Hydrolysis of Human Horny Cells by Alkaline Protease: Morphological Observation of the Process

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ABSTRACT: The interaction of highly purified alkaline protease from *Bacillus* sp. KSM-K16 with the horny cells of human skin contained in skin grime was directly visualized by electron microscopy. It became clear that the protease first penetrates the horny cells and then adsorbs, mainly onto the internal structure of the cells at the initial stage of hydrolysis, and directly hydrolyzes the keratin filaments, though the marginal band surrounding them retains its original shape. Then, hydrolysate produced from the keratin filaments flows out of the cell, and early in the hydrolysis process keratin filaments decrease and then disappear, leaving a marginal band, i.e., the cell turns to a hollow state. As a result, the remaining marginal band loses support from inside the cell, thus promoting cleavage and dispersion. Until this stage in the protease reaction, the remarkable liberation of hydrolysis products as water-soluble protein does not occur.

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KEY WORDS: Alkaline protease, *Bacillus* sp. KSM-K16 (K16 protease), electron microscopy, horny cell, keratin filament, laundry detergent, marginal band, morphological observation, skin grime.

Horny cell of human epidermis, contained in skin grime that adheres to a person's collar and sleeves, is one of the typical protein soils that are transferred from the human body by frictional contact between skin and fabric. Epithelial cells combine with human secretions, such as sebum and perspiration, or other external solid particles to form complex soils (1). These soils adhere unevenly to the fiber surface of fabric or are trapped between fibers (2). Each horny cell is a sheet-like, relatively rigid water-insoluble protein, and is highly resistant to the ingredients of heavy-duty detergents. Therefore, once this protein is trapped between fibers, it becomes a soil that is very hard to remove by ordinary home laundering in Japan (2,3).

Conventionally, various alkaline proteases, which exhibit their activities in an alkaline region, have been incorporated into heavy-duty detergents for washing clothes to remove protein soils. Some alkaline proteases show a remarkable detergent effect against not only water-soluble protein soils but also against water-insoluble protein soils, such as skin grime that includes human horny cells (3). The effect of protease on human stratum corneum has been investigated (4,5). However, the mode of action of protease in the degradation of horny cells has not been fully elucidated under laundering conditions. Namely, the relationship between the enzymatic hydrolysis of horny cells and the detergent effect of protease, the detergent mechanism of protease, has not been fully established.

We recently isolated the alkaline protease for laundry detergent produced from *Bacillus* sp. KSM-K16 (K16 protease), which exhibits high activity for hydrolyzing human horny cells. This protease shows excellent detergency against skin grime on collar and sleeves (6,7).

In the present study, the degradation process of human horny cells in skin grime by K16 protease was investigated morphologically by transmission electron microscopy (TEM) and by scanning electron microscopy (SEM), and the distribution of the enzyme on the cross-section of horny cells during the proteolysis was directly visualized by double-labeling immunoelectron microscopy to elucidate the detergent mechanism of protease at the macromolecular level.

EXPERIMENTAL PROCEDURES

Enzyme source. The alkaline protease (K16 protease) used in the present work was obtained in a highly purified state through several steps of column chromatography as reported previously (6), starting from a two-day culture broth of *Bacillus* sp. KSM-K16. The molecular weight was estimated to be approximately 28,000 by sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis. The specific activity of the purified protease was 117.1 Anson units/g-protein (8). The reactivity of the enzyme in 10-min assay with casein (Art. 2242; E. Merck, Darmstadt, Germany) as a substrate was maximum at pH 12.3 and at 55°C.

Enzymatic hydrolysis of horny cells. The specimen of horny cells was prepared as a model substrate of accumulated cells on a naturally soiled collar after home laundering in Japan—cotton cloth was scraped against human neck epidermis, and was suspended in 0.1 M sodium carbonate buffer (pH 10.5, a standard pH of laundry detergent's liquor). Horny

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cells were collected from the cloth by centrifugation. The collected cells were washed with distilled water, and then dried in a vacuum desiccator. The reaction mixture consisted of 0.02 wt% of the dried horny cells, 0.05 M sodium carbonate buffer (pH 10.5), and the enzyme (12.2 nM of K16 protease) in a total volume of 10 mL. After incubation for an appropriate period at 20°C under mechanical shaking (50 strokes/min), 0.6 mM of phenyl methyl sulfonyl fluoride was added to the reaction mixture to stop the enzymatic hydrolysis, and then the mixture was centrifuged at 5°C. The liberated soluble protein in the supernatant was determined as bovine serum albumin (Sigma Chemical, St. Louis, MO) by the method of Lowry *et al.* (9). The residual cells collected on a glass filter were subjected to electron microscopy after being washed thoroughly with distilled water.

Electron microscopic observation of degraded horny cells. Structural change of horny cells during the treatment with K16 protease was followed by electron microscopy procedures referred to in our previous papers (10,11). The collected cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde dissolved in a phosphate-buffered saline (PBS), Dulbecco's PBS (Nissui Pharmaceutical, Tokyo, Japan), for 30 min, and then washed five times with PBS. After being postfixed for 2 h in 2% osmium tetroxide dissolved in PBS, they were rinsed three times with PBS (12). They then were dehydrated by 30-min incubation with 25, 50, and 75% ethanol at 5°C, and incubated in 100% ethanol at 5°C. The prepared samples thus obtained were embedded in Durcupan epoxy resin (Ladd Research Ind., Burlington, VT) at 5°C, and the resin was polymerized at 37, 40, 50, and 60°C for 6 h each. Ultrathin cross-sections (60–100 nm thick), prepared with diamond knives (Diatome, Bienne, Switzerland) on an ultramicrotome (MT2-B; Sorvall, Newtown, CT), were stained with uranyl acetate for 10 min, followed by lead staining solution for 10 min by the method of Sato (13), and finally examined under a transmission electron microscope (H-7000; Hitachi, Tokyo, Japan) at 75 kV accelerating voltage.

The surface condition of the residual cells, collected on a glass filter as described previously, was also examined with a scanning electron microscope (T-330A; JEOL, Tokyo, Japan) at 15 kV after critical-point drying.

Double-labeling immunoelectron microscopic observation of adsorbed protease. The distribution of adsorbed proteases on the cross-section of horny cells at the initial stage of hydrolysis was observed by double-labeling immunoelectron microscopy based on the method of Geuze *et al.* (14). The horny cells, treated with K16 protease under the standard conditions, were fixed with 2% paraformaldehyde for 1 h and were washed five times with PBS. They were then dehydrated and embedded in resin (as mentioned previously). Ultrathin cross-sections of horny cells were then soaked in 1% bovine serum albumin (Sigma Chemical), dissolved in PBS containing 0.1% Triton X-100 (Sigma Chemical), at 20°C for 30 min to cover the parts without adsorbed enzyme. Then the purified anti-protease antibody (diluted 1:10 with PBS) prepared from rabbit serum was used to treat the ultrathin cross-section at 30°C for 1 h in PBS. Goat anti-rabbit IgG antibody, labeled with colloidal gold (G-3766; Sigma Chemical) as the second antibody (15) at the dilution ratio of 1:50, was reacted with the ultrathin cross-section at 30°C for 1 h. After being stained with uranyl acetate and lead staining solution, the distribution of colloidal gold in the ultrathin cross-sections was observed by TEM.

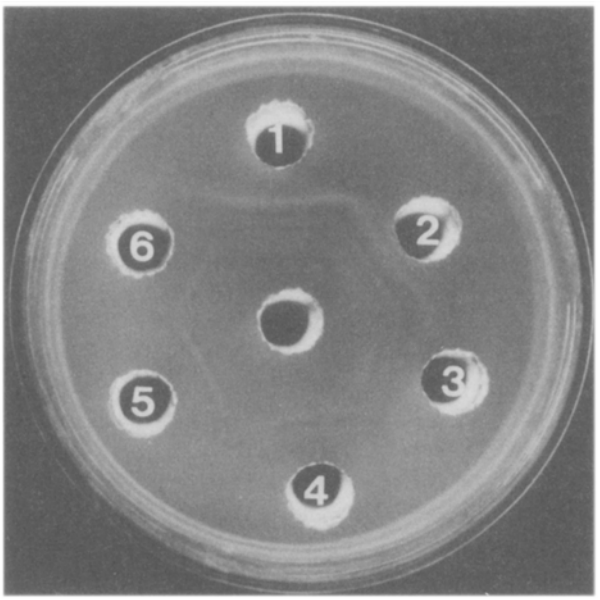

FIG. 1. Reactivity of the anti-protease antibody examined by Ouchterlony double-diffusion. The central well contained 36 μM of purified K16 protease. Wells 1, 2, 3, 4, 5, and 6 contained undiluted antibody against purified K16 protease (2.0 mg/mL), diluted 1:2, 1:4, 1:8, 1:16, and 1:32 with phosphate-buffered saline (PBS). All samples were dissolved in PBS.

Preparation of anti-protease antibody. Rabbits (Japanese whites) were immunized by subcutaneously injecting 1 mg of purified K16 protease three times (at weekly intervals) in the presence of Freund's complete adjuvant. The IgG fraction was precipitated from the serum with ammonium sulfate and further purified by ion-exchange chromatography (16). Reactivity of the antibody was examined by Ouchterlony double-diffusion (17) on 1% agarose plates in PBS (pH 7.4) (Fig. 1).

RESULTS AND DISCUSSION

Horny cells, which are transferred from human epidermis to fabric, show a characteristic pattern of soiling; that is, they get caught between fibers. This differs sharply from the permeation-type soiling of water-soluble proteins, which are easily removed by ordinary home laundering in Japan (2,3). To clarify the mode of action of alkaline protease that contributes to the removal of water-insoluble horny cells, the hydrolysis process of the cells by alkaline protease was investigated. The alkaline protease, which was newly isolated for laundry detergent, K16 protease from *Bacillus* sp. KSM-K16, was allowed to react on horny cells sampled from human neck epidermis under typical low-temperature laundering conditions in Japan [at pH 10.5 and 20°C (18)].

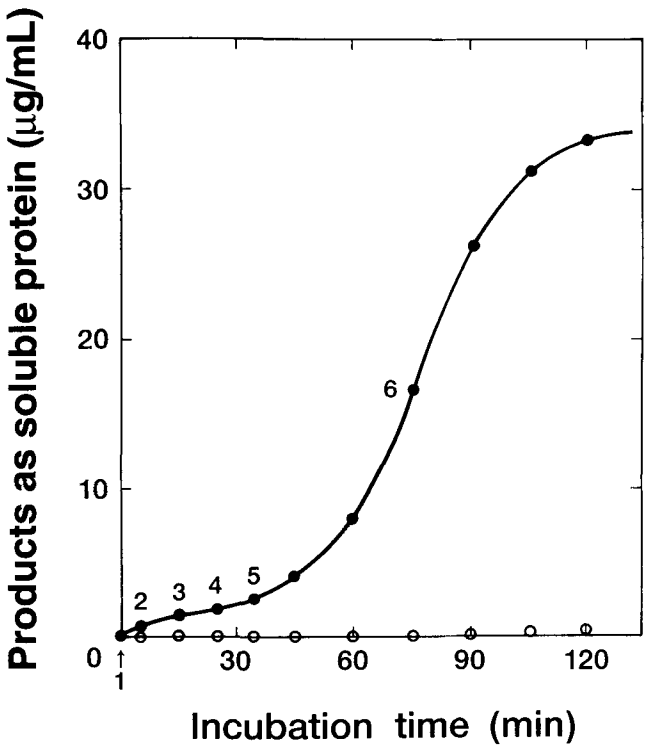

FIG. 2. Time course of the production of soluble protein from horny cells by K16 protease. ●, Incubation with K16 protease; ○, incubation without protease (control) at pH 10.5 and 20°C. The reaction mixture consisted of 0.02 wt% of horny cells and 12.2 nM of K16 protease. The numbers above the curve show the hydrolysis stages at which morphological observations were carried out. The results of the observations are shown in Figures 3, 4a, and 4b for stage 1, Figures 5 and 9 for stage 2, Figure 6 for stage 3, Figures 4c, 4d, and 7 for stage 4, and in Figure 8 for stage 5.

Figure 2 shows the time course of the production of soluble protein formed from horny cells during the incubation with K16 protease. The soluble protein was released slightly at earlier stages of the enzymatic hydrolysis (before 35-min incubation), and it is clear that production of the hydrolysate was remarkably increased at later stages (after 35-min incubation). Consequently, a two-step production of the hydrolysate was observed. In contrast, trace liberation of soluble protein was observed during 2-h incubation without the enzyme.

Morphological changes of the horny cells with the progress of enzymatic hydrolysis were observed by means of electron microscopy to investigate the characteristic pattern of hydrolysate production. Figure 3 shows a result of TEM observation of the cross-section of horny cells before being affected by protease action, that is, immediately after being suspended in an alkaline buffer containing K16 protease (at stage 1 of Fig. 2). It is generally accepted that, as observed with an electron microscope, a horny cell consists mainly of keratin filaments (KF) inside the cell (19), and a marginal band (MB) surrounding them (20). The MB, located along the outer frame of the cell, and the KF are clearly observed in Figures 3b and 3c, respectively. The surface condition of horny cells at the same stage is also visualized by SEM (Figs. 4a and 4b). Thick, sheet-like horny cells are shown in these photographs.

Figure 5a shows a TEM photograph of horny cells at an early stage of protease action (after 5-min incubation; stage 2 of Fig. 2). A higher magnification view of one of the cells, which was typical at this stage, is shown in Figure 5b. Cross-sections of external and internal parts of the cell [note the sites with (E) and (I) in Fig. 5b] at higher magnification are

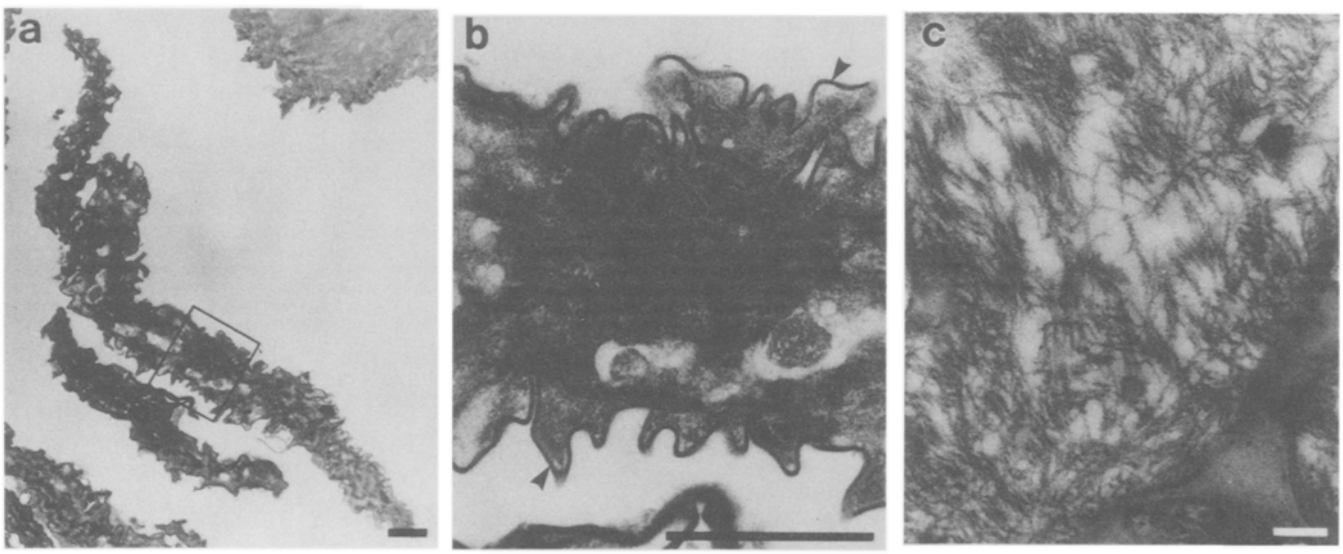

FIG. 3. Transmission electron micrographs of the ultrathin cross-section of horny cells before being affected by enzymatic proteolysis. a, Horny cells incubated with K16 protease for 10 s at pH 10.5 and 20°C; b, high-magnification view of a; c, high-magnification view of keratin filaments inside the cell. The reaction mixture contained the same components as that in the legend to Figure 2. Arrowheads in b indicate a marginal band. Bar: a and b, 1.0 μm; c, 0.1 μm.

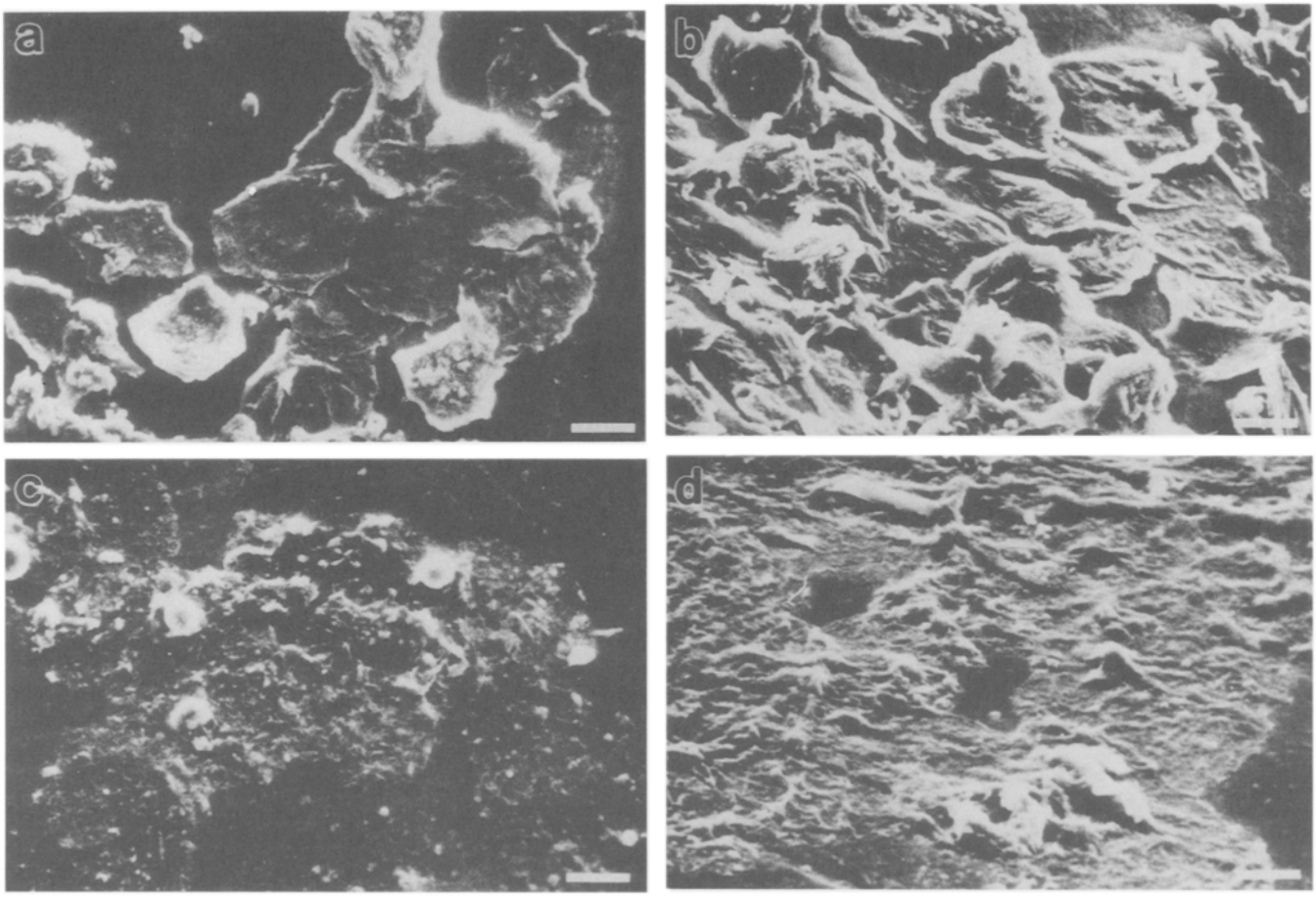

FIG. 4. Scanning electron micrographs of the surface condition of horny cells. a and b, Horny cells incubated with K16 protease for 10 s; c and d, horny cells incubated with K16 protease for 25 min at pH 10.5 and 20°C. The reaction mixture contained the same components as that in the legend to Figure 2. Front and oblique views of the cells are shown in a, c, and b, d, respectively. Bar, 10 μm.

shown in Figures 5c and 5d, respectively. It is clear that some clusters flow out of the cell (Fig. 5c), and a keratin pattern inside the cell becomes obscure (Fig. 5d), despite the early stage of hydrolysis. A cross-section of the cells after 15-min incubation with the protease (at stage 3 of Fig. 2) is shown in Figure 6, and it shows that the KF located in the cells has already decreased substantially. As shown in Figure 7, it is clear that almost all KF has disappeared, leaving the MB and hollowing the cell after 25-min incubation (at stage 4 of Fig. 2). Scanning electron micrographs of the cells at the same stage shown in Figure 7 indicate that the thickness of the cells has decreased and the morphology is rather flat (Figs. 4c and 4d). After this stage, i.e., at stage 5 of Figure 2 and later, cleavage of MB accompanied by dispersion was observed (Fig. 8).

The morphological changes of horny cells were observed only under the influence of protease action. Separation of aggregated cells into individual ones was observed during incubation without enzyme, but hollowing or disintegrating of individual cells was not observed. This fact is consistent with the trace liberation of soluble protein produced from horny cells without protease action (Fig. 2). Moreover, the previously mentioned mode of action of K16 protease in the hydrolysis of horny cells is common to other alkaline proteases because alkaline protease from *Bacillus licheniformis* (Type VIII: Bacterial; Sigma Chemical) showed a similar mode of action against horny cells, although its hydrolysis velocity for KF was smaller than that of K16 protease. The activities of K16 and Type VIII proteases were 5,620.8 and 542.8 KF units/g-protein, respectively. One KF unit of protease activity is defined as the amount of enzyme that catalyzes the liberation of solubilizable proteins equivalent to 1 mg of tyrosine per min from a 0.1% solution of KF, which was prepared from human horny cells, as reported previously (6,7), at 30°C and pH 10.5. It has also been reported that the horny layer became transparent upon treatment with bacterial proteases, although detailed transformation of each cell was not clearly observed (3).

Figure 9 shows the localization of adsorbed K16 proteases in the cross-section of horny cells at an early stage of enzymatic hydrolysis corresponding to Figure 5 (after 5 min of incubation under the standard conditions), as observed by double-labeling immunoelectron microscopy. It is clear that K16 protease is adsorbed mainly on internal cell structures, although a horny cell has a MB with a strong crosslinking struc-

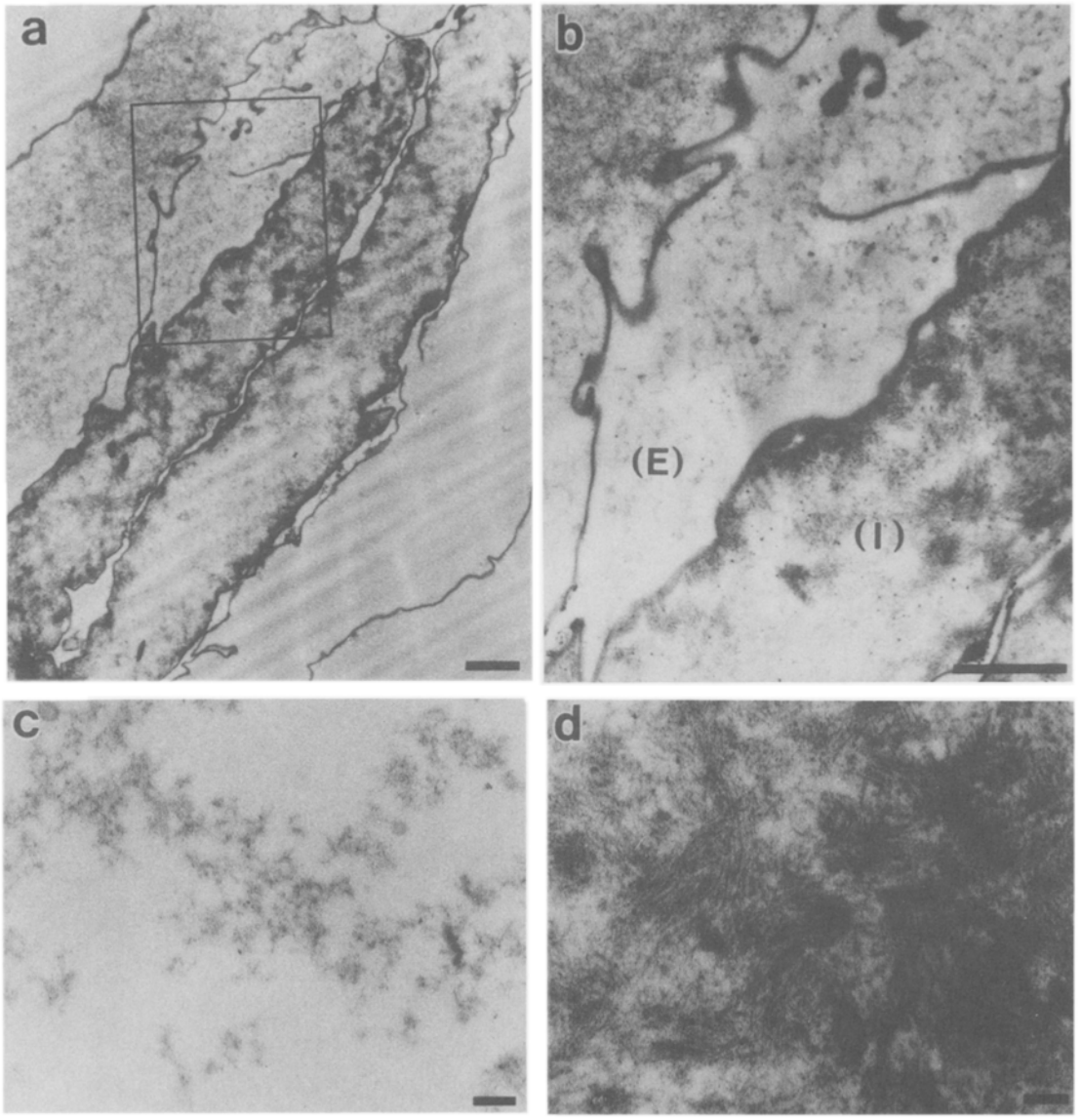

FIG. 5. Transmission electron micrographs of the ultrathin cross-section of horny cells at the early stage of enzymatic proteolysis. a, Horny cells incubated with K16 protease for 5 min at pH 10.5 and 20°C; b, high-magnification view of a; c, higher-magnification view of external part (E) of the cell shown in b; d, higher-magnification view of internal part (I) of the cell shown in b. The reaction mixture contained the same components as that in the legend to Figure 2. Bar: a and b, 1.0 μm; c and d, 0.1 μm.

ture (20). Hence, it is suggested that the protease penetrates the cells to perform enzymatic hydrolysis of internal KF even at the early stage of hydrolysis. Because MB isolated from horny cells is so resistant to protease action that the hydrolysis velocity of K16 protease for MB is very slow (Hoshino, E., K. Maruta, Y. Wada and K. Mori, unpublished results), it can be inferred that the protease passed through MB swelled in alkaline buffer (21) or through the cracks of MB that had already been damaged without enzymatic hydrolysis.

A possible process of enzymatic proteolysis against horny cells, which are pretreated under alkaline conditions, is summarized in Figure 10. At the initial stage of enzymatic reaction, protease passes through MB and directly hydrolyzes KF in the cell (stage 2). Then, hydrolysate produced from KF flows out of the cell, and early in the reaction process KF decreases and disappears, that is, the cell is turned to a hollow state (stage 3–4). As a result, the remaining MB loses support from inside the cell, thus promoting cleavage and dispersion (stage 5). This hydrolytic mode of the enzyme may contribute to the removal of horny cells during laundering process. It also appears that later in the enzymatic hydrolysis, after stage 5, the hydrolysate produced from KF that has flowed out of the cell is digested further by protease action. It is not until this stage that a drastic liberation of water-soluble protein occurs (stage 6), and a characteristic two-step liberation of the products seems to be observed (Fig. 2). This appears to be

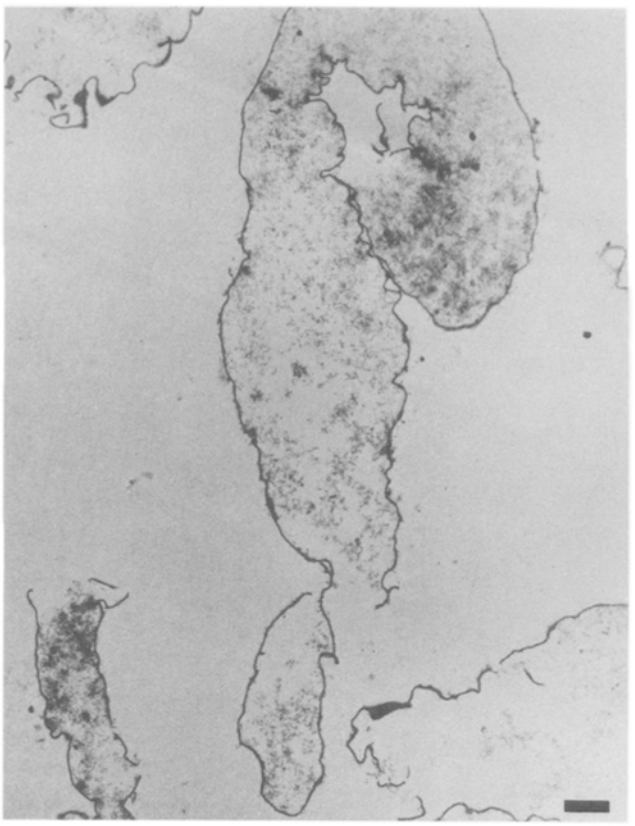

FIG. 6. Transmission electron micrograph of the ultrathin cross-section of horny cells after 15-min incubation with K16 protease. Reaction conditions were the same as those in the legend to Figure 2. Bar, 1.0 μm.

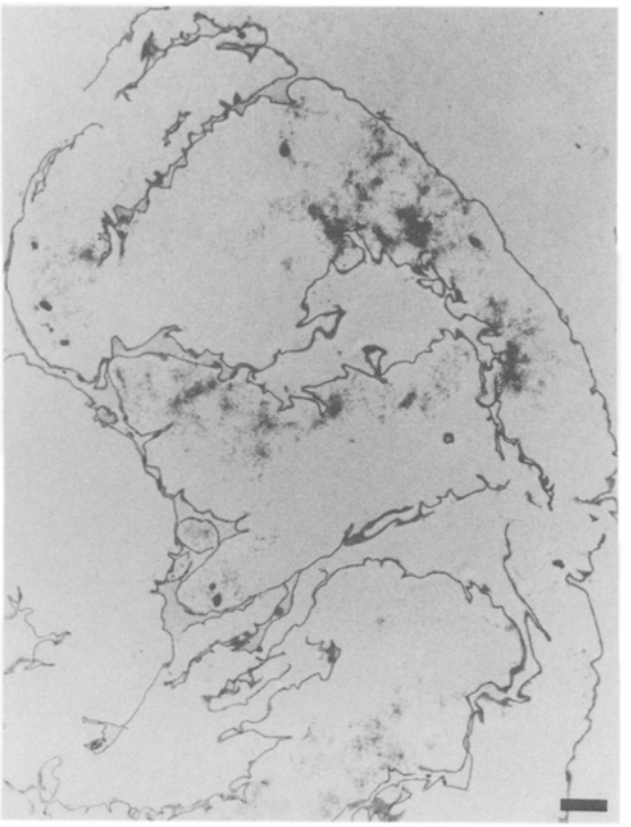

FIG. 7. Transmission electron micrograph of the ultrathin cross-section of horny cells after 25-min incubation with K16 protease. Reaction conditions were the same as those in the legend to Figure 2. Bar, 1.0 μm.

caused by the protease reaction's priority for KF inside the cell to that for the hydrolysate from KF. Details, however, remain to be determined.

It has been widely accepted that protease hydrolyzes the outside of horny cells first and then destroys the cells in the laundering process. However, the present work suggests that protease promotes the disintegration and dispersion of the cells by hollowing them, and that an alkaline protease, which exhibits a high activity for hydrolyzing KF inside the cell, contributes to excellent detergency against skin grime.

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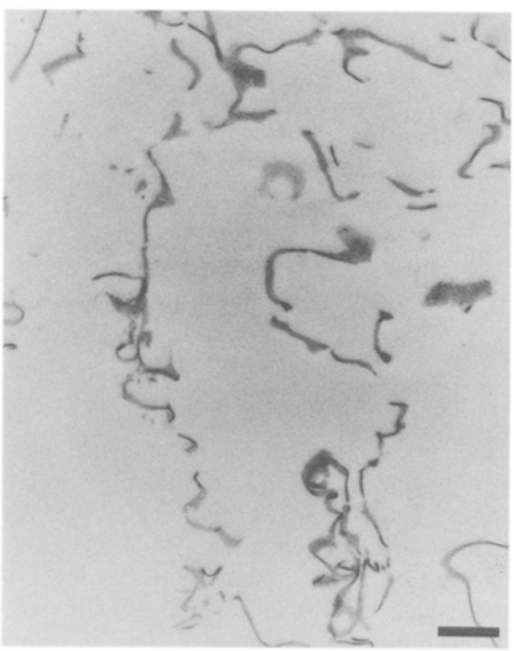

FIG. 8. Transmission electron micrograph of the ultrathin cross-section of horny cells after 35-min incubation with K16 protease. Reaction conditions were the same as those in the legend to Figure 2. Bar, 1.0 μm.

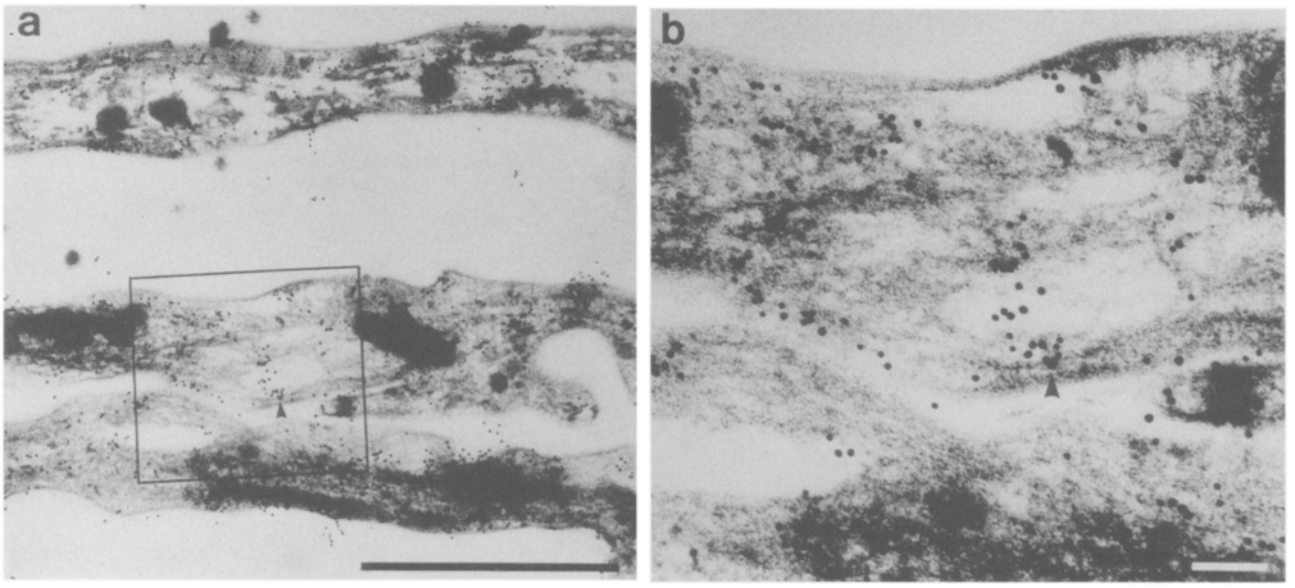

FIG. 9. Double-labeling immunoelectron micrographs of adsorbed proteases on ultrathin cross-section of horny cells. a, Horny cells incubated with K16 protease for 5 min at pH 10.5 and 20°C; b, high-magnification view of a. The reaction mixture contained the same components as that in the legend to Figure 2. Arrowheads indicate some of the colloidal gold particles. Colloidal gold particles, which are due to nonspecific binding to horny cells, were not observed. Bar: a, 1.0 μm; b, 0.1 μm.

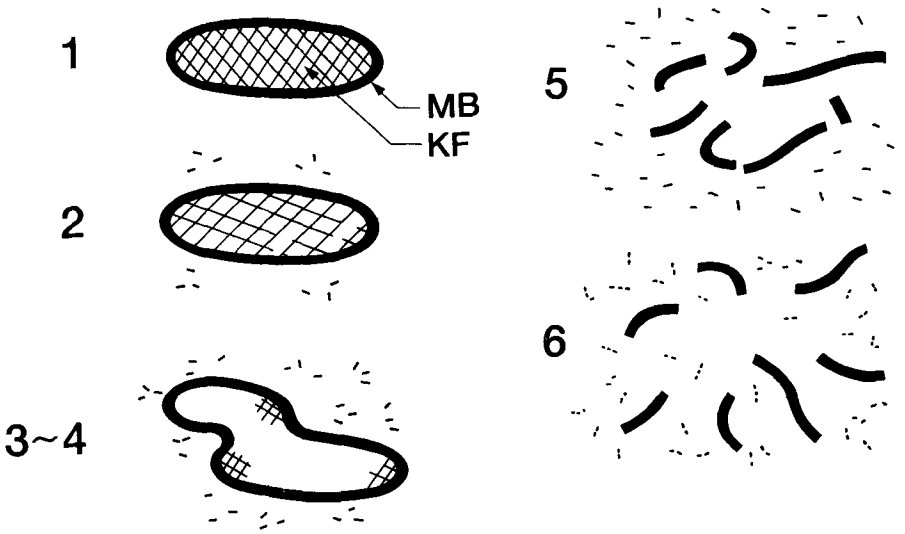

FIG. 10. Proposed mechanism for the enzymatic degradation of horny cell by alkaline protease. MB, marginal band; KF, keratin filaments.

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