit antiserum to human fibrinogen and antiserum to cold insoluble globulin.

Molecular weight determination: Protein samples were heated to 96°C for three minutes in 1% SDS with or without 1% β-mercaptoethanol (or 2 mM dithiothreitol) prior to electrophoresis in 5% SDS acrylamide gels according to the method of Shapiro, et al. (13).

Staining of completed gels was accomplished with Coomassie Brilliant Blue and destaining with 7% acetic acid. Matched unstained gels were cut into 1.2 mm discs in a gel slicer; protein was extracted from the discs into 2 ml of .5% SDS-8 M urea-2 mM dithiothreitol.

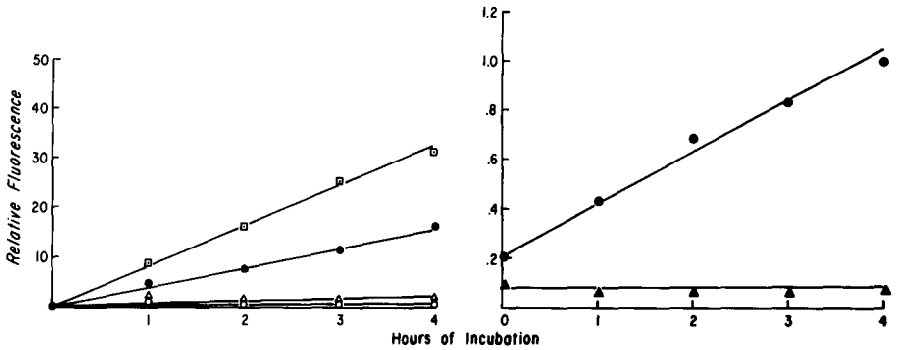

Fig. 1. Extraction of dansyl cadaverine-labeled fluorescent proteins from bovine epidermis. Each point represents an average of triplicate samples with subtraction of controls incubated without calcium. Values on abscissa represent duration of incubation of homogenate with dansyl cadaverine.

Fig. 1, left. Fluorescence solubilized by serial extraction with the following solvents: (●——●) tris-acetate, pH 7.5; (○——○) citric acid-sodium citrate, pH 2.5; (△——△) SDS-urea-dithiothreitol, pH 8.0; (⊡——⊡) 0.2 N NaOH.

Fig. 1, right. Extraction of insoluble fluorescent protein with cyanogen bromide (●——●) prior to treatment with .2 N NaOH. Note that subsequent NaOH extraction (▲——▲) yields no fluorescence.

Crosslinking of purified substrate: A nonfluorescent tris buffer extract of bovine epidermis was passed over the Sepharose-anti-fibrinogen column, and the protein eluting with thiocyanate was adjusted to a concentration of 1.4 mg/ml. Volumes of .5 ml were incubated with .25 ml dansyl cadaverine-10 mM $CaCl_2$, and 50 µl epidermal transglutaminase, purified as previously described (4). Crosslinking proceeded for 0-60 minutes at 37°C and was terminated with EDTA. After overnight dialysis against tris buffer, fluorescence was read in the spectrophotofluorometer.

RESULTS

As shown in Fig. 1 (left), significant time and calcium-dependent increases in TCA-precipitable, nondialyzable fluorescence over controls were demonstrated only in the tris buffer and cyanogen bromide extracts. No fluorescence was seen in citrate buffer extracts, which solubilize the α-helical fibrous protein of epidermis (14). Adjustment of the pH of this extract to neutrality resulted in precipitation of a nonfluorescent fibrous protein.

There was also no fluorescence increase over controls in SDS-urea-dithiothreitol extracts, which solubilize sulfur-rich matrix proteins of keratinizing tissues (15). If cyanogen bromide treatment was omitted, time and calcium-dependent

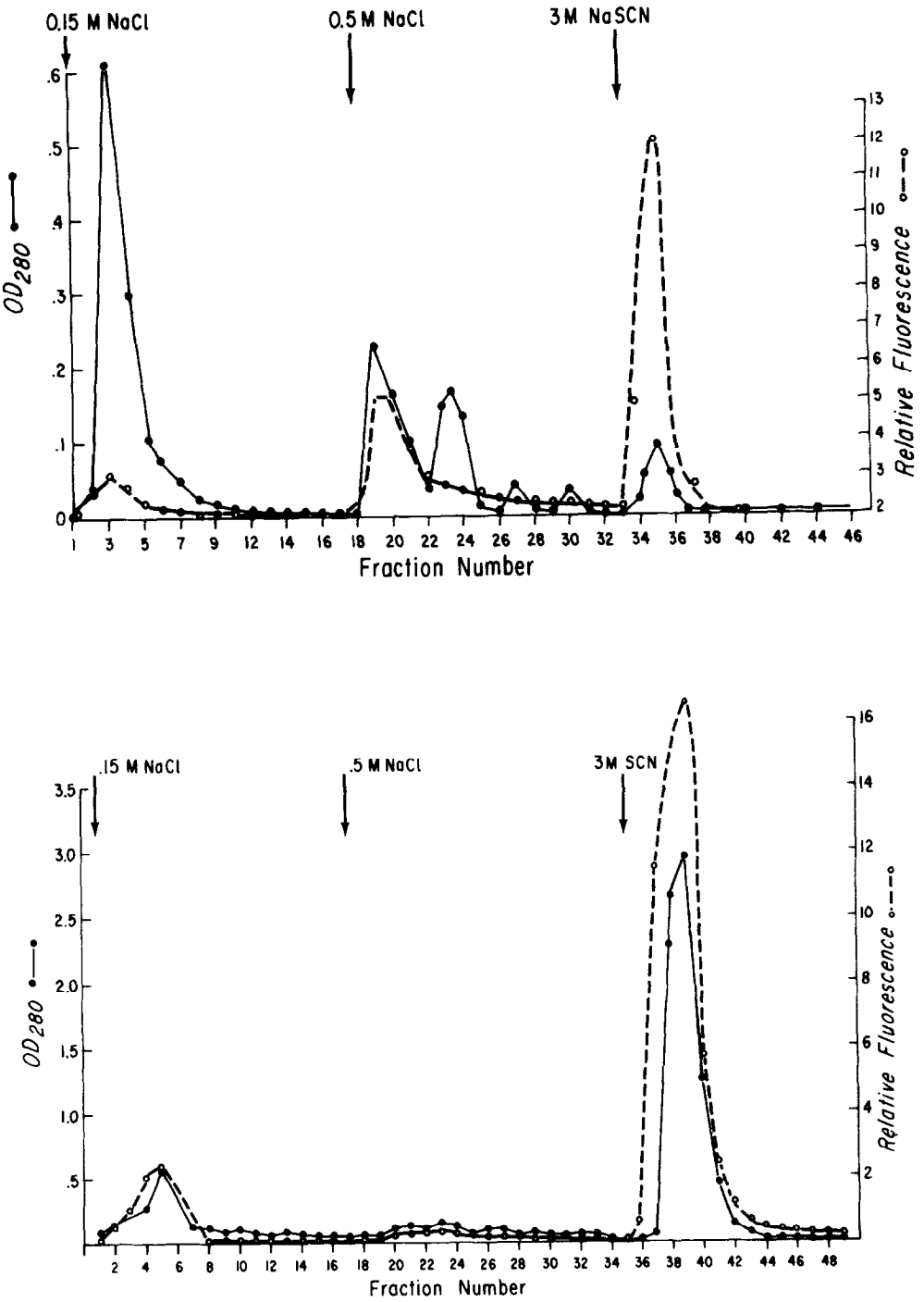

Fig. 2. Affinity chromatography of dansyl cadaverine-labeled epidermal extracts on Sepharose-anti-fibrinogen. Top, tris-acetate extract. Bottom, cyanogen bromide extract.

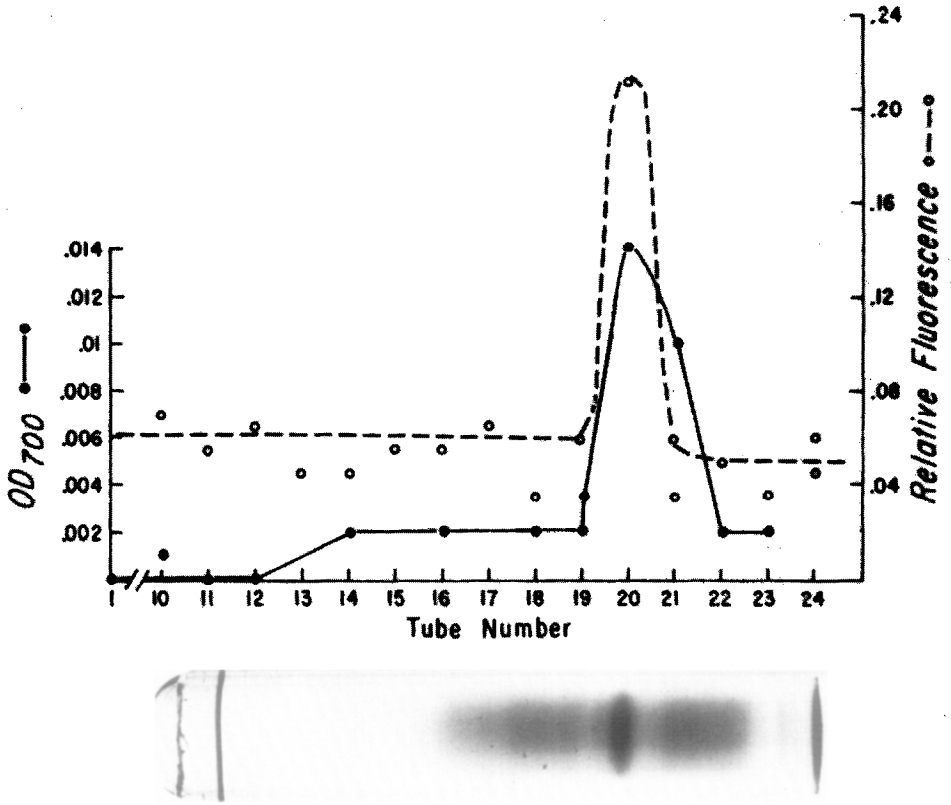

Fig. 3. Electrophoresis of buffer-soluble substrate (isolated by affinity chromatography) in 5% SDS acrylamide with accompanying fluorescence elution pattern.

incorporation of dansyl cadaverine was seen in the .2 N NaOH extract. If, however, sodium hydroxide extraction followed cyanogen bromide treatment, negligible fluorescence was present in this extract as shown in Fig. 1 (right). This indicates that acidic cyanogen bromide, which ruptures methionine bonds preferentially (16) was able to release the highly insoluble substrate protein from epidermal residues.

The major fluorescent species of both the soluble supernatant and cyanogen bromide extracts was strongly bound by Sepharose-anti-fibrinogen, from which it was released by thiocyanate (Fig. 2). The soluble fluorescent protein was homogeneous and had a molecular weight of 150,000 by SDS acrylamide gel electrophoresis in the presence of dithiothreitol. The insoluble cyanogen bromide-

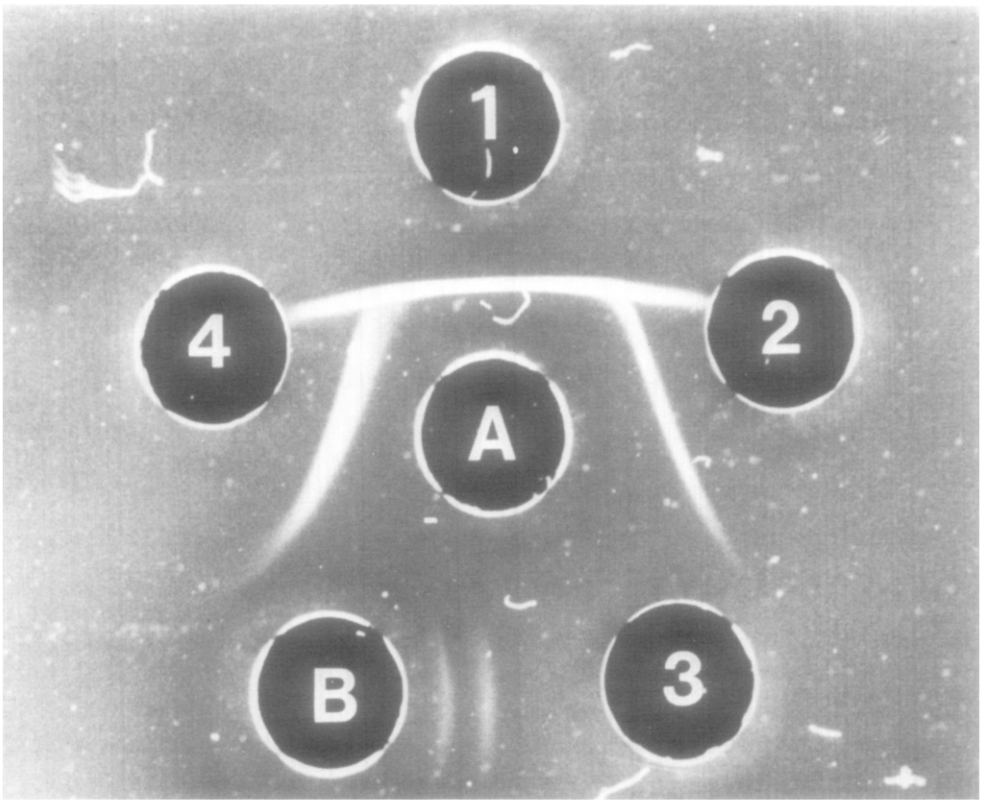

Fig. 4. Precipitation reactions of soluble substrate (well 3), bovine fibrinogen (wells 2, 4) and human fibrinogen (well 1) with antiserum to fibrinogen (well A) and antiserum to insoluble substrate (well B).

extracted fluorescent protein remained at the origin of 5% SDS gels. Neither of these substrate proteins precipitated in agar with anti-fibrinogen or antiserum to cold insoluble globulin. Fibrinogen controls, when passed through the same columns, bound to the column but were eluted with the .5 M NaCl wash. Sepharose conjugated to nonimmune rabbit serum did not bind epidermal or plasma proteins.

The buffer-soluble substrate for epidermal transglutaminase bound to Sepharose-anti-fibrinogen even when it had not been previously crosslinked to dansyl cadaverine and was readily eluted with thiocyanate. This protein accepted dansyl cadaverine in the presence of purified epidermal transglutaminase and calcium and had a molecular weight of 150,000 in 5% SDS acrylamide gels.

Immunization of rabbits with purified insoluble substrate elicited an

antiserum which gave two separate precipitin lines in agar with the dialyzed cyanogen bromide extract. The antiserum also gave two precipitin lines with the purified soluble substrate (Fig. 3). It did not precipitate with bovine fibrinogen or bovine cold insoluble globulin. Antiserum to fibrinogen did not precipitate with either epidermal substrate but gave reactions of partial identity with human and bovine fibrinogen.

DISCUSSION

In these studies, we have demonstrated that there are a limited number of preferential natural substrates for epidermal transglutaminase in bovine epidermis. The relative specificity of transglutaminases for their natural substrates has been demonstrated by Lorand (17) and re-emphasized by Mosher (18), in experiments with plasma transglutaminase. These investigators have shown that dansyl cadaverine can be incorporated both into the precursor fibrinogen and crosslinked fibrin during coagulation.

The two major substrates for transglutaminase in epidermis differ markedly in solubility. The highly insoluble substrate is not an α-helical fibrous protein, as it is not soluble in citrate buffer at pH 2.5. It is also not a sulfur-rich matrix protein as such, since it is not released into solution with denaturing-disulfide-reducing solvents alone. It is necessary to rupture peptide bonds either with sodium hydroxide or with acidic cyanogen bromide, to release this insoluble substrate.

Surprisingly, both the soluble substrate and the major fragment of the insoluble substrate showed high affinity for Sepharose-conjugated antiserum to fibrinogen, although neither precipitated in agar with anti-fibrinogen. This suggests that the two proteins are both related to fibrinogen immunologically, although they are not identical to fibrinogen.

The soluble substrate showed affinity for antiserum to fibrinogen even when it had not been crosslinked to dansyl cadaverine, indicating that adherence to the column was not an artifact produced by crosslinking of the protein to dansyl cadaverine but was intrinsic to the molecule itself. The ability of this protein

to accept dansyl cadaverine identified it as a natural epidermal substrate for the enzyme.

The soluble and insoluble substrates are not only related by a common affinity for anti-fibrinogen, but by common precipitation in agar with antiserum to the insoluble substrate. Thus we propose that the soluble substrate is a precursor of the insoluble one.

The remarkable insolubility of the insoluble substrate correlates with the known extreme insolubility of many components of keratin, notably the thickened inner layer of the cell membrane of the maturing keratinocyte (19). This layer is felt to lend structural stability and chemical resistance to skin.

That ε-(γ-glutamyl)lysine crosslinking may contribute stability to membrane-associated proteins has been recently suggested by Lorand (20) and has also been proposed to occur in bacterial systems (21).

Preliminary amino acid analysis of the purified soluble substrate (Buxman and Wuepper, unpublished data) has shown very low half cystine content and high serine, glutamic acid and lysine contents. This correlates well with analysis of other proteins rich in gamma glutamyl bonds, as well as with a recent analysis of a highly insoluble membrane-associated protein isolated from bovine epidermis by Matoltsy[1]. We therefore propose that the natural substrate for epidermal transglutaminase is manufactured as a soluble precursor protein, and is transformed into a highly insoluble structural protein, possibly the highly insoluble protein lining the inner aspect of the keratinocyte cell membrane. This latter protein is known to be laid down in the upper Malpighian and granular layers of the epidermis, regions in which high transglutaminase activity has been identified histochemically (10).

Studies to identify the ε-(γ-glutamyl)lysine bond in enzymatic digests of the insoluble substrate are in progress, as are studies to further characterize both transglutaminase substrates.

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[1] A. G. Matoltsy, personal communication.

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