The Effect of Builders on the Activity of Protease Enzymes

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The effect of builders on the stability of protease enzyme activity was studied in an effort to identify superior builders which are soluble in water and compatible with enzymes formulated into heavy duty laundry powders. Various poly(styrenesulfonate-methacrylate) copolymers, polyacrylate and tripolyphosphate anionic builders, as well as various poly(vinylalcohol-vinylacetate) nonionic copolymers, namely PVAs, were used. Zeolite 4A was also used as a typical nonphosphate particulate builder in the detergents. The protease used is from *bacillus stearothermophilus*. The calcium content was determined to be 16.7 mole/mole of protease by atomic spectrophotometry.

In binary systems composed of a fixed concentration of 10 U/mL protease and varied concentrations of compound, builder or surfactant, it was found that compounds having the larger calcium ion binding capacity (C.B.C.) lowered the relative activity of protease enzyme. The activity of protease enzyme alone was lowered about 20% by addition of 0.02% sodium dodecylbenzene sulfonate (DBS).

The anionic builders added to the binary system of fixed 10 U/mL protease and 0.02% DBS reduce the protease enzyme activity in proportion to the magnitude of their C.B.C. Addition of anionic builders further lowered the protease enzyme activity. The nonionic builders and the nonionic surfactant can enhance the protease enzyme activity by protection of protease against the inhibitor, DBS.

It is certain that calcium atoms contained in the protease must play an important role for the protease enzyme activity and its stability. Calcium atoms must have a great influence on the formation of protease-substrate complex, protease-compound complex and substrate-compound complex, because the protease, protein substrate and anionic compound would all be negatively charged in alkaline solutions. Builders for enzyme-containing detergents should be constructed to be insensitive to calcium ion.

KEYWORDS: Builder, calcium, detergent, enzyme activity, polyelectrolyte, polystyrenesulfonate copolymer, polyvinylalcohol, protease, stability.

Great effort in seeking environmental preservation and energy savings have developed some special heavy duty detergents containing zeolites and enzymes in Japan (1,2). Nonphosphate, enzyme-containing detergents have been estimated to have equivalent performance to phosphate detergents. A few problems had been pointed out concerning use of zeolites because of undesirable effects resulting from their water-insolubility, such as deposition onto the washed garments(3-5) and agglomeration and sedimentation in drainage(6,7). Although these problems had been minimized by production of the fine particle size zeolites (8), a new water soluble builder is desired.

In the study of the nonphosphate detergents, we previously found that not only protein or sebum soils but also particulate soils are effectively removed from cotton fabrics by detergents containing protease and/or lipase enzymes (9,10). A superior builder for enzyme-containing detergents would have the characteristic of compatibility with enzymes being as important a builder function as other functions such as sequesteration, alkalinity, buffer action, dispersive power, and so on.

The purpose of this study is to identify some builders that are soluble in water and compatible with enzymes. In the present paper, the effect of the builder on the stability of protease enzyme activity was studied using various water-soluble compounds.

MATERIALS AND METHODS

Protease enzyme. The protease enzyme from *bacillus stearothermophilus*, named Toyozyme NP, was supplied by Tosoh Co., Ltd. (Tokyo, Japan) and was used without further purification. It is not tailored for detergent application. The properties of this protease are as follows: The activity optimum is at pH 8; the stability region is about from pH 5-10; the protease enzyme activity is 58.8 $\times 10^4$ U/g at alkaline pH 9.5 and at 30°C with Casein-Folin B method (11); and the molecular weight is 3.7 $\times 10^4$ by GPC, SDS-PAGE method.

Builders. Various anionic compounds and various poly(vinylalcohol)s were studied as builders. The properties of those studied are presented in Tables 1 and 2. The homologous styrenesulfonate compounds were supplied from Tosoh Co., Ltd. Various poly(styrenesulfonate-methacrylate) copolymers were developed for the purpose of our studies through the courtesy of Tosoh Co., Ltd. Active contents of these samples are from 17.7 to 80.5%.

Sodium polyacrylate is a powdery ingredient and assayed more than 95%. It was supplied by Nihon Kayaku Co., Ltd. (Tokyo, Japan). Sodium tripolyphosphate, STP, was used as a typical reference of detergent chelating agents. It was purchased from Wako Pure Chem. Co., Ltd. (Osaka, Japan). Sodium type of Zeolite 4A was also used as a typical reference of nonphosphate builder formulated in detergents. The diameter of particles of Zeolite 4A is 0.4-0.8 μm. This was supplied by Mizusawa Chemical Co. Ltd. (Niigata, Japan).

Various PVAs above 95% purity were supplied by Kuraray Co., Ltd. (Osaka, Japan). Saponification degree, polymerization degree, the charge on PVA resulting from copolymerization with anionic or cationic monomer, and the addition of alkyl ($C \fallingdotseq 12$) groups at the terminal of the polymer chain were investigated as variables. As the mole fraction of ionic comonomer, itaconic acid or quarternary ammonium compound are low, the charge on the PVAs are only weakly anionic or weakly cationic, respectively. All builders were used without further purification at a 100% dry builder basis.

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TABLE 1

Properties of Electrolytes Used

Abbrev.	Composition		M.W.	C.B.C.[a] ($CaCO_3$ mg/g)	pK_{Ca}[b]
	NaSS[c]	NaMAA[d]			
US-1	10	90	6.7×10^4	36.6	—
US-2	20	80	7.9×10^4	40.5	—
US-3	30	70	6.8×10^4	25.4	—
US-4	50	50	6.9×10^4	23.1	2.9
US-5	70	30	6.7×10^4	17.2	—
PS-5	NaSS homopolymer		$5\text{-}8 \times 10^4$	2.3	—
NaSS	NaSS monomer		206	—	—
SPA	Sodiumpolyacrylate		$6\text{-}7 \times 10^3$	46.9	3.1
STP	Sodiumtripolyphosphate		368	333.0	6.1

[a]C.B.C., calcium binding capacity.
[b]pK_{Ca}, stability constant.
[c]NaSS, sodium styrenesulfonate $CH_2{=}CH$ SO_3Na.
[d]NaMAA, sodium methacrylate $CH_2{=}C(CH_3)COON_a$.

TABLE 2

Properties of Polyvinyl Alcohols Used

Abbrev.	Composition	n(P.D.)	M.W.
	$(CH_2{=}CH{\cdot}OH;CH_2{=}CH{\cdot}OCOCH_3)_n$		
203		300	1.47×10^4
205	88.0;12.0	500	2.45×10^4
210		1000	4.90×10^4
217		1700	8.36×10^4
405	81.5;18.5	500	2.59×10^4
117-1	99.6; 0.4	1700	7.51×10^4
117-2	98.5; 1.5	1700	7.59×10^4
117-3	97.5; 2.5	1700	7.66×10^4
117-4	96.0; 4.0	1700	7.77×10^4
217	88.0;12.0	1700	8.36×10^4
R-205	Alkyl group adduct	500-600	
A-600	Anionic group sub. (1 mole)	600	
C-600	Cationic group sub. (0.2 mole)	600	

Surfactants. Sodium dodecylbenzene sulfonate (DBS), was used as a typical anionic surfactant formulated into heavy duty powders. It is a standard reagent for the test of detergents for garment washing and was purchased from Wako Pure Chem. Co., Ltd. Nonionic stearyl poly-(oxyethylene) ether, $C_{18}\bar{P}_{20}$, was also used. It was a pure chemical assaying 100%, and was supplied by Nippon Emulsion Co., Ltd. (Tokyo, Japan).

Estimation of calcium ion binding capacity of the builder. The calcium ion binding capacity of the builder was determined electrometrically employing an Orion Calcium Ion Electrode 93-20-01 and an Orion Digital Ionalyzer Type 601A (Orion Research, Inc., Boston MA). The free calcium ion concentration in 50 ppm $CaCO_3$ hard water was measured as a function of added builder concentraion at pH 10 and at 30°C. Calcium ion binding capacity (mg $CaCO_3$/g builder) was calculated from the builder concentration required to lower the initial 50 ppm hardness to 20 ppm hardness.

Assay of the protease enzyme activity and its stability. The protease enzyme activity $[PU]^{Cas,FRB}_{r.t,yf}$ was assayed on casein hydrolytic activity by the Casein-Folin B method (11). The stability of protease enzyme activity was determined as follows: Individual 100 mL solutions were made up containing 10 U/mL Toyozyme NP, various amounts of compound, builder and/or surfactant, and 0.05 M borate buffer at pH 9.5 and kept in the thermobath at 30°C. After 30 min., 1 mL of the protease solution was sucked out and added to 5 mL of 6% casein substrate solution preheated in a test tube. The mixture was kept for 10 min at 30°C. The reaction was stopped by the addition of 5 mL of a mixed solution of 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid. After standing 30 min at 30°C, the reaction mixture was filtered through Whatman No. 542 paper. The optical density was measured at 660 nm for the filtrate colored by 1 N Folin-Ciocalteu reagent. The effect of the compound on the stability of protease enzyme activity (i.e.) the residual activity after 30 min was estimated by dividing the optical density of the compound/protease solution by that without the compound.

Determination of calcium atom content in Toyozyme NP. Calcium atoms in Toyozyme NP were determined on the aqueous protease soultion by a Hitachi Atomic

Spectrophotometer Type 508 (Hitachi Co., Ltd., Tokyo, Japan). All experiments were done using distilled water, passed through an ion exchanging resin followed by filtration thorugh an RO membrane filter.

RESULTS AND DISCUSSION

Chelating properties of the builder. Figure 1 shows the lowering of water hardness as a function of the logarithm of the concentration of added electrolyte in 50 ppm $CaCO_3$ water hardness at pH 10 and at 30°C. From this figure, C.B.C. and pK_{ca} of the anionic compounds were calcuated and summarized in Table 1, together with other properties. By contrasting performance of SPA, US-1-US-5 and PS-5, it is predicted that the C.B.C. of polyelectrolytes should be much more influenced by carboxyl groups than by sulfonate groups in the polymer structure. Performance of monomer NaSS and its homopolymer PS-5 indicates the interaction between sulfonate and calcium ion must be enhanced by polymerization. Since the water hardness vs concentration curve of STP is lower at lower concentration, STP is shown to be a very superior chelating agent. Calcium ion exchanging capacity of Zeolite 4A is estimated to be 70 mg $CaCO_3$/g at pH 10 and at 30°C (12).

Calcium atom content in Toyozyme NP. Although Toyozyme NP is not a product supplied for use as a detergent ingredient, the amount of calcium atoms contained in it was determined to be 16.7 mole/mole of protease by atomic spectrophotometry. That is to say, the content corresponds to 1.93×10^{-2} g/g of protease or 2.68×10^{-8} g/active units of protease.

Two kinds of commercially available protease manufactured for detergent use were assayed for calcium content by the same method as was Toyozyme NP. The calcium atom contents of protease A and protease B were determined to be 0.6×10^{-8} and 17.9×10^{-8} g/active unit of protease, respectively. We thus confirmed that the calcium atom content of Toyozyme NP is not much different from that of the detergent grade proteases.

It is well known that the active site of protease would be constructed by calcium ionic bridges between the three amino acid residues, such as Asp., His. and Ser. at the specific positions of amino acid sequence (13). Kubo *et al.* (14) have deduced the three dimensional structure of Toyozyme NP by the determination of nucleotide sequence and amino acid sequence, and reported four calcium atoms and a zinc atom in a molecule of Toyozyme NP. These atoms would be intensely bound to protein ligands and held in the three dimensional molecular structure. Of course, 16.7 mole of calcium atoms per mole of protease as determined is significantly higher than 4 mole of calcium atoms per mole of protease. We assume that most of the calcium atoms in Toyozyme NP would be supplied by the calcium chloride which was added in the process of purification of this extracellular protease (14). The other unbound calcium atoms must be also attracted to negatively charged protease in the alkaline solution. Therefore, those free calcium ions would be surrounding the protease molecules in the solution.

The stability of protease enzyme activity in the binary system of protease and builder. The effect of the builder on the stability of protease enzyme activity was investigated with 10 U/mL protease solution and the relative activity vs the concentration curves for various anionic compounds are shown in Figure 2 and Figure 3 and, for nonionic compounds, in Figure 4. As shown in Figure 2, the relative activity in presence of STP, which has the highest C.B.C. and pK_{ca}, is markedly lower and nearly becomes zero at extremely low STP concentration. On the other hand, the relative activity of PS-5, which has the lowest C.B.C., is only slightly lowered in the region of PS-5 concentration tested. As with other ionic compounds, the larger the C.B.C. of the ionic compounds and the higher their concentration, the lower the relative activity.

Although Zeolite 4A has a larger C.B.C. than SPA, it shows nearly equal relative activity of protease to that of

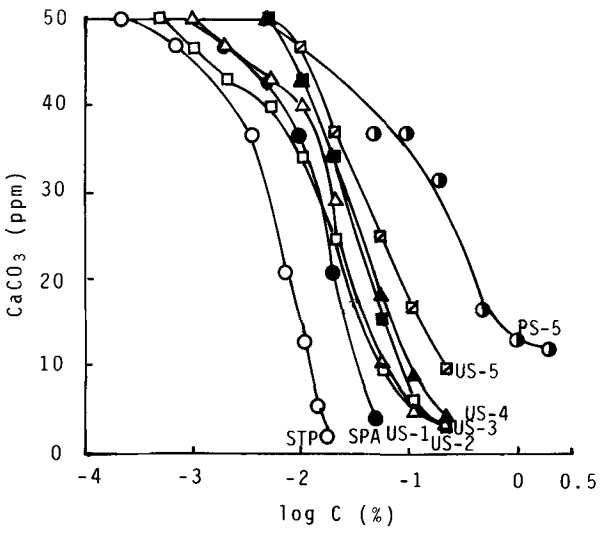

FIG. 1. Lowering of the water hardness as a function of added concentration of electrolyte in 50 ppm $CaCO_3$ at pH 10 and at 30°C.

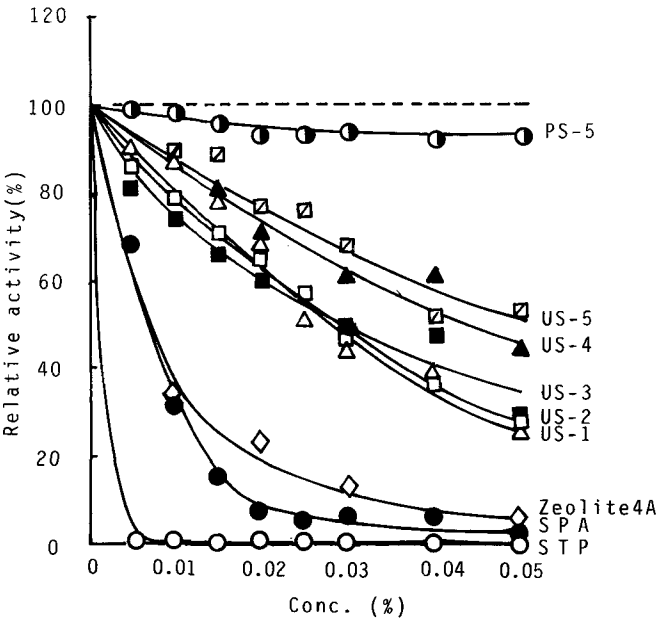

FIG. 2. Effect of electrolyte on the stability of protease enzyme activity of 10 U/mL protease solution at pH 9.5 and at 30°C.

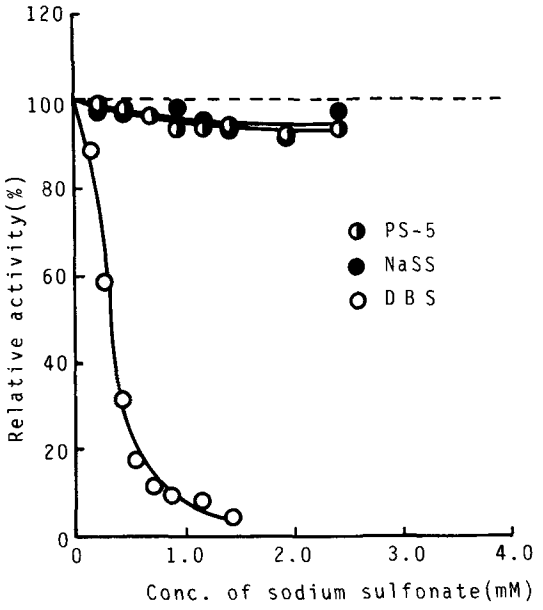

FIG. 3. Effect of sodium sulfonate compound on the stability of protease enzyme activity of 10 U/mL protease solution as a function of equivalent molar concentration of sodium sulfonate at pH 9.5 and at 30°C.

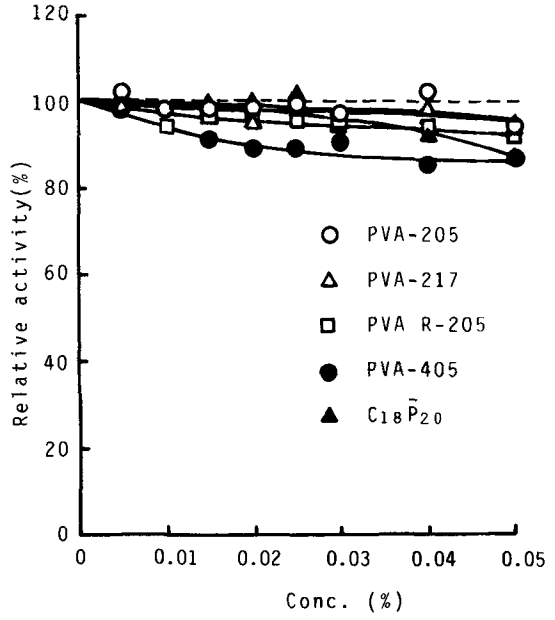

FIG. 4. Effect of nonionic compound on the stability of protease enzyme activity of 10 U/mL protease solution at pH 9.5 and at 30°C.

SPA; because Zeolite 4A cannot exchange calcium ion bound up with a protease structure (13).

Figure 3 shows the effects of three sulfonate compounds on protease activity as a function of the equivalent molar concentration of sodium sulfonate. The relative activity curve of anionic surfactant DBS decrease markedly relative to that of PS-5 and NaSS. The C.B.C. of DBS also was determined to be practically the same as that of US-1, using the calcium ion electrode method (15). Combining this information, we propose that the calcium ion binding property and, consequently, the stability of protease enzyme activity is not only greatly influenced by the kind of ionic groups but also by the hydrohobic moiety in the structure of anionic compound because of their adsorption properties.

The effects of nonionic compounds, four kinds of PVAs and a nonionic surfactant on the stability of protease enzyme activity are shown in figure 4. Because of their low interation with calcium ion, all these compounds have little effect on protease activity even at the higher concentration ranges. Kravetz *et al.* (16) similarly reported that sulfonate surfactants strongly deactivate protease. We suggest that these results indicate interactions between calcium contained in protease and the added compounds, builder and/or surfactant, significantly influences the stability of protease enzyme activity.

The stability of protease enzyme activity in the ternary system of protease, DBS and builder. Because heavy duty powders necessarily formulate anionic surfactant, the effect of builder on the stability of protease enzyme activity was also investigated in the ternary systems composed of fixed 10 U/mL protease, 0.02% DBS and varied concentrations of builder. Figure 5 shows the relative activity of protease as a function of electrolyte in the ternary system. As mentioned above, the result on the ordinate, which is a binary system composed of protease and DBS, show low relative activity of about 20%. The electrolytes added to that binary system lowered the protease enzyme activity further in proportion to the magnitude of the chelating property of the electrolyte. Ionic compounds, electrolytes and anionic surfactant additionally lowered the stability of protease enzyme activity.

The effects of PVAs on the stability of protease enzyme activity in the ternary systems were studied as functions of degree of saponification and the degree of polymerization. Both Figures 6 and Figures 7 show the relation between the relative activity and the saponification

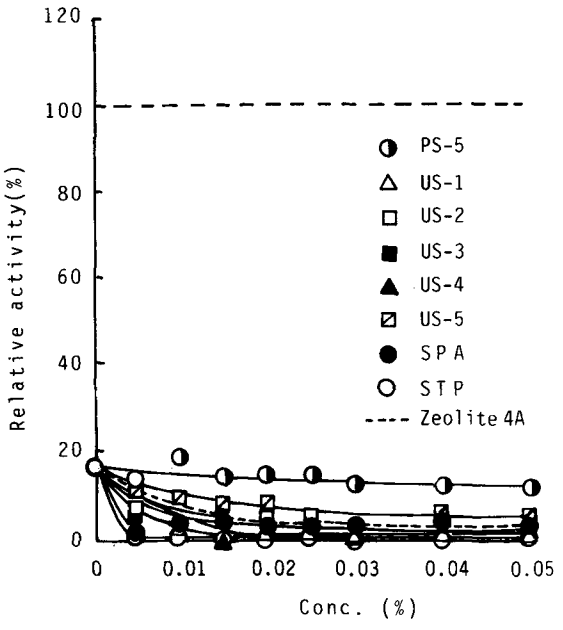

FIG. 5. Effect of electrolyte on the stability of protease enzyme activity of 10 U/mL protease solution containing 0.02% DBS at pH 9.5 and at 30°C.

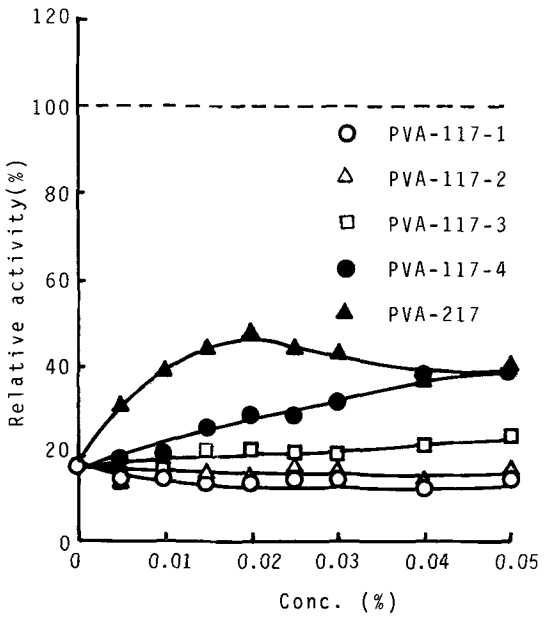

FIG. 6. Effect of saponification degree of PVA on the stability of protease enzyme activity of 10 U/mL protease solution containing 0.02% DBS as a function of concentration of PVA at pH 9.5 and at 30°C.

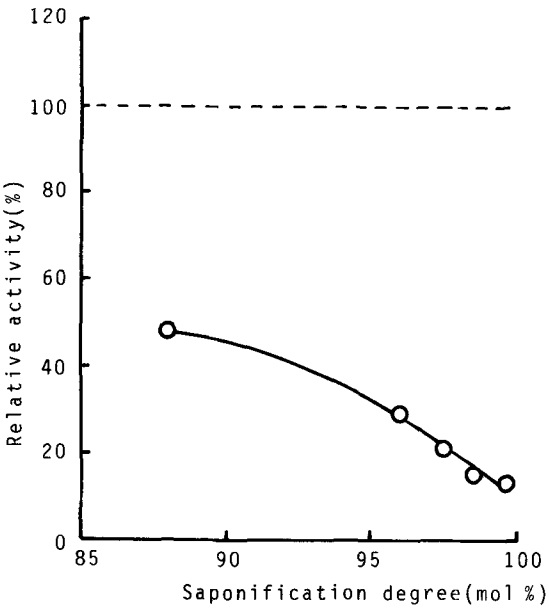

FIG. 7. Effect of saponification degree of PVA on the stability of protease enzyme activity of 10 U/mL protease solution containing 0.02% DBS and 0.02% PVA at pH 9.5 and at 30°C.

degree at fixed 1700 polymerization of PVAs. The former is shown as a function of concentration of PVA, and the latter is as a function of saponification degree at fixed 0.02% concentration of PVA. The relative activity of protease was significantly enhanced by the addition of PVA into the protease solution containing inactivator DBS. The lower the saponification degree the higher the molar fraction of hydrohobic moiety of PVA the higher the relative activity.

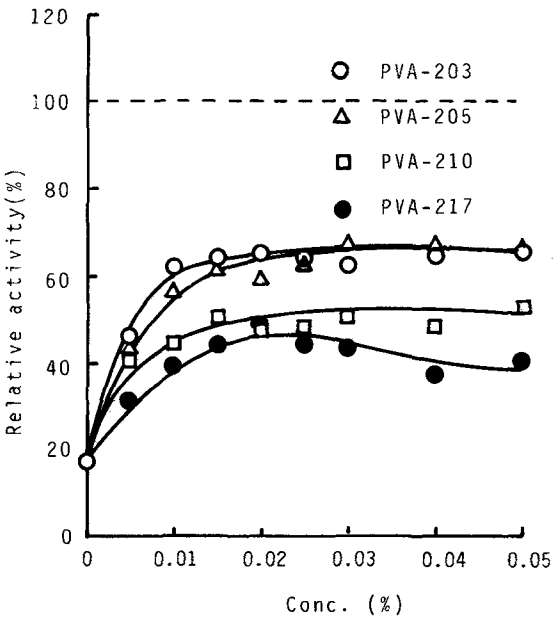

FIG. 8. Effect of polymerization degree of PVA on the stability of protease enzyme activity of 10 U/mL protease solution containing 0.02% DBS as a function of added concentration of PVA at pH 9.5 and at 30°C.

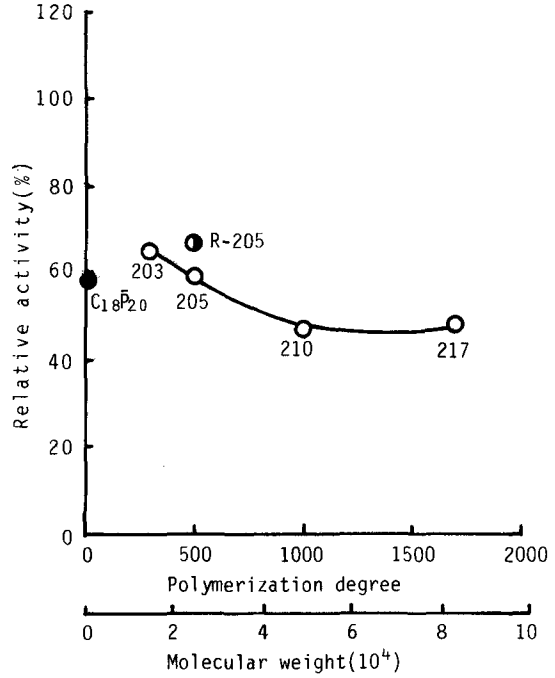

FIG. 9. Effect of polymerization degree of PVA on the stability of protease enzyme activity of 10 U/mL protease solution containing 0.02% DBS and 0.02% PVA at pH 9.5 and at 30°C.

Both Figures 8 and Figures 9 show the relation between the relative activity and the polymerization degree at fixed 88% saponification of PVAs. The former is shown as a function of concentration of PVA, and the latter is as a function of polymerization degree and of molecular weight at a fixed 0.02% concentration of PVA. For reference to nonionic PVAs, the results for the nonionic

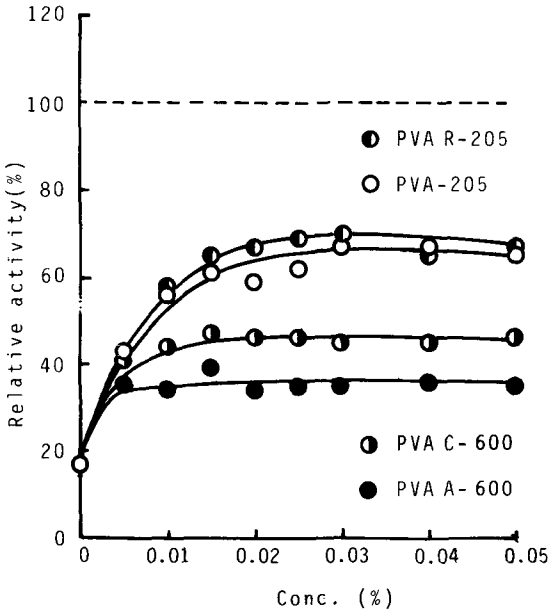

FIG. 10. Effect of modified PVA on the stability of protease enzyme activity of 10 U/mL protease solution containing 0.02% DBS as a function of added concentration of PVA at pH 9.5 and at 30°C.

surfactant, $C_{18}\bar{P}_{20}$, are also plotted in Figure 10. It is clear that the lower the polymerization degree of PVA, the more stable the protease enzyme activity. But the effect of PVA on protease protection against inactivator DBS would be less influenced by the degree of polymerization than by saponification.

Figure 10 shows the effects of modified PVAs. Both anionic and cationic PVAs show the contribution to the stability of protease enzyme activity. We especially note the behavior of anionic PVA as compared to that for those of chelating compounds as shown in Figures 2 and 3. These weakly ionic PVAs behave as a nonionic compound rather than as an ionic compound. PVA R-205, having an added alkyl group, shows a slightly greater effect than PVA 205. It was shown that nonionic compounds, as well as the weakly ionic PVAs, are not only compatible with protease but protect the protease enzyme activity against DBS, and effectively enhance the stability of protease enzyme activity in the detergent solution.

Interaction between the three components, protease, DBS and builder must be very complex, and become even more complex in presence of calcium. At the present time, the mechanism of protease deactivation and protection against deactiviation in those systems is not understood. However, calcium atoms definately play an important role in protease enzyme activity and its stability. Currently we are studying the adsorption behavior of the builder, surfactant and calcium atom onto the protease molecule from the view point of protease enzyme activity (17).

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