Detergency and Mechanism of Soil Removal in Detergent-Enzyme System

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ABSTRACT

Enzyme effects on detergency were studied at a relatively low temperature (20 C) using naturally soiled fabrics (collars on working clothing) and 2 types of artificially soiled cotton cloths, milk-soiled and carbon-milk-soiled. Results of the test showed that protease has a favorable effect on detergency even at 20 C and improves the removal of common soils, solid or oily, as well as protein-based soils. The protease also was proved fully effective under mechanical agitation in the presence of detergents. Furthermore, the role of enzyme was discussed based upon gel filtration of the washing bath which treated the milk-soiled cloths. The degradation of protein by protease to a molecular weight of ca. 10,000 to 50,000 was proved to be fairly important for removal of soil protein. It also was found that endo-peptidase action is more effective than exo-peptidase action. Based upon these findings, a problem in activity evaluation method for enzymes as detergent components also was presented.

INTRODUCTION

As a result of a remarkable progress in biochemical industries, alkaline proteases have been developed, and various kinds of enzyme-containing detergents have been marketed in many countries, especially in Europe and North America.

Effects of enzymes in detergents have been discussed in many papers. Oldenroth (1) tested protease effectiveness to remove protein-based soils, such as blood, milk, cocoa, and egg yolk under various washing conditions. Langguth (2) studied the detergency of enzyme in the presence of detergent raw materials by using blood, milk, and ink soil (EMPA-116). Hoogerheide (3) described synergism between washing temperature and washing time. Minagawa (4-6) studied the effects of various factors, such as substrate, pH, temperature, washing time, metallic ions, and detergent ingredients. Most of these studies, however, were performed at 40-60 C. To our knowledge, only 1 investigator, Minagawa, has dealt with the effect of enzyme at lower temperatures, e.g. 20 C, because the average washing temperature in Japan is about 20 C.

There are many methods of measuring the activity of protease in a presoaking agent or in a detergent product. Many reports (7-10) on these subjects have lacked standardization. Most of this research is based upon quantitative determination of soluble protein hydrolysate, and few refer to detergency. The present paper reports our studies of the detergency of detergent-enzyme systems using naturally soiled fabrics and 2 types of artificially soiled ones. Our aim is to elucidate the performance of enzyme at lower temperatures (e.g. 20 C) and the correlation between removal of protein-based soil and that of common soils. Further, the soil-removal mechanism in detergent-enzyme systems are discussed on the basis of gel filtration data, which give information about decreased mol wt of hydrolyzed protein in the detergent bath after washing.

Generally it is believed that protein in the presence of an enzyme might be changed in space structure by heating or with the elapse of time to form denatured protein which seems to bind various soils to the surface of fabrics. If the denatured protein then is changed into water-soluble protein, it could be readily removed from the surface of fabrics by a common detergent, together with other nonprotein soils. Hoogerheide (3) proposed a similar hypothesis.

TABLE I

Protein and Lipid Content (%) of Milk-soiled Cloth

Cloth \ Component	Protein (mg/g cloth)	Lipid (mg/g cloth)
Original cloth	0	2.1
Soiled cloth	15.3	16.4
Confidence interval (95%)	±1.7	±1.3

MATERIALS USED FOR TEST

Enzyme: Protease A is an endo-peptidase obtained from *B. subtilis* which contains ca. 10% crude protein and ca. 90% inorganic Ca and Na salts. Its activity is 68,000 PU/g at pH 9.5 and 55,000 PU/g at pH 8.6, according to Anson-Hagihara (11). Protease B extracted from *Streptomyces griseus* is a mixture of endo-peptidase and exo-peptidase. Its activity at pH 8.6 is 280,000 PU/g.

Washing materials: Detergent product is a powder composed of 18% sodium alkylbenzene sulfonate, 27% sodium tripolyphosphate, 4.5% sodium silicate (SiO_2/Na_2O = 2:1), 1.8% sodium carboxymethyl cellulose (technical grade), 38.6% sodium sulfate, and 10.1% water.

Sodium dodecyl sulfate (SDS) is prepared by sulfating dodecyl alcohol (purity 98%) with oleum, neutralizing, and then removing inorganics and unreacted organic residues by treating with ethanol and petroleum ether respectively.

Naturally soiled fabrics: Cotton swatches (prepared from Kanakin A-2023, Kanebo Co., Ltd., Japan, 7 x 37 cm) were sewed on collars of men's working clothes and submitted to normal wearing conditions for 3 days. Then the swatches were removed and stored in a desiccator for a week.

Carbon-milk soiled fabrics: Carbonblack (1 g) was dispersed into 70 ml commercial milk (solid content 0.8%) diluted with 210 ml water, by means of a homogenizer (HV-M. Tokushu Kikakogyo, Japan). The cotton cloths (Kanakin A-2023) were immersed in this soiling solution for 5 min and squeezed out between a pair of rollers. After being predried at room temperature, the soiled cloths were dried at 105 C for 3 hr and conditioned in a desiccator for more than 2 weeks before test. The surface reflectance of the soiled fabrics was controlled within the range of 33±1%.

Milk soiled fabrics: Commercial evaporated milk (Nestle,

TABLE II

Test Solution[a]

Test solution	Detergent product (g/l)	Protease A (PU/ml)	Washing temperature (C)
A	2.0	---	20
B	2.0	0.2	20
C	2.0	---	40
D	2.0	0.2	40

[a]Water hardness, 30 ppm ($CaCO_3$); washing time, 30 min; liquor ratio (cloth/soln.), 1:25; rpm of Terg-O-tometer, 100.

Japan) was diluted with water to prepare a soiling solution containing 3% protein. The cotton cloths (same as specified above) were immersed in this solution for 10 min and then squeezed, predried, oven-dried, and conditioned by the same procedure as carbon-milk soiled fabrics. Protein and lipid content of the soiled swatches are shown in Table I.

Dextran-gel: Sephadex G-200 (Pharmacia Fine Chemicals) was used.

Protein of known mol wt: Lysozyme (7 x crystalline, Seikagaku Kogyo Co.), egg albumin (2 x crystalline, N.B.C.), bovine serum albumin (Fract V, Armour Co.), and γ-globulin (Fract II, Armour Co.) were used for the calibration curve.

Casein: Hammersten, Merck.

Folin reagent: Prepared according to Lowry and Folin (12).

PROCEDURES AND RESULTS

Detergency Test by Naturally Soiled Fabrics (1 Bath Method)

Each naturally soiled fabric was cut into halves, and each of these pieces was washed in each of the paired test solutions. Test solutions used are shown in Table II. After washing, rinsing, and drying, the halves were rejoined to make an ordered pair (i, j, Table III) for visual evaluation of cleanliness. Visual evaluation in 5-point scoring system for each pair was carried out according to Scheffe's method. For each combination of enzyme-detergent samples from (A:B) to (C:D), 20 pieces of naturally soiled fabrics were used, with an equal number of (i,j) and (j,i) pairs to eliminate the influence of order. Each paired swatch was judged by 5 trained inspectors according to the following scoring system: +2: i looked whiter than j; +1: i looked slightly whiter than j; 0: no difference was observed; -1: j looked slightly whiter than i; -2: j looked whiter than i.

Experimental conditions, results of judgment, and the analyses of variance are shown in Table II, Table III, and Table IV respectively. Order effect and deviation from subtractivity were pooled into error, because they were regarded as insignificant in visual judgment. Main effects were estimated on their significance under the reliability of 95%. The results are shown in Figure 1. Y (0.05) shows the degree of variance of difference between 2 main effects, $|\alpha_i - \alpha_j|$, which can be calculated from mean square of error.

Detergency Test by Carbon-Milk Soiled Fabrics (1 Bath Method)

Washing was carried out in a Terg-O-tometer, for 30 min. As for test conditions, 5 levels of reciprocation speed (0, 35, 50, 100, and 150 rpm), 2 levels of temperature (20 and 40 C), and 3 levels of protease concentration (0, 0.2 and 0.4 PU/ml) were applied. For each set of conditions, 5 pieces (10 x 10 cm) of carbon-milk soiled fabrics were washed in 5 separate pots each containing 500 ml test solution. After washing, each swatch was rinsed with 500 ml water for 5 min and dried by ironing. Then the swatches were submitted to nitrogen-content determination, as well as surface reflectance measurement. Nitrogen content of each swatch was determined by Kjeldahl's method and then the removal efficiency (Dp) was calculated by the equation:

$$Dp(\%) = (Ns - Nw)/(Ns - No) \times 100$$

where Ns, Nw, and No represent respectively nitrogen content of swatch before washing, after washing, and control value, i.e. nitrogen content of unsoiled cotton fabric.

Carbon removal efficiency (Dc) was calculated with surface reflectance by Kubelka-Munk equation:

$$Dc(\%) = \{(K/S)s - (K/S)w\}/\{(K/S)s - (K/S)o\}$$
$$(K/S)i = (1 - Ri)^2/2Ri \quad (i = S, W \text{ or } O)$$

where Rs, Rw, and Ro represent respectively surface reflectance of soiled swatches before washing, after washing, and control value. The results are shown in Figures 2 and 3. In Figure 4, Dc, Dp data are plotted to show correlation between protein removal (Dp) and carbon removal.

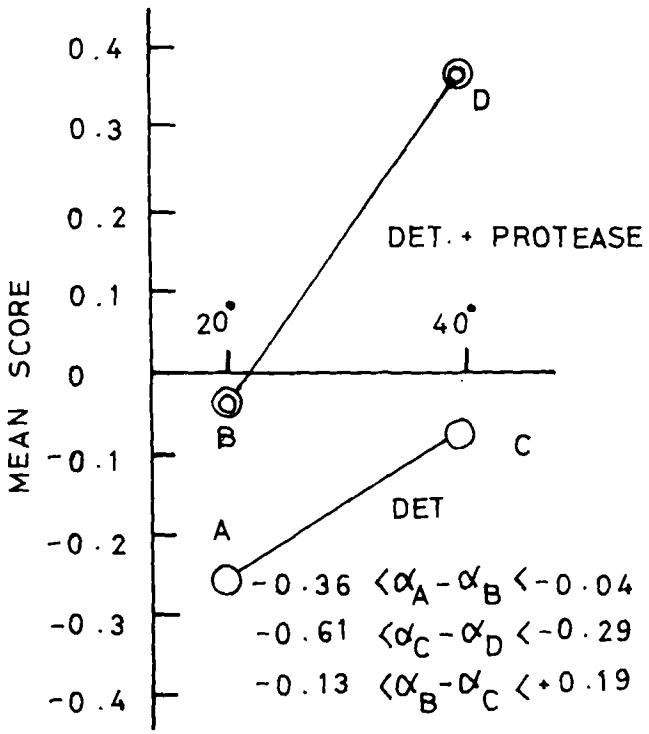

FIG. 1. Estimate of main effects using naturally soiled cloths.

TABLE III

Result of Judgments

Pair (i, j)	+2	+1	0	-1	-2	Total score
(A,B)	0	6	32	10	2	-8
(B,A)	4	14	23	9	0	13
(A,C)	1	9	23	13	4	-10
(C,A)	3	11	28	7	1	8
(A,D)	1	5	14	22	8	-31
(D,A)	12	10	25	3	0	31
(B,C)	1	8	39	2	0	8
(C,B)	3	2	42	3	0	5
(B,D)	2	5	21	10	12	-25
(D,B)	8	12	20	9	1	17
(C,D)	0	7	20	15	8	-24
(D,C)	9	12	21	7	1	21
Total	44	101	308	110	37	

TABLE IV

Analysis of Variance[a]

Source	Sum of squares	Degree of freedom	Mean square	F ratio
Main effect	84.1132	3	28.04	37.0**
Error	450.8868	597	0.757	
Total	535.0000	600		

[a] $Y\ 0.05 = q\ 0.05\sqrt{\frac{Ve}{2nk}} = 0.16.$

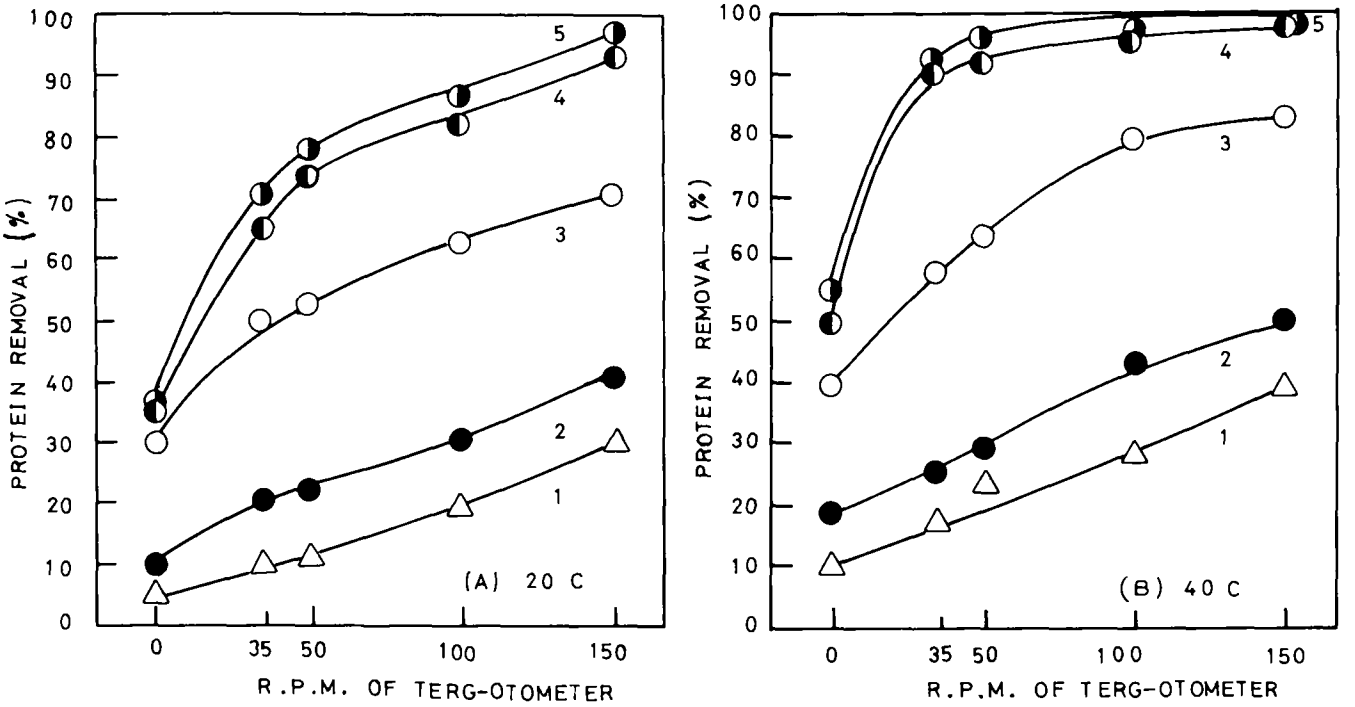

FIG. 2. Effect of mechanical action on protein removal for carbon-milk soiled fabric. (1) Water, (2) protease A solution (0.2 PU/ml), (3) detergent product solution (1.7 g/liter), (4) detergent (1.7 g/liter) + protease A (0.2 PU/ml), (5) detergent (1.7 g/liter) + protease A (0.4 PU/ml).

Detergency Test by Milk-Soiled Fabrics (1 Bath Method)

The 1 bath washing test was carried out with a Terg-O-tometer at various temperatures, reciprocation rates, and concentrations of protease to find some relation between Dp and lipid removal (DL). Dp was calculated from nitrogen content determined by the same procedure as specified above, and DL was obtained from content of diethyl ether soluble material determined by Soxhlet extraction method, applying the equation:

$$DL(\%) = (Ls - Lw)/(Ls - Lo) \times 100$$

where Ls, Lw, and Lo are lipid content of soiled fabric before washing, after washing, and control value. The result is shown in Figure 5. We could not obtain the relation between Dp and DL with carbon-milk soiled fabrics, because oil in carbon black also might be extracted.

Detergency Test by Milk-Soiled Fabrics (2 Bath Washing Test)

Milk-soiled fabrics were washed in aqueous solution containing sodium dodecyl sulfate and protease through the steps specified below. Dp was determined after Step I and Step III. Figure 6 shows the relation between Dp and concentration of protease A at 20 C, and Table V shows the difference between protease A and protease B.

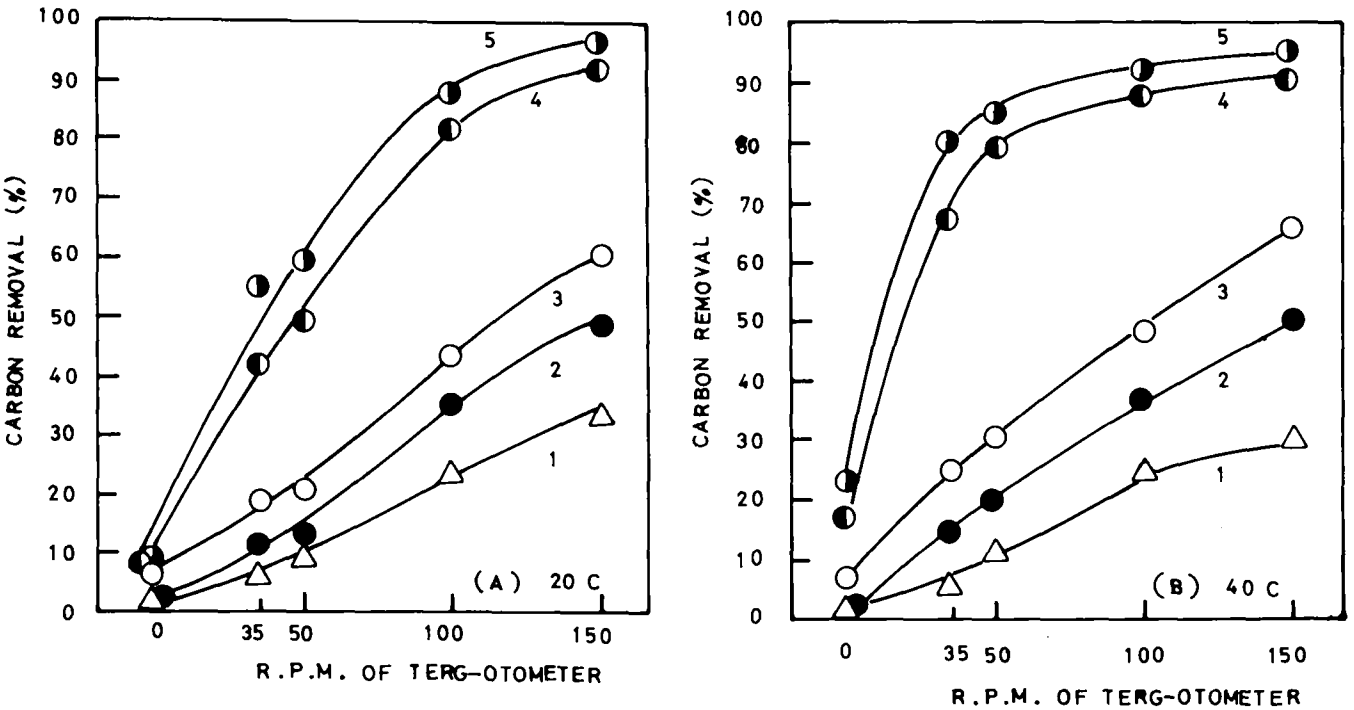

FIG. 3. Effect of mechanical action on carbon removal for carbon-milk soiled fabric. Parameter is the same as Figure 2.

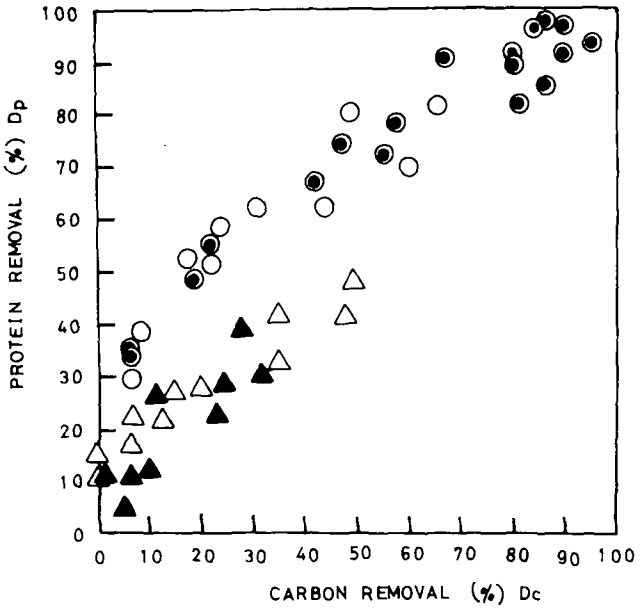

FIG. 4. Relation between protein removal and carbon removal for carbon-milk soiled fabric.

Non-detergent system: (▲ water, △ enzyme solution.

Detergent system: (○ detergent solution, ◉ detergent-enzyme solution.

Step I (presoaking): Soiled fabrics were soaked in 0.01 M tris-HCl buffer (pH 8.6) containing protease at 20±1 C for 30 min, liquor ratio (fabric:solution), 1:100. Protease concentration 2 levels (0.3 and 3.0 PU/ml) were applied.

Step II (deactivation of protease after soaking): Presoaked fabrics were taken out carefully and then were resoaked in MacIlvaine buffer (pH 3.2) and heated at 105 C for 1 hr.

Step III (washing): The deactivated fabrics were washed in 0.01 M Na_2CO_3 buffer solution (pH 9.5) containing 0.05% sodium dodecyl sulfate by means of Terg-O-tometer at 30±1 C for 15 min. Liquor ratio was 1:100.

Step IV (drying): The washed swatches were oven dried at 105 C for 1 hr.

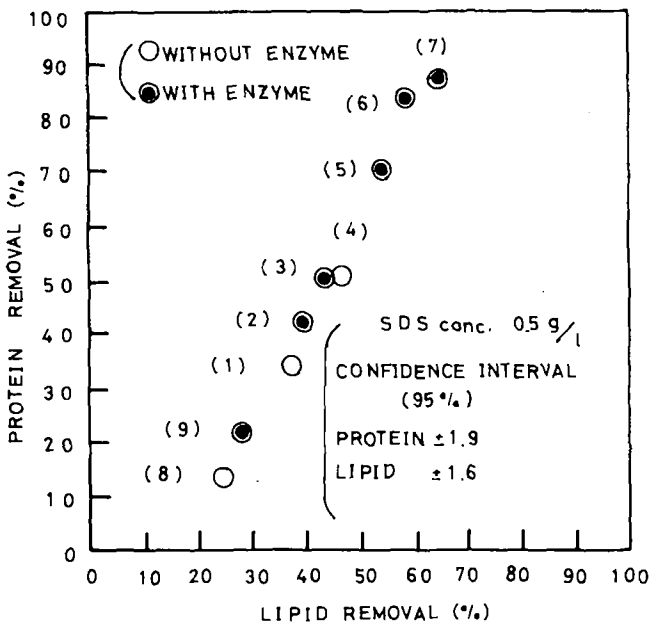

FIG. 5. Correlation between removal of protein and removal of lipid for milk-soiled fabric. (1) 20 C, 100 rpm; (2) 0.3 PU/ml, 20 C, 100 rpm; (3) 3.0 PU/ml, 20 C, 100 rpm; (4) 40 C, 100 rpm; (5) 0.3 PU/ml, 40 C, 100 rpm; (6) 3.0 PU/ml, 40 C, 100 rpm; (7) 3.0 PU/ml, 40 C, 140 rpm; (8) 20 C, 50 rpm; (9) 0.3 PU/ml, 20 C, 50 rpm.

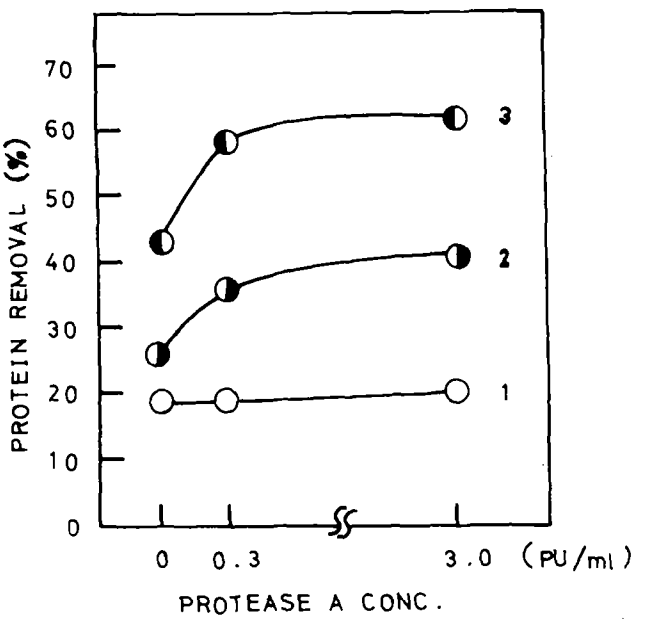

FIG. 6. Protein removal in presoaking and washing for milk-soiled fabric. (1) Protein removal during presoaking (Step I), (2) protein removal during washing (Step III), (3) total removal (Step I + Step III).

Gel filtration of washing bath: Waste sodium dodecyl sulfate solution, after Step III in 2 bath washing method, was passed through filter paper to remove solid contaminants and through anion exchange resin (IRA-120) to remove detergent. Then the solution was concentrated under reduced pressure at 50 C. The concentrated liquor was centrifuged at 3000 rpm for 15 min to remove lipids which interfere with the gel filtration.

The refined solution was charged into a gel filtration column containing Sephadex G-200 gel and developed. The eluates were collected as fractions of each 6 ml. Protein content of each fraction then was determined by UV absorption at 280 mμ. The column void volume was checked using Blue dextran 2000. Calibration curve showing the relation between elution volume (Ve) and logarithmic mol wt (log MW) were obtained with proteins of known mol wt, lysozyme, egg albumin, bovine serum albumin, and γ-globulin, in the same procedure as Andrews' method (14). As shown in Figure 7 Sephadex G-200 gave a linear relation between Ve and log MW over the mol wt range 8,000-200,00. This indicates that the mol wt relates to the bulkiness of aggregated protein. Gel filtration curves of various washing solutions are shown in Figure 8.

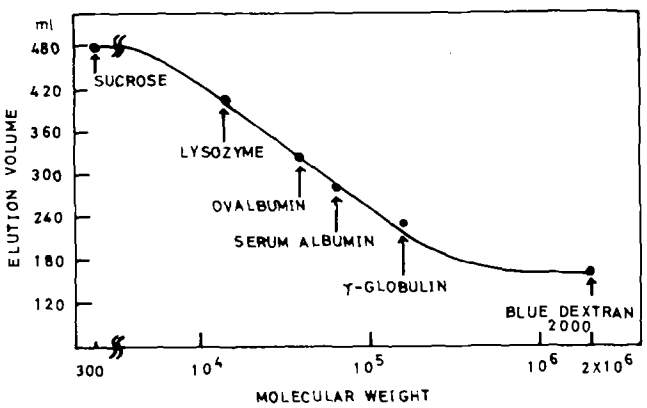

FIG. 7. Plots of elution volume against log M.W. for proteins on Sephadex G-200, column size; 2.5 x 90 cm eluant; 0.01 M tris-HCl buffer (pH 8.6) containing 0.1 KCl, sample volume; 3 ml. Upward flow method at room temperature was used to maintain a constant flow rate 10 ml/hr.

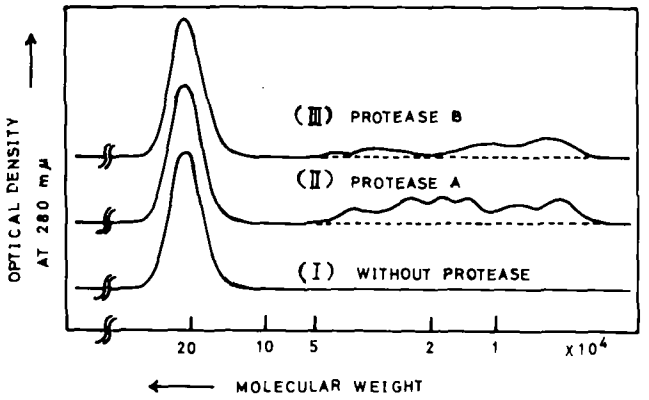

FIG. 8. Gel filtration patterns of filtered washing solution. (1) Presoaking in water and washing with sodium dodecyl sulfate (SDS), (2) presoaking 0.3 PU/ml protease A solution and washing with SDS, (3) presoaking 0.3 PU/ml protease B solution and washing with SDS.

Gel filtration pattern of pure casein hydrolysates: Pure casein solution, pure casein hydrolysate by protease A, pure casein hydrolysate by protease B, and trichloroacetic acid-soluble fraction of the hydrolysate by protease B were analyzed separately by gel filtration. The results are shown in Figure 9.

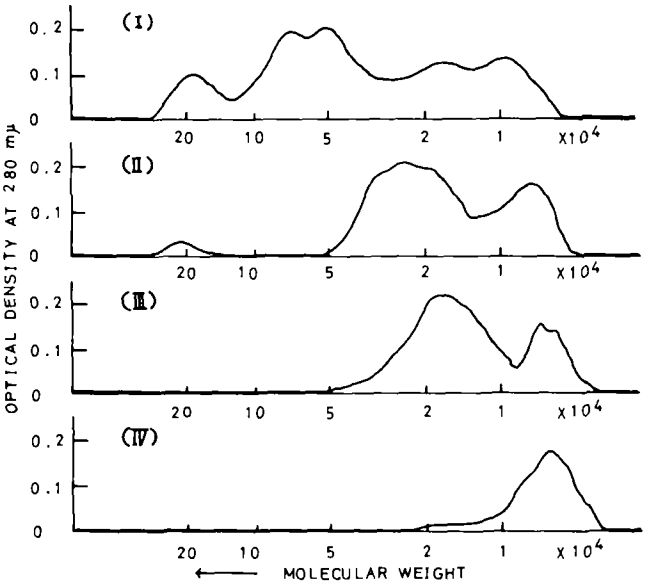

FIG. 9. Gel filtration patterns of casein and its hydrolysate by protease. (1) Casein, (2) hydrolysate by protease A, (3) hydrolysate by protease B, (4) trichloroacetic acid-soluble casein hydrolysate by protease B.

DISCUSSION

A. As shown in Figure 1, $|\alpha A\text{-}\alpha B|$ and $|\alpha C\text{-}\alpha D|$ values compared with Y (0.05) indicate that protease effect on detergency is significant at 20 C, as well as 40 C. The protease concentration applied in this series of tests, 0.2 PU/ml, was ca. the same concentration used in practice in enzyme-detergent solutions. Thus it was recognized that protease contributes to detergency, even at 20 C under usual conditions. Of course the effect of protease was more remarkable at 40 C. Most solid soils observed on naturally soiled fabrics are colored particles composed of clay or dust (15). Consequently, removal of solid soil may have a remarkable effect on detergency rate based upon visual judgment. Therefore it may be presumed that protease effect on detergency is due mainly to decomposition of protein which binds solid soils to fiber surface.

B. To verify the presumption, effects of mechanical agitation on soil removal were investigated. Curves 3 and 4 in Figure 2, as well as those in Figure 3, show the positive effect of protease A on protein and carbon removal. In detergent-enzyme system, Dp and Dc curves at 40 C have inflection points at relatively lower agitation rates, while at 20 C both curves increase with the increase of agitation rate, with less remarkable inflection point. These show that detergency at 20 C is more remarkably dependent upon agitation than at 40 C and that protease would not show its full effect at 20 C without a sufficient agitation. Figure 4 shows the close correlation between Dp and Dc. It is, therefore, inferred that protease has an indirect effect on removal of solid soils as a result of peptide chain cleavage. Furthermore, curve 2 in Figures 2 and 3 shows that protease A cannot improve Dp or Dc by itself, and the effect of protease A would be observed clearly only in the presence of detergent in the system. There are some differences in soil removal mechanism between detergent-containing system and detergent-free system, since plots in Figure 4 can be divided into 2 groups relating to each system.

Probably this difference results from wetting and swelling of protein caused by detergent. Therefore it can be concluded that the effect of protease on solid soil removal is most remarkably exhibited when it is backed up with surface effect of detergent and mechanical effect of agitation.

C. In a series of washing tests using milk-soiled fabrics, a linear correlation between removal of protein and that of lipid is observed over a wide range, as shown in Figure 5. Furthermore, both removals increase with PU of protease A. This suggests the indirect effect of protease A on the lipid removal.

D. The 2 bath washing system and gel filtration technique are applied to investigate the soil-removing mechanism of an enzyme-detergent system. The reason we used the 2 bath system is the necessity of separating the washing process with surfactant from hydrolysis process with enzyme. In 1 bath washing system, enzyme activity remains during washing and gel filtration processes, and removed protein-hydrolysate is hydrolyzed further by the enzyme. It is, therefore, difficult to find out the relation between soil removal and degree of protein-hydrolysis. In contrast,

TABLE V

Effect of Endo-peptidase and Exo-peptidase[a]

Protease	Concentration (PU/ml)	Total removal (%)	Note
A	0.3	55.1	Endo-peptidase
A	3.0	65.6	Endo-peptidase
B	0.3	51.2	(Endo-peptidase + Exo-peptidase)
B	3.0	54.4	(Endo-peptidase + Exo-peptidase)
Without enzyme	---	46.8	---

[a]Confidence interval (95%); ±1.6.

protease on soaked swatches is deactivated completely in Step II in our 2 bath washing, and no protease activity remains in sodium dodecylsulfate solution in Step III.

Protein removal during Step I (soaking) shows no increase with protease concentration up to 3.0 PU/ml, whereas Dp during Step III (washing) shows an obvious dependence upon protease concentration in the soaking bath, as shown in Figure 6. These data suggest that protein is hydrolyzed only partially during the soaking, and the hydrolyzed peptide, which still remains on the fabric, is removed easily by mechanical force in the presence of detergent.

Table V shows that protease A, endo-peptidase, has a superior effect for Dp than protease B, which is a mixture of endo-peptidase and exo-peptidase. Gel filtration data show that there is a difference in hydrolysis products between protease A and protease B, and protease B gives hydrolysates of lower mol wt than protease A, as shown in charts II and III, Figure 9. It is noteworthy that protease-producing hydrolysates of lower mol wt are not always more effective for washing.

Gel filtration curves in Figure 9 are obtained with pure milk casein. Since protein of our milk-soiled fabrics are denatured by oven-drying, gel filtration curves show corresponding peaks shifted to higher mol wt.

E. The 3 gel filtration patterns illustrated in Figure 8 show remarkable differences. The elution pattern (I) obtained from control solution (detergent solution which treated swatches soaked with plain water) exhibits one large peak indicating the aggregation of milk casein by heat treatment. Patterns II and III exhibit the same large peak, as well as small peaks indicating partially hydrolyzed products. The former corresponds to mol wt of more than 200,000 and the latter correspond to ca. 200-50,000. Several lower peaks of pattern II and 2 lower peaks of III correspond to mol wt of 10,000-50,000 and 200-10,000, respectively. As shown in Figures 6 and 8, an enzyme decomposing protein into hydrolysates of relatively higher mol wt (10,000-50,000) is, in this case, more effective for washing than another, producing fragments of lower mol wt (less than 10,000). Regarding the mechanism of Dp, Hoogerheide and coworkers(3) suggested that if protein on the fabrics were degraded into protease or peptone, it would lose its function as a binder and could be removed easily by surface active agent; our data described above substantiated their hypothesis.

F. There is some doubt about the method of assay which estimates protease activity by determining the amount of hydrolysate, soluble in trichloroacetic acid. Pattern IV in Figure 9 is a curve of trichloroacetic acid soluble fraction of hydrolyzed casein. It contains only lower molecular peptides (mol wt, less than 10,000) and amino acids. Hydrolysates of higher mol wt (10,000-50,000) are not included in trichloroacetic acid-soluble fraction. However, trichloroacetic acid method is not a satisfactory assaying method for proteinase activity, because decomposition of protein in soil to relatively higher mol wt (10,000-50,000) is also important for soil removal. Therefore, some other assaying method for protease activity should be applied if possible.

G. Our conclusions in discussions D. and E. are based upon milk-soiled fabrics. However, as described in discussion A., similar effects of protease are observed also with naturally-soiled fabrics. Consequently, these conclusions may be applicable to practical washing.

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