The Effect of Synthetic Detergents on Malt Amylase

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Received July 6, 1957

Synthetic detergents of all types are now widely used domestically, and in many branches of medicine and industry. The biological effects of these detergents are only partially understood, and it is considered that a systematic examination of the effect of some of these detergents on enzymes may be of value.

INTRODUCTION

Moreland[1] (1939) reported that surface active substances (including a detergent) had little effect on several enzymes tested. Putnam and Neurath[2] (1944) observed that sodium dodecyl sulfate (SDS) precipitated crystalline pepsin at acid pH. Since then several studies have been made on the combination of SDS with protein by them. Recently, Wills[3)4)5)6)] (1950-1955) has made systematic studies on the effect of detergents on enzymes.

Most commercial detergents are complex mixtures and often of secret formula, but some classifications have been presented, for instance, anionic, cationic, non-ionic and amphoteric detergents. The present work deals with those detergents of all types. Since the year 1953, the author[7)8)9)10)] has reported several studies on the relation between detergents and malt amylase. By the examinations which have been made during the time the author, as well as the other researchers, have found the detergent a useful agent for the study of enzyme and protein. As an extension of this work, a study has now been made of separation of α-amylase from β-amylase accompanied with.

In the investigations described here, a few experiments have been made on commercial detergents, but in most cases those detergents which are obtainable in a fairly pure state have been used, and the effect of detergents on amylases has been compared with each other.

EXPERIMENTAL

Materials. Detergents were obtained from the following sources.

Anionic. SDS (Sodium dodecyl sulfate), SDBS (Sodium dodecyl benzen sulfonate) from Kishida chemicals Ltd.

Cationic. HD (Dodecyl pyridinium Chloride) from Daiichi Kogyo Seiyaku, Hyamin (Benzethonium Chloride) from Sankyo Ltd, M_2-100 (tetradecyl-dimethyl benzyl Ammonium Chloride) from Nihon Yushi, and Osvan (Alkyl-dimethyl-benzyl-ammonium chloride) from Takeda Chemicals Ltd.

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Non Ionic. N-Y (Polyoxy ethylene-laurylate), T-20 (polyoxy ethylene sorbitan monolaurate) and T-60 (Polyoxyethylene sorbitan monostearate) were given by the courtesy of Daiichi Kogyo Seiyaku Ltd.

Amphoteric. Am-2 (Dodecyl-amino-dicarboxylic acid-Na-salt) and Am-16 (Hexadecyl amino dicarboxylic acid-Na-salt) from Daiich Kogyo Seiyaku Ltd.

Amylase Preparations. α-and β-amylase were prepared, as described by Schwimmer[11] and Fischer[12] respectively and the enzyme solution containing 459 γ of protein per 1 ml. for α-amylase, and 3000 γ of protein per 1 ml. for β-amylase were used.

Amylase Activity Estimation. These amylases prepared as described above were diluted suitably with distilled water, to estimate their activities. 2% soluble starch (20 ml.) N/20 acetate buffer soln. (5 ml.) were mixed in a Erlemmeyer flask which was placed in a water bath at 40°C. The reaction was started by the addition of amylase soln. (5 ml.). Samples (5 ml.) were withdrawn. after 0 and 30 min., and then reducing sugars formed during the reaction time were measured with Willstatter method[13] (hypoiodite method).

Remaining Activity. After the treatment, activity remained was calculated as percentage of the initial activity.

pH-Measurements. pH value of the solution experimented was measured with Coleman's pH meter (glass electrode), for it was difficult to determine an exact value of the solution contained detergent by the pH test paper.

RESULTS

Inhibition of Amylase by Detergents. The effect of varions concentrations of detergents on amylase was studied. These detergents were mixed with amylase solution in final concentrations of 0,002% to 0.5%, and allowed to stand for 60 minutes at 20°C. Thereafter amylase activity remained was estimated at pH 6.0 for anionic-(SDS) and amphoteric-(Am-2) treatment, at pH 4.7 for non-

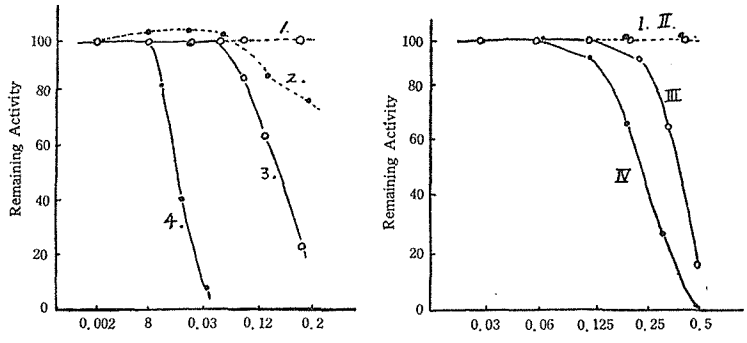

Fig. 1. Comparison of the effect of varying concn. of detergent on amylase at 20° for 60 min.

1, α-amylase treated with Osvan.
2, β-amylase treated with Osvan.
3, α-amylase treated with SDS.
4, β-amylase treated with SDS.
I, α-amylase treated with N-Y.
II, β-amylase treated with N-Y.
III, α-amylase treated with Am-2.
IV, β-amylase treated with Am-2.

ionic-(N-y), cationic-(osvan) treatment and control (not treated with detergent), considering the effect of pH on detergent inhibition as described in the following.

From the results of Fig. 1, it was evident that the ranks of inhibiting power on amylase, were SDS, Am-2 and Osvan : and non-ionic (N-Y), did not show any inhibition. Generlly, α-amylase seems more stable than β-amylase against detergent.

Effect of pH. The effect of detergents on amylases at different pH's is shown in Fig. 2.

Amylase and detergent were left in contact for 30 min. at various pHs, and 20°C ; and the estimation of activity remained was carried out at pH 6.0 for anionic- and amphoteric-, at 4.7 for cationic- and nonionic-detergent treated solution.

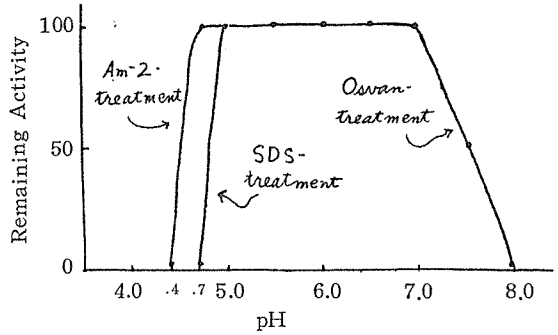

Fig. 2. Effect of pH on inhibition of amylase by detergent.

The pH/inhibition curve for amylase which was sharp on the acid side in the cases of SDS and Am-2, though Osvan on the alkaline side. The enzymes were unaffected by N-Y at all pH range tested.

Effect of Temperature. Most chemical and enzymatic reactions are influenced by temperature. The occurrence of complex of detergent and enzyme may be affected by temperature, and so, the enzyme was left in contact with detergents at various temperatures for 10 min., thereafter the remaining activity was estimated as described above.

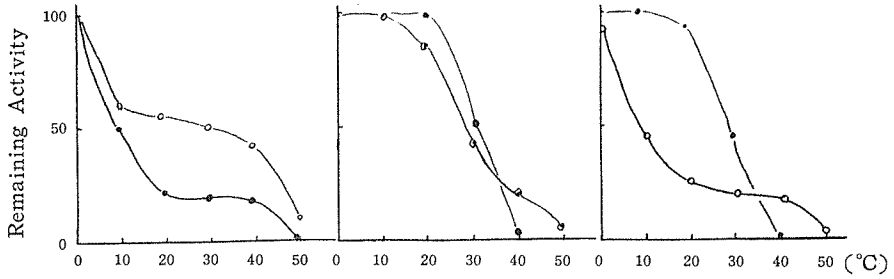

Fig. 3. Effect of temperature on inhibition of amylase by detergent.
—○— α-amylase —●— β-amylase

In general, enzymes are very susceptible to thermal inactivation, the higher the temperature, the more rapidly are the catalytic properties of the enzyme destroyed. The thermal inactivation on enzyme treatment with detergent is very interesting, for it is a so low temperature as shown in Fig. 3, i.e, at 10° on SDS inhibition of both amylases and Am-2 inhibition of α-amylase, at 30° on Osvan inhidition of both amylases and Am-2 inhibition of β-amylase.

Effect of Time. The inhibition of detergent on enzyme is affected with concentrations of enzyme and detergent, pH, temperature and the length of time contacted. The effect of time of contact with amylase on inhibition by detergent was examined as follows. Pure α-and β-amylase solution 5 ml was left in contact with detergent 5 ml. which concentration is SDS 0.1%, Osvan 0.025%, N-Y 0.05% and Am-2 0.125%, for varying length of time, at 20° acid and alkaline pH adjusted with acetic acid and ammonium solution, and at various time intervals afterwards the remaining activity was estimated as described above.

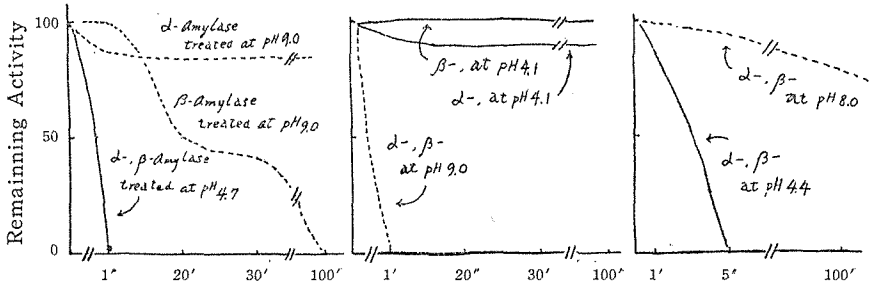

Fig. 4. Effect of the time of contact with amylase on inhibition by detergent. Ordinate : Remeining Activity ; Abscissa : Time (min.) contacted with detergent.

The inhibition occured in a short time at acid pH by SDS and Am-2, and at alkaline pH by Osvan. Non-ionic detergent could not inhibited enzyme on the side of acid and alkali.

Reversibility of Inhibition. Inhibition of amylase by detergent in more concentrated state than CMC (critical micelle concentration) and at 55° for 5 min. showed to be irreversible with any treatment, that is, dilution with water, cooling, passing through ion-exchange resin, and adding of detergent adversely charged.

Inhibition produced by Osvan at pH 8.0 was found, however, to be reversible when the pH was adjusted. For example, amylase solution 5 ml. was mixed

Table. 1. Reversibility of Osvan inhibition of amylase with the change of pH from 8.0 to 6.5.

Time (min.)*	Reversibility (%)				
	0′	0.5′	10′	20′	40′
α-Amylase	98	90	70	42	20
β-Amylase	85	73	48	18	0

* Time length of contact of amylase with Osvan at pt pH 8.0.

with 0.1 % Osvan 5 ml at pH 8.0 and left at various time intervals afterwards it was neutralized to pH 6.5 with 0.1 N acetate buffer (pH 4.1) 5 ml., then activity restored was estimated.

Under the present condition, formation of complex of amylase and Osvan will be slow and loose, and by changing the pH from 8.0 to 6.5 the complex will dissociate and make the activity reversed.

Inhibition of amylase by SDS at pH 4.7, however, could not be reversed by the change of the pH to 6.5, and it was assumed that the complex of amylase and SDS would be considerably more powerful than that of Osvan.

The author observed a marked precipitate when SDS was mixed with Osvan, as a consequence of which the inhibition of enzyme was abolished. These experiments suggest that it is possible to protect enzymes against inhibition by detergent with adversely charged one.

Amylase solution 5 ml. was mixed with 0.05% anionic detergent (SDBS) 4.5ml and kept for varying time at 23°, and then added 0.25% Osvan 0.5 ml. The activity restored was estimated as described above. The result is shown in Table 2.

Table 2. Reversibility of SDBS inhibition of amylase with Osvan.

Time (min.)*	Reversibility (%)				
	0′	0.5′	2′	5′	15′
α-Amylase	75	70	70	66	40
β-Amylase	67	60	60	52	12

* Time of contact of amylase with SDBS.

Effect of Ca-ion. In general, with Ca-ions α-amylase is protected and β-amylase is inhibited on heat treatment. In this examination the author found that α-amylase was protected with Ca-ions on the inhibition by Osvan at pH 8.5.

A mixture of amylase solution 5 ml. and 0.05% Osvan 5 ml. was kept at pH 8.5 and 28°C for a several minutes, then 0.4*M* $CaCl_2$ solution 5 ml. was added into, and allowed to stand for 5 min. more. The remaining activity was measured at pH 4.7 as mentioned above.

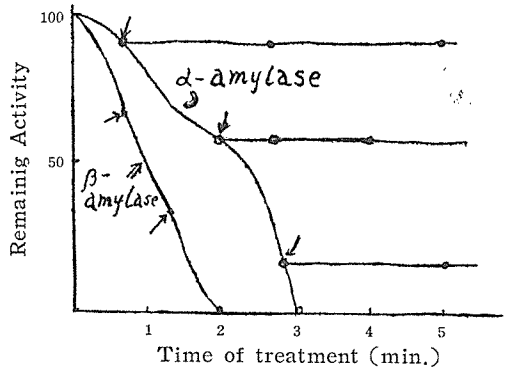

Fig. 5. Effect of Ca-ion on Osvan-inhibition of amylase (arrows show the time of adding of $Cacl_2$)

As shown in Fig. 5. when $CaCl_2$ was added after Osvan had been in contact with α-amyiase for a short time, the inhibition stopped immediately, but it was not able to reverse the inhibition. Ca-ion seemed to protect native α-amylase but could not reactivate denatured one. Contrary to the case of α-amylase, on the inhibition of β-amylase by Osvan Ca-ion showed no protection.

DISCUSSION

It has been suggested that most of enzymes belong to globular proteins and that the enzyme action depends on the specific internal structure of their molecules which are stabilized by a great number of weak secondary intramolecular bonds. On the other hand the denaturation of a globular protein is thought to arise from an alternation of this intramolecular structure and deformation of enzyme molecule.

Now, the results of the investigations on the effect of detergents of all types on amylase have indicated that these detergents, except non-ionic ones, combined with the enzyme so strongly that the distortion of enzyme molecule and irreversible inactivation occured.

Interpretation of the results on the effect of concentration of the detergent on amylase (Fig. 1) is greatly facilitated by considering the behaviour of the detergent molecule in solution. Physicochemical investigations[14] of detergent solutions, by the measurement of electrical conductivity, osmotic pressure, solubilization and by other methods, have shown that the molecule of the detergent may exist in solution either as a free ion or as a micelle. In dilute solutions the detergent exists mainly as the free ion, but in concentrated solutions the micelle predominates. Now, for instance, according to Hartley[15] (1936) and Ralston[16] (1946), it is thought to be approximately spherical and to be consisted of several associating molecules of SDS with a negatively charged surface layer. The transition between two forms, i. e., free ion and micelle, is fairly sharp and occurs at a critical concentration which depends on the salt concentration as well as the temperature.

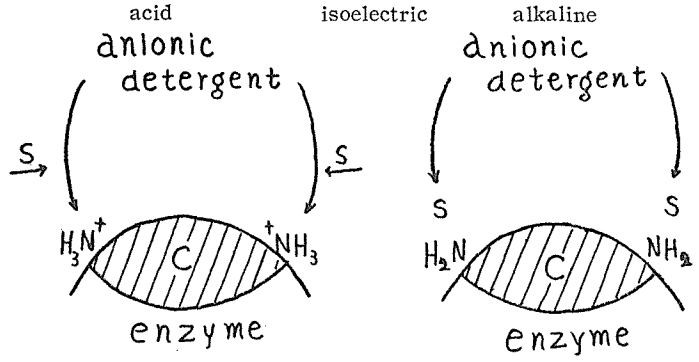

Fig. 6. Schematic illustration of the possible mode (bridge type) of action of detergent on enzyme (after Wills[3] (1950)).
C : active center or center of enzyme
S : substrate molecule

It is considered that the inhibition of enzyme with the detergent will be stimulated rapidly in solutions the micelle predominates, and as its mechanism Wills[17] showed the conception of a bridging mechanism, that is, binding of the enzyme and detergent by the bridge type of combination causes distortion of the molecule and denaturation.

A bridge type combination of the detergent occurs on the one side of isoelectric point of enzyme protein as shown in Fig. 6.

pH may affect the enzyme property and an ionization at the active site of the enzyme may affect its catalytic activity. The enzyme protein increases positive and negative charge on acid and alkaline side respectively. Therefore, when a complex of detergent and enzyme in electrostatistically occurs, anionic- and cationic detergents increase the affinity against enzyme protein on acid side and alkaline side, respectively (Fig. 2). In this respect, the sharp pH/inhibition curve may be due to the increasing of more complex at inhibiting pH.

Reversibility of the detergent inhibition of amylase with several techniques seems to show that these detergents on treating with lower concentration is probably liberated from the complex with enzyme when the pH of the solution tested is adjusted and the detergent is discharged with adversely charged one (Tables 1, 2). Then, the native state is found again.

A probable explanation of the difference of detergent inhibition between α- and β-amylase is that, in addition to the simple ionic binding, there is a possibility of association of the long hydrocarbon chain with the peptide chains of the enzyme protein. This is known to occur in the case of SDS according to Putnam[18].

Now, it is concluded that the inhibitory effect of the detergent on amylase is influenced by the concentration of the former, the pH, the temperature and the time of their contact. Its mechanism is not clarified yet, because the state of detergent and enzyme protein is so complex that they have not been understood as yet.

SUMMARY

The effect of synthetic detergents on malt amylases has been studied. All detergents showed characteristic behaviors on the treatment of amylase. The inhibiting power of detergents on amylases were SDS (anionic)>Am-2 (amphoteric)>Osvan (cationic-), while N-Y (non ionic-) did not show any inhibition. These detergents, with the exception of the nonionic one, inhibited powerfully the amylases under certain conditions, which were related with the concentration of the detergent, the pH, the temperature and the Ca-ion. In general, α-amylase was more stable than β-amylase against detergent treatment.

ACKNOWLEDGMENT

The author wishes to express his sincere thanks to Prof. H. Katagiri for his valuable guidance concerning this investigation, and to acknowledge the experimental assistance of Mr. H. Anyoji and Mr. J. Yagi.

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