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Kim, Hee Chul, Ph.D.

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MODULATION OF CELLULAR TRANSGLUTAMINASE: ISOLATION AND CHARACTERIZATION OF TRANSGLUTAMINASES FROM GUINEA PIG EPIDERMIS

By

Hee Chul Kim

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Faculty of the College of Arts and Sciences

of The American University

in Partial Fulfillment of

the Requirements for the Degree

of

Doctor of Philosophy

in

Chemistry

Signatures of Committee:

Chair: _____

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Dean of the College

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Date

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Washington, D.C. 20016

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ABSTRACT

Transglutaminases are calcium-dependent enzymes that catalyze the formation of covalent ε(γ-glutamyl)lysine cross-links in proteins. A cationic proenzyme form of epidermal transglutaminase (protransglutaminase E) has been isolated from epidermis of new born mouse and from adult guinea pig. Upon activation, this zymogen is the source of most of the extractable enzyme activity in epidermis and skin. The molecular mass of protransglutaminase E determined by sedimentation equilibrium was 77,800 and was similar to the value estimated by SDS-PAGE. Dispase or thrombin treatment of the proenzyme resulted in an 80-fold activation of transglutaminase activity. In the nondenatured state, the Dispase-activated enzyme was shown by gel permeation and sedimentation equilibrium methods to be the same molecular size as the proenzyme. In the denatured state, the enzyme consisted of two peptide fragments of M_r 50,000 and M_r 27,000, as analyzed by SDS-PAGE under non-reducing conditions. The activated epidermal

transglutaminase showed a thiol dependency for full enzyme activity as well as a low Ca^{2+} ion requirement (Ka < 10^{-10} M). High levels of Ca^{2+} ion induced a concentration dependent activation of proenzyme in a reversible manner. The purified zymogen from guinea pig skin displayed higher glycine, serine, and alanine contents then guinea pig liver transglutaminase. Westernbolt analysis of three different forms of transglutaminase present in epidermis, with monospecific polyclonal antibodies to protransglutaminase E, showed non-identity with either the membrane-associated transglutaminase or the cytosolic tissue transglutaminase. These results indicate that a substantial portion of the total transglutaminase in skin occurs as proenzyme form of the epidermal transglutaminase and that activation could be induced by neutral protease and Ca^{2+} ions that are made available during the terminal differentiation of epidermal calls into the cornified layers of skin.

To my parents

To Eum Mi, Myung Hee, and Jae Whan

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TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS iv

LIST OF FIGURES vii

LIST OF TABLES ix

ABBREVIATIONS x

LITERATURE REVIEW 1

Introduction 1

Epidermis 2

1. Structure of Epidermis 3
2. Structural Markers of Keratinocyte Differentiation 7

Hair 10

1. Structure of the Hair Follicles 12
2. Structural Markers of Hair Follicle Differentiation 17

Transglutaminases 22

1. Introduction 22
2. Multiple Forms of Transglutaminase. . . 23
3. Substrates of Transglutaminases . . . 29
4. Regulation of Transglutaminases . . . 33

Research Objectives 35

MATERIALS AND METHODS 36

Materials 36

Methods 37

Protein Determination 37

Transglutaminase Assay 38

Preparation of Succinylated Casein . . . 39

Preparation of Epidermis and Hair Follicles . . 40

Purification of Epidermal Transglutaminase . . 43

Preparation of Dispase-activated Enzyme from Epidermal Protransglutaminase . . . 44

Determination of Molecular Weight of Protransglutaminase and Dispase-activated Enzyme 45

Determination of Sedimentation Coefficient and Frictional Ratio 48

Agarose Gel Electrophoresis and Activity Staining . 49

Active Site Labeling of Epidermal Transglutaminase 50

Determination of Amino Acid Composition of Epidermal Transglutaminase 50

Detection of Soluble Substrate of Epidermal Protransglutaminase 51

Preparation of Immunoglobulin Specific for Various Transglutaminase. 52

Electroblotting 53

Detection of the Protein Band by Antibody Probe . 54

RESULTS 56

Biochemical and Physical Studies 56

Immunological Studies 83

DISCUSSION 88

REFERENCES 93

LIST OF FIGURES

Figure		Page
1.	Schematic diagram showing the layering of the epidermis	5
2.	The structures of hair follicles	13
3.	Schematic diagram for the preparation of epidermis and hair follicles	42
4.	Activation of transglutaminase in epidermis by Dispase treatment of whole skins from 4-day-old mice .	57
5.	Distribution of transglutaminase activity and activation of transglutaminase from guinea pig skin . .	58
6.	Anon exchange chromatography of guinea pig skin extracts	59
7.	Distribution of transglutaminase activity from DE 52 ion exchange chromatography and activation of transglutaminases by Dispase treatment . .	61
8.	Affinity chromatography of transglutaminase on Heparin-Sepharose 4B	62
9.	Exclusion chromatography of transglutaminase on Bio-Gel A-0.5m	64
10.	Cation exchange chromatography of transglutaminase on S-Sepharose	65
11.	Polyacrylamide gel electrophoretic comparison of protein pattern of isolated proTGase (Lane 1), activated TGase E (lane 2) and pattern of radioactivity (Lane 3) after radio [^{14}C] acetamide inactivation	68

12. Comparison of exclusion chromatography of native and activated epidermal transglutaminase on Bio-Gel A-0.5m 69

13. Sedimentation equilibrium study plotted as optical density at 280 nm versus radius . . . 71

14. Sedimentation velocity study plotted as radius versus time 72

15. Agarose gel showing the transglutaminase activity visualized by enzyme-catalyzed incorporation of a fluorescent substrate, monodansyl-cadaverine, into casein 76

16. Protease-induced activation of proTGase E . . 79

17. Activation of native and activated transglutaminase by dithiothreitol and calcium 80

18. Comparison of ion exchange chromatography on Mono-S in the presence and in the absence of calcium ion 81

19. Schematic diagram of identification of soluble substrate from TGase preparation . . . 84

20. Purification of the soluble substrate on Bio-GelA-0.5m and (Inset) visualized by enzyme-catalyzed incorporation of fluorescent substrate, monodasnylcadaverine, into the soluble proteins 85

21. Westernblot analysis of various transglutaminases 86

LIST OF TABLES

Table		page
1.	Mammalian Transglutaminase	24
2.	Purification of Protransglutaminase from Guinea Pig Skin	66
3.	Physical Properties of Protransglutaminase . .	74
4.	Amino Acid Composition of Guinea Pig Epidermal Protransglutaminase and Guinea Pig Liver Transglutaminase	75

ABBREVIATIONS

ATPase	Adenosyltriphosphatase
BCA	Bicinchoninic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbant assay
IRS	Inner root sheath
kDa	Kilodalton
Mr	Molecular weight
NIH	National Institutes of Health
ORS	Outer root sheath
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonylfluoride
proTGase E	Epidermal protransglutaminase
SDS	Sodium dodecyl sulfate
TBS	Tris acetate buffer solution, pH 7.5 containing 0.5 M NaCl
TCA	Trichloroacetic acid
TGase C	Tissue transglutaminase or Liver transglutaminase
TGase E	Active epidermal transglutaminase
TGase K	Keratinocyte-specific transglutaminase

Tris	Trihydroxyaminomethane
Tyr-Tyr	Dipeptide containing tyrosine

LITERATURE REVIEW

Introduction

Cross-linking of proteins, an event which occurs post-translationally, is one of the vital physiological processes involved in stabilization of tissues and cellular matrices. Various cross-links in a variety of tissues have been identified i.e., disulfide bonds of many proteins (Anfinsen, 1973), aldol aldimine condensation in collagen and elastin (Tanzer, 1976), -Tyr-Tyr- bonds in fertilized egg membrane (Gross and Sizer, 1959), N-ε(β-aspartyl)lysine bonds in human urine (Lou, 1975), and N-ε(glycyl)lysine bonds in histone 2A from calf thymus tissues (Goldknof et al., 1959). The N-ε(γ-glutamyl)lysine cross-links, which were first identified in the stabilized fibrin clot (Pisano et al., 1968; Matactic et al., 1968; Lorand et al., 1968), are the most abundant intermolecular cross-links in the physiological system. Similar cross-links have been found in epidermal appendage such as wool, porcupine quill medullar protein, guinea pig hair medullary protein and the inner root sheath protein of guinea pig hair follicles (Asquish et al., 1970; Harding and Rogers, 1971; Harding and Rogers, 1972). Harding and Rogers have shown that citrulline-containing fractions of porcupine quill medullary protein, guinea pig hair medulla protein and the inner root sheath protein of guinea pig hair follicles are cross-linked

by N-ε(γ-glutamyl)lysine bonds. During the terminal differentiation of epidermal cells, cornified envelope is formed as a result of N-ε(γ-glutamyl)lysine cross-link formation of cellular proteins, i.e., involucrin (Rice and Green, 1979), histidine-rich glycoproteins (Dale, 1985), keratolinin (Buxman et al., 1980; Zettergen et al., 1984), and other envelope proteins (Baden et al., 1987). Rice and Green (1977) have estimated that approximately 18% of the total lysine residues from stratum corneum of human planter callus were found as the cross-linking dipeptide N-ε(γ-glutamyl)lysine.

The formed intermolecular N-ε(γ-glutamyl)lysine cross-links between proteins in vitro were shown to provide proteins with greater structural stability; increased mechanical resilience, and resistance in protease-susceptibility. For example, cross-linked fibrin has been shown to be more mechanically stable than noncross-linked fibrin (Roberts et al., 1973) and increased molecular elasticity has been shown to be correlated to the amount of cross-linking between the α-chains of fibrin (Shen et al., 1974, 1975). Cross-linked fibrin has also been shown to be more resistant to spontaneous lysis (Bidwell, 1953; Gottlieb et al., 1959). and cleavage by plasmin (McDonagh et al., 1971; Pizzo et al., 1972) than noncross-linked fibrin. Thus, formation of N-ε(γ-glutamyl)lysine cross-linking is an essential process in the cornification of epidermis.

Epidermis

Skin is the first barrier between the organism and its environment in the resistance to environmental insult, and the

adaptation to the thermal regulatory requirement of the organism using the vasculature and sweat gland. It contains an extensive and remarkable neuroreceptor network which serves as a complex transducer of environmental information. The skin is composed of two major tissue layers, the epidermis and dermis. The former, the outlayer, is a thin stratified epithelium which varies relatively little (75 μm to 150 μm) in thickness over most of the body except on the palms and soles where its thickness may be 0.4-0.6 mm (Rushmer et al. 1966). The latter, underlying the epidermis, is a dense fibroelastic connective tissue, which constitutes the mass of the skin, contains vascular and nerve networks, and encloses specialized excretory and secretory glands and keratinized appendage structures, such as hair and nail. Beneath the skin is the subcutaneous tissue, or hypodermis, which is composed of loose areolar connective tissue or fatty connective tissue displaying regional variations in thickness. Fibrous bands, continuous with the fibrous structure of dermis, spread and form continuous attachments of the skin to the underlying fibrous skeletal components such as fascial sheets and periosteum (Breathnach, 1971; Odland, 1983).

1. Structure of Epidermis

The epidermis consists of four distinct cell types: keratinocyte, melanocyte, Langerhans' cell, and Merkel's cell. The principal cell type in this epithelial continuum, the epidermal cell, is most commonly called the keratinocyte, so named because of the fibrous

proteins, keratins, which constitute the end product of epidermal differentiation. There are three distinct phases to the life of the keratinocyte: (1) growth and proliferation, (2) maturation and outward displacement, and (3) desquamation (Stenn, 1988).

The epidermis (Fig. 1) is recognized as composed of two principal cellular region: an outermost laminated sheet of dry anucleated cornified cells, the stratum corneum, and the nucleated or living inner cells, the stratum Malphgii (or the 'rete mucosae' from which the surface cells arise by differentiation). The inner living cells are subdivided commonly into three cell layers. (i) The stratum germinativum (basal layer), (ii) The stratum spinosum (spinous layer, also called the Malpighian layer), and (iii) The stratum granulosum (granular layer) (Odland, 1983).

The basal cells form a single layer, are columnar in shape, and lie with their long axis perpendicular to the living line between the epidermis and dermis. Most of mitotic activity of the epidermis is limited to this layer. They have a deeply basophilic cytoplasm and a dark-staining oval or elongated nucleus. They are connected to each other and to the overlying squamous cells by intercellular bridges or desmosomes. These desmosomes are less distinct than those in spinous cell layer (Breathnach, 1971). At their base, the basal cell are attached to the basal lamina by hemidesmosomes. The amount of melanin present in the basal cell parallels the skin color (Lever and Schaumburg-Lever, 1975). The 50 kDa and 58 kDa keratins are present in all cell layers including the relatively undifferentiated basal layer (Sun et al. 1979).

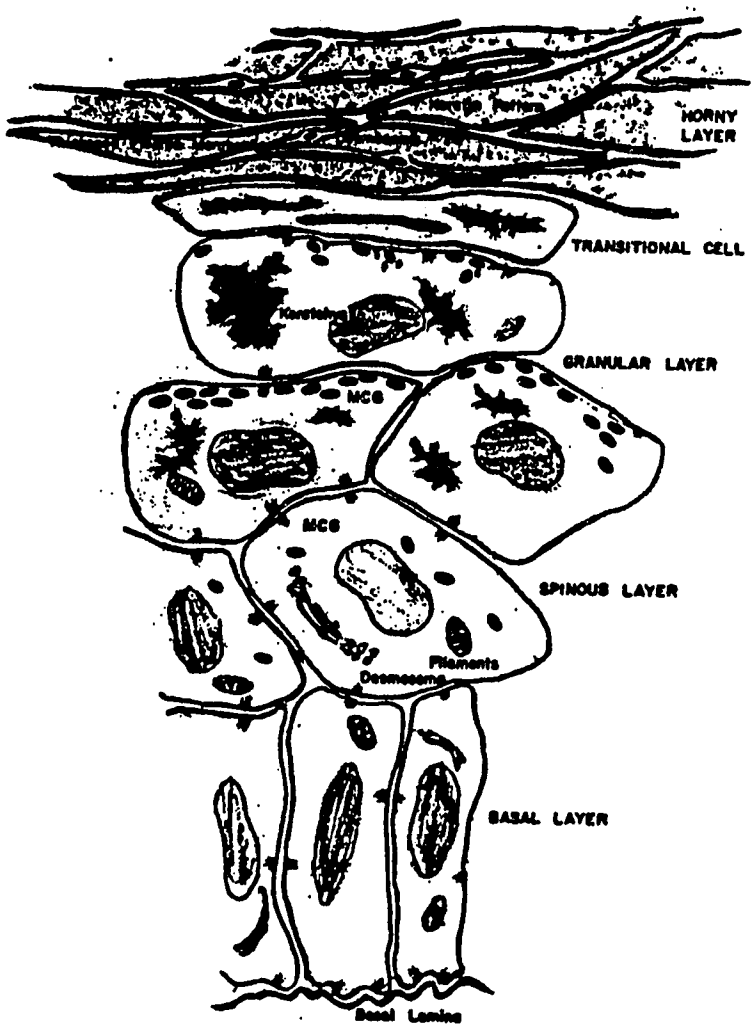


Figure 1. Schematic diagram showing the layering of the epidermis. MCG; membrane-coating granules

The cells of the spinous layer are polygonal and form a mosaicusually 5 to 10 layers thick (Breathnach, 1971). They become flattened toward the surface, with their long axis arranged parallel to the skin surface. The cells are separated by spaces that are traversed by intercellular bridges. Examination of the epidermis with the polarizing microscope reveals the cytoplasm of the cells in the stratum malpighi to the composed of numerous doubly refractive tonofibrils which form a tridimensional network around the nucleus and radiate out to the cell broader (Nieuwmeijor, 1953). These tonofibrils contain sulfhydryl and disulfide groups (Montagna et al., 1974) and show the X-ray diffraction pattern of keratin (Nelemans et al., 1952). The intensive study of the keratins shows that the spinous cells start to express 56.5 kDa and 65-67 kDa keratins (Moll et al., 1982; Franke et al., 1981; Tseng et al., 1982).

The cells of the granular layer are diamond-shaped or flattened and filled with keratohyalin granules that are deeply basophilic and irregular in shape (Breathnach, 1971). The thickness of the granular layer in normal skin is proportional to the thickness of the stratum corneum: it is only one to three cell layers thick in areas where the stratum corneum is thin, but measures up to 10 layers thick in areas with a thick stratum corneum, such as on the soles and palms of the hand (Holbrok & Odland, 1974). The granular cell layer represents the keratogenous zone of the epidermis where preparation for the dissolution of nucleus and other cell organelles occurs. In contrast with basal and spinous cell layers where lysosomal enzymes, acid phosphatase and aryl sulfatase are present, there are only a few

granular aggregates. The granular layer is diffuse staining for lysosomal enzymes (Rees, 1967; Jonson and Daniels, 1969).

As result of their abrupt and complete keratinization, the cells of the stratum corneum, visible layer of the epidermis, are anuclear and filled with keratins. This layer consists of layers of flattened keratinized cells. The number of layers varies with body region, ranging from 15 to 20 on the abdomen and several hundreds on the palm and soles (Holbrook and Odland, 1974). In the intercellular spaces, variable amounts of granules or reticular material may be seen through electron microscopy. This is thought to represent the contents of lamellar granules discharged at a lower level (Breathnach, 1971).

2. Structural Markers of Keratinocyte Differentiation

Basal cells contain a distinctive array of intermediate cytoplasmic filaments and organelles of synthesis, such as Golgi complexes, mitochondria, endoplasmic recticulum and ribosomes. These cells also contain replication markers such as centrioles and prominent nucleoli (Montagna and Parakkal, 1974). The growth of cells begins in the basal layer of the epidermis where there is a population of proliferating cells. Most normal keratinocyte cell divisions occur within this cell layer, however, it is recognized that all basal cells don't have an equal proliferative ability. The rate of division of cells in the basal layer has been difficult to establish. Nevertheless, several investigators have measured that about one or two mitoses are found per 1,000 basal cells and determined that it

takes about 28 days for the epidermis to renew itself (Baker and Kligman, 1967; Halprin, 1972). After division, a cell may remain in the basal layer or move outward. Of daughter cells, one must move outward in order to maintain epidermal thickness. The mechanism and selection for vertical displacement is not known, nor clearly understood for vertical displacement independent of cell division. Determination of the mitotic activity of epidermis has followed where there are distinctive markers for the synthetic activity and differentiation of keratinocyte, and nonantomical parameters of cellular differentiation such as mitotic potential, enzymes and their products, and surface antigens (Odland, 1983). Among molecular markers of keratinocyte differentiation are the polysaccharide coat as demonstrated by electronmicroscopic cytochemistry, and pemphigus and pemphigoid antigens (Diaz, 1979).

The stratum spinosum contains keratin filaments or tonofilament. As observed by light microscopists more than a century ago. In the cytoplasm, the keratin filaments loop about the perinuclear spaces are commonly aggregated in bundles. These bundles of filament are called tonofilament (Zelickson, 1962; Rothberg, 1964). There appears to be an important correlation between desmosomes and tonofilaments both in number and in strength of intercellular adhesion. The shape of the spinous cell progressively becomes more flattened as it moves outward. These flattened cells appear to accumulate irregular non-membrane-bound electron-dense lamellar granules at a latter stage of differentiation. Two types of granules are visible in mouse epidermis by electron

microscopy (Breathnach, 1971). Uniformly dense in appearance, the smaller type is called the dense homogeneous deposit. Its properties are poorly defined, but it is known to be rich in sulfhydryl groups. Larger, more irregular and granular, the second type is called keratohyalin granule. It contains histidine-rich proteins, RNA, polysaccharide, and lipids (Fukuyama et al., 1966). As examined by Matoltsy et al. (1965), these keratohyalin granules are seen in the region of the Golgi apparatus, appear to migrate towards the periphery of the cell, and are found predominantly at the outer surface of flattened cells in the stratum granulosum. Subsequent to their movement towards the cell envelope, the lamellar granules are extruded into the intercellular compartment. They are seen in lacunae between cells of stratum granulosum but more commonly between the stratum granulosum and the lowermost stratum corneum cells. These granules may provide the matrix that embeds keratin filaments in the stratum corneum (horny layer). This hypothesis is supported by the finding that in vitro, tonofilaments clump when mixed with a purified keratohyalin proteins called filaggrin - a cationic protein, histidine rich protein of 50 kDa molecular weight, produced in the upper spinous or granular layers from a larger (600 kDa) phosphoprotein precursor (Dale, 1985). Their lipid components of the granules are thought to be rearranged into lipid layers between cells of stratum corneum (Elias & Friend, 1975). Their broader function is unknown.

At a subsequent stage of differentiation, the intracellular morphology of keratinocytes shows the degradation of mitochondria

and ribosomes, the disappearance of the nuclear envelope, and ultimately the degradation of nucleus as the cytoplasm of the flattened cells becomes almost filled by keratohyalin and filaments. These residual cells appear as a dense marginal band adjacent to the inner face of plasma membrane called the cornified envelope. The cornified envelope is not solubilized by dissociating and reducing agents whereas the mature keratin, stabilized by disulfide bonds, can be solubilized with reducing agents (Sun et al., 1979). So following treatment with these agents, the envelope, the proteinaceous skeleton of the stratum corneum, can be preferentially isolated. In cultured human epidermal cells, analysis of the cell envelope showed that 17% of lysines in the cell envelope were bonded in ε(γ-glutamyl)lysine cross-links catalyzed by epidermal transglutaminase (Rice and Green, 1977). More recently, involucrin a protein substrate of transglutaminase in keratinocytes has been isolated from epidermis. It has a molecular weight of 92 kDa and is immunologically and chemically distinct from the keratin proteins (Rice, 1979). However to date the amino acid composition of cornified envelope correlates poorly with that of involucrin.

The final step in epidermal maturation is desquamation. The Morphology of the intercellular space in the lowermost layers of the stratum corneum has been shown to consist of a continuum of leaflets of lipid perforated by the desmosomal attachment devices characteristic of keratinocyte (Elias, 1981), while the outermost layers of the stratum corneum appear to be a disassembly of the

lipid leaflets and disintegrated desmosomes associated with the desquamation of surface cells into environment.

Hair

Hairs are keratinous fibers growing out from epithelial follicles distributed over the skin surface, except for on the palms of the hand and soles of the feet. In the human, the male and female have an equal number of hair follicles at birth, approximately two million, 100,000 of which are on the scalp. The apparent difference in hair density between the sexes is due to the type of hair and its distribution, not due to the number of hair follicles (Montagna et al., 1969). Hair is classified into two types: (1) a very fine, nonpigmented hair identical with the lanugo hair, now called vellus hair, which covers most of the body, and (2) a terminal hair, which is coarse, usually pigmented, and found in the scalp, eyelid, and eyebrow areas (Montagna and Parakkal, 1974). Vellus hair follicles are thin and extend only as deep as the middermis. The vellus hair shaft is correspondingly thin, colorless, and soft. The terminal hair shaft is pigmented and coarse, and it often contains an additional central structure, a medulla. Hair length varies from region to region because there are differences in growth period and growth rate of hair follicles. Terminal hairs may achieve lengths of more than 50 cm, whereas the vellus hair rarely achieves lengths greater than 1 cm and is often much shorter in area such as the eyelid and forehead. All hair growth is cyclical and in humans is asynchronous, so that in any specific area growing hair follicles are adjacent to

resting hair follicles. Three phases of the hair growth cycle are recognized: the resting hair follicle (telogen), the growth phase (anagen), the regression phase (catagen) (Stenn, 1988). These cycles of metabolic activity of the hair are not synchronous in the skin of humans. Each follicle maintain an independent rhythm of growth and rest. The ratio of growing to resting follicles varies with ages, and with the individual from month to month (Stenn, 1988).

The hair shaft is produced by mitotic activity in the intracutaneous follicle during the growth phase. The follicular epithelium achieves an extraordinarily high mitotic rate to account for the rapid synthesis of hair. Van Scott & Ekel (1958) have shown that there is a constant relationship between the volume of the growing portion of the hair and the size of the dermal hair papilla, and that the size of hairs appears to be determined by information in the connective tissue papilla.

1. Structure of The Hair Follicles

The typical structure of the hair is shown in Fig. 2. To describe the cellular phenomena associated with hair growth a more precise system has been developed (Orwin, 1976) which obviates the difficulties of correlating events in follicles of different lengths and copes with the detailed ultrastructural studies. In this system, the dermal papilla assigned to the zone A refers to cells in the mitotic zone, and appears to be an essential component of the hair follicle although its function is little understood. The precursor dermal cells which later form the dermal papilla are also present from the

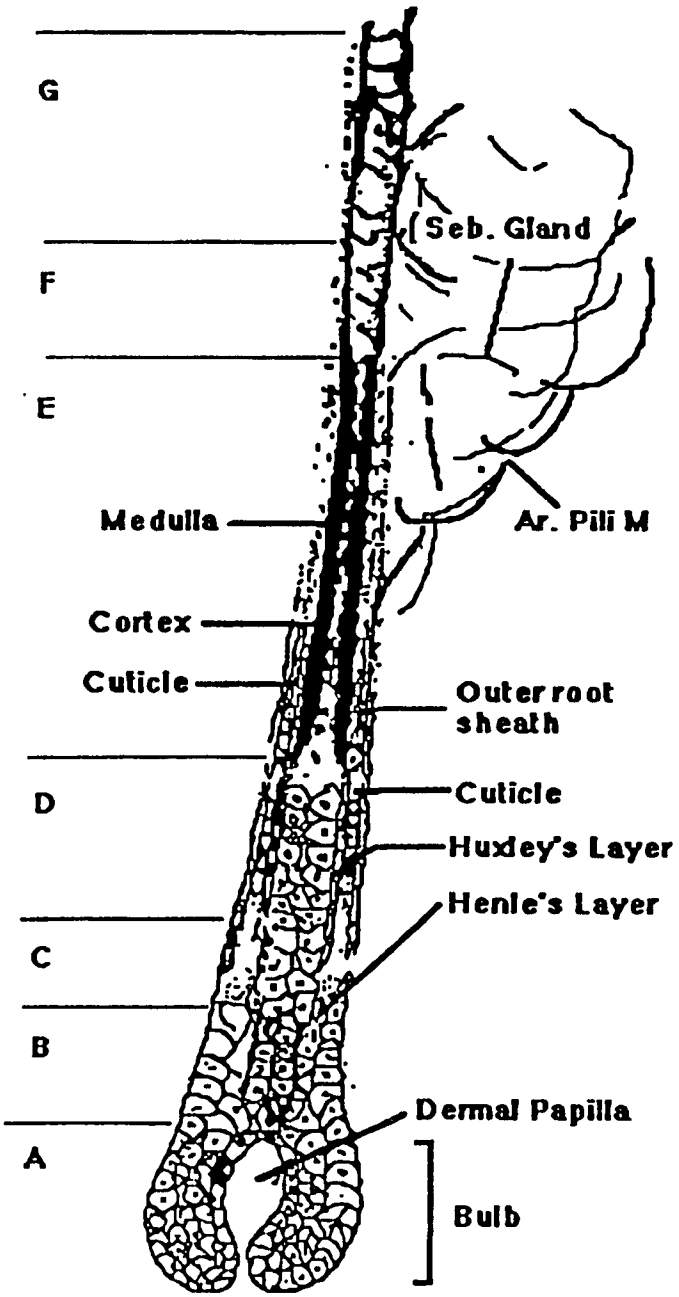


Figure 2. Structure of the hair

earliest stages of follicle development (Orwin, 1979). The formation of new dermal papilla is induced by lower follicle tissue after the removal of the existing papilla and prior to regeneration of rat vibrissae (Oliver, 1966, 1967). Normal fibers do not form in the dermal papilla although the production of fragile hairs from some depapillated follicles has been reported in the rat (Butcher, 1965). Ultrastructural studies have shown that the dermal papilla is composed of fibroblasts in the mouse, guinea pig, and human hair follicles (Roth et al.,1964a; Parakkal, 1966; Carlsen, 1974). These cells are about 40 nm in thickness and contain rough endoplasmic reticulum and well-developed Golgi complexes. Collagen, acid mucopolysaccharides, and glycogen can be observed in ground substance (Braun-Falco, 1959; Sasai, 1976; Parakkal, 1966; Bell,1969). However to date little evidence has been produced to indicate the mechanism of interaction of the dermal papilla with the epithelium-derived zone A cells.

Surrounding the dermal papilla, the cells of the bulb are mitotically active and are the progenitor cells which differentiate to form the various cell lines of the fiber: the inner root sheath (IRS), and the outer root sheath (ORS). In the animal hair follicles, the bulb and the dermal papilla are typically angled away from the axis of the fiber (Auber, 1952). Studies of the orientation of the mitotically active cells of the bulb and the movement of labelled cells out of the bulb suggest that the region around the lower dermal papilla gives rise to the ORS and the IRS, while the cells round the upper dermal papilla produces the fiber (Auber, 1952; Epstein et al., 1969; Orwin,

1971). Both wool and hair follicles bulb cells adjoining the dermal papilla are columnar, and have larger nuclei, many ribosomes and mitochondria, and relatively small amounts of endoplasmic reticulum, vacuoles, and Golgi complexes (Birbeck et al., 1957; Roth et al., 1964a; Forslind et al., 1966). There are intercellular spaces between many cells and plasma membrane shows desmosomes and gap junctions (Orwin et al., 1973a,b). The bulbs of hair follicles are rich in a variety of enzyme such as glycolytic and glycogen-synthesizing enzymes, aminopeptidase, cytochrome oxidase, and ATPase etc. (Adachi et al., 1969; Chapman et al., 1971; Sasai, 1977). Little is known about control of cell division in the bulb, although it is known to be influenced by factors such as photoperiodicity and nutrition (Short et al., 1965; Hutchinson, 1965).

Compared with the mature epidermis, the hair wall is more complex. It is made up of three vertically oriented concentric cellular cylinders: the fiber, inner root sheath, and outer root sheath. The fiber is composed of three major cell types: the fiber cuticle, the cortex, and the medulla. The last is usually present in coarse fibers only (Orwin, 1970). In the fiber, the medulla consists of the hardened remnants of highly vacuolated cells and occupies the central axis of some keratinized fibers. These cells change the light-reflective properties of nonpigmented fibers so that medullated fibers appear whiter than nonmedullated fibers (Orwin, 1979). The cortex constitutes the bulk of most fibers and is responsible for the properties of strength, elasticity, and waviness. It is formed from cells located around dermal papilla in the upper region of the bulb

in nonmedullated fibers and from those regions adjacent to the widest areas of the dermal papilla (the mid region) in medullated fibers (Orwin, 1979). The cell of the fiber cuticle form the external single cellular layer over the periphery of the fibers and are responsible for its surface properties (Rogers, 1959a,b).

The IRS is composed of three different concentric layers, each normally one cell thick (Auber, 1952; Germell et al., 1971). Surrounding the fiber cuticle is the IRS cuticle followed by Huxley's layer and Henle's layer, the last being adjacent to the outer root sheath. The IRS does not form part of the emergent fiber but is degraded and sloughed in the upper follicle (Fig. 2; Zone F). The IRS cuticle appears to be closely associated with the formation of the scale pattern on the surface of the fiber cuticle (Auber, 1952). Huxley's layer may be more than one cell deep. This variation appears to be associated with preventing major changes in the diameter of the IRS/fiber complex when marked changes occur in the diameter or shape of the fiber. Further, the Huxley's layer may have a role in the transport of metabolites as there are gaps between hardened Henle's cells through which Huxley's cells make contact with companion cells of ORS (Happey et al., 1962; Gemmell et al., 1971). Henle's layer may have a role in determining cell shape in other layers (Auber, 1952). More likely, it is involved in the movement of the IRS/fiber complex toward the skin surface because of its association with companion cells in the ORS (Straile, 1962; Orwin, 1971).

The outer root sheath forms the nonkeratinizing or nonhardening region of the follicle and is continuous with epidermis. It has potentialities not associated with fiber growth. Straile (1962) has distinguished several zones within the ORS according to their probable function. Apart from the bulb, these are the zone of proliferation (zone B through D), zone of migration (zone E), zone of sloughing (zone F), and zone of the upper ORS which is similar to and continuous with the epidermis (zone G). In the animal hair follicles with deflected bulbs, the IRS/fiber complex is located eccentrically so that the ORS is thicker on the deflected side of the follicle.

2. Structural Markers of Hair Follicle Differentiation

When cells pass above a "critical level" (Auber, 1952) in the dermal papilla and the bulbs, they enter zone B (Fig. 2) where differentiation begins. This zone is characterized by changes in cell size and shape and the initiation of keratin formation in cortical cells. Zone C begins where Henle's layer hardens, a stage at which most changes in shape have been completed. This is a region of major protein synthesis for the cortex. Zone D commence where the plasma membrane of the fiber cuticle cells apposed to the inner root sheath is associated with a continuous layer of cuticle keratin.

In contrast to other follicle cell types, mitosis has not been observed in the medullary cell line even in those cells adjacent to the dermal papilla (Auber, 1952). The first signs of differentiation are an increase in cytoplasm and ribosomes (Parakkal and Matoltsy, 1964; Parakkal, 1969). Tonofilaments from the few desmosomes

present increase during differentiation and appear to be carried over into the hardened state (Roth and Clarke, 1964; Hojiro, 1972). Medullary granules about 30 nm in diameter appear in the cytoplasm of cells during the early stages of differentiation (low zone B) (Roth and Heiwig, 1964b; Parakkal, 1969; Hojiro, 1972). They gradually increase in size until, at about the level where the keratinization of the cortex is initiated (start of zone E), they fuse into an amorphorous mass, usually around the periphery of the cell (Rogers, 1964; Roth and Heiwig, 1964b; Parakkal, 1969a; Hojiro, 1972). Associated with medullary granule production is the formation of vesicles and vacuoles, some of which are derived from degenerating mitochondria during the latter stages of differentiation (Roth and Heiwig, 1964b; Parakkal, 1969). Differentiation of the cortical and fiber cuticle cell plasma membranes seem to have as an ultimate aim of stabilization of cell to cell contacts. The intercellular spaces in zone A rapidly decrease as the cells move through zone B and the plasma membranes become closely apposed (Birbeck and Mercer, 1957; Roth and Heiwig, 1964a; Orwin, 1970).

The three layers of the IRS in hair follicles show marked similarities in their differentiation and are characterized by the production of a precursor protein, trichohyalin, which transforms into the filamentous matrix component of the hardened cells. Henle's layer differentiates first and hardens at the state of zone C (Auber, 1952; Gemmell and Chapman, 1971). Huxley's layer forms trichohyalin earlier than IRS cuticle, but hardens later according to Gemmell and Chapman (1971). Hardening occurs in these two cell

lines at about the level where keratinization commences (start of zone E). Henle's and Huxley's cells become lanceolate in shape while the IRS cuticle has one face which interdigitates with the apposing fiber cuticle surface. These cells are circumferentially wider than long (Auber, 1952). Trichohyalin is usually regarded as one of the earliest differentiation products which distinguish IRS cells from other types. It is normally associated with fibrils about 7-8 nm wide, but whether the trichohyalin or the fibrils arise first is disputed. In Henle's layer, which differentiates first, filaments are reported to precede the appearance of the associated amorphous trichohyalin droplets in follicle of the mouse (Roth and Heiwig, 1964a; Parakkal, 1969a), and man (Charles, 1959). Birbeck and Mercer (1957) reported that the trichohyalin transforms into a filamentous matrix component about the middle of zone B. On hardening, the trichohyalin disappears and the major part of the cytoplasm becomes filled with material within which tubular filaments can be observed in a matrix of low electron density. The filaments are oriented parallel to the axis of the fiber (Steinert et al., 1971). Similar events have been observed in the differentiation of Huxley's cells and IRS cuticle cells. It has been noted that, in the later stages of differentiation of the IRS cuticle, the filaments are predominantly located near the plasma membranes apposing the fiber cuticle (Gemmell and Chapman, 1971). Histochemical tests have shown that hair follicle trichohyalin is rich in arginine but not citrulline, whereas hardened IRS cells react strongly for citrulline but not arginine (Rogers, 1963). Biochemical studies of hair follicle

have shown that isolated hardened IRS cells and their tryptic digests have amino acid compositions which are very low in cystine and high in glutamic acid, aspartic acid, and lysine (Steinert et al., 1971). Further biochemical studies have shown that IRS proteins of hardened cells are covalently bound by peptide linkages (Rogers, 1959b, 1962; Steinert et al., 1969). Specifically, the ε(γ-glutamyl)lysine cross-links formed by transglutaminase are present in IRS proteins (Harding and Rogers, 1971).

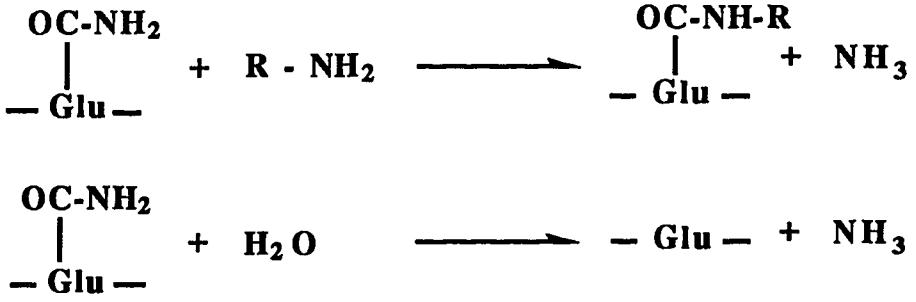
Electron microscopic observation has shown quite clearly that two distinct ORS cell layers differentiate at the periphery of the wool follicle bulb (Orwin, 1971). Although the cells are still mitotically active, the two layers can be distinguished from the base of the dermal papilla. The cell layer next to Henle's cells adheres more closely to Henle's layer than to the outer layer of the ORS. At higher levels in the follicle, i.e., zone B and above, cells have a flattened form and the location distinguishes them from the other ORS cells. A similar cell layer at these upper levels have been recognized in the follicles of man (Montagna and Parakkal, 1974). The cells of the peripheral layer of the bulb, the presumptive ORS layer, although initially flattened, become more cuboidal by the top of zone A (Fig. 2). They are characterized by marked convolutions in their surfaces suggesting that they do not adhere closely to neighboring cells of the same type or to companion cells. However, they are closely apposed to the basement membrane enclosing the epithelium-derived cells of the follicle and continuous with that of the dermal papilla. In zone B, these cells may form several layers according to the thickness of the

overall ORS. This region forms the beginning of the zone of proliferation within which ORS cells may undergo further division (Auber, 1952; Straile,1962, 1965; Chapman, 1971). These cells move up toward the skin surface through the zone of migration (Straile, 1962). In so doing, they move first toward the IRS side of the ORS and become elongated (Chapman, 1971). In the later stages of differentiation (zone F), they undergo a form of cornification and finally disintegrate into fragments which are sloughed into the piary canal along with the degraded IRS cells (Straile, 1962; Gemmell and Chapman, 1971). In comparison with other cell lines, ORS cells contain many vacuoles, Golgi complexes, both smooth and rough endoplasmic reticulum, mitochondria, ribosomes, lybosomes, and multivesicular bodies (Rogers, 1964; Roth and Helwig, 1964a; Orwin, 1976b). They also have a more extensive membrane system. An interesting feature of both companion and other ORS cells is the development of fibrillar material, often associated with desmosomes, which reaches its greatest extent in zone E (Rogers, 1964; Orwin, 1971). At later stages of development (zone B to F), bundles of more electron dense filaments accumulate (Rogers, 1964; Orwin, 1971). The cornification of ORS cells prior to sloughing is reported to involve little keratohyalin and is different from epidermal keratinization (Gemmell and Chapman, 1971).

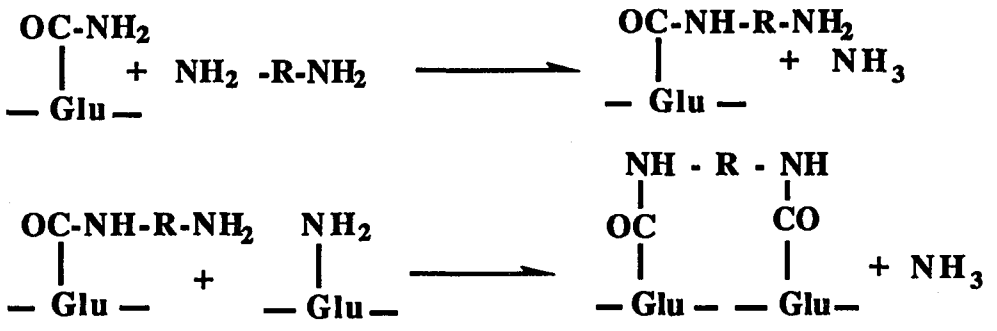
Transglutaminases

1. Introduction

The N-ε(γ-glutamyl)lysine cross-links in cornified envelope of stratum corneum and inner root sheath of hair follicle are formed by specific enzymes termed the transglutaminases. Transglutaminases (E.C. 2. 3. 2. 13. protein-glutamine: amine γ-glutaminyltransferase) are calcium dependent enzymes that catalyze an acyl transfer reaction in which the γ-carboxamide groups of peptide bound glutamine residues, acting as acyl donors, and react with the primary amino groups from a variety of compounds to form monosubstituted γ-amides of peptide-bound glutamic acid (see reviews Folk and Chung,1973; Folk, 1980). The representative reactions in the presence or in the absence of an amine is denoted as follows:



Furthermore, the polyamines, putrescine, spermidine and spermine, found in both intracellular and extracellular systems, have been found to serve as amine substrates, resulting N(γ-glutamyl)amine and N,N-bis(γ-glutamyl)amine cross-links (Beninati, et al., 1985):



This Ca^{2+}-dependent enzymatic activity was first discovered in the livers of a number of mammals (Clark et al., 1959). Studies leading to this discovery were stimulated by earlier reports of a Ca^{2+}-dependent enzymatic system in guinea pig livers that promoted the covalent attachment of L-lysine through its ε-amino group to proteins (Borsook, 1959; Schweet, 1955). Since then, different transglutaminases have been isolated and characterized (see review, Folk, 1980). The distribution of enzyme in specific tissue and body fluids suggest that each enzyme may be involved in specific physiological functions (see review, Folk, 1980).

2. Multiple Forms of Transglutaminase

Transglutaminases exist in both extracellular and intracellular forms and are found in cells and body fluids of a number of mammals. A summary of distinguishing characteristics of known transglutaminase in mammalian systems is shown in Table 1.

A. Transglutaminase C (TGase C)

Transglutaminase C (also called tissue transglutaminase or liver transglutaminase) was first isolated by Folk and Cole (1966) from

Table 1

Mammalian Transglutaminases

Type Enzyme	Common Name or Source	Mol. Wt. ($X10^{-3}$)	References
TGase C	Liver TGase or Tissue TGase	76.620	Folk & Cole (1966) Ikura et al., (1988)
	Red blood cell, Macrophage, Ubiquitous		Murtaugh et al., (1983)
TGase B	Particulate-associate TGase, Rat swarm chondrosarcoma, lung	95	Chang & Chung (1986) Cocuzzi & Chung (1986)
TGase E	Hair follicle TGase, hair follicle	54	Chung & Folk (1972) Peterson & Buxman (1981)
	Epidermal TGase, epidermis	50-55	Goldsmith & Martin (1975) Buxman & Wuepper (1976)
TGase K	Keratinocyte-specific TGase, Type I, human keratinocytes	92	Thacher & Rice (1985)
Factor $XIII_a$	Plasma	80.488	Loewy et al., (1961)
	Megakaryocytes, platelets, Placenta, Monocytes, Macrophage, Promonocytes (U937) Fibroblasts,		Kiesselbach et al., (1972) Bohn (1970) Bohn & Schwick (1971) Henriksson et al., (1985) Kardin et al., (1987) Nickoloff et al., (1989)

guinea pig liver homogenates. Transglutaminase C is found in the cell cytosol of numerous mammalian cell types, tissues, and organs (Chung, 1972,1975). These included macrophages (Murtaugh et al., 1983), fibroblasts (Dell'Orco et al., 1985), mast cells (Fesus et al., 1985), lung (Cocuzzi and Chung, 1986), and brain (Selkoe et al., 1982). This transglutaminase is a monomeric protein with a molecular weight of 76,620 deduced from amino acid sequence (Ikura et al., 1988) and exists in the active state. The enzyme contains 17 1/2 moles of -SH per mole of enzyme, but enzymatic activity is dependent upon a single, specific sulfhydryl group (Folk and Cole, 1966; Ikura et al., 1988). The particular functions of the transglutaminases C have not been fully elucidated.

B. Transglutaminase B

Transglutaminase B was isolated from the malignant chondrocytes, rat swarm chondrosarcoma (Chang and Chung, 1986). This enzyme was found to be distinct from transglutaminase C in its electrophoretic mobility, molecular size, substrate specificity, and immunologic reactivity. Reaction with monospecific antibodies to guinea pig and rabbit transglutaminase C showed non-identity with transglutaminase B. Upon treatment with thrombin enzymatic activity of transglutaminase B increased 3-4 fold with concomitant changes of molecular weight to one-half of that of untreated transglutaminase B.

C. Hair Follicle Transglutaminase (TGase E)

Transglutaminase in Guinea pig hair follicle contains two enzymes that catalyze incorporation of amines into proteins (Chung and Folk, 1972). One of these enzymes was found to be indistinguishable from the transglutaminase C on the basis of its physical, chemical, and immunological properties. The other enzyme has not been detected in other organs and tissues. It had a molecular weight of 54,000 dalton by gel filtration. The enzyme was found to catalyze incorporation of methylamine into the acetylated B-chain of oxidized insulin to form peptide-bound γ-glutamic acid methylamide. Harding and Rogers (1972) also observed the amine incorporating activity in the homogenates of hair follicles from guinea pigs and rats, and from the wool follicles from sheep. Guinea pig hair follicle homogenates catalyzed the incorporation of gycine ethyl ester into casein through γ-glutamyl amide bond. Rat hair follicle transglutaminase has been purified and characterized (Peterson and Baxman, 1981). It has molecular weight of 52,000 ± 3,000 on gel filtration and 26,000 ± 2,000 with SDS-gel electrophoresis. There was no immunological identity between the epidermal transglutaminases from rat skin, hair follicle transglutaminase or plasma protransglutaminase (factor XIII). These results have been corroborated by Ogawa and Goldsmith (1977) who reported that human follicle transglutaminase is immunologically distinct from plasma and epidermal transglutaminase.

D. Epidermal Transglutaminase (TGase E)

The epidermal transglutaminase has been identified in skin tissue from a variety species. The discovery of ε(γ-glutamyl)lysine bonds and hair follicle transglutaminase in the hair structure led to the investigation of an enzyme that catalyzes incorporation of amines into casein as well as the cross-linking of fibrin (Goldsmith and Baden, 1973). A partially purified enzyme from cow snout with molecular weight between 40,000 and 60,000 by gel filtration was first isolated by Goldsmith et al. (1974). Buxman and Wüepper (1975) isolated a transglutaminase in essentially homogeneous form from the soluble protein of glabrous cow snout epidermis. They reported the molecular weight of the enzyme was 55,800 dalton obtained by sedimentation equilibrium. This value was in close agreement with a value of 54,600 dalton estimated by size-exclusion chromatography. The enzyme was a single component of 4.4 S and migrated a single band in gel electrophoresis.

Goldsmith and Martin (1975) have identified an transglutaminase activity in human callus. They demonstrated that the molecular weight of the protein was between 50,000 and 55,000 by gel filtration and achieved a 50-fold purification. These enzyme shared many of the same properties as guinea pig hair follicle and cow snout transglutaminase. Ogawa and Goldsmith (1976) purified human epidermal transglutaminase to a homogeneity, and reported its molecular weight as 50,000 by SDS electrophoresis. Ogawa and Goldsmith(1977) showed human epidermal transglutaminase showed that the enzyme was immunologically distinct from other

transglutaminases. An unusual feature of the epidermal transglutaminase is its activation by heating to 56° C in the presence of calcium or by the treatment with dimethylsulfoxide in the presence of calcium (Ogawa and Goldsmith, 1976), chaotropic salts, alcohols, and trypsin (Plishker et al., 1978). By the immunoblotting of crude extract with monospecific antibody, Negi et al. (1985) presented a possible precursor form of the enzyme from stratum corneum in human lamellar ichthyosis.

E. Transglutaminase K

Transglutaminase K (keratinocyte-specific transglutaminase, type I) was isolated from cultured human keratinocytes (Thacher and Rice, 1985). Thacher and Rice reported the molecular weight of the enzyme to be 92,000 daltons. Monoclonal antibodies against transglutaminase K did not cross-react with the transglutaminase C from skin tissues. In their immunohistochemical staining of skin with monoclonal anti-TGase K, the enzyme was distributed evenly from basal layer to stratum corneum.

F. Human Coagulation Factor XIII

Human coagulation factor XIII exists in an inactive zymogen (Factor XIII, protransglutaminase) form. The proenzyme, converted to active transglutaminase (Factor XIIIa) upon incubation with thrombin, are found in blood plasma and platelets, uterus, placenta, prostate gland, liver (see review, Folk and Chung, 1973; Folk and Finlayson, 1977; Folk, 1980), macrophage (Adany et al., 1985; Henriksson et al., 1985), promonocyte cells (U937, Kardin et al.,

1987), fibroblasts (Nickoloff and Griffiths, 1989). The zymogen form of plasma factor XIII is composed of two identical catalytic subunits, termed a-chain, and two identical noncatalytical subunits, termed b-chain (Schwartz et al., 1971b; Chung, 1972; Takagi and Konishi, 1972; Chung, 1975). The tetrameric subunit structure of the plasma factor XIII is designated a_2b_2 and active form of Factor XIIIa is designated a'_2b_2. However, the factor $XIII_a$ from platelets, uterus, macrophages and fibroblasts, was found to exist as a dimer of a-subunits (Chen and Doolittle, 1971). It has been suggested that the role of plasma factor XIII b-chains is to stabilize the catalytic subunit a_2 of factor XIII in plasma by forming a tight complex. The half-life of the plasma zymogen (a_2b_2) in rabbits is known to be 52-55 hours, whereas the a-subunits in b-chain-deficient rabbits is only 2.5 hours (Lee and Chung, 1976; Bohn and Schwick, 1971). The b-chain may also have a function subsequent to the activation of plasma factor XIII. Cooke (1974) observed that when the plasma enzyme formed from the zymogen in the absence of Ca^{2+} was incubated with a casein substrate and a fluorescent amine at low concentration of Ca^{2+}, a pronounced lag occurred before the steady state was attained, but that the platelet enzyme showed no such lag period. This lag time could be shortened, and eventually abolished, if the enzyme were preincubated with high concentrations of Ca^{2+}.

3. Substrates of Transglutaminases

A citrulline-rich protein located in the inner root sheath of hair follicles, medulla from hair, and related structures such as porcupine

quills was found to contain a very high glutamic acid and low cysteine content (Rogers, 1962). It was very different from the surrounding keratin proteins of the cortex. Steinert et al., (1969) demonstrated that this citrulline-rich protein was covalently cross-linked. The disulfide bond appeared as an unlikely candidate for cross-links in the IRS and medulla because the cyteine-cysteine content was known to be very low in the proteins in the IRS and medulla and, additionally, the differences in solubility between the keratin proteins of the cortex and citrulline-rich proteins of IRS and medulla were very striking. Asquish et al., (1970) discovered ε(γ-glutamyl)lysine bonds in the digests of wool keratin using an enzymatic hydrolysis. A more detailed study was done by Harding and Rogers (1971) in which they found a value of 13 moles of ε(γ-glutamyl)lysine/1,000 moles of amino acid residues in the soluble proteins from hair medulla. This corresponds to 25% of the lysines residues being cross-linked.

Several precursor proteins for the envelope of stratum corneum have been described by many laboratories. These precursor proteins were identified through their immunological reactivity with antibody raised against cornified envelope protein or by their substrate properties (capability to accept primary amines) for transglutaminase. Rice and Green (1979) found a cell envelope precursor protein that can incorporate dansylcadaverine from the neutral salt extracts of cultured epidermal cells and referred as involucrin. The purified substrate had a molecular weight of 92,000 by gel filtration and SDS-gel electrophoresis, and an isoelectric point

of 4.5 ± 0.3. The sedimentation coefficient was 2.8S in sucrose and had an estimated molecular weight of 83,000. This substrate is distributed within the cytoplasm with predominant localization in the periphery where its antibody is completely adsorbed by the purified cell envelope. As a putative substrate for transglutaminase, it has been demonstrated that 17% of the lysines in the cell envelope from cultured human keratinocyte are in ε(γ-glutamyl)lysine cross-links (Rice and Green, 1977) which in stratum corneum 18% of the lysine are in such cross-links.

Buxman and Lobitz (1980) found a 36 kDa dalton protein (keratolinin) which is labeled by dansylcadaverine when the neutral salt extracts of cow snout epidermis were incubated with dansylcadaverine. This substrate was dissociated by chaotropic agents or detergents into noncovalent subunits. The molecular weight of these subunits was 6,000-6,200 on electrophoresis in 15% acrylamide. Their isoelectric points of human and bovine keratolinins were 5.4/5.0 and 6.3/6.0, respectively. Antibodies to human keratolinin had no cross-reactivity with its bovine counterpart, and the antibodies to involucrin did not cross react with keratolinin by immunodiffusion (Zettergren et al., 1984).

Soluble basic precursors of the cornified envelope of mammalian epidermis were identified using a monoclonal antibody which reacts with the periphery of the cells in the upper layers of human epidermis. These cells were visualized by indirect immunofluoresence following immunization of mice with cornified envelope of cultured human keratinocytes. The polypeptides

derived from these cells were shown to have a pI of about 9.0. Under nonreducing conditions, these peptides had molecular weight of 30,000 and 60,000 (Baden et al., 1987).

Filaggrin, a 50 kDa cationic protein, rich in histidine residues, and produced in the upper spinous or granular layers was identified as a precursor which does not aggregate keratin filaments in vitro (Dale, 1985). Recently, a glycine-rich cornified envelope precursor, loricrin, characterized from a cDNA clone encoding a major component of the human cornified envelope was discovered. The amino acid sequence deduced from the nucleotide sequence is very similar to the amino acid composition of mature cornified envelopes (Hohl et al., 1989).

Human fibrinogen comprises three types of peptide chain: Aα, Bβ, γ, each of which occurs twice in the structure of the molecule (Chen and Doolittle, 1970). Fibrin monomer, which can be denoted $(\alpha\beta\gamma)_2$, is produced under the influence of thrombin by removal of fibrinopeptide A and, subsequently, of fibrinopeptide B from the NH_2-termini of the appropriate chains. Under physiological conditions, fibrin monomers assemble to form a fibrin clot (gel), the monomeric units of which can undergo ε(γ-glutamyl)lysine cross-linking by plasma factor XIII in the presence of thrombin (Pisano et al., 1969; Collen et al., 1970). Chen and Doolittle (1970, 1971) found that the carboxy-terminal region of the γ-chain, which contains an acceptor function, also contains a donor. Specifically, The lysyl residue in the position 6 (from the carboxy-terminus) and the glutaminyl residue in the position 14 undergo cross-linking with the

corresponding residues on another γ-chain to form a pair of reciprocal ε(γ-glutamyl)lysine bridges (Chen and Doolittle, 1971). Examination of the subunit structure of fibrin by polyacrylamide gel electrophoresis in SDS extended the scope of the cross-linking investigations and increased their convenience and accessibility. The SDS-PAGE result allowed a clear distinction to be made between the reciprocal bridging of γ-chains and the second type of cross-linking, α-polymerization (Lorand et al., 1969a; Takagi and Iwanaga, 1970). In contrast to the pattern exhibited by the γ-chains in SDS-PAGE, α cross-linking resulted in a polymer which did not enter a 10% acrylamide gel. Since the fibrin monomer contains only two α-chains, the α cross-linking estimated to result in a polymers consisting of at least 5 or 6 α-chains must be an intermolecular event (McDonagh et al., 1971).

4. Regulation of Transglutaminases

Although cellular regulation and inter-relationship of each of the molecular forms of the enzymes is not clearly understood, numerous studies have described the regulation of the tissue transglutaminase. The tissue transglutaminase activity in human keratinocytes was found to decrease following trypsin treatment (Thacher and Rice, 1985), and SV-40 induced transformation of baby hamster kidney cells and human lung fibroblast (WI-38) cells resulting in down-regulation of the enzyme (Birckbichler et al., 1977; Birckbichler et al., 1980). Retinoic acid (Lichti et al., 1985) and butyric acid (Prasad and Sinha, 1976) induced the synthesis of tissue transglutaminase in

mouse and human keratinocytes, human promyelocytes, and mouse peritoneal marophages. In contrast, phorbol esters induced the level of the particulate transglutaminase (Yuspa et al., 1980). Ca^{2+} ion, a known modulator of cell activation, also appears to affect the transglutaminase catalyzed reactions in the cell. At low Ca^{2+} concentration (0.03 mM), mouse keratinocytes grow as a monolayer with characteristics of basal cells with base levels of transglutaminase activity. Whereas the keratinocyte incubates in high Ca^{2+} concentration (1.6 mM), cells differentiate to form cornified envelope (Rice and Green, 1979).

Proteases play important roles in many physiological reactions: blood coagulation cascade, the complement activation system, fibrinolysis, kinin generation, terminal differentiation, etc. (see reviews, Neuah and Walsh, 1976; Reich et al., 1975). Protease also participates in modulation of the transglutaminase activity. Treatment of cells and tissues with protease resulted in an enhancement of transglutaminase activity i.e., trypsin treatment of WI-38 cells (Birckbichler et al., 1980), thrombin treatment of rat chondrosarcoma cells (Chang and Chung, 1986), and Dispase treatment of mouse epidermis (Chung et al., 1989; Martinet et al., 1988). These results suggest that cellular transglutaminases might be modulated during the terminal differentiation or activated from precursor that exists in the epidermal cells and hair follicles.

Research Objectives

The evidence indicates that the cross-linking reaction catalyzed by transglutaminase is an obligatory process in the formation of cornified envelope of the stratum corneum. A recent study by Michel et al. showed that the cornified envelope prepared from outer layers of stratum corneum in normal skin was structurally rigid and polygonal, but the envelope from the inner layers was fragile and irregular. This fragile structure is also observed in envelopes prepared from cultured keratinocytes and psoriatic scales. These observations suggest that the decrease in cross-linking process may be associated with the pathobiology of skin. The present study was undertaken: (1) to identify of protease-activatable transglutaminase in the epidermis, (2) to study the biochemical and enzymatic characteristics of transglutaminases present and to understand the specific role of each enzyme in epidermis, and (3) to study the modulation of enzyme activity in the stratum corneum to gain information about the physiological regulation of the cornification process.

MATERIALS AND METHODS

Materials

Iodo[^{14}C]acetamide (57 mCi/mmol) and [1,4 ^{14}C]Putrescence hydrochloride (118 mCi/mmol) and were purchased from Amersham (Arlinton Heights, IL). Monodansylcadaverine, phenylmethyl sulfonyl fluroride (PMSF), Freund's adjuvant, 3,3'diaminobenzidine, and aprotinin were purchased from Sigma (St. Louis, MO). Benzamidine hydrochloride, succinic anhydride, and dithiothreitol were obtained from Aldrich (Milwaukee, WI). Dispase, proteinase K, chymotrypsin, trypsin, endoproteinase Glu-C, and leupeptin were purchased from Boehringer Mannheim (Indianapolis, IN). Reacti-Gel (HW-65), Micro protein assay reagent, bovine serum albumin, and disposable column were purchased from Pierce (Rockford, IL). Glass-fiber filters (type GF/A, 25 mM), DE 52 cellulose, and 3 MM chromatography paper (produced by Whatman, Inc.) were purchased from Chemtrix (Hillsboro, OR). Casein was purchased from United States Biochemical Corp. (Cleveland, OH). Heparin-Sepharose 4B and S-Sepharose were purchased from Pharmarcia (Piscataway, NJ). Hydrofluor was obtained from National Diagnostics (Manville, NJ). Bio-gel A-0.5m, gelatin, HRP color development reagent, peroxidase conjugated goat anti-rabbit IgG, and peroxidase conjugated protein A were purchased from BioRad (Richmond, CA). Electrophoresis apparatus and precast gels were purchased from Novex (Encinitas, CA). Vector-A-stain was

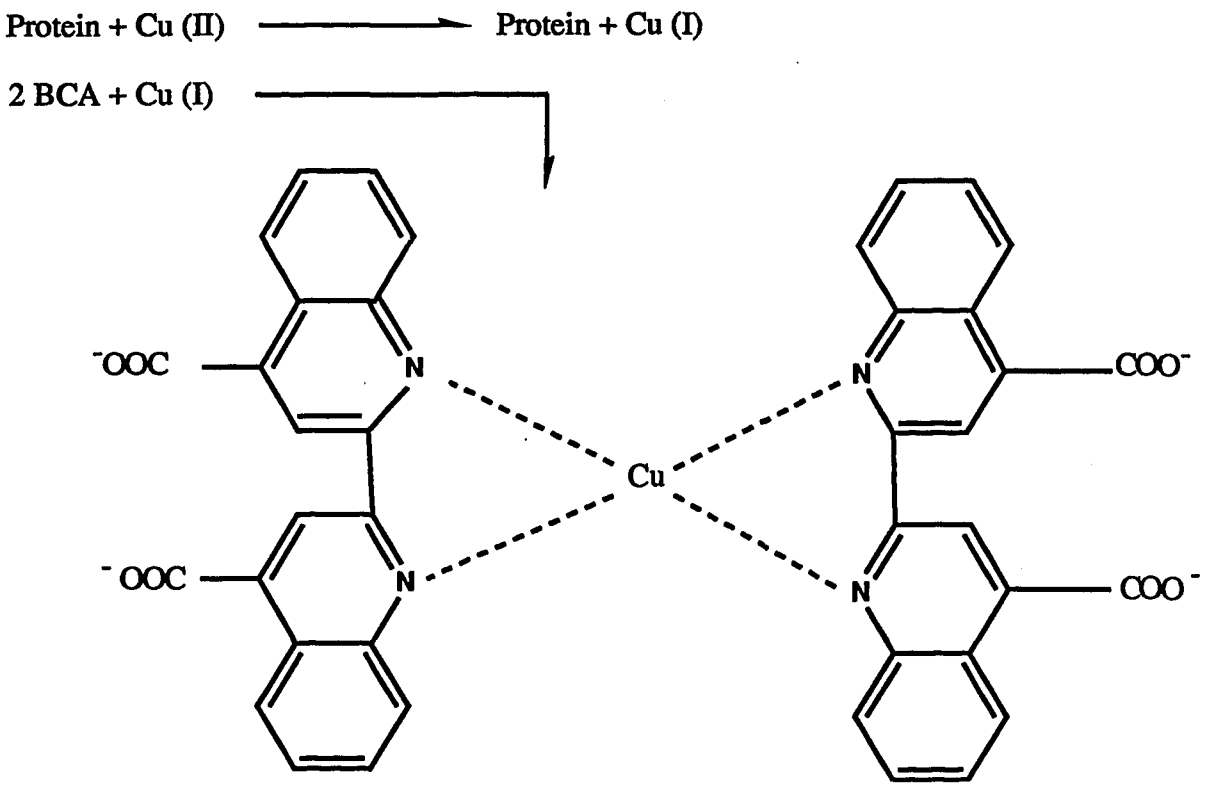
purchased from vector Laboratories (Burlingame, CA). New born mice, 4-day-old, were obtained from Jackson Laboratory (Bar Harbor, ME). Specific pathogen free Rabbits (New Zealand White 2.2 kg - 2.6 kg), used for production of antibody, were provided by Animal Care Unit of the National Institutes of Health. Human thrombin was generously provided by Dr. G. Murano (Food and Drug Administration, Bethesda, MD). TGase C (tissue transglutaminase or liver transglutaminase from guinea pig) was provided by Dr. S.I. Chung (National Institute of Dental research, NIH, Bethesda, MD). Keratinocyte-specific transglutaminase from cultured human keratinocyte and its monoclonal antibody was generously provided by Dr. S.M. Thacher (Texas A&M, College Station, TX). Epidermal transglutamiase from human foreskin was provided by Dr. S.C. Park (Seoul, Korea).

Methods

Protein Determination

Protein concentration was measured colorimetrically according to a modified Lowry's method (Smith et al, 1985) based on metal chelate protocols. In the presence of proteins, copper II ions are reduced to copper I. The copper I ion will be chelated by two bicinchoninic acid (BCA) molecules. As the working reagents are being chelated, color of the solution will change from green to purple. The higher the protein concentration, the deeper the purple color. The absorbance of the samples was measured with a spectrophotometer (DU 20, Beckman, Fulleton, CA) at 562 nm and

compared to a standard curve to determine the total protein concentration.



Transglutaminase Assay

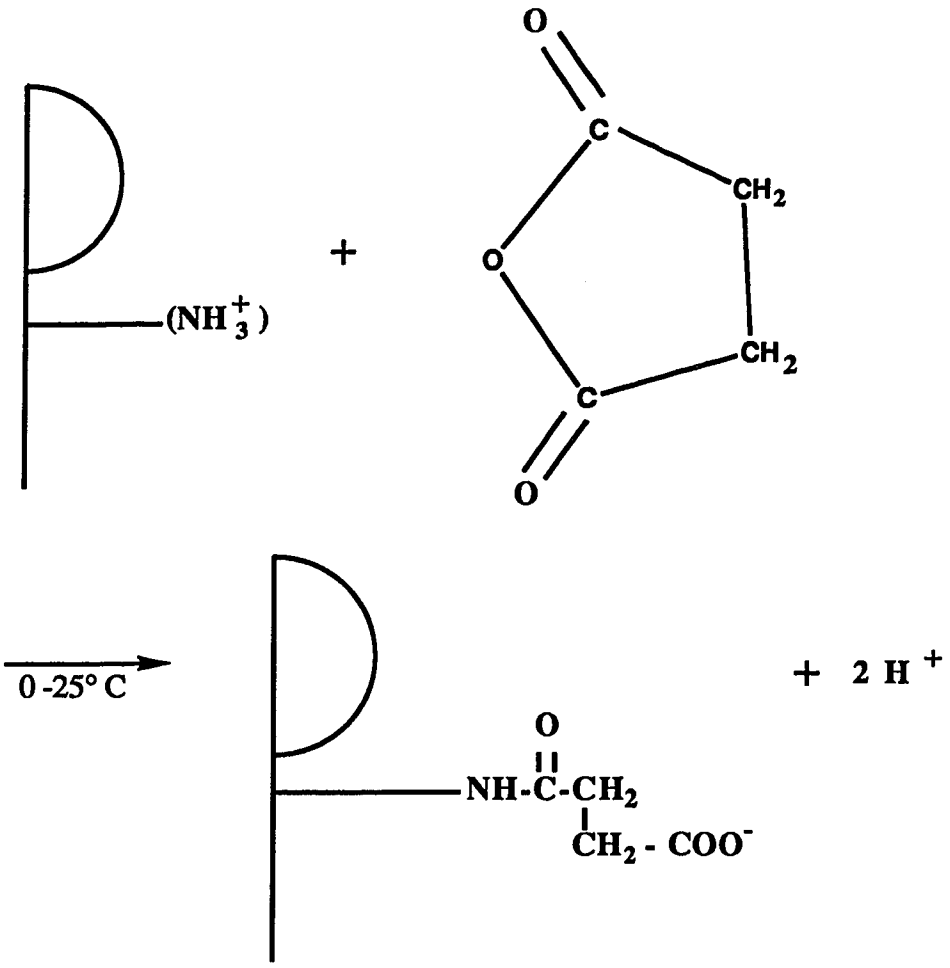
A modified transglutaminase assay method (Chung and Folk, 1972) was used to determine the enzyme activity of the various fractions. The enzyme activity is measured by determination of the amount of [1,4 ^{14}C] putrescine incorporated into the protein acceptor, succinylated casein. The samples, 20-50 µL, were mixed with 0.5 mL of the reaction mixture containing 0.1 M Tris acetate buffer (pH 7.5), 1% succinylated casein (prepared as described below), 1 mM EDTA,

10 mM $CaCl_2$, 0.5 % lubrol PX, 5 mM Dithiothreitol, 0.15 M NaCl, 0.5 μCi of [1,4 ^{14}C] putrescine (118 mCi/mmol). The reactions were carried out at 37°C for 1 hour and were terminated by addition of 4 mL of 7.5% cold trichloroacetic acid (TCA). After 30 minutes in the cold, the trichloroacetic acid-insoluble precipitates were filtered on GF/A glass filters and washed with about 30 mL of 5% cold TCA. The filters containing TCA-precipitates were dried and transferred into scintillation counting vials with 10 mL of scintillation counting fluid (Hydrofluor). The vials were vigorously shaken to pulverize the filters. The radioactivity was measured vials in a liquid scintillation counter (Beckman, Fulleton, CA) and transglutaminase activity expressed as: cpm/h/mL.

Preparation of Succinylated Casein

Succinylation of casein, an acylation reaction, was performed to block the free amino group of lysine residues of casein, since they serve as acyl acceptors for transglutaminase catalytic action. Succinylated casein was prepared according to the method of Klotz (Klotz, 1959). Casein (20g) was dissolved in 1 liter of distilled water and the pH was adjusted to 7 with 0.2 M NaOH. Solid succinic anhydride (100 g) was added in small increments to the proteins solution over a period of 1 hour. Whenever the pH dropped below 6.5, it was adjusted to 6.5-7.5 by adding 10 M NaOH. After all succinic anhydride was added, the solution was allowed to stand for about 20 minutes at room temperature. The succinylated casein was

dialyzed against 1 mM EDTA (pH 8.0) with several changes. The solution was then lyophilized, and stored at 4°C.



Preparation of Epidermis and Hair Follicles

1. Preparation of Native Epidermis and Native Dermis

4-day-old mice (C57 BL) were sacrificed and the skins removed surgically. The separation of epidermis from dermis was achieved by heating the whole skin for 40 seconds at 60°C.

2. Preparation of Dispase-treated Epidermis and Dermis

Whole mouse skin was removed from the body and floated on a 0.25% solution of Dispase in Hank's balanced salt solution at 4°C. The length of protease treatment varied from 6 to 14 hours. Epidermis was separated from the dermis with forceps, and both were washed three times in cold-distilled water and stored at -20°C (Fig. 3).

3. Isolation of Hair Follicles

Hair follicles were isolated from dermis by a described method (Martinet et al, 1988). Dispase-treated dermis samples from 100 whole skins of 4-day-old mice, obtained after 12 hours Dispase treatment at 4°C, were further digested at 37°C for 1.5 hours with 50 mL of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mg/mL collagenase, 1 mg/mL deoxyribonuclease, and 0.1 mg/mL elastase. The digest was filtered through cheesecloth in order to remove the larger debris. To the filtrate was added 1 volume of Dulbecco's modified medium containing 1 mg/mL deoxyribonuclease (washing medium), the hair follicles were collected by centrifugation for 10 min at 40 x g in Sorvall RT 6,000 centrifuge at 4°C. The hair follicles were resuspended in washing medium and collected as described above. The supernatant was discarded and this step was repeated once again. The washed hair follicle-rich pellet was sieved using a nylon gauze of pore size 100 μm. The single cells passed through the gauze, whereas the hair follicles remained on the gauze. The latter was collected by washing with phosphate-buffered saline (pH 7.4).

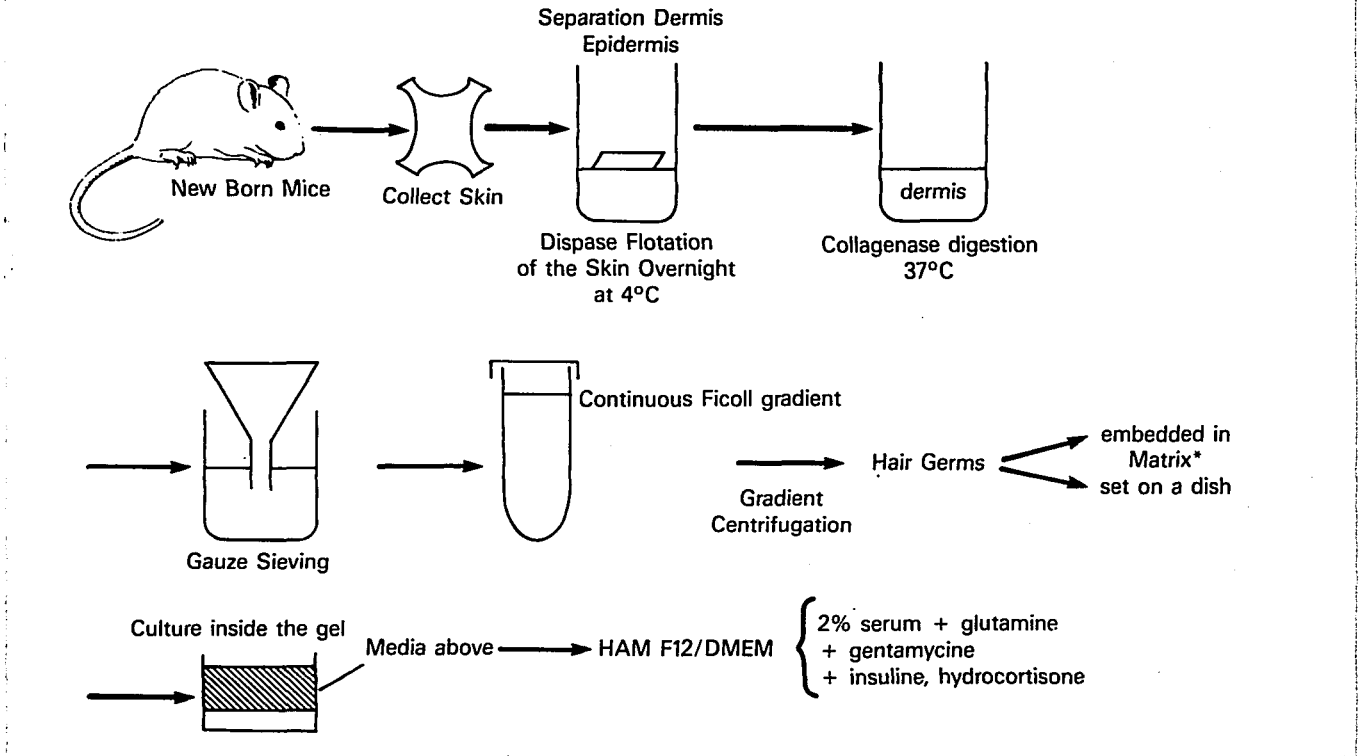


Figure 3 Schematic diagram of the preparation of epidermis and hair follicles

Purification of Epidermal Protransglutaminase

Guinea pig skins (Pel-Freez, Rogers, AR) were shaved, cut into approximately 1 cm^3 pieces, and pulverized under liquid nitrogen using a microanalytical mill (A-10, Tekmar, Cincinnati, OH). The resulting powder (wet weight 200g) was suspended in 1 liter of buffer A (0.1 M Tris acetate buffer pH 7.5, 10 mM EDTA, 2 mM benzamidine hydrochloride, 1 mM PMSF, 2 mg/mL of leupeptin, and 0.02 trypsin inhibitor unit/mL of trasylol) and homogenized with a Polytron Homogenizer (Brinkmann, Switzland) for 3 min on setting 6. After centrifugation for 30 min at 30,000 x g (J 21 rotor, Beckman, Fulleton, CA), the supernatant solution was saved. The resultant pellet was re-homogenized in 1 liter of buffer A. The combined supernatant solutions were passed through a column (5 X 10 cm) of DE 52 cellulose previously equilibrated with buffer B (0.05 M Tris acetate buffer, pH 6.0, and 1 mM EDTA). The column was washed with 2,500 mL of equilibrating buffer (3 mL/min), and the total column eluate was pooled. Ammonium sulfate was added to yield 75 % saturation. The precipitate formed after 1 hour of storage at 4°C was collected by centrifugation, dissolved in 150-200 mL buffer C (5 mM Tris acetate, pH 6.0, and 1 mM EDTA), and dialyzed against buffer C. The enzyme solution was clarified by centrifugation and then applied to a column (5 X 14 cm) of Heparin-Sepharose CL 4B equilibrated with buffer C. The column was washed with buffer C until the effluent solution showed an absorbance of less than 0.02 at 280 nm and eluted with 1 liter linear salt gradient (0 - 0.25 M) in the same buffer. The 20 mL fractions were collected at a flow rate of

3 mL/min. The fraction containing transglutaminase activity were pooled and concentrated to 1.5 - 2 mL in an ultrafiltration cell with a YM - 10 membrane (Amicon, Danvers, MA). The concentrated sample was applied to a column (2.6 X 98 cm) of Bio-Gel A-0.5 m, which had been equilibrated with 5 mM Tris acetate buffer pH 6.0, containing 0.15 M NaCl and 1 mM EDTA (buffer D), and 3.8 mL fractions were collected at a flow rate of 0.2 mL/min. The fractions containing transglutaminase activity were pooled and concentrated by ultrafiltration as described above. The concentrated transglutaminase solution was applied to a second column of Bio-Gel A 0.5m. The fractions containing transglutaminase activity were pooled and dialyzed against buffer C. The dialyzed sample was applied to a S-Sepharose (2.6 X 17 cm) column equilibrated with buffer C. The column was washed with 100 mL of buffer C. The elution was carried out with 5 mM Tris acetate buffer, pH 7.5 and pH 8.0, containing 1 mM EDTA and 1.2 mL fractions were collected at flow rate of 1 mL/min. The fractions containing transglutaminase activity were pooled and stored at -20°C. SDS-polyacrylamide gel of the enzyme solution showed a single band after staining with Coomassie blue.

Preparation of Dispase-activated Enzyme from Epidermal Protransglutaminase

The Dispase-activated enzyme was prepared by treatment with 0.5 units Dispase for 5 min. After activation, the sample was passed through a Superose 12 column equilibrated with 5 mM Tris acetate, pH 6.0, containing 0.15 M NaCl. The fraction containing enzyme

activity were collected and concentrated by Amicon stir cell on YM-10 membrane.

Determination of Molecular Weight of Protransglutaminase and Dispase-activated Enzymes

1. Electrophoretic Method

Molecular weight of purified transglutaminase was determined by the method of Shapiro and Marizel (1967). Purified transglutaminase from new born mouse and guinea pig skin were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% separating gel) at 30 mA, using a discontinuous buffer system (Laemmli, 1970). Samples (2-5 mg) for electrophoresis were diluted in an equal volume of nonreducing sample buffer (0.125 M Tris acetate pH 6.8, 4% sodium dodecyl sulfate (SDS), 20 % glycerol, and 0.001 % bromophenol blue). The samples were boiled for 5 min and applied. Electrophoresis was carried out at 30 mA for 1-2 hours. After electrophoresis, proteins were visualized by staining with 0.1 % Coomassie Brilliant Blue solution containing 40 % methanol and 10 % acetic acid for 20 minutes and destaining solution containing 10 % methanol and 7 % acetic acid. Under the SDS electrophoretic condition, migration is determined not by intrinsic electrical charge of proteins but by molecular weight (Shaprio et al., 1967), and a linear relationship exists between the log of molecular weight of a protein and its R_f (Shapiro and Marizel, 1969). The molecular mass of the enzymes was estimated by its electrophoretic mobility (R_f) relative to standard marker proteins.

2. Gel Filtration

Unlike electrophoretic techniques, gel filtration provides a means of determining the molecular mass or size of globular protein under a wide range of concentration, pH, ionic strength, and temperature etc. Samples of proteins (2-5 mg) and standard proteins (2-5 mg each) were dissolved in 2 mL of buffer G (5 mM Tris acetate, pH 6.5,1 mM EDTA, and 0.15 M NaCl). Each sample was applied to a column (2.6 X 98 cm) of Bio-Gel A 0.5m equilibrated with buffer D and the elution volume (V_e) based on the middle of the peak was determined. The elution of a solute is best characterized by a distribution coefficient (K_d) and a linear relationship exists between K_d and log of the molecular weight.

$$K_d = \frac{V_e - V_o}{V_t - V_o}$$

where V_e : elution volume

V_o : void volume

V_t : total bed volume

The molecular weight of the sample was determined from the standard curve of the proteins using gel filtration calibration kits (13,700-669,000) from Pharmacia Fine Chemicals (Piscataway, NJ).

3. Sedimentation Equilibrium

Determination of molecular mass by sedimentation equilibrium was carried out by the method of Cherverka (1969). Either 160 μg of proTGase E or 110 μg of Dispase-activated enzyme was filled in a conventional 12 mm ultracentrifuge cell. Before filling with enzyme solution, 0.1 mL of Dow-coring No 555 silicone fluid is introduced into the cell by using 250 μL Hamilton syringe with No 22 needle. After the cell is sealed and placed in the rotor, the rotor is fastened onto the coupling of the drive shaft and the temperature of the rotor is measured by use of external thermometer. When a vacuum was reached about 1.0 μ, the rotor was accelerated to 10,000 rpm. As soon as the rotor was attained the desired operating speed, pictures are taken at various angles of the Schlieren diaphragm with 8 min intervals. The molecular weight was calculated from the equation (1).

(1) $$C_r = C_b \exp (A M (r^2 - r_b^2))$$

where C_r is the concentration at the cell round,

C_b is the concentration at the cell bottom,

A is a constant,

r is radial distance in centimeters,

r_b is radial distance at bottom in centimeters,

M is the molecular mass of the sample.

Determination of Sedimentation Coefficient and Frictional Ratio

Sedimentation coefficient was determined by the method of Schachman (1957) to analyze the molecular properties of proTGase E. The sample (0.45 mL) in salt solution (ionic strength = 0.1) was placed in the right hand sector and 0.46 mL of solvent in the left hand sector. The sample cell was adjusted to equal the weight of 0.5 g of the filled cell. Temperature of the rotor was near 20°C before the sample cell was placed in the chamber. When the rotor speed reached operating speed (60,000 rpm), the first Schlieren photo was taken, followed by 8 min intervals. The first plate was developed immediately after the five frames to check whether exposure times was satisfactory. The photography was taken at 8 min interval until all boundaries had sedimented sufficiently from that the plateau region had disappeared. The sedimentation coefficient was calculated from the results. The plot of logarithmic distance versus time in seconds was made from the results and determined the slope of the best line through the points. S_{obs} was calculated from the equation (2). Sedimentation coefficient in Svedberg (S) was obtained by dividing by 10^{-13}. Frictional radio were calculated using the equation (3).

(2) $$S_w = \frac{2.303\ d \log \chi}{60\ \omega^2\ dt}$$

Where ω is the angular velocity in radians per second,

χ is the distance of the boundary from the axis of rotation,

t is the time in seconds,

(d $\log\chi/dt$) is a slope of log χ versus t plot.

(3) $$f/f_o = 1.19 \times 10^{-5} (1-V)_{w,20} M^{2/3} / S_{w,20} V^{1/3}$$

where V is partial specific volume,

M is the molecular weight of the sample,

$S_{w,20}$ is sedimentation coefficient at 20°C

Agarose Gel Electrophoresis and Activity Staining

Horizontal agarose gel electrophoresis was carried out on a LKB Mutipor unit (Piscataway, NJ). The agarose (1g) was dissolved in 0.15 M barbital, 0.075 M Tris glycine buffer, pH 8.8. The gel was equilibrated for 1 hour in the same buffer and placed on a glass plate. The native sample (~10 µL) was applied and the electrophoresis was carried out in 0.15 M barbital, 0.075 M Tris glycine buffer, pH 8.8 using an electric field of 10 V/cm for 1-2 hour at 10°C. After electrophoresis, the transglutaminase activity was detected by the dansylcadaverine method (Lorand, 1979). The staining solution contained 0.1 M Tris acetate (pH 8.5), 2 mM monodansylcadaverine, 1% succinylated casein, and 50 mM calcium chloride. The gel was incubated for 2-4 hour at 37 °C in this solution and Whatman # 1 paper soaked with the same solution was overlayed on the gel. The gel was removed and fixed in 5%

trichloroacetic acid for 2 hours at room temperature. The excess dansylcadaverine was removed by washing 7.5% trichloroacetic acid. The gel was soaked in 0.5 M Tris solution and the fluorescent protein band identified under UV light.

Active Site Labeling of Epidermal Transglutaminase

The catalytically active component of Dispase-activated enzyme was determined by the method of Folk and Chung (see reviews 1973; Folk, 1983) based on incorporation of radioactive iodoacetamide into the enzyme. Approximately 10 μg of activated enzyme prepared as previously described was incubated in 40 μL of 0.1 M Tris acetate (pH 7.5) containing 10 mM calcium chloride, 1 mM EDTA, and 5 μCi iodo[^{14}C]acetamide (24 Ci/mol). After 20 min at room temperature, the reaction was stopped by addition of 40 μL of 25 mM EDTA. A portion of 20 μL of this inactivation mixture was assayed for activity and another 20 μL portion of the mixture was analyzed by SDS-PAGE Radiolabeled enzyme was detected by autoradiography on Kodak X-R2 film.

Determination of Amino Acid Composition of Epidermal Protransglutaminase

The amino acid composition was determined by the modified method of Moore and Stein (1951). The sample (0.1 mg) of purified transglutaminase was dialyzed against distilled water for 30 hours and lyophilized. Two samples of lyophilized powder were dissolved with 0.5 mL 6 N HCl and hydrolyzed at 105°C for 24 and 48 hours,

respectively. The hydrolyzed samples were dried in a speed concentrator (Savant, Farmindale, NY) and dissolved with 50 μL of Na buffer for the amino acid analysis. 20 μL portions were injected into the automated amino acid analyzer (System Gold, Beckman, Fulleton, CA). The data was normalized for 77,000 molecular weight.

Detection of the Soluble Substrate of Epidermal Protransglutaminase

The soluble substrate was determined by the modified method of Hanigan and Goldsmith (1978) based on incorporation of dansylcadaverine into soluble proteins of various fractions. The final concentration of various fractions of the preparation was adjusted to 5 mM dithiothreitol, 10 mM $CaCl_2$, and 10 mM dansylcadaverine. The mixtures were incubated at 37°C for 18 hours. At end of incubation period, 20% TCA was added for 1 hour at 4°C and centrifuged 12,000 x g for 10 min. The pellets were washed twice in 5% TCA to remove the free dansylcadaverine. The pellets were extracted twice in ethylether to remove excess TCA. The resulting pellets were dissolved in SDS-sample buffer (0.192 M Tris HCl, pH6.8, 10% SDS, 5 mM β-mercaptoethanol, 20% glycerol). An aliquot (20 μL) were electrophoresed on SDS polyacrylamide gels. After the electrophoresis, the dansylated band was identified by viewing with ultraviolet A radiation (254 nm).

Preparation of Immunoglobulin Specific for Various Transglutaminases

Antibody specific for epidermal protransglutaminase was prepared by the modified method of Hurn et al. (1976). Purified transglutaminase (0.5 mg) was diluted with phosphate buffered saline (0.5 mL), mixed and emulsified with an equal volume of Freund's complete adjuvant, using a double-hub connector and two syringe. For the first injection, 1 mL of emulsion was given intramuscularly into the limb of each rabbit (near the lymph node). The second injection was made into the other side of foreleg after 2 weeks of the first injection. The booster injection of the protein (0.5 mg per rabbit) emulsified with Freund's incomplete adjuvant was made intramuscularly three weeks after second injection. The rabbits were bled 10 days after the booster injection. The blood was collected in clean dry glass bottles and allowed to clot at room temperature. The antiserum was separated by centrifugation, and stored at -20 °C after 0.1 % sodium azide was added as an antibacterial agent.

Antibody specific for transglutaminase was prepared by passing antiserum through an immunoabsorbent column containing antigen chemically coupled to the Reacti-Gel (HW-65). The column was prepared as follows: the Reacti-Gel (HW-65) (20 mL of suspension) was washed on a Buchner funnel, excess acetone was removed and the gel was equilibrated in 100 mL of cold 0.1 M sodium borate buffer (pH 9.0). The wet Reacti-Gel cake was directly added to a solution containing 2 mg of purified transglutaminase to be coupled.

The solution was maintained the pH 9. The solution was gently stirred at 4°C for 30 hours. The Immunogel was washed with 1 M NaCl and water sequentially. In order to block unreacted ligand, the Immunogel was filtered on a Buchner funnel and the resulting wet-gel was directly added to 1.0 M ethanolamine solution. The blocked Immunogel was wash with 1 M NaCl, cold distilled water, and phosphate buffered saline sequentially. The antiserum solution was mixed with Immunogel previously equilibrated with phosphate buffered saline (pH 7.2) at 4°C. The antibody adsorbed Immunogel was packed into a column (1 X 5 cm) and washed with equilibrating buffer containing 0.5 M NaCl. Proteins were eluted with 1 M Glycine HCl buffer (pH 3.0). The fractions were collected in 2 mL of 1 M Tris acetate buffer (pH 8.0), to reduce the risk of denaturation due to the low pH. The pooled fractions were dialyzed against phosphate buffered saline (pH 7.2) at 4°C for 24 hours, 1 % bovine serum albumin was added as a stabilizer, and stored at -20°C. Antiserum against guinea pig liver transglutaminase was prepared as described above.

Electroblotting

Proteins separated upon SDS-PAGE were electrophoretically transferred onto transmembrane paper (nitrocellulose or Immobilon PVDF) according to the method by Towbin et al. (1976). Gels were positioned in contact with transmembrane, backed with 3 MM chromatography paper and porous sponges, and placed in a transblot chamber containing transfer buffer. Proteins were transferred for 30

minutes at 120 mA (constant current) immediately following electrophoresis.

Detection of the Protein Bands by Antibody Probe

Transglutaminase was detected via an indirect enzyme-linked immunoabsorbant assay (ELISA) employing a rabbit antiserum to guinea pig epidermal protransglutaminase and other transglutaminase as the primary antibody, and peroxidase-conjugated protein A or peroxidase-conjugated goat anti-rabbit IgG as secondary antibody by modified method of Smith and Fisher (1984). Following transfer, blots were immediately incubated in 3 % gelatin solution for 1 hour with one change. The blots were then rinsed thoroughly with distilled water followed by a 10 min wash in 20 mM Tris-HCl containing 0.5 M NaCl (TBS). The blots were incubated 4 hours or overnight at room temperature with the primary antibody diluted 1: 300 in 1 % gelatin in TBS containing 0.1 % Nonidet P-40, washed three times for 10 minutes each in 0.05 % Tween-20 in TBS (TTBS), and then incubated for two hours at room temperature with secondary antibody diluted with 1:3,000 in 1% Gelatin in TBS containing 0.1 % Nonidet P-40. After incubation with the secondary antibody, the blots were again washed three times for 10 min each in TTBS, and then three times for 10 minutes each in TBS. For the detection of peroxidase conjugated antibody or peroxidase conjugated Protein A, staining was carried out by placing the transmembrane into the HRP color development solution containing 0.05 % hydrogen peroxide and 0.5 mg/mL 4-chloro-1-

naphthol. Staining time varied according to the rate of appearance of bands, normally less than 20 minutes, and the transmembrane was washed several times with distilled water.

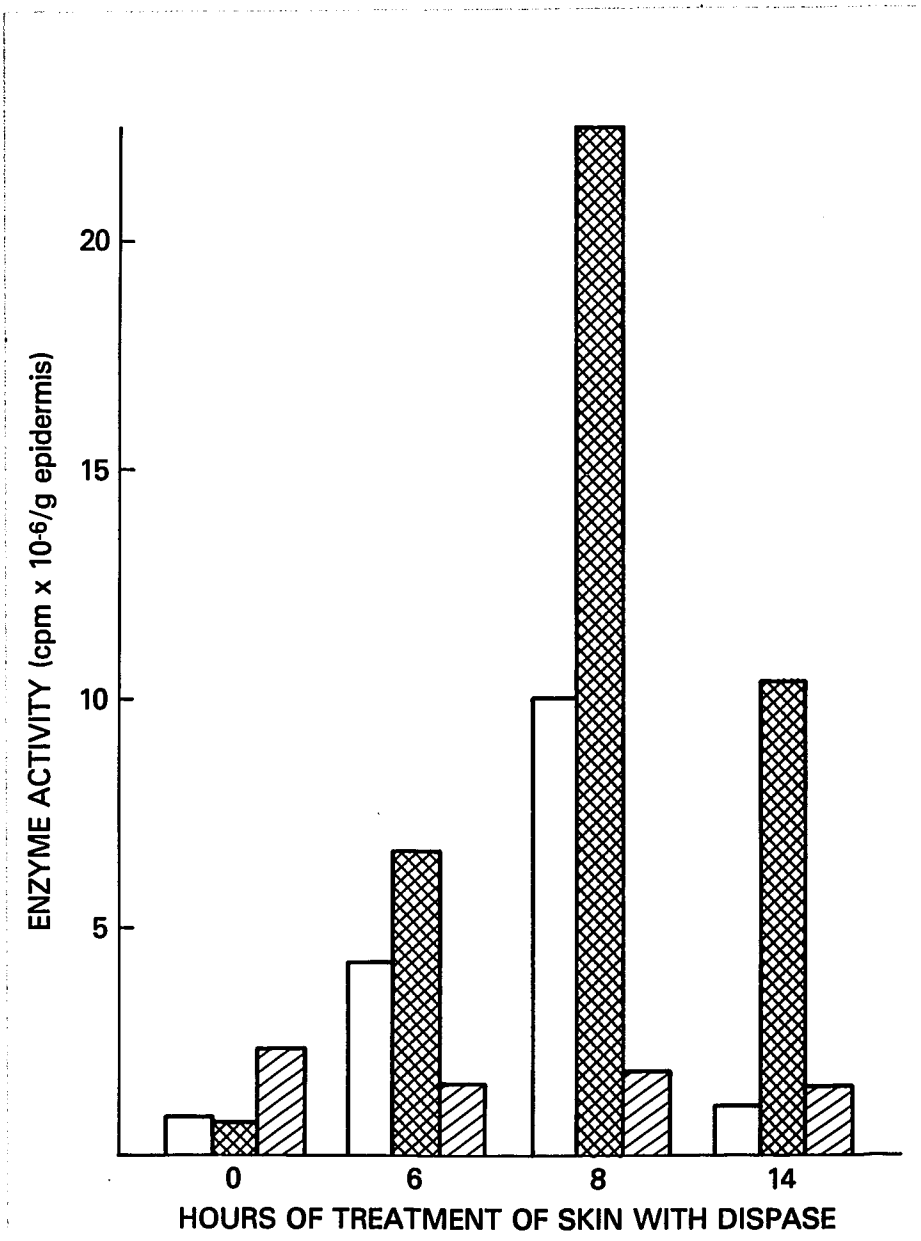
RESULTS

Biochemical and Physical Studies

Purification of Protransglutaminase from Guinea Pig Skin and Mouse Epidermis

Treatment of whole skin of newborn mice with neutral protease, Dispase, in order to separate dermis and epidermis causes pronounced changes in the levels of both the anionic and cationic forms of transglutaminase activity in the epidermis. As shown in Fig. 4, total enzyme activity was increased about 35-fold after 8 hours incubation in 0.25% Dispase solution. This preliminary experiment suggests that Dispase-activatable transglutaminase exists in the skin. In order to purify a large quantity of the precursor form of Dispase-activatable transglutaminase and compare its immunological properties with well-characterized guinea pig liver transglutaminase (tissue TGase or TGase C). Guinea pig skins have been used as the source of the enzyme. The crude extracts contained close to 45-48% of the total transglutaminase activity in the skin (Fig. 5). Repeated extraction of the particulates with buffer A solubilized of 10-15% more of the bound-enzyme activity. Preincubation of the extracts with Dispase caused a 4 to 5-fold increase in the level of enzyme activity, but only a slight (3-5%) increase in the particulate-bound enzyme activity. Fig. 6 shows chromatography of a crude extract on

Figure 4. Activation of transglutaminases in epidermis by Dispase treatment of whole skins from 4-day-old mice. Epidermis was extracted and chromatography on DE 52 cellulose was performed as decribed in "Methods". The open bars depict the enzyme activity bound to and eluted from the DE 52 column (anionic transglutaminase); the cross hatched bars depict that unbound to the DE 52 column (cationic transglutaminase); and the hatched bars depict that activity remaining in the pellet as measured by adding a suspension of pellet to the assay solution. The 0-h treatment denotes the heat treatment employed to prepare native epidermis


ENZYME ACTIVITY (cpm x 10-6/g epidermis)
20
15
10
5
0
6
8
14
HOURS OF TREATMENT OF SKIN WITH DISPASE

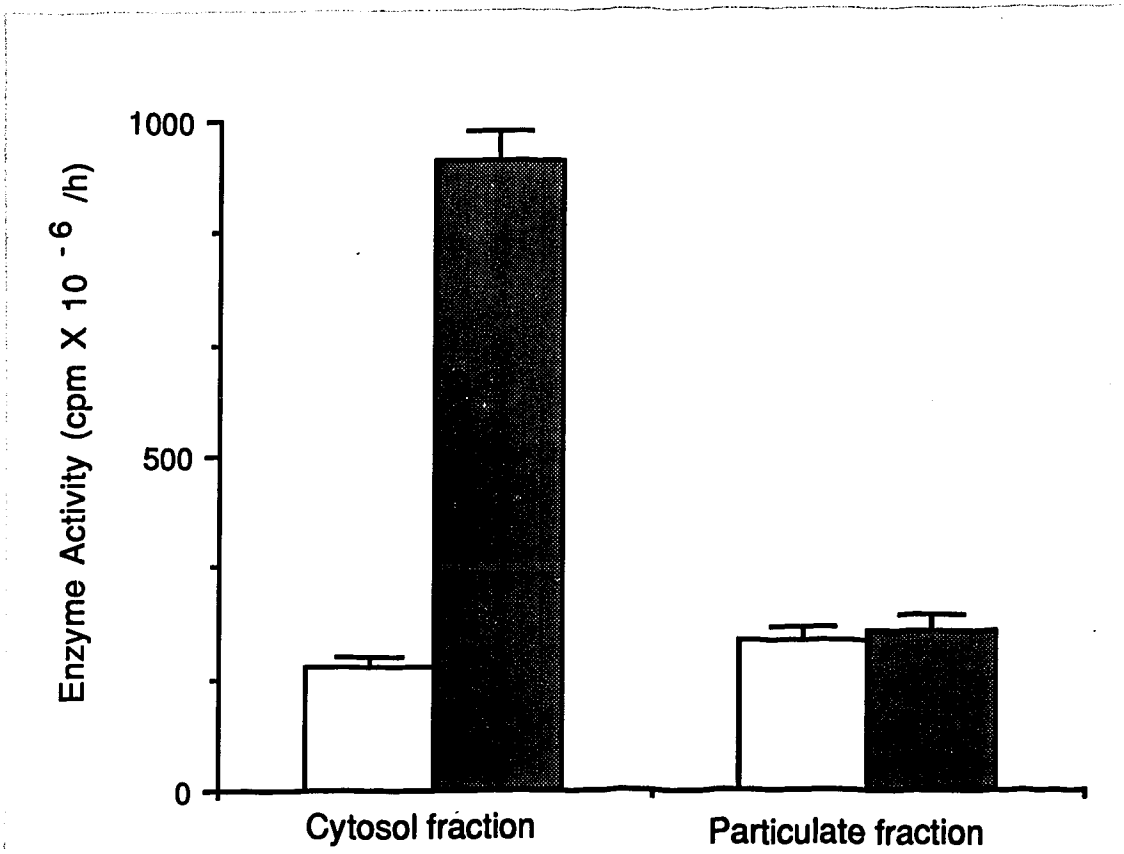
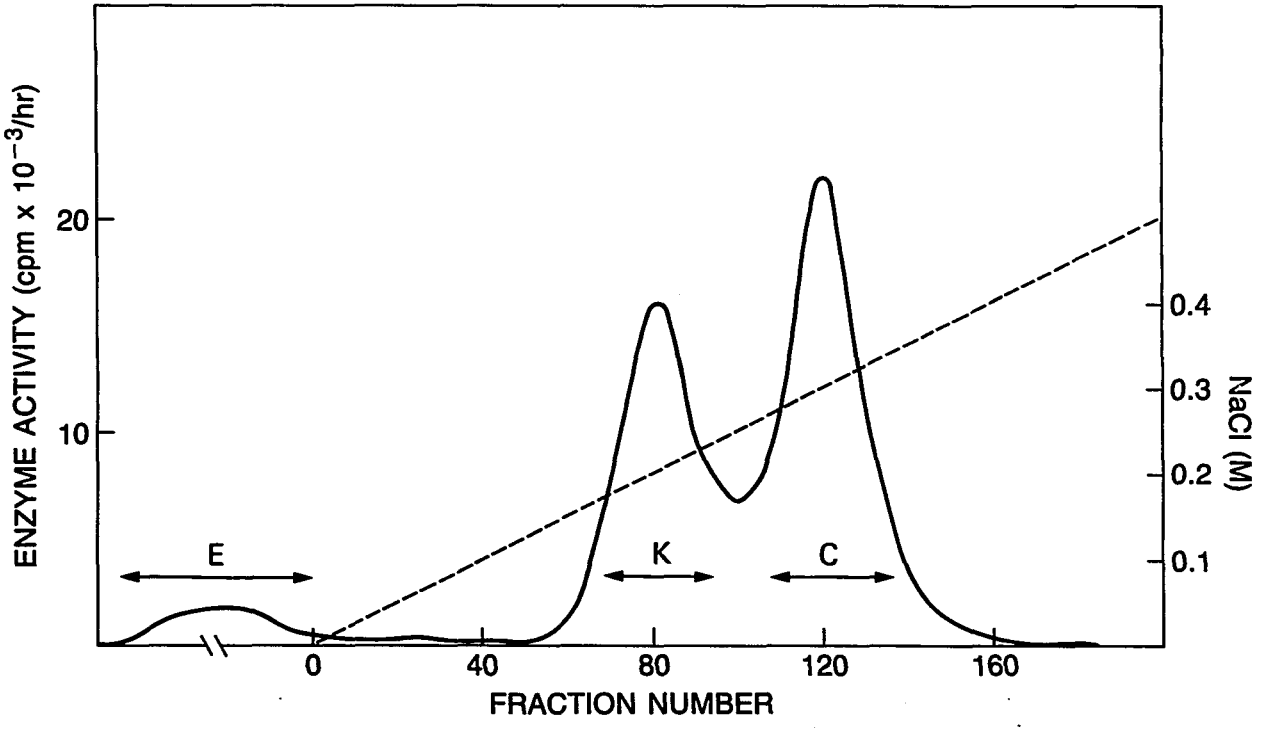


Figure 5. Distribution of transglutaminase activity and activation of transglutaminase from guinea pig skin. The skin extracts (200 g, wet weight) were extracted as decribed in "Methods". The transglutaminase activities here are those after two extractions, with the 30,000 x g supernatant representing the cytosol fractions and the particulate fraction representing the residual pellet which was resuspended in 1 liter of buffer A. The open bars depict cytosol and particulate transglutaminase activities; The cross-hatched bars depict the samples of the cytosol and particulate fractions that were preincubated with 0.05 unit Dispase for 10 minutes prior to assay.

Figure 6. Anion exchange chromatography of guinea pig skin extracts. The cytosol fraction of the skin extracts (200 g, wet weight) was diluted with 5 volumes of distilled water and applied on a 5 X 7 cm column of DE 52 cellulose equilibrated with 5 mM Tris acetate buffer (pH 6.0) containing 1 mM EDTA, washed with equilibrating buffer (2,500 ml), and eluted with a 1 liter of a linear salt gradient from 0.0 to 0.5 M NaCl in equilibrating buffer. Fractions of 5 ml were collected and 25 μL aliquots were assayed for enzyme activity. The pooled fractions of unbound activity was designated TGase E, the first activity peak was designated TGase K, and the second activity peak was designated TGase C.


ENZYME ACTIVITY (cpm x 10^{-3}/hr)
20
10
0.4
0.3
0.2
0.1
NaCl (M)
E
K
C
0
40
80
120
160
FRACTION NUMBER

a DEAE-cellulose column. After application of the extract, the column was extensively washed with the equilibrating buffer until the wash fraction showed negligible Dispase-activatable transglutaminase activity. This DE-52 column wash fraction contained most of the Dispase-activatable transglutaminase activity (will be referred to as proTGase E). Two peaks of enzyme activity were eluted from the DE-52 column with a linear salt gradient. The first peak of transglutaminase activity, which eluted at 0.19 M salt concentration, has previously been identified as the 92 kDa particulate-associated transglutaminase (TGase K)(Thacher and Rice, 1985; Chang and Chung, 1986) and the second peak of enzyme, eluted at 0.3 M salt concentration, was identified as tissue transglutaminase (TGase C)(Folk and Cole, 1966; Chung and Folk, 1972). The relative levels of each of the transglutaminase activities eluted from the DE-52 column is shown in Fig. 7. Treatment of proTGase E with Dispase resulted in a 25-fold increase in the level of transglutaminase activity whereas there was a partial loss in the enzyme activity of TGase C following the treatment with Dispase. Greater than 80-85% of total cytosol transglutaminase activity was shown to be present in the unbound fraction of DE 52.

The enzyme of the non-adsorbed fraction from the DE 52 column was further purified using affinity chromatography on a Heparin sepharose 4B column (Fig 8). The transglutaminase activity was bound on Heparin sepharose 4B, and then eluted with a linear salt gradient. As shown in the activity curve, the transglutaminase activity showed a peak at 0.15 M NaCl concentration whereas a peak

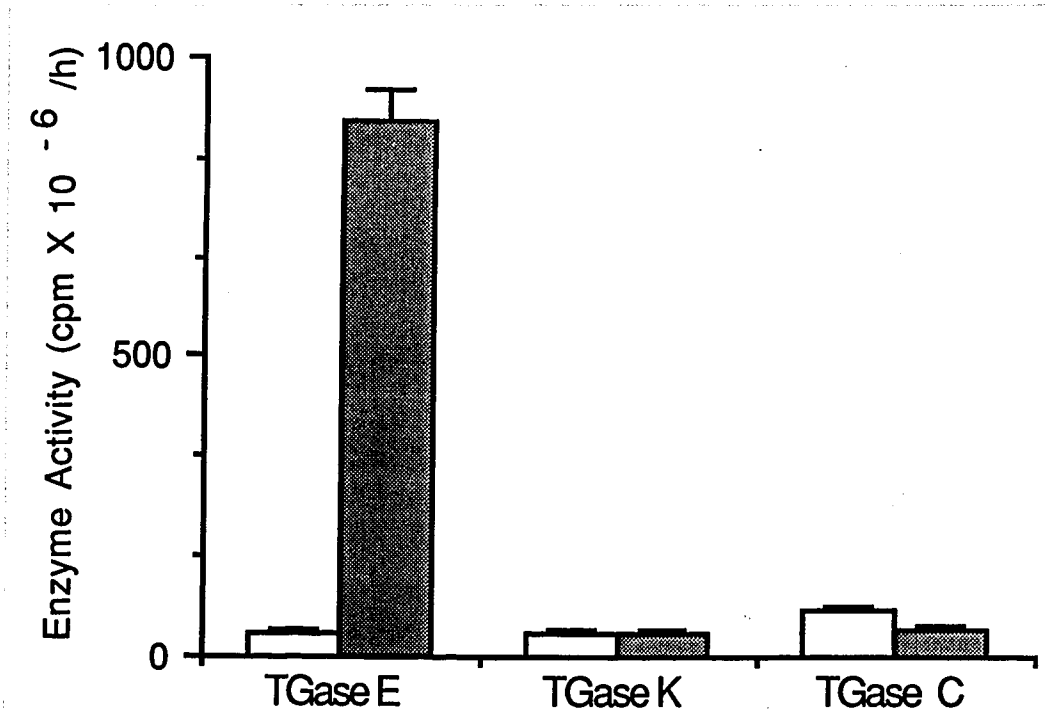


Figure 7. Distribution of transglutaminase activity from DE 52 ion exchange chromatography and activation of transglutaminases by Dispase treatment. The pooled fractions of DE 52 ion chromatography were designated TGase E, TGase K and TGase C, respectively. The open bars depict the enzyme activities without prior treatment; The cross-hatched *bars* depict samples that were treated with 0.05 unit Dispase for 10 minutes prior to assay.

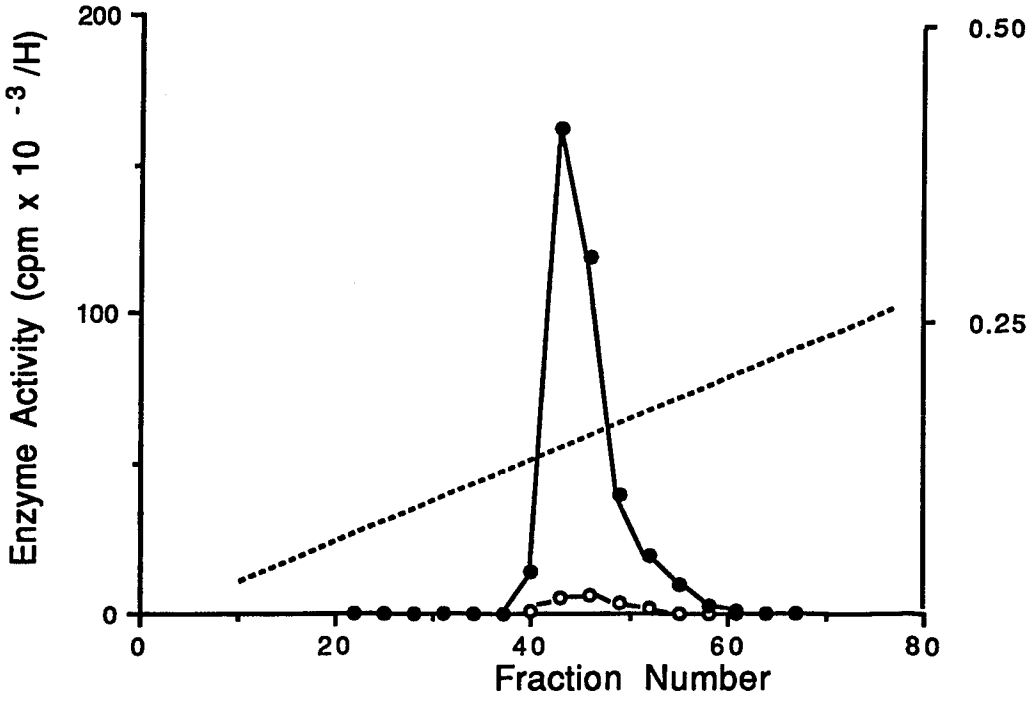


Figure 8. Affinity chromatography on Heparin sepharose 4B. Transglutaminase activity of fractions eluted from Heparin sepharose 4B at linear salt gradient (0-0.25 M NaCl). o——o, Transglutaminase activity; •—•:enzyme pretreated with Dispase prior to activity assay, ····· molarity of NaCl.

of proTGase activity appeared at a slightly lower salt concentration. The fractions eluting at salt concentrations between 0.05 M and 0.2 M were rich in transglutaminase activity and were pooled. The pooled fraction was concentrated to 2 mL using a YM-10 membrane under 55 psi nitrogen pressure.

Concentrated samples from the Heparin sepharose 4B were next applied onto a Bio-Gel A-0.5m column previously equilibrated with 5 mM Tris acetate, pH 6.0, containing 0.15 M NaCl and 1 mM EDTA. The peak of enzyme activity eluted at 293 mL. The fractions rich in enzyme activity were pooled and concentrated to 2 mL as described above. The concentrated sample was applied to a second column. The transglutaminase activity was found in the same elution volume (293 mL) as in the first gel filtration. The fractions rich in transglutaminase activity were pooled and dialyzed against buffer C. A typical chromatogram of gel permeation on Bio Gel A-0.5m is shown in Fig. 9.

Dialyzed samples were loaded onto a S-Sepharose cation exchange column and eluted with a stepwise pH gradient. Fractions eluting at 5 mM Tris acetate, pH 7.5, as well as 50 mM Tris acetate, pH 8.0 showed peaks of transglutaminase activity as shown in Fig. 10. Fractions that eluted at pH 8.0 and were rich in transglutaminase activity, were pooled and stored frozen at -20°C. A summary of the purification of guinea pig epidermal transglutaminase is shown in Table 2. The final yield of enzyme activity was 53 % with a 2850 fold increase in enzyme specific activity. All purification steps were performed at room temperature.

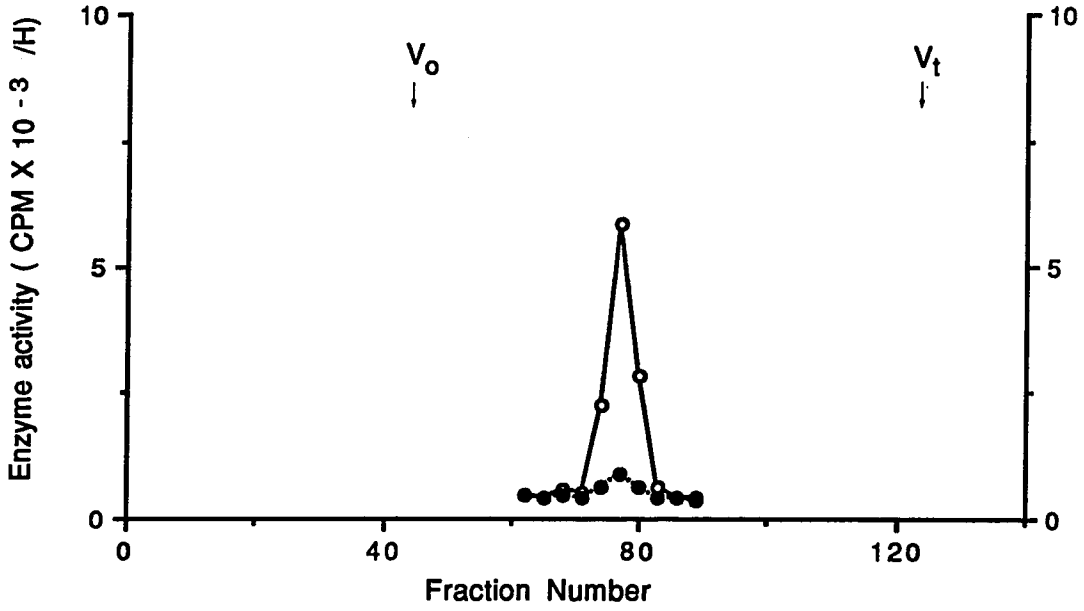


Figure 9. Exclusion chromatography of transglutaminase on Bio-Gel A-0.5m. •—•, transglutaminase Activity; o—o, Dispase-activated transglutaminase activity as described in "Methods".

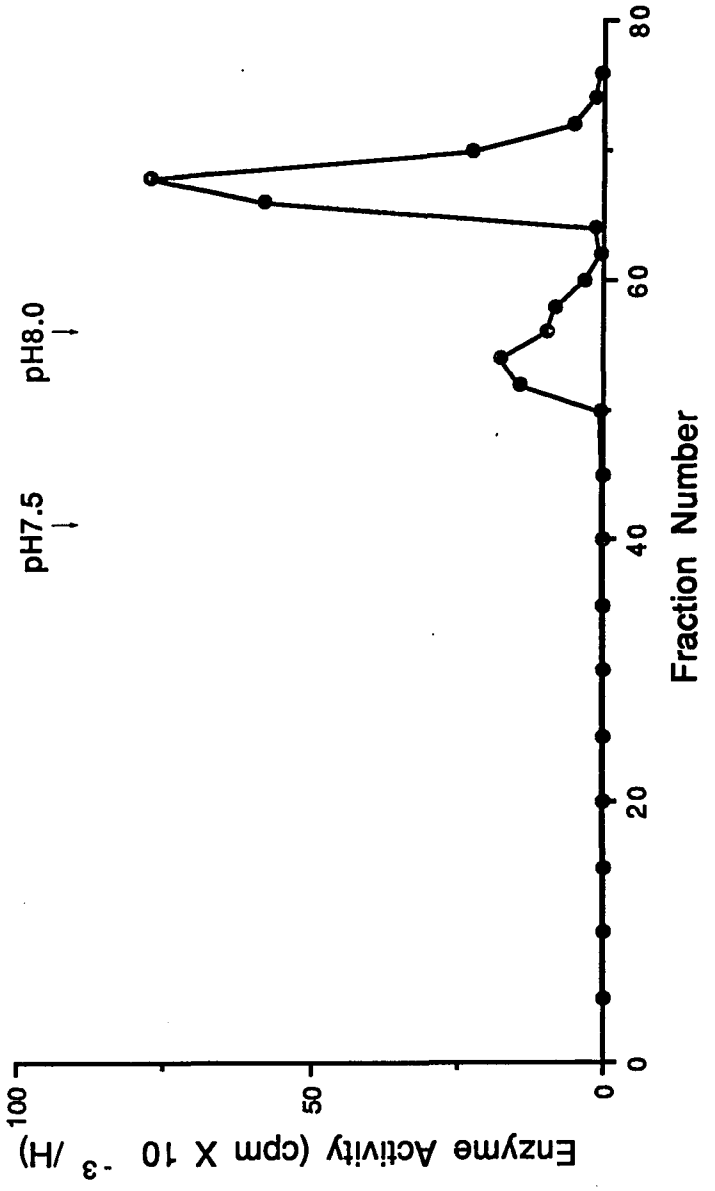


Figure 10. Cation exchange chromatography of transglutaminase on S-sepharose. •—•: Dispase-activated transglutaminase activity at indicated pH as described in "Methods".

Table 2

Purification of Protransglutaminase from Guinea Pig Skin

Step	Total Activity* ($cpm \times 10^{-6}/H$)	Total Protein (mg)	Specific Activity ($cpm \times 10^{-6}/H/mg$)	Yield (%)	Purification (fold)
Homogenate supernatant fluid	942	74,000	0.012	100	
DEAE-cellulose Unbound fraction	899	40,500	0.022	95	1.7
Heparin-Sepharose eluate	802	893	0.897	85	71
Bio-Gel A-0.5m Gel filtrate	706	62.5	11.30	58	941
S-Sepharose eluate	508	14.1	36.1	53	2842

* The total activities are determined by activation of protransglutaminase by Dispase.

Molecular Weight of ProTGase E and Dispase-activated TGase E

SDS-PAGE analysis of the isolated proTGase E and Dispase-activated enzymes are shown in Fig. 11. Coomassie Blue staining of the polyacrylamide gel showed a single protein band with a mobility equivalent to 77 kDa for proTGase E (lane 1) and two protein bands, one with mobility equivalent to 50 kDa and the other equivalent to 27 kDa for the Dispase-activated enzyme (lane 2). The molecular mass of the proTGase E estimated from SDS-PAGE is comparable to the molecular mass of 75,500 ± 4,000 daltons estimated from the K_d values obtained from gel permeation studies on the standardized column of Bio-Gel A 0.5m (Fig. 12). The molecular mass is estimated from the logistic distribution of K_d values as function of molecular masses of the known globular proteins as described method in Chang and Chung (1986). Gel filtration of the Dispase-activated enzyme also showed a K_d value similar to that of proTGase E (Fig. 12). Analysis of the fractions (75-79) containing the Dispase-activated TGase E from the gel column on SDS-PAGE under non-reducing conditions showed the presence of 50 kDa and 27 kDa and fraction 77 appear to contain a highest concentration of each peptide (Inset of Fig. 11). These results suggest that the Dispase-activated enzyme retains the same molecular mass as that of proTGase E, despite cleavage of the peptide chain.

The molecular weight of proTGase E was also measured by the equilibrium ultracentrifugation (Chervenka, 1969). The molecular mass was calculated by direct fitting the data of concentration

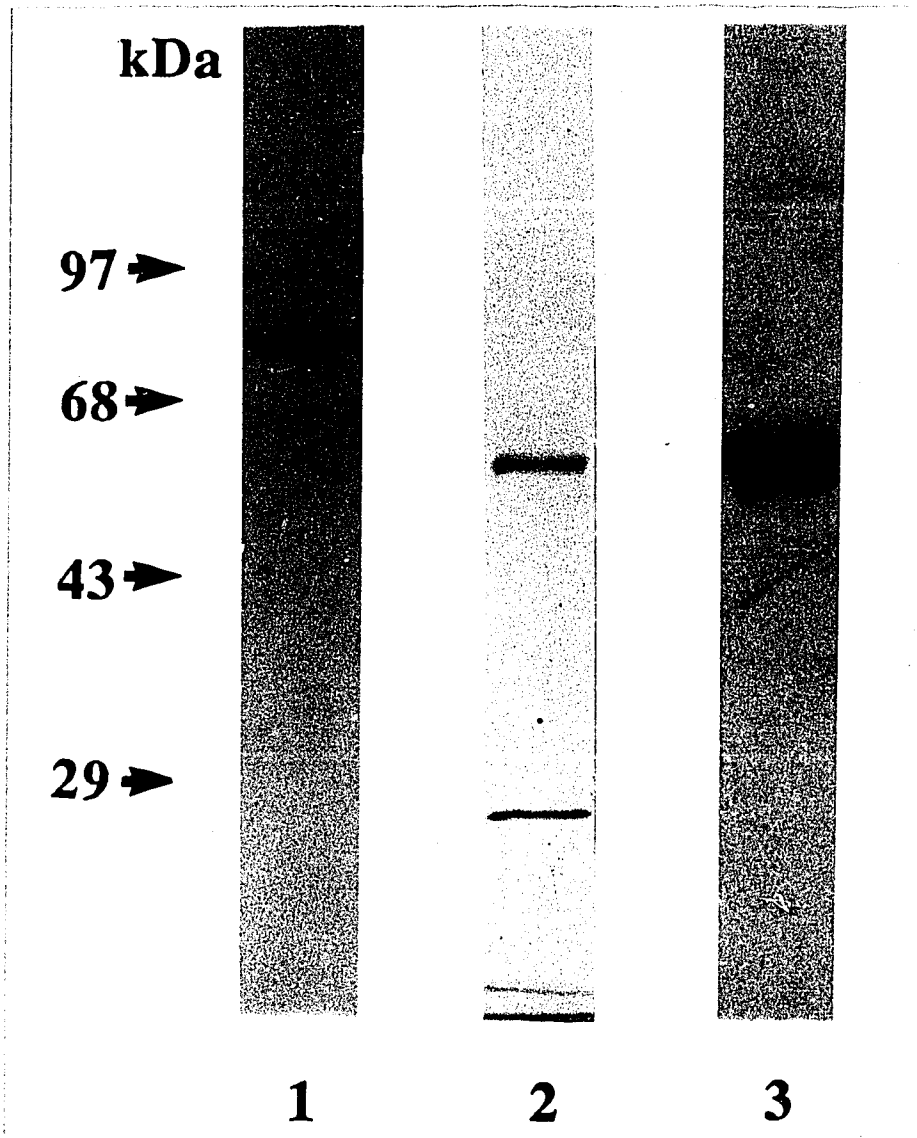


Figure 11. Polyacrylamide gel electrophoretic comparison of protein pattern of isolated proTGase (lane 1), activated TGase E (lane 2) and pattern of radioactivity (lane 3) after radio [^{14}C] acetamide inactivation. In lane 3, approximately 20 µg of enzyme was incubated in 40 µl of 0.1 M Tris acetate buffer (pH 8.0) containing 10 mM $CaCl_2$, and 5 mM iodo[^{14}C]acetamide (24 Ci/mole). after 20 min at room temperature, the reaction was stopped by addition of 40 µl of 25 mM EDTA.

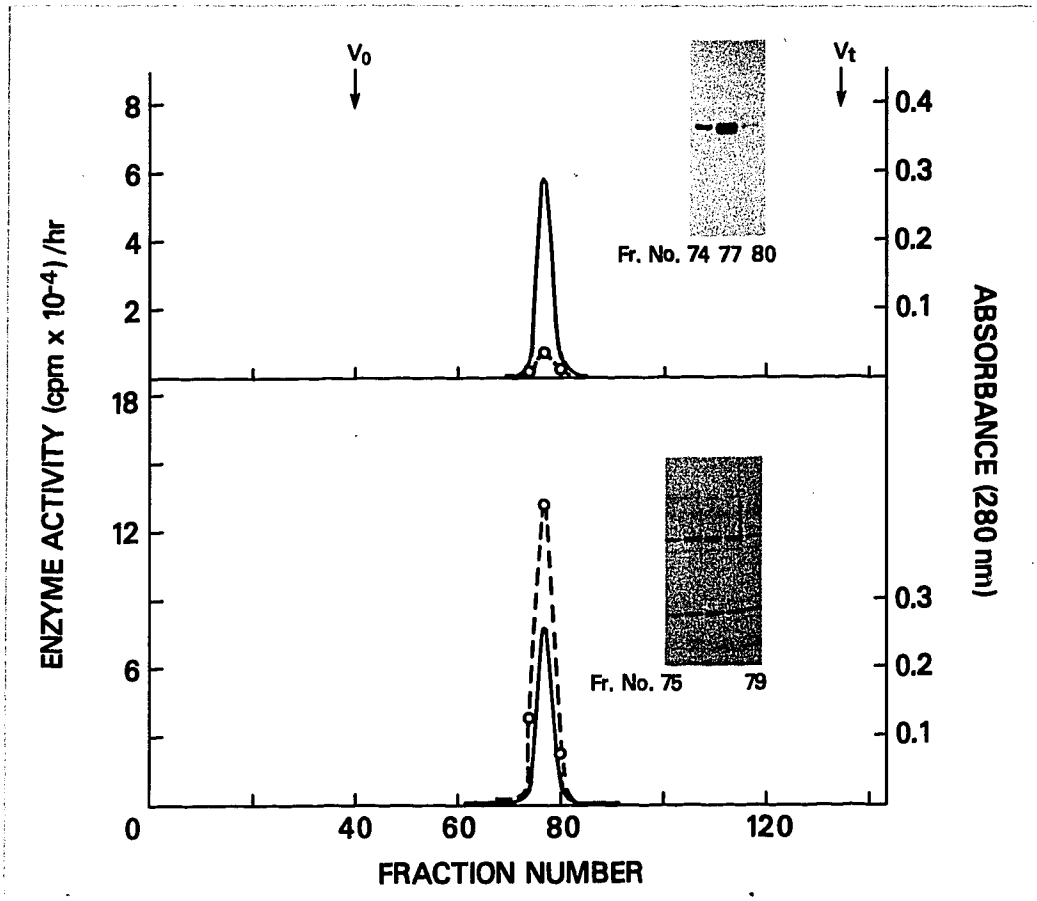


Figure 12. Comparison of exclusion chromatography of native and activated epidermal transglutaminase on Bio-Gel A-0.5m. The activated enzyme was prepared by preincubation with 0.05 unit Dispase for 10 min prior to application on the gel exclusion column. The purified and activated enzyme were applied on a 2.6 X 98 cm Bio-Gel A 0.5 m column equilibrated with phosphate buffered saline. Samples of 3.8 ml were collected and 50 µl portion were assyed for activity(o····o) and absorbance at 280 nm (———). (Inset) SDS-PAGE of the fractions after gel filtration was performed under non-reducing conditions using 10 % acrylamide as the separating gel.

distribution as a function of radial position obtained from the ultracentrifuge using the equation (1). The activated protein solution exhibited the presence of more than one component; the data could be suitably fit using a two-exponent model. The fits are shown in Fig. 13. Plots of the distribution of the data with respect to the fitting line demonstrated the absence of systematic deviations, indicating that the one and two component models were appropriate for the native and Dispase-activated enzyme, respectively.

A compositional partial specific volume of 0.702 cm^3/g was calculated from the amino acid composition (Schachman, 1957). The concentrations were determined spectrophotometrically using a value of $E_{287.5}$ = 10.0 for the extinction coefficient, which was determined from dry weight measurements. The molecular mass of proTGase E was 77.8 ± 0.7 kDa. The Dispase-activated enzyme had a major component (82%) with a molecular mass of 76.5 ± 1.5 kDa and a minor component (18%) of 763.8 ± 8.5 kDa. This was most probably an aggregate of the major component since the amount of Dispase introduced to activate the enzyme was far to small to account for the minor component.

The sedimentation coefficients for the proTGase E and for the Dispase-activated enzyme were obtained from the natural logarithm of the radius of the half plateau region concentration as a function of time of appropriate sedimentation velocity experiments. The values were corrected to standard conditions of water at 20°C. The proTGase E had a sedimentation coefficient of 3.49 ± 0.19 S and the activated enzyme had a sedimentation coefficient of 3.09 ± 0.11 S (Fig. 14).

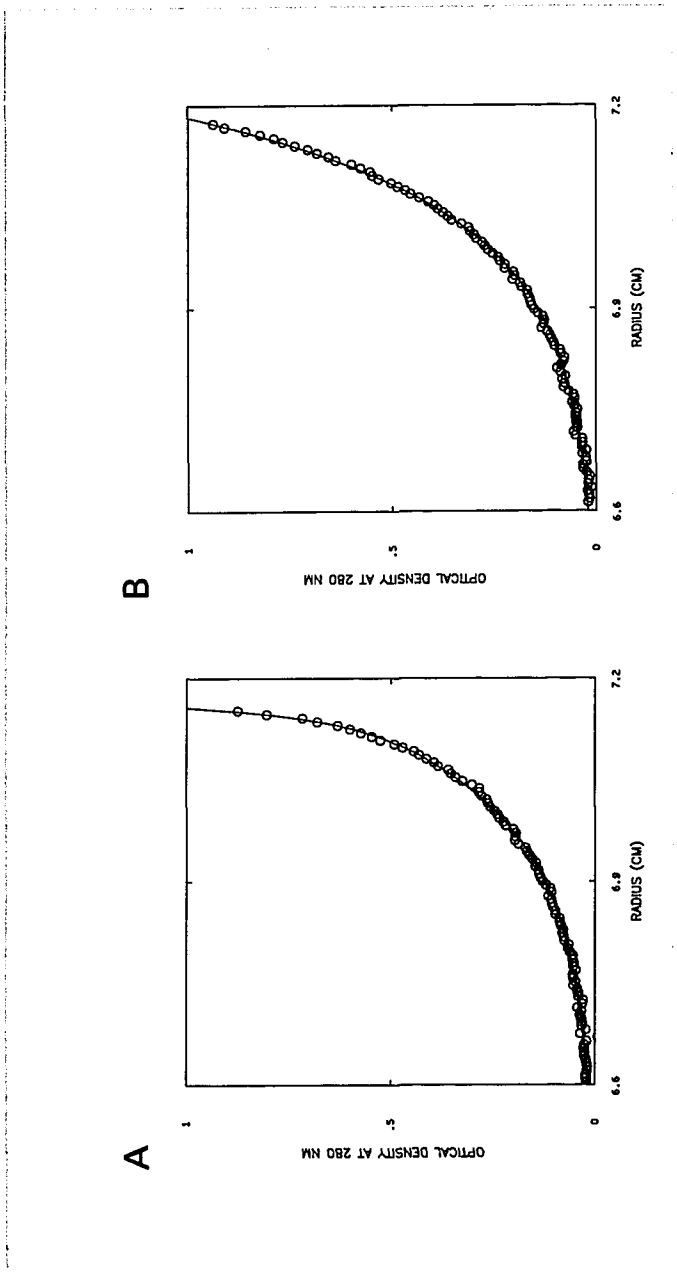


Figure 13. Sedimentation equilibrium study plotted as optical density at 280 nm versus radius. A. proTGase E; B. Dispase-activated TGase E.

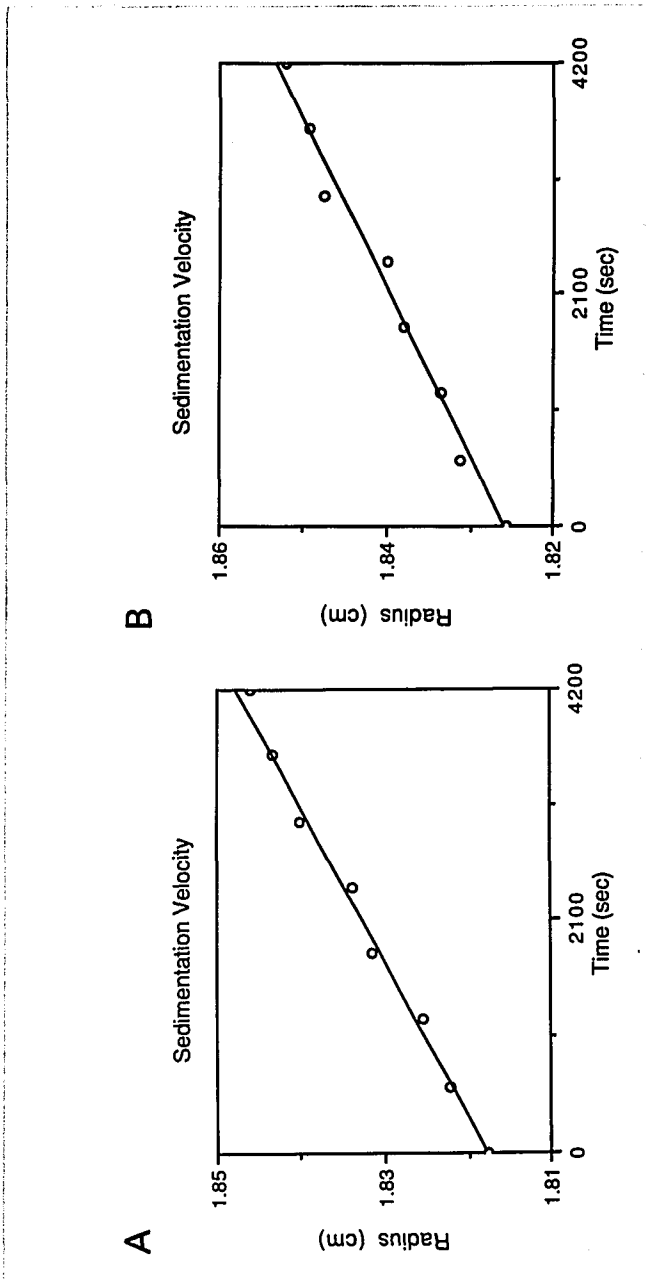


Figure 14. Sedimentation velocity study plotted as radius versus time. A. proTGase E; B. Dispase-activated TGase.

Frictional coefficients were calculated using equation (3) giving values of 1.66 and 1.87 for the proTGase E and activated enzyme, respectively (Table 3). These values correspond to axial ratios of 12:1 and 18:1, respectively, in the absence of hydration. Since the extent of hydration is not known here, probably the only significance which can be attributed to these differences is that they imply the possibility of a change in the shape of the molecule following activation.

Amino Acid Analysis

The amino acid composition of proTGase E, shown in Table 4, is quite different from that of cytosolic TGase C (Ikura et al, 1988). The proTGase E contains higher glycine (17.5%), serine (10.25%) and alanine (9%) than TGase C. The high glycine and serine levels of proTGase E appear to be unique to the proteins synthesized in differentiated keratinocytes, i.e. keratins (Steinert et al., 1985), filaggrin (Dale, 1985).

Comparison of Transglutaminases by Activity Staining

A comparison on agarose gels of the electrophoretic mobilities of the various transglutaminase - proTGase E from guinea pig skin, epidermal transglutaminase from human foreskin, particulate-associated transglutaminase (TGase K) from cultured human keratinocytes, and tissue transglutaminase (TGase C) from guinea pig liver - are shown in Fig. 15. The mobilities of the four enzymes were quite distinct. Protransglutaminase from guinea pig skin migrated

Table 3

Physical Properties of Protransglutaminase

	proTGase E	TGase E
Molecular Mass	kDa	kDa
SDS-PAGE	77	50 & 27
Gel permeation	75.5 ± 4.0	75.5 ± 4.0
Sed. Equilibrium	77.8 ± 0.7	76.5 ± 1.4 (82%)
		763.8 ± 8.5 (18%)
	S	S
Sed. Coefficient	3.49 ± 0.19	3.09 ± 0.11
	f/f_0	f/f_0
Frictional Ratio	1.656	1.872

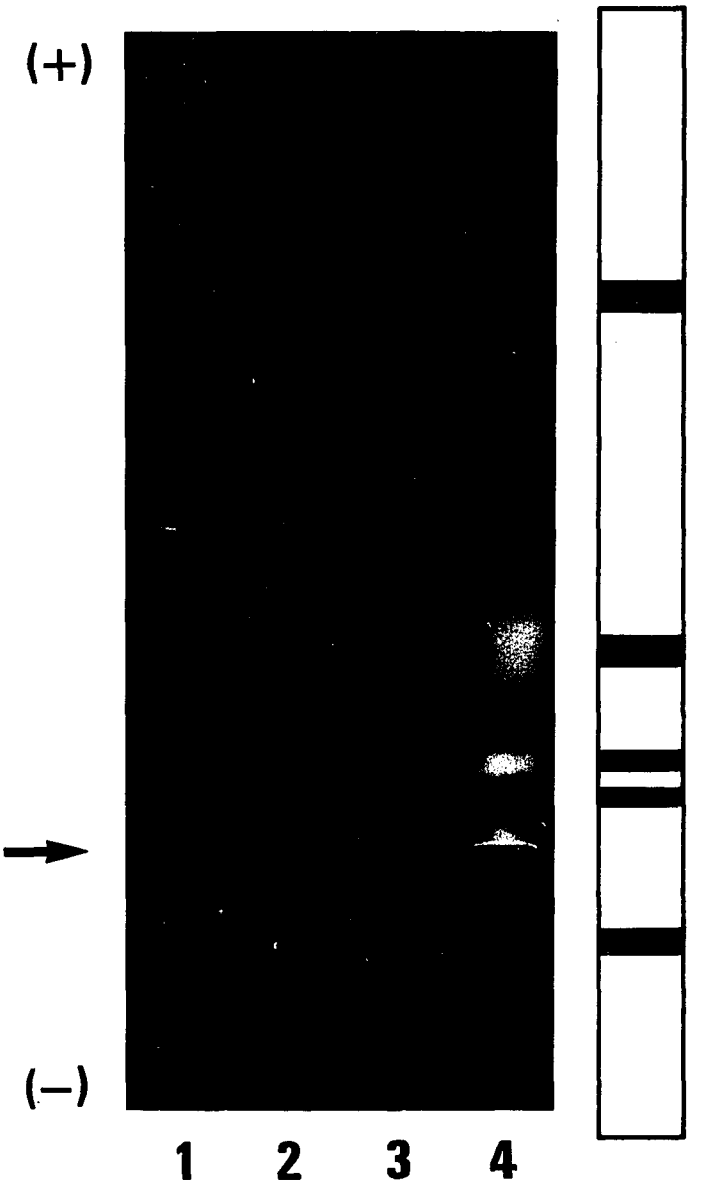
Table 4

Amino Acid Composition of Guinea Pig Epidermal Protransglutaminase and Guinea Pig Liver Transglutaminase

	proTGase E	TGase C
Asx	74.1	78[1]
Thr	34.2	31
Ser	64.9	43
Glx	54.9	77[2]
Pro	34.5	32
Gly	111.7	57
Ala	57.3	45
Val	48.8	60
Met	8.9	9
Ile	20.8	34
Leu	40.6	69
Tyr	7.1	24
Phe	14.8	23
Lys	25.2	29
His	7.1	12
Arg	28.2	40
Cys	>1.5	17
Trp	N.D.	10
Total	>634.6	690

1. Asn 37, Asp 41
2. Gln 27, Glu 50

Figure 15. Agarose gel showing the transglutaminase activity visualized by enzyme-catalyzed incorporation of a fluorescent substrate, monodansylcadaverine, into casein. lane 1, liver transglutaminase; lane 2, epidermal transglutaminase from human foreskin; lane 3. epidermal transglutaminase from guinea pig; lane 4, keratinocyte-specific transglutaminase from human cultured keratinocyte extracts.


(+)
(–)
1
2
3
4

toward the negative pole, whereas the other transglutaminases moved to the positive pole. The electrophoretic mobilities at pH 8.8, largely a function the total charge of the protein, correspond closely with the elution profiles from DE 52 cellulose chromatography, which utilizes a similar principle of separation. The relative mobilities of each transglutaminase did indeed correspond well with the behavior of the respective enzymes on a DE 52 column during the linear salt gradient buffer application. Most notably, the particulate-bound transglutaminase migrated less than the tissue transglutaminase. The epidermal transglutaminase from human foreskin showed an activity peak at 0.05 M NaCl concentration on a DE 52 column.

Identification of Catalytic Peptide Fragment

The transglutaminases contain catalytically essential thiol (-SH) group at their active sites (see review, Folk and Chung, 1973). The activated transglutaminase prepared from gel filtration after 0.05 units of neutral protease treatment reacted with radiolabeled alkylating agents under catalytic conditions. The enzyme activity was lost as a result of concomitant incorporation of radioactivity due to selective derivatization of the active site thiol (-SH) group. When the active enzyme treated with iodo[^{14}C]acetamide was examined by autoradiography after polyacrylamide gel electrophoresis in sodium dodecyl sulfate, a single band, corresponding in position to that of the 50 kDa fragment, appeared (Fig. 11. lane 3). This finding defines the catalytically active portion of the activated enzyme and gives an independent estimate of enzyme molecular mass.

Specificity of Protease-induced Activation of ProTGase E

Dispase, a neutral metallo-protease from Bacillus polymyxa, is known to have a broad substrate specificity. In order to learn the possible identity of the amino acids in the protease-sensitive peptide region involved in the activation of proTGase, other proteases with different substrate specificities were examined. Proteinase K and trypsin were able to induce activation of proTGase E, but chymotrypsin and endoproteinase Glu-C were not (Fig. 16). Thrombin, the enzyme generated from the cascade of activation of coagulation factors, is known to have a limited substrate specificity and to catalyze the hydrolysis of the Arg-Gly peptide bond (Takagi and Doolittle, 1974). Treatment of proTGase E with 1 unit of thrombin resulted in partial generation of enzyme activity. Kinetic studies of proTGase E activation with 50 units of thrombin showed that emergence of 50 kDa and 27 kDa peptide fragments coincided with the generation of enzyme activity (inset of Fig. 18). These results suggest that the thrombin-sensitive bond, Arginine-X, is likely present in the cleavage site of the proTGase E.

Ca^{2+} Ion Requirement and Ca^{2+} Ion-induced Activation of ProTGase E

Transglutaminases are known to require Ca^{2+} ion for their catalytic activity (See review, Folk and Chung, 1973). TGase E is no exception. However, the Ca^{2+} ion requirement is so low that it was difficult to measure experimentally (Fig. 17). Since succinylated casein was used as the substrate for the transglutaminase assay and

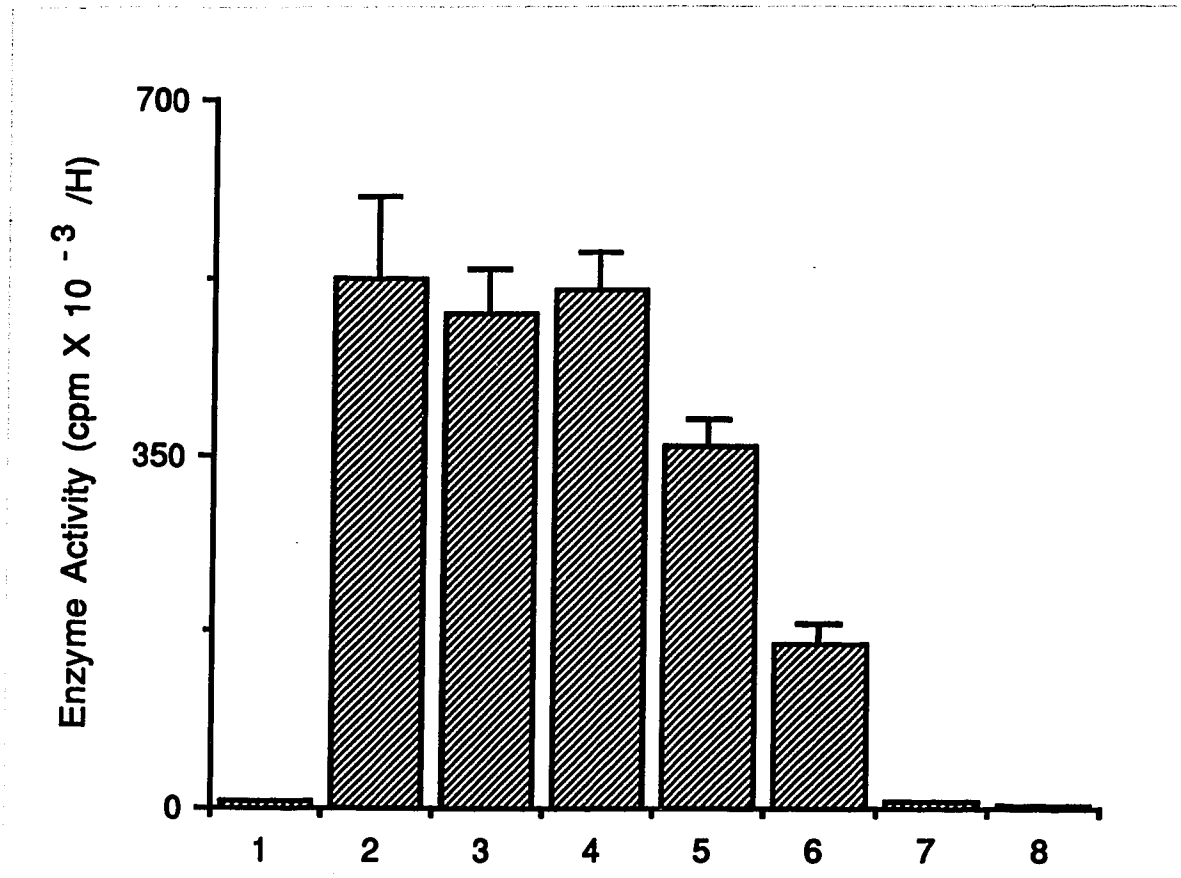
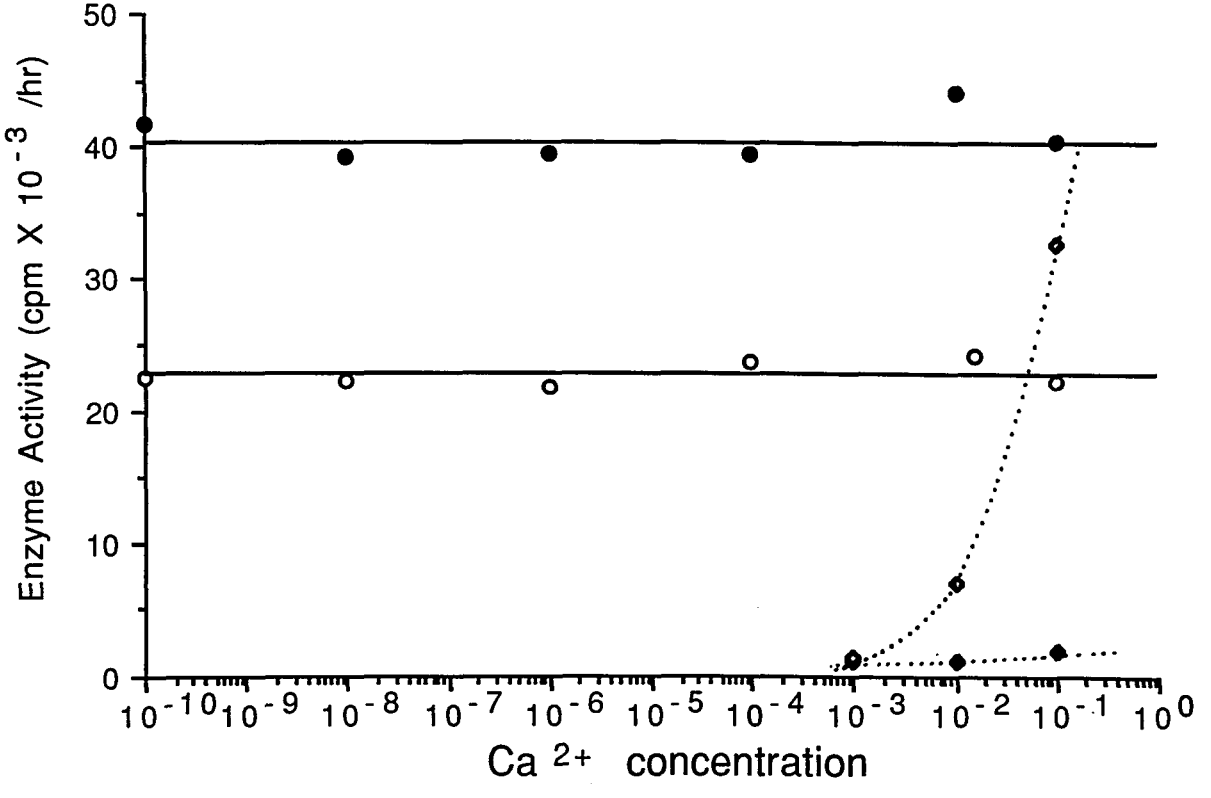


Figure 16. Protease-induced activation of proTGase E. The enzyme was preincubated with various proteases for 10 min at 25° C prior to activity assay. lane 1, control; lane 2, Dispase; lane 3, trypsin; lane 4, proteinase K; lane 5, human thrombin; lane 6, Enzyme activity was assayed in the presence of 20% ethanol; lane 7, chymotrypsin; lane 8, endoproteinase Glu-C.

Figure 17. Activation of native and activated enzyme by dithiothreitol and calcium. The purified enzyme (10 µl) was mixed with 240 µl of 0.1 M Tris acetate (pH 7.5) buffer containing various calcium concentrations and incubated for 25° for 30 min. After preincubation, 0.2 ml of 2% succinylated casein (pH7.5) was added and the activity assay was conducted as described in "Methods". •—•, enzyme pretreated with 0.05 unit of Dispase assayed in the presence of DTT; o—o, enzyme pretreated with 0.05 units of Dispase assayed in the absence of DTT; ♦—♦, enzyme pretreated with various concentrations of calcium ion assayed in the presence of DTT; ◊—◊, enzyme pretreated with various concentration of calcium ion assayed in the absence of DTT.


Enzyme Activity (cpm X 10 -3 /hr)
50
40
30
20
10
0
10-10 10-9 10-8 10-7 10-6 10-5 10-4 10-3 10-2 10-1 100
Ca 2+ concentration

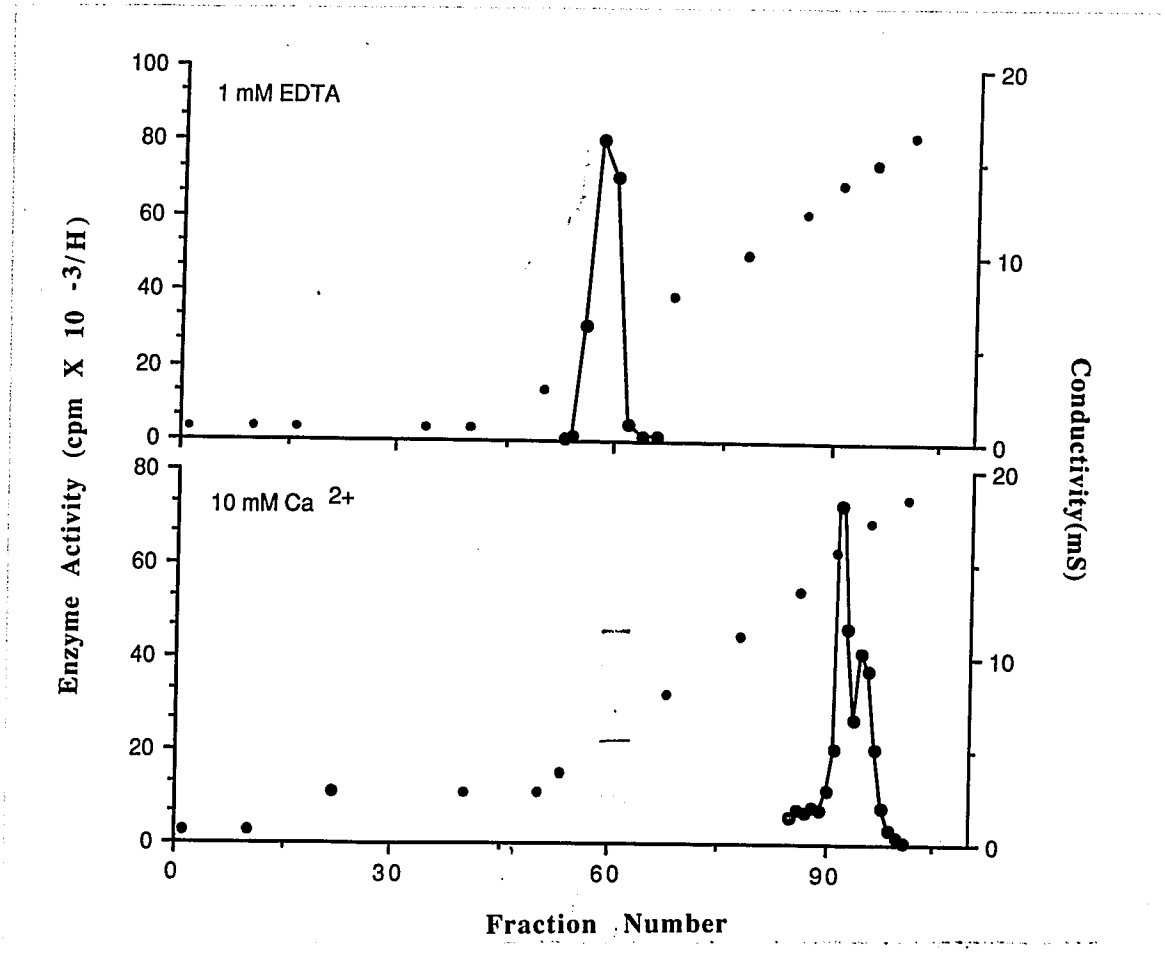


Figure 18. Comparison of ion exchange chromatography of transglutaminase on Mono-S in the presence and in the absence of calcium ion. The Dispase-activated proTGase E (1 mg) was applied to a Mono-S HR 5/5 column previously equilibrated with 5 mM Tris acetate containing 1 mM EDTA or 10 mM $CaCl_2$. The enzyme was eluted with a 40 ml linear salt gradient from 0 to 0.2 M NaCl in equilibrating buffer. Enzyme activities of the fractions were assayed. (•——•), in the presence of 1 mM EDTA; (o—o), in the presence of 10 mM $CaCl_2$; (····), conductivity.

casein is known to have high affinity Ca^{2+} ion binding sites, the casein was dialyzed against 1 mM EDTA and then with distilled water. With this mobilied casein, purified proTGase E showed 75-80% of the level of transglutaminase activity in the absence of added $CaCl_2$ in comparison with the assay solution containing 1 mM $CaCl_2$. In the presence of 1 mM EDTA, no catalytic activity was observed. As shown in Fig. 18, 10^{-10} M Ca^{2+} concentration appeared to be saturating. Treatment of proTGase E with 100 mM Ca^{2+} ion generated full potential catalytic activity in the absence of protease. Similar activation of potential catalytic activity induced by high Ca^{2+} ion concentration (100 mM) was observed with factor XIII (platelets or plasma protransglutaminase)(see review, Folk and Finlayson, 1977). Addition of sulfhydryl groups in the assay solution also enhanced the catalytic activity nearly two fold. The mechanism of such thiol(-SH) activation is not known. Pretreatment of the enzyme with 5 mM dithiothreitol, and subsequent removal of thiol (SH) groups by gel filtration did not resulted in activation of catalytic activity. Another reducing reagent, asorbic acid, did not show any effect.

With the limited quantity of proTGase E available, it was difficult to employ circular dichroism or UV spectrophotmetric methods to examine any conformational properties of proTGase. Since adsorption of the enzyme to a cation exchange matrix depends on the net anions on the surface, then the extent of adsorption to cation exchange matrix will likely be affected by any conformational change. ProTGase E was equilibrated with buffer containing 10 mM $CaCl_2$ and chromatographed on a Mono S column that was equilibrated with

buffer containing 10 mM $CaCl_2$. The adsorbed proTGase E was eluted with a increasing linear gradient of NaCl in buffer containing 10 mM $CaCl_2$. A comparison of the elution of proTGase E from Mono S column in buffer with EDTA to its elution in buffer with $CaCl_2$ is shown in Fig. 18. ProTGase E in the buffer containing EDTA eluted from the column at 0.05 M NaCl concentration whereas proTGase in the buffer containing Ca^{2+} eluted at 0.16 M NaCl concentration. This suggests that Ca^{2+} ions induced an increase in the anions on the surface of proTGase, probably as a result of conformational change.

Identification of Soluble Substrate

Soluble substrates for the Dispase-activated transglutaminase were identified by incorporation of amine substrate, dansylcadaverine. SDS-PAGE analysis of the soluble substrates is shown in Figures 19, 20. The fluorescent bands observed correspond to molecular masses of 67,000, 50,000, 25,000 and 6,000. The band at 6,000 correlated well with an epidermal substrate that had been reported as keratolinin (Zettergren, 1984). The acidic protein with a fluorescent band at 25,000 and basic proteins between 43,000 and 68,000 are possible substrates for the Dispase-activated enzyme.

Immunological Studies

Rabbit anti-serum to guinea pig proTGase E was purified on the immobilized proTGase E column. This monospecific polyclonal immunoglobulin cross-reacted only with proTGase E and not with other cellular transglutaminases (Fig. 21). Immunoblot staining of proTGase E with polyclonal antibody to guinea pig liver TGase C

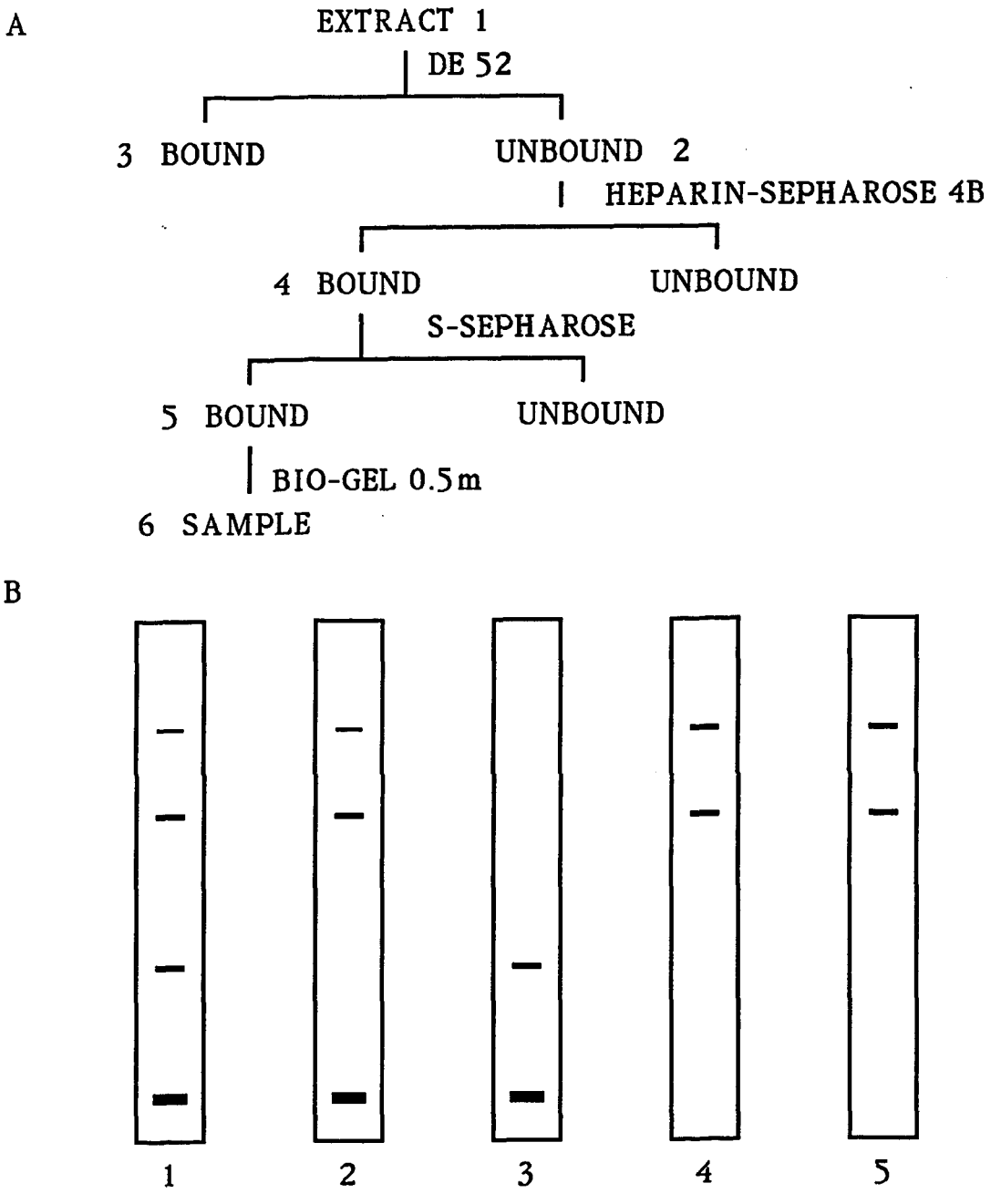


Figure 19. Schematic diagram of identification of soluble substrate from TGase preparation. Top (A) shows the preparation diagram. Bottom (B) is a schematic diagram of the fluorescent pattern of the each of the fractions viewed with ultraviolet A radiation.

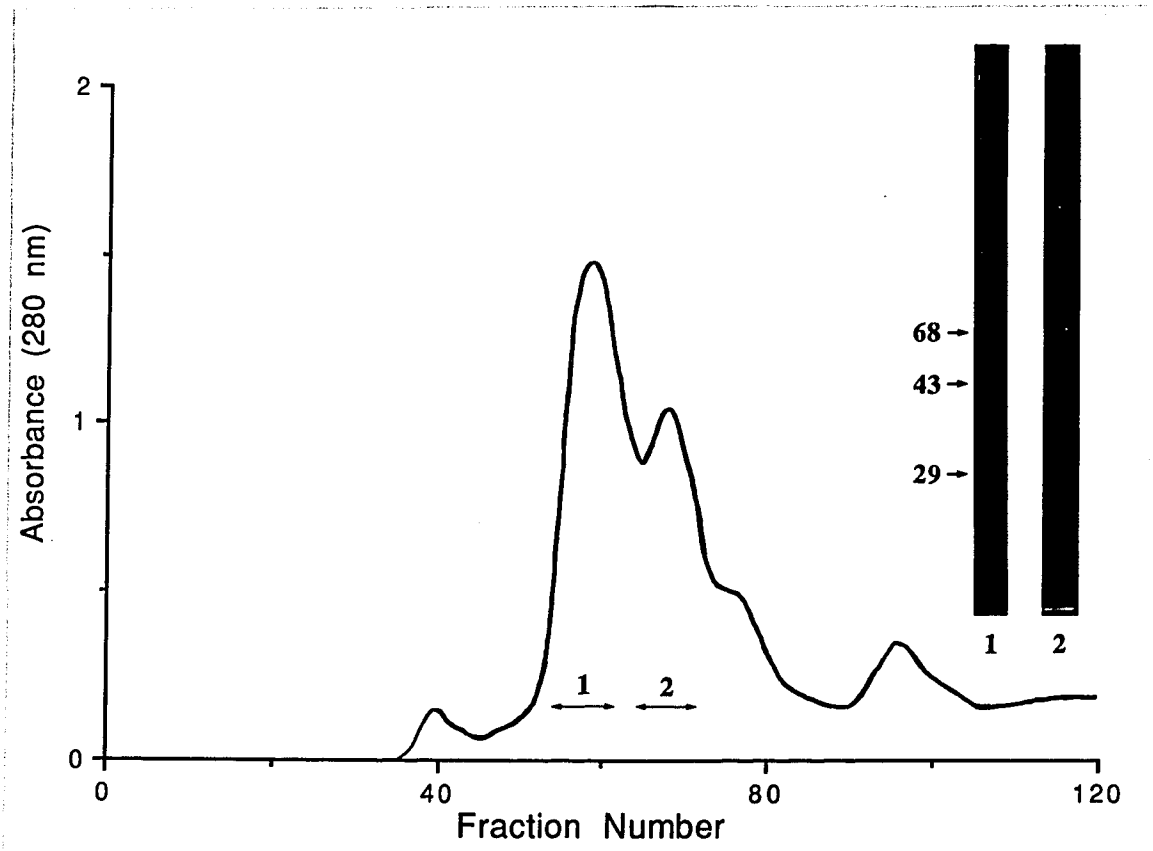


Figure 20. Purification of the soluble substrate on Bio-Gel A-0.5m and (inset) visualized by enzyme-catalyzed incorporation of fluorescent substrate, monodasnylcadaverine, into the soluble proteins.

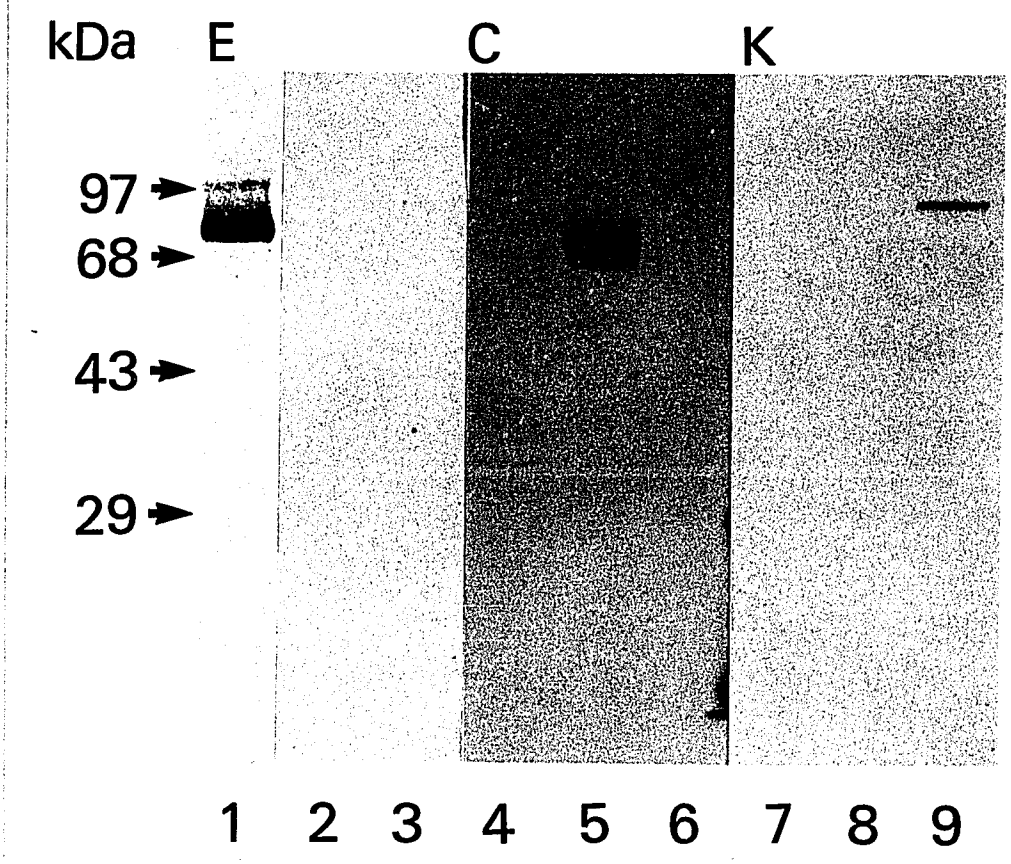


Figure 21. Westernblot analysis of various transglutaminases. lane1,4,7, proTGase E; lane2,5,8, TGase C; lane3,6,9, TGase K.

and with monoclonal antibody to human keratinocyte TGase K showed no cross-reactivity. TGase C and TGase K only reacted with their respective antibodies and not with antibody to other types of TGases. These results suggest that each TGase present in skin extracts (TGase C, TGase K, and proTGase E) is immunologically distinct. Western blot analysis of Dispase-activated enzyme showed both 50 kDa and 27 kDa bands that reacted with the anti-proTGase E. Monospecific antibody recovered from the antibody that complexed with the 50 kDa peptide from the blots reacted with the 77 kDa proTGase but not with 27 kDa peptide. Western blot analysis of skin homogenate with this monospecific antibody to proTGase E showed a single reactive band with the same electrophoretic mobility as that of isolated proTGase E. These results indicate that proTGase is the largest molecular form of this proTGase E in skin and that most of the antigen in skin is in this proTGase E form.

DISCUSSION

We reported earlier that the level of transglutaminase activity in epidermis increased following protease treatment as function of the time in newborn mouse skin (Chung et al., 1989; Martinet et al., 1988). Further examination showed that the transglutaminase activity was present essentially in two soluble forms: a labile high molecular weight anionic enzyme and a stable lower molecular weight cationic enzyme. To the test of possibility of protease-induced de novo synthesis of transglutaminase, the newborn mouse skin was incubated with cycloheximide prior to Dispase treatment. Incubation with cycloheximide did not inhibit the Dispase-induced increase in the level of transglutaminase activity which suggests an alternative mechanism for cell mediated activation of preformed enzyme. The finding that treatment of skin extract with dispase caused a pronounced increase in transglutaminase activity (Fig. 4) did support a possible proenzyme mechanism and suggests that the cationic Mr 50 kDa enzyme might be the terminal product drived from cell mediated activation of a proenzyme.

A one step separation of the three different forms of transglutaminase in epidermis was achieved by anion exchange chromatography on a DE-52 cellulose (Fig. 6). When the skin extracts prepared in the presence of protease inhibitors were

chromatography on a DE-52, at neutral pH and low ionic strength, most of transglutaminase activity was adsorbed and only a small amount of enzyme activity was found in the unbound fraction. All of the inducible transglutaminase activity following Dispase treatment was contained in the unbound fractions. Following dispase activation, calculation of the transglutaminase activity for the bound and unbound fractions showed that 80-85% of the extracted enzyme activity was present in the unbound fraction. Most of the enzyme required Dispase activation (Fig. 7). Purity of the isolated proTGase E was determined by gel filtration where the assay of the peak fractions showed the same specific activity for each protein containing fraction. Further, antibody raised against this proTGase E gave a single immuno-reactive protein band in the crude skin extract as examined by western blot analysis. The appearance of a single proteins band in SDS-PAGE analysis of proTGase E with Coomassie blue staining provided additional evidence of its apparent homogeneity.

Examination of the chemical and physical properties of proTGase E showed some unique features quite different from the other well-characterized tissue transglutaminase (TGase C). Measurement of the frictional coefficient give a value of 1.66 which corresponds to a 12:1 axial ratio in the absence of hydration. This large difference in axial ratio suggests that the proTGase E is extremely asymmetric. This fact is corroborated by the observation that the molecular mass of denatured proTGase E estimated from SDS-PAGE is equal to the molecular mass of non-denatured proTGase E estimated from the

sedimentation equilibrium and gel filtration. Protease-induced activation of proTGase E resulted in fragmentation of the 77 kDa peptide chain into 50 kDa and 27 kDa peptides as observed by SDS-PAGE analysis under non-reducing conditions. However, the molecular mass of the enzyme as measured by sedimentation equilibrium and gel filtration methods showed a value almost equal to the molecular mass of the native proTGase E (Table 4). By labelling the active site (-SH) group of transglutaminase E with iodo[^{14}C]acetamide, the catalytic site amino acid residue was localized to the larger peptide chain (Fig.11 lane 3). Whether the small peptide chain participates in enzyme catalysis is not known. The small peptide chain was always found to be present in the purified transglutaminase E (Chung and Folk, 1972; Peterson and Buxman, 1981). This suggests that the 27 kDa peptide chain is tightly associated with the catalytic peptide chain and may be an integral subunit of the transglutaminase E. An indication of possible conformation changes associated with proTGase E activation by protease is observed in the slight change in values of sedimentation and frictional coefficient of the protease-activated enzyme from the values obtained for proTGase E.

This protransglutaminase E is distinct in many respects from both the particulate-associated 92 kDa transglutaminase (TGase K) and tissue transglutaminase (TGase C). These differences are indicated by the elution properties on anion-exchange chromatography, western blot analysis with each antibody, molecular mass, and enzymatic properties. The proTGase E is the proenzyme of previously isolated

50 kDa epidermal transglutaminase (Buxman and Wuepper, 1975; Martin and Goldsmith, 1975) and hair follicle transglutaminase (Chung and Folk, 1972). A high molecular weight transglutaminase identified in human is likely the same proTGase of the human M_r 50 kDa epidermal enzyme (Negi et al., 1985).

Preliminary studies on the expression of proTGase E show that it is not expressed in normal skin tissue. Both TGase C and TGase K are expressed in the cultures keratinocytes (Lichti et al., 1985). Immuno-staining of the guinea pig skin, mouse skin and human skin with monospecific polyclonal anti-proTGase E drived from guinea pig showed only the suprabasal granular layers and hair follicles were positively stained. This suggest that proTGase E isolated from epidermis and skin which represents 80-85% of total extractable activity and 65-75% of total transglutaminase activity remain a zymogen. Further, the localization of proTGase E to the suprabasal layer suggest that expression of proTGase E is associated with the terminal differentiation of epidermis (stratum granulosum) concurrent with the expression of K-1, K-10 keratins and is activated most likely during the death of the epidermal cell and its entry into the stratum corneum (Sun et al., 1979). Morphologically, these events occur during the formation of dense accretions at the inner leaflet of the plasma membrane called the marginal band (Odland, 1983). The finding that the soluble envelopes prepared from the outer layers of stratum corneum are structurally more rigid and thicker than those from inner layer of stratum corneum (Michel et al, 1988) suggest that cross-linking of envelope proteins continues in the layers of the

stratum corneum. The inherent properties of proTGase, activation of proTGase E by high Ca^{2+} ions, organic solvent, and high temperature, and the stability of protease-activated enzyme in comparison with the extreme lability of the active enzyme as shown by TGase K and TGase C, suggest that proTGase may be the enzyme responsible for the cornified envelope formation.

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