New Detergent Mechanism with Use of Novel Alkaline Cellulase

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Microscopic studies of naturally soiled cotton undershirts showed that there was sebum in the microscopic spaces in the interior of the cotton fibers. Ordinary detergents did not remove this soil satisfactorily, although they readily removed sebum on the exterior of the fibers. Alkaline cellulase, which was compatible with the alkaline ingredients of detergents and which interacted selectively with celluloses in interfiber space in the interior of fiber, effectively removed sebum soil in the interfiber spaces in the presence of usual detergent ingredients. The removal of soil by the hydrolysis of the amorphous regions of fibers with cellulase is a new detergent mechanism.

KEY WORDS: *Bacillus* sp., cellulase, cotton fiber, cotton undershirt, interfiber space, microscopic study, detergent mechanism, sebum soil.

Synthetic heavy-duty detergents now commercially available have good detergency as a result of numerous technical improvements over the forty years since the invention of detergents. At present, the degree of detergency seems to have peaked; all detergents contain similar ingredients and are based on the same mechanisms. In these mechanisms, soil adsorbed onto the surfaces of fibers or in their interstices is removed by surfactants and builders, which lower interfacial tension and enhance the repulsive force between the soil and the fabric (1,2). Proteases are often used to hydrolyze proteins in soil (3). All of these mechanisms involve interaction between the ingredients of the detergent and the surfaces of the fabric and the soil. In order to find a new, drastically effective ingredient, more microscopic study on soiled fiber, especially natural fiber such as cotton, and a new detergent mechanism are required.

EXPERIMENTAL PROCEDURES

The alkaline cellulase to be used was first obtained as the crude enzyme from a two-day culture broth of *Bacillus* sp. KSM-1001 precipitated with a 75% saturated solution of ammonium sulphate and dissolved in 200 mL of 10 mM Tris/HCl buffer at pH 7.5. The enzyme solution was dialyzed for 24 hr against 2 L of 10 mM Tris/HCl buffer at pH 7.5. Then, 100 mL of the concentrated solution was put on a DEAE-Toyopearl (Toyo Soda Mfg. Co., Tokyo, Japan) ion-exchange column (4.4 cm × 33 cm) equilibrated with 10 mM Tris/HCl buffer at pH 7.5. After the column was thoroughly washed with the same buffer, the crude enzyme was eluted with 0.1, 0.2 and 0.3 M NaCl in the same buffer at the flow rate of 180 mL/hr. The CMCase (a cellulase activity against carboxymethyl cellulose) containing the main cellulase component was eluted at 0.3 M NaCl. The fractions of the enzyme were collected and concentrated. Then 100 mL of the concentrated solution was put on a hydroxyapatite (Seikagaku Kogyo Co., Tokyo, Japan) column (3.2 × 23 cm) equilibrated with 5 mM phosphate buffer at pH 7.0, and eluted with 100, 125 and 150 mM of the same buffer at the flow rate of 20 mL/hr. The purified enzyme was eluted at 150 mM buffer and collected. A single peak of protein was found in ultracentrifugation and SDS-polyacrylamide gel electrophoresis, whereby the molecular weight was estimated to be 130,000. No β-glucosidase activity was detected during the hydrolysis of *p*-nitrophenyl β-D-glucoside and cellobiose in 24-hr assays. The production of reducing sugars by the enzyme in 10-min assays with the use of CMC was maximum at pH 9.5 and at 40°C. The CMCase activity was stable for 24 hr at 5°C between pH 5 and 11, but decreased rapidly at pH below 5. Thermal stability decreased linearly between 40 and 60°C.

The activity of the alkaline protease (*Bacillus licheniformis*, Type VIII: Bacterial, Sigma Chemical Co., St. Louis, MO) was 32.5 Anson units/g (4). One gram of the alkaline cellulase (*Bacillus* sp. KSM-1001) had 1500 units of activity. One unit of activity of the alkaline cellulase was defined as the amount of enzyme that catalyzed the liberation of reducing sugar equivalent to 1.0 μmol of D-glucose per min from a 0.35% solution of CMC (A01MC, DS 0.68, DP 200, Sanyo Kokusaku Pulp Co., Tokyo, Japan) at 40°C and pH 9.0.

The heavy-duty detergent consisted of 25% sodium linear alkyl benzene sulphonate (mean length of alkyl chain, 11.7), 20% zeolite 4A, 10% sodium silicate (Na_2O/SiO_2, 1/2.3 by weight), 10% sodium carbonate, 1% polyethyleneglycol (MW 6000), 1% sodium polyacrylate (MW 10,000), 0.5% enzyme (protease or cellulase), and 5% moisture; the remainder was sodium sulphate.

Two cotton undershirts (100% cotton, Gunze Co., Tokyo, Japan) were extracted with chloroform for 9 hr in a Soxhlet extractor, one of which had been worn for two days without being laundered. Twelve swatches measuring 5 × 5 cm were cut from the front of each undershirt. Four swatches of soiled undershirt were washed with the detergent incorporating the alkaline cellulase or that incorporating the alkaline protease in a Terg-O-Tometer (5). The swatches to be washed were soaked in 200 mL of 0.67% detergent solution for 1 hr at 30°C. After the solution was diluted with 800 mL of water, the swatches were agitated for 10 min at 20°C in a Terg-O-Tometer. Ordinary Japanese tap water with about 55 ppm of $CaCO_3$ was used.,

The yarn taken from the swatches were treated in 1% osmium tetroxide buffered with 20 mM phosphate at pH 7.0 for 3 hr (6,7). They were then rinsed three times in purified water. After being dried, the yarns were embedded in resin of low viscosity (Aquembed, Ladd Co., Burlington, VT). Ultrathin sections 600–1000 × 10^{-10} m thick were prepared with diamond knives (Diatome Co., Bienne, Switzerland) on an ultramicrotome (MT2-B, Sorvall Co., Newtown, CT). The presence of osmium tetroxide combined with unsaturated oil was looked for under an optical microscope (Microphot-FX, Nikon Co., Tokyo, Japan; Fig. 1) and transmission electron microscope (JEL-100CX, JEOL Co., Tokyo, Japan; Fig. 2). The cross-

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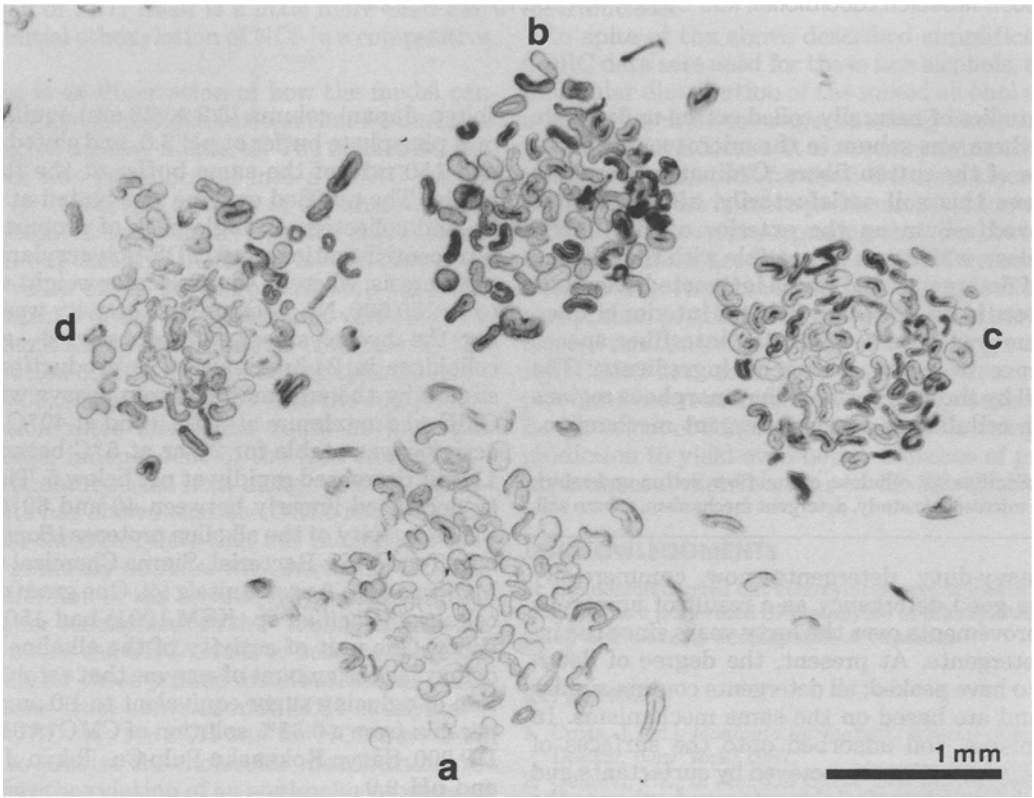

FIG. 1. Optical micrographs of ultrathin sections of yarns in cotton undershirt stained with osmium tetroxide. a, Yarn of unsoiled cotton undershirt; b, naturally soiled undershirt; c, undershirt washed with a detergent incorporating alkaline protease; and d, washed with a detergent incorporating alkaline cellulase. For evaluation of the whiteness of the yarn, the gray-scale of fibers on a photograph was measured by use of an image analyzer (Quantime T-720, Leica Cambridge Ltd., Cambridge, England) (8). The mean value on the gray-scale for each yarn was 11.56 for the unsoiled yarn, 9.57 for the naturally soiled yarn, 9.64 for yarn washed with a detergent incorporating alkaline protease, and 11.37 for yarn washed with a detergent incorporating alkaline cellulase.

sections of fibers in cotton undershirts stained with osmium tetroxide were also examined by scanning electron microscopy (JSM-35C, JEOL Co., Tokyo, Japan) and an energy-dispersive X-ray microanalyzer (Kevex 7000, Kevex Co., San Carlos, CA). Fractured surfaces of the fibers were prepared by cutting with a glass knife. Counts of X-ray emissions for osmium at the energy levels of 1.91, 8.91 and 10.35 keV were recorded at selected locations within and around the fiber by using a spot probe (0.1 μm^3). White points on the pictures of the cross sections were referred to osmium complexed with sebum soil as shown in Figure 3.

Double-labelled immunoelectron micrographs of ultrathin sections of cotton fibers were observed as follows: First, 1 g of cotton yarn was sampled from the cotton undershirt treated with chloroform, and soaked in 100 mL of enzyme solution buffered with 0.05 M sodium carbonate at pH 9 for 1 hr at 30°C. The activity of alkaline protease was 8.3×10^{-3} Anson units/mL of solution, and that of the alkaline cellulase was 6.2 units/mL of solution. The yarn treated with enzyme was incubated in Karnovsky fixative (7) for 3 hr to fix the adsorbed enzyme. Ultrathin sections of fibers were then soaked in 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) for 30 min to cover the parts without adsorbed enzyme. Then 320 ppm of purified anti-cellulase antibody or purified anti-protease antibody prepared from rabbit serum was used to treat the ultrathin section for 2 hr at 20°C in phosphate-buffered saline (20 mM of buffer with 150 mM NaCl at pH 7.5). Goat anti-rabbit IgG antibody labelled with colloidal gold (Auro Probe EM, Janssen Life Sciences Products Co., Beerse, Belgium) as the second antibody was reacted with the ultrathin section under the same conditions mentioned above. The distribution of colloidal gold in the ultrathin sections was observed by transmission electron microscopy (Fig. 4).

The composition of the soil in the naturally soiled undershirt was found by gas chromatography of chloroform extracts. Natural soil was 26.3% triglycerides, 9.8% diglycerides, 1.5% monoglycerides, 10.2% squalene, 16.1% waxes, 4.5% cholesterol esters or other sterol esters, 2.5% cholesterol or other sterols, and 29.1% other. Residual soil after washing of the swatches in the detergent with alkaline protease was 29.9% triglycerides, 11.5% diglycerides, 0.4% monoglycerides, 9.1% squalene, 8.4% waxes, 6.0% cholesterol esters or other sterol esters, 3.9% cholesterol or other sterols, and 30.8% other.

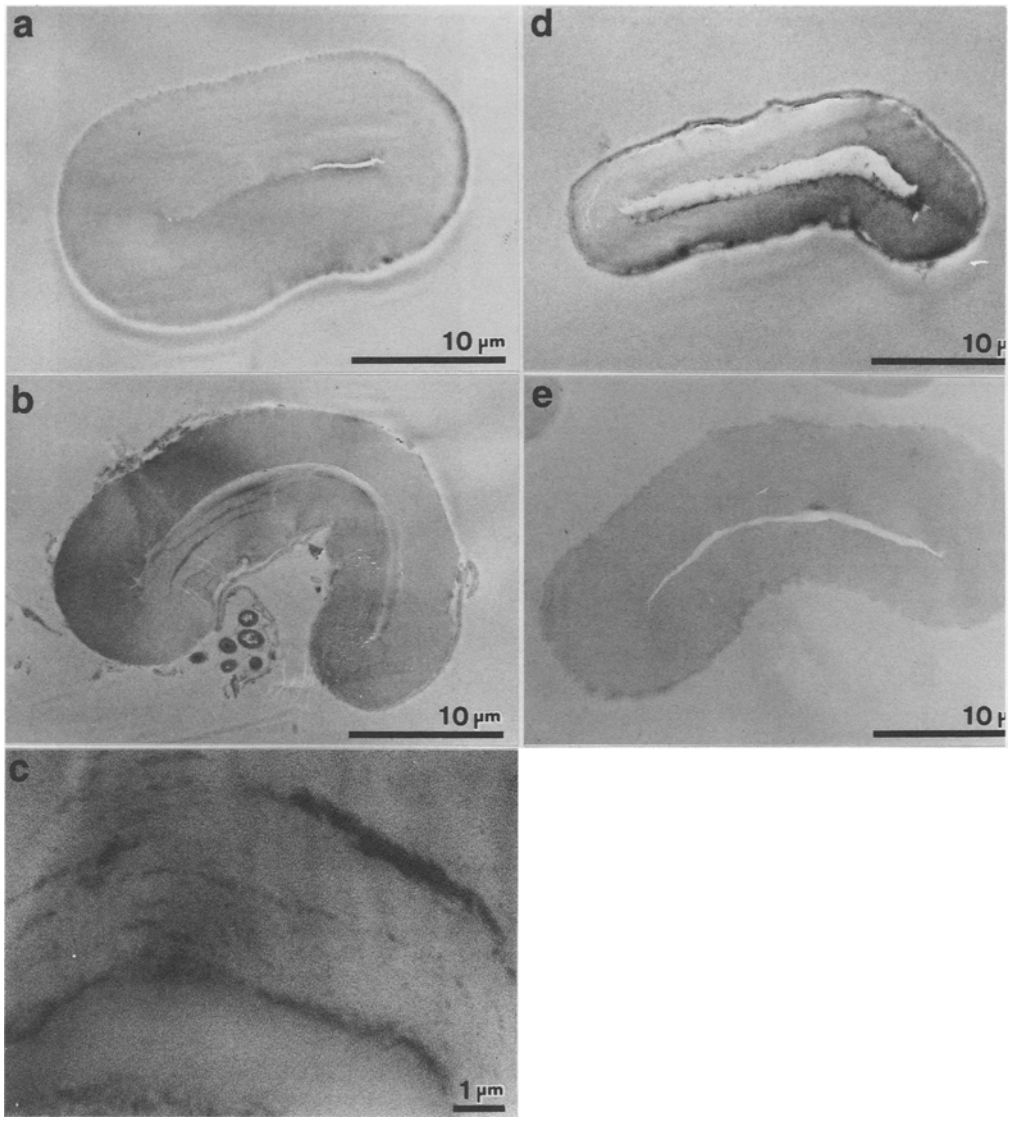

FIG. 2. Transmission electron micrographs of ultrathin sections of fibers in cotton undershirt stained with osmium tetroxide. The cotton fibers were a, unsoiled; b and c, naturally soiled; d, washed with a detergent incorporating alkaline protease; and e, washed with a detergent incorporating alkaline cellulase. The fibers shown were taken from yarns with samples shown in Figure 1, being selected so as to have the same gray-scale value as the mean gray-scale value of the corresponding yarn.

RESULTS AND DISCUSSION

When cotton fibers were soiled with triolein dissolved in toluene, much triolein was deposited in the interfiber spaces in the interior of individual cotton fibers (9). We studied soil in the interior of cotton fibers soiled naturally. Optical micrographs of ultrathin sections of cotton yarns in unsoiled and naturally soiled cotton undershirts, which were stained by osmium tetroxide, are shown in Figure 1a and b, respectively. Electron micrographs of the same preparations of fibers are shown in Figure 2a and b. Both the interior and exterior of the soiled fiber was stained. The unsoiled fiber was very weakly stained. An ultrathin section of the same naturally soiled fiber at more magnification is shown in Figure 2c. Black curved lines in the shape of interfiber spaces (10) were seen in the interior of the fiber, and there was no staining of the highly crystalline lamellae of cellulose, where even water cannot enter (11). Fractured surfaces of unsoiled and naturally soiled cotton fibers observed with an electron probe microanalyzer are shown in Figure 3a and b, respectively. White spots arising from X-rays specific to osmium were seen in the interior of the soiled fibers. While in the unsoiled fibers, only traces of contaminating osmium were found. The soil was extracted with chloroform from the naturally soiled undershirt used in Figure 1b. After extraction, osmium tetroxide did not stain the interior of the fibers. The composition of the extracted soil as identified by gas chromatography was in good agreement with that of sebum reported previously (12). Thus, the stains in Figures 1b and 2b, the curved stain in Figure 2c, and

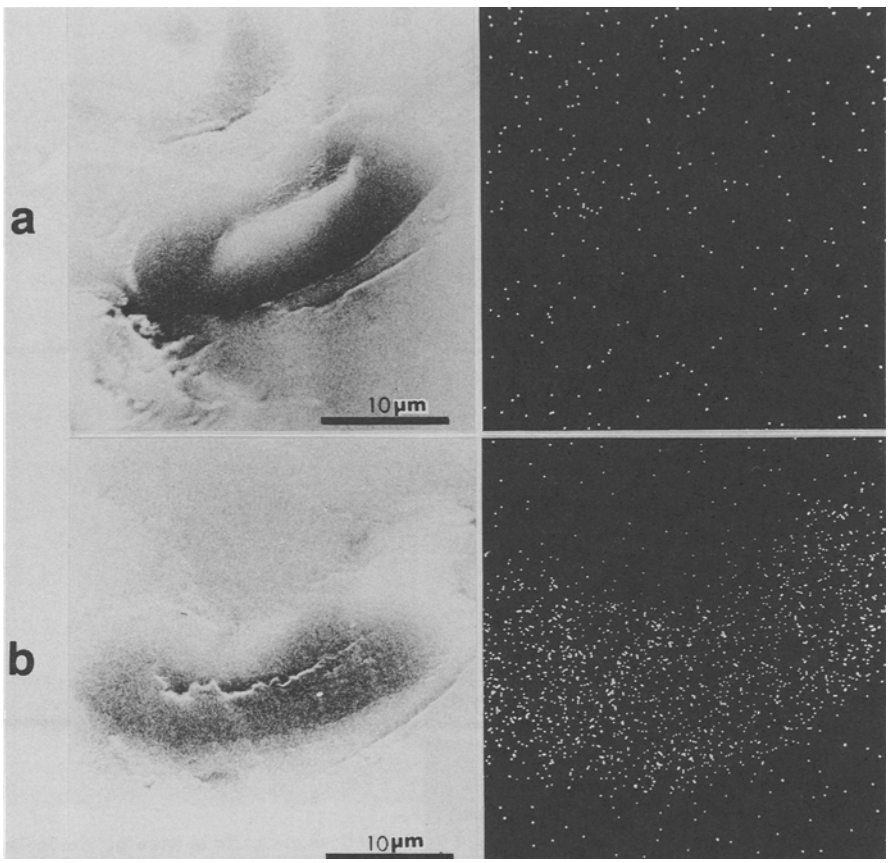

FIG. 3. Electron probe microanalysis of cross-sections of fibers in cotton undershirts stained with osmium tetroxide. The cotton fibers were a, unsoiled; and b, naturally soiled. The samples and experimental procedures were the same as those in Figure 1.

the white spots in Figure 3b correspond to unsaturated sebum complexed with osmium. Therefore, there was sebum in the interfiber spaces of cotton fibers of naturally soiled cotton undershirt.

The naturally soiled cotton undershirt pictured in Figure 1b was washed in a solution of an ordinary heavy-duty detergent incorporating alkaline protease with the presoaking and washing conditions generally used in Japan. Optical and electron micrographs of ultrathin sections of the washed fibers in Figures 1c and 2d showed that much soil remained in the interior of the fibers. The composition of the soil remaining after washing with the detergent incorporating alkaline protease was in good agreement with that of sebum shown in experimental procedues. Sebum soil trapped in the interfiber spaces was not removed by this detergent, which functions by the usual detergent mechanisms.

The amorphous layers of cellulose molecules in the interfiber spaces, unlike the crystalline lamellae (11), are probably viscous when hydrated by perspiration during wearing and by water during washing. If so, soil would be trapped by a viscous layer of hydrated cellulose molecules. If some compound that interacted with these hydrated molecules disrupted the viscous layers, the soil would be released from the inside of the fibers. Cellulase might act in this way. For such use, a cellulase compatible with the alkaline ingredients of heavy-duty detergents and suitable for use in washing at low temperatures was needed. A novel alkaline cellulase with these properties was screened for, and found in *Bacillus* sp. KSM-1001. This enzyme, a typical cellulase in that it catalyzed endohydrolysis, had an optimum pH at about 9.5 and an optimum temperature at about 40°C; it was compatible with surfactants and builders (manuscript in preparation). The maximum amount of alkaline cellulase that adsorbed on insoluble cellulose was calculated by use of the Langmuir isotherm at 5°C, at which temperature no hydrolysis was found, and the saccharification by the cellulase of insoluble cellulose was measured by the *p*-hydroxybenzoic acid hydrazide method (13) (manuscript in preparation) at 40°C. Both values increased as the relative crystallinity (14) of the insoluble cellulose decreased (manuscript in preparation).

Ultrathin sections of cotton fibers that were untreated (Fig. 4a), treated with alkaline protease (Fig. 4b), or treated with alkaline cellulase (Fig. 4c and d) were observed by double-labelling immunoelectron microscopy (15). The use of colloidal gold showed that alkaline cellulase had adsorbed onto parts in the interior of the cotton fibers, but that alkaline protease had not. The alkaline cellulase could penetrate into the interfiber spaces and adsorb with cellulose molecules there because the cellulose there is not as crystalline as that of the lamellae (11).

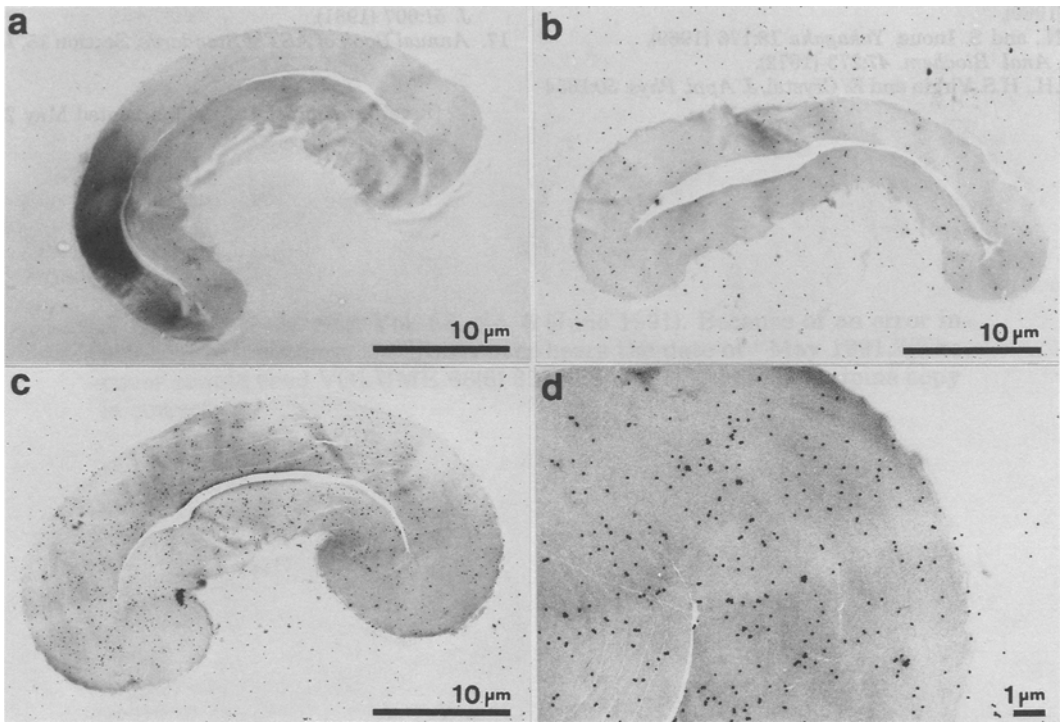

FIG. 4. Double-labelled immunoelectron micrographs of ultrathin sections of cotton fibers treated by alkaline enzymes. The cotton fibers were a, untreated with enzyme; b, treated with alkaline protease; or c and d, treated with alkaline cellulase.

The amount of water (w/w) bound to the cellulose of the unsoiled cotton undershirt was measured by differential scanning calorimetry (16). With increased concentrations of alkaline cellulase, the amount of bound water decreased; the 18% water in the cotton was reduced to about 12% by the reaction of 400 units of alkaline cellulase with 1 g of cotton in 1 L of reaction mixture for 2 hr at pH 9 and 30°C. Water adsorbs to cellulose molecules only in the amorphous region of cotton fibers (16), so the alkaline cellulase seemed to react with cellulose molecules bound with water in the amorphous region of interfiber spaces and on the exterior of the cotton fibers.

The naturally soiled cotton undershirt used in Figure 1b was washed with a detergent incorporating alkaline cellulase. Optical and electron micrographs of ultrathin sections of the washed fibers in Figures 1d and 2e showed that the sebum soil had been removed from the interior of the fiber. The detergency effect of the alkaline cellulase was tested by another method. The mean 33.5 ± 4.7 mg of residual sebum soil per gram of cotton undershirt collected from twenty homes after home laundering was reduced to 5.9 ± 2.0 mg by use of the detergent with the alkaline cellulase and to 29.5 ± 3.6 mg by use of the detergent with the alkaline protease by ten wear-and-wash cycles in controlled laundry tests in the laboratory on a paired comparison basis (17), in which the washing conditions were the same as those in the legend of Figure 1, except for the washing machine (VH-1330, Toshiba Co., Tokyo, Japan) and the liquor ratio (1.5 kg of undershirt in 30 L of detergent solution). The contribution of the alkaline cellulase to the cleanliness of cotton undershirt, judged visually, was greater than that of the alkaline protease; use of alkaline cellulase resulted in cleaner undershirts than use of alkaline protease. The difference in cleanliness depended on the amount of residual sebum soil. Thus, when alkaline cellulase indirectly contributes to remove the sebum soil trapped in the interfiber spaces of cotton fibers by reaction with the cellulose molecules of the cotton, not by reaction with the soil, detergency is excellent. This detergent mechanism is a new one.

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