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ACANTHOLYSIS INDUCED BY PROTEOLYTIC ENZYMES II. ENZYME FRACTIONS PRODUCED BY TRICHOPHYTON MENTAGROPHYTES

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Abstract. Trichophyton mentagrophytes produces three enzyme fractions of molecular weight 65 000, 92 000 and 18 000, respectively. Normal human skin was cultivated and the specimens were subjected to electron microscopy. Enzyme fraction I showed the capacity to induce epidermal acantholysis and dermo-epidermal separation, while the other two fractions showed no proteolytic nor keratinolytic action in cultivated skin.

Key words: Enzyme fractions; *T. mentagrophytes*; Acantholysis; Ultrastructure

Certain kinds of dermatophytes produce proteolytic enzymes and affect the horny layers of skin or hairs (1, 3, 6, 9, 17, 20). A keratinolytic enzyme, the so-called keratinase, has been studied from the biochemical point of view (10–12, 14–17, 19–21). Some authors have reported that keratinase can induce dermo-epidermal separation and acantholysis in epidermis (4, 5).

In the present paper, we cultured *Trichophyton mentagrophytes*, isolated a crude proteolytic enzyme fraction from the culture fluid and used it in culture of skin.

MATERIAL AND METHODS

A. *Isolation of a proteolytic enzyme fraction*

1. *Culture of T. mentagrophytes.* According to Yu et al. (19, 20) and Weary et al. (16, 17), *T. mentagrophytes* was inoculated into a keratin medium composed of washed and sterilized wool 2.5 g, glucose 0.9 g, $MgSO_4 \cdot 7H_2O$ 0.6 g, thiamine 0.01 g, pyridoxine 0.01 g and inositol 0.05 g in one litre of 0.028 M phosphate buffer, pH 7.8. After inoculation, the culture was left at 25°C for 14 days, and shaken for another 10 days at room temperature. The fungi and the residual wool were then removed by filtration.

A control material was prepared similarly, only without fungi.

2. *Preparation of enzyme fractions.* The filtrate of the culture was applied onto a diethylaminoethyl (DEAE) Sepharose CL-6B column (Pharmacia Fine Chemicals AB, Sweden). The effluent was transferred onto a carboxymethyl (CM) Sepharose CL-6B column (Pharmacia). The crude enzyme fraction, i.e. fraction I, was eluted from the CM-Sepharose column with 1 M NaCl, 0.01 M phosphate buffer, pH 7.8, dialysed against distilled water and lyophilized.

The fungi and the residual wool were washed with 0.028 M phosphate buffer, pH 7.8, homogenized and filtered. The filtrate was applied to a DEAE-cellulose column. The adsorbed crude enzyme fraction, i.e. fraction II, was eluted with 1 M NaCl, 0.01 M phosphate buffer, pH 7.5, dialysed against distilled water and lyophilized.

Fraction III adsorbed onto the CM-cellulose was eluted with 1 M NaCl, 0.01 M phosphate buffer, pH 7.8, dialysed against distilled water and lyophilized.

Two control materials were prepared. Control I was the filtered medium incubated with wool and without fungi, and control II was the filtrate of homogenized wool incubated with no fungi.

3. *Protein contents.* The protein contents of the three fractions and two controls were examined by the methods

Table I. *Protein contents and molecular weight*

	Protein contents		
	Total protein (mg/ml)	%	Molecular weight
Enzyme fractions			
I	0.635	27.67	65 000
II	0.484	23.83	92 000
III	0.317	21.57	18 000
Controls[a]			
I	0.025	1.45	
II	0.117	2.93	

[a] Control I: Filtrate of medium with wool incubated without fungi, before homogenization. Control II: Filtrate of medium with homogenized wool incubated without fungi.

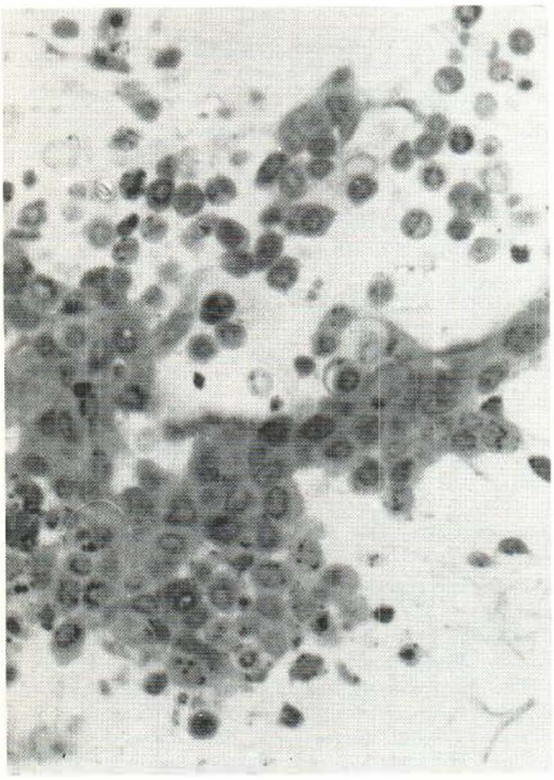

Fig. 1. 72 hours after start of culture. Light microscopy. Typical acantholysis in epidermis. ×800.

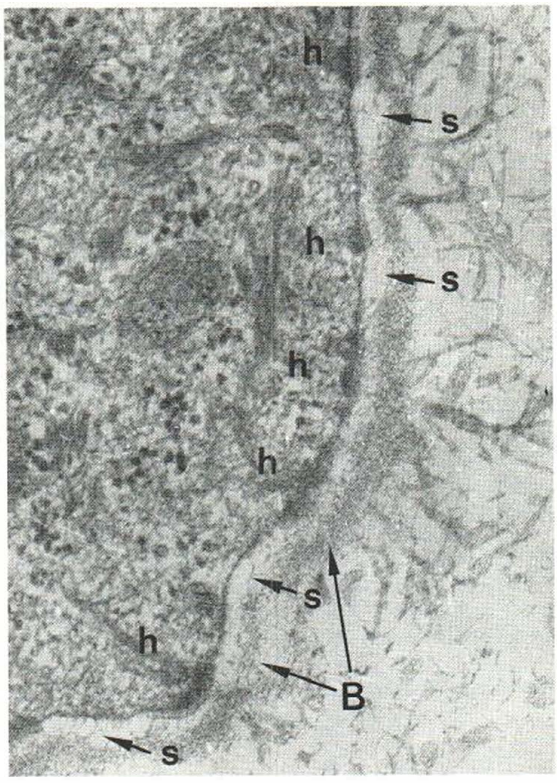

Fig. 2. 18 hours. Widening at subepidermal space (*s*) between basal cells and basal lamina (*B*). The anchoring filaments are elongated. No changes in hemidesmosomes (*h*). ×60000.

of Blumenkrantz (2). The material was dissolved in 0.9% isotonic NaCl solution and assayed with a Technicon Auto-Analyzer (Technicon Instruments Co., New York).

4. *Molecular weights.* The molecular weights of the three fractions were estimated on a Sephadex G-100 column (Pharmacia Fine Chemicals), according to the method of Whitaker (18). 0.05 M NaCl, 0.028 M phosphate buffer, pH 7.8, was used for elution. The column was 2.6×17 cm, and the elution speed was 3 ml/min.

B. *Effects of the enzyme fractions on human skin in organ culture*

1. *Skin culture with enzyme fractions.* The skin material of a middle-aged woman was obtained at plastic surgery. The preparation of culture medium and methods of cultivation were described in a previous paper (7). Samples of skin were cultured separately with each enzyme fraction dissolved in the medium. The final concentration of fraction I in the culture medium was 4.34 mg/ml, while that of fractions II and III was 5.04 and 5.56 mg/ml, respectively. Each medium for culture contained the same amount of protein i.e. 1.2 mg/ml.

Three control materials were prepared as follows. Controls I and II were cultivated in the media containing the productions from the above-mentioned controls I and II, respectively. Control III was cultivated in the ordinary medium.

2. *Light microscopical observation.* Before preparing ultrathin sections for electron microscopy, the sections, 4000–6000 nm thick, were stained with 1% toluidine blue in 0.1 M phosphate buffer, pH 7.0.

3. *Electron microscopical observation.* After the culture was started, at 1, 3, 6 hours and, thereafter, every 6 hours, up to 96 hours, 18 times altogether, the specimens were fixed with 3.0% glutaraldehyde in a 0.15 M cacodylate buffer at pH 7.4 containing 7.5% sucrose for 6 hours. After postfixation with 0.5% osmium tetroxide in the same buffer for 1 hour, they were dehydrated in a series of alcohols of increasing concentration, and embedded in Epon 812. Ultrathin sections were obtained by using an ultramicrotome (Reichert Ultracut), stained with uranyl acetate and lead citrate, and studied in an electron microscope (JEOL 100CX).

RESULTS

A. *Enzyme fractions*

Table I shows the protein contents and molecular weights of enzyme fractions I, II and III, and controls I and II.

Enzyme fractions I, II and III contain a larger quantity of protein than the controls. On comparing the three enzyme fractions, however, the percentages of protein are very similar, i.e. 27.67, 23.83 and 21.57%, respectively.

Fraction II has the largest molecular weight, of 92 000, while fractions I and III have 65 000 and 18 000, respectively.

B. *Effects of the enzyme fractions on human skin in organ culture*

After 96 hours, the specimens cultured in the media containing enzyme fractions II and III, controls I, II and III showed no specific changes of the dermo-epidermal junction and epidermal desmosomes. After 72 hours, the specimen cultured in the medium containing fraction I showed typical acantholysis on light microscopy (Fig. 1).

On electron microscopy, after 18 hours from

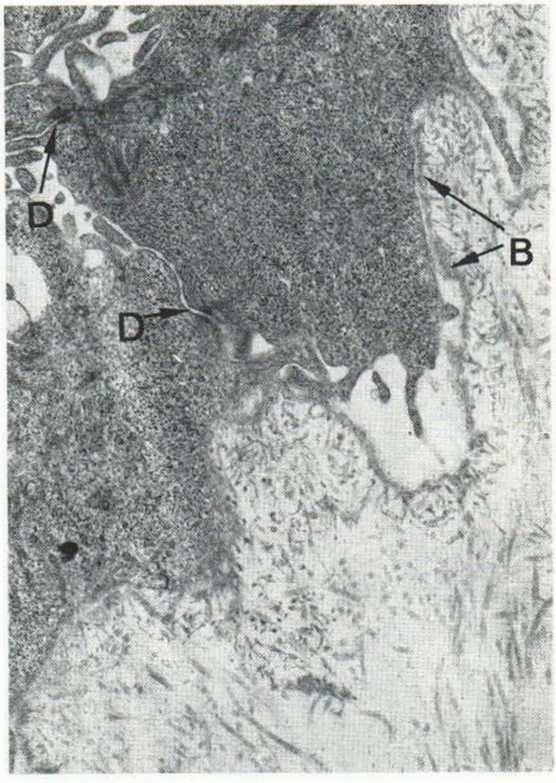

Fig. 3. 30 hours. Space between basal cells and basal lamina (*B*) is partly widened, the intercellular substance of desmosomes (*D*) degraded. ×15 000.

Fig. 4. 54 hours. Separation at subepidermal space more distinct. Basal lamina (*) is ruptured and degraded; some parts are left with anchoring fibrils (arrows). ×30000.

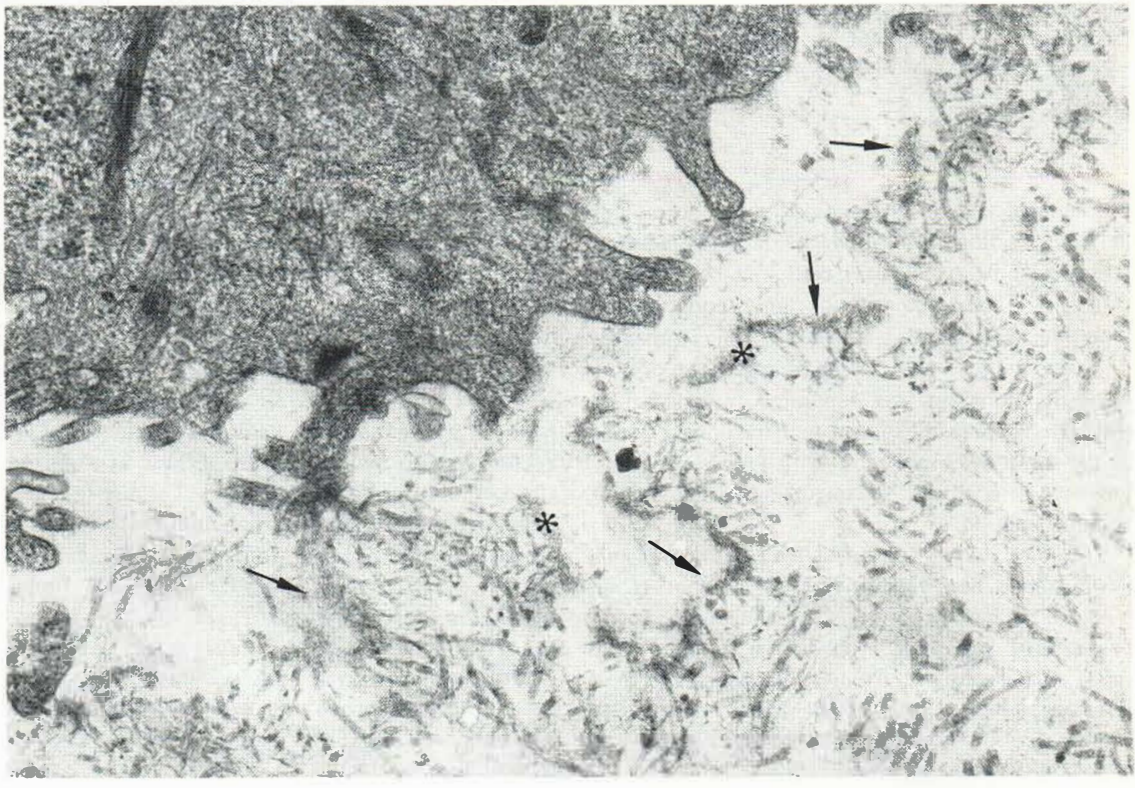

Fig. 5. 72 hours. The dermo-epidermal junction separated. Hemidesmosomes are no longer found on the surfaces of basal cells. Most basal lamina material has disappeared. Some parts are left with anchoring fibrils (arrows). ×15 000.

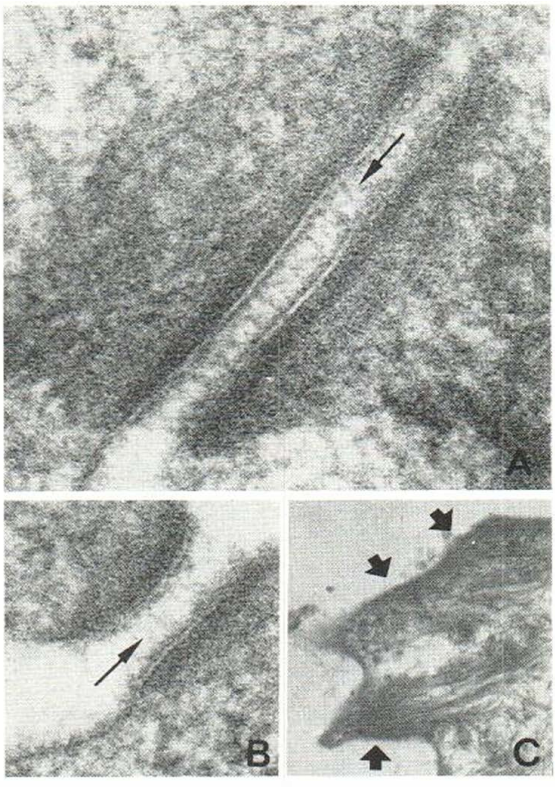

Fig. 6. Desmosomes. (A) 30 hours. The intercellular contact layer has disappeared (arrow). ×150000. (B) 42 hours. The intercellular filamentous structures diminished (arrow). ×150 000. (C) 72 hours. On the surface of acantholytic cells, many ruptured desmosomes with attachment plaques and tonofilament masses are seen (arrows). ×30 000.

the start of culture, separation of the dermo-epidermal junction was seen at the subepidermal space, viz. between basal cells and basal lamina (Fig. 2). This separation became more distinct with time (Figs. 3, 4, 5). The basal lamina appeared ruptured and degraded. After 72 hours, the dermo-epidermal junction was separated in most areas (Fig. 5). Hemidesmosomes were no longer found on the surface of basal cells. With a few exceptions, the anchoring fibrils remained undigested by this enzyme fraction.

The intercellular contact layers of epidermal desmosomes had disappeared by 30 hours (Fig. 6 A). The filamentous intercellular substance disappeared (Fig. 6 B) and, at 72 hours, the epidermal cells were separated from each other. On their surfaces numerous ruptured desmosomes were observed, still maintaining attachment plaques and masses of tonofilaments (Fig. 6 C).

The horny layer of epidermis and hair follicles, as well as envelopes of keratinocytes remained intact.

DISCUSSION

Noval & Nickerson (12) studied *Streptomyces fradiae* which showed keratinolytic activity directed against native keratin. They crystallized the keratinolytic enzyme called 'keratinase', and examined its properties. The optimum pH was 9 when directed against wool; it hydrolyses one peptide, viz. poly-L-lysine (12, 13). Other fungi, e.g. *Microsporum canis*, *M. gypseum*, *Trichophyton schoenleini*, *T. rubrum* and *T. mentagrophytes* also show keratinolytic activity (14–17, 19–21). We used *T. mentagrophytes* which, according to Takiguchi et al. (14, 15), shows higher activity than others.

Yu et al. (19–21) studied keratinases produced by *T. mentagrophytes*. They reported on three kinds of keratinase. One extracellular enzyme was called keratinase I, and two cell-bound enzymes keratinases II and III. Keratinase I digested guinea pig hair, and incubation resulted in the release of the hair protein into the medium. It hydrolysed several different peptides.

In the present paper, we identified three crude enzyme fractions produced by *T. mentagrophytes*, viz. one extracellular enzyme fraction and two cell-bound enzyme fractions, which seemed identical with the keratinases I, II and III of Yu et al. (19–21). In comparison with previous authors, our protein content results were low (14, 15, 19–21). One of the reasons may be that we did not perform gel filtration. Although the molecular weight values which were estimated by the previous authors (14, 15, 19–21) and by us differed, the discrepancy could have been caused by the strain of dermatophyte, the methods of estimation, or the purity of enzymes.

Dobson & Bosley (4) and Einbinder et al. (5) used keratinase for organ culture and observed the specimens by light microscopy. After 30 min, keratinase 0.1% produced dermo-epidermal separation, and by 2 hours acantholytic cells were seen. Einbinder et al. (5) reported that the basal lamina and the mature structure of hair keratin were intact. In this study, however, the basal lamina was degraded by this enzyme fraction, while, prior to the present study, when the influence of fungi on wool was studied by scanning electron microscopy (6), two of us verified that *T. mentagrophytes* destroyed the wool structure and peeled off the cuticle.

The changes in desmosomes and dermo-epidermal junction induced by enzyme fraction I are similar to those induced by elastase (7), papain (8), and trypsin (13), and seem to be characteristic phenomena induced by proteolytic enzymes. Fractions II and III which, according to the previous authors (14–17, 19–21) also showed keratinolytic activity, were expected to induce such phenomena on the skin. However, in the present study, they showed no specific influence on the cultured skin.

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