BBA 66530

EFFECT OF TRITON X-100 ON SWEET POTATO β-AMYLASE

YASUHITO TAKEDA AND SUSUMU HIZUKURI
Faculty of Agriculture, Department of Agricultural Chemictry, Kagoshima University, Kagoshima (Japan)

(Received October 4th, 1971)

SUMMARY

The inactivation of sweet potato β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) by high dilution of the enzyme solution was prevented and most of the inactivated enzyme was restored by the addition of Triton X-100 above the critical micelle concentration. Triton X-100 also protected the enzyme from denaturation by heat and sodium dodecyl sulfate. Various proteins and synthetic polymers, such as polyethylene glycol and polyvinyl alcohol, had a similar stabilizing effect as Triton X-100, but the synthetic polymers of low polymerized products were less or not effective. These facts suggest that Triton X-100 is effective in maintaining and restoring the native folded structure of the enzyme and that the effect is due to its macromolecular behavior.

INTRODUCTION

β-Amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) has been found abundantly in some cereals, tubers and roots[1] and has been purified in the crystalline state from sweet potato[2], barley malt[3], wheat[4] and soy bean[5]. On account of its strict substrate specificity to the hydrolysis of only a penultimate α-1,4-glucosidic linkage of starch-type molecules from their non-reducing ends to yield β-maltose, the enzyme is useful in the determination of the structure of starch, glycogen and their derivatives, as well as for the industrial production of maltose.

Since the β-amylase of sweet potato was purified by Balls *et al.*[2] in 1948, the enzyme has been studied by many workers from various aspects. The enzyme appears to be composed of 3 or 4 polypeptide chains[6-8] and the molecular weight of the associated form has been reported to be 156 000 (ref. 9), 197 000 (ref. 6) and 215 000 (ref. 10). The enzyme contains many sulfhydryl groups[2] and at least some of them are presumed to be directly or indirectly involved in its activity[6]. It has been suggested that all the sulfhydryl groups are buried in the molecule[2,7,11]. The enzyme is inactivated easily in diluted solution. The nature of this inactivation is not clear yet

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

but some sulfhydryl compounds, proteins[12], polycations[13] and glycerol[14] have been used to protect the enzyme from inactivation. In the previous paper[15] we suggested that a neutral detergent, Triton X-100, was a good protector of the enzyme. In the present paper, we deal with the effect of Triton X-100 on the activity and structure of the enzyme.

MATERIALS AND METHODS

Materials

Sweet potato β-amylase was purified by the method described in the previous report and crystallized from aqueous solution[15].

Bovine serum albumin and Sephadex G-25 (coarse) were obtained from Sigma Chemical Co. and Pharmacia Fine Chemicals, respectively. Soluble starch was obtained from Junsei Pure Chemicals and Co. Triton X-100 and other reagents of the highest grade were purchased from Kako Pure Chemical Industries.

Glucoamylase and α-amylase were generously donated by Dr. Tsujisaka and Nagase and Co., respectively.

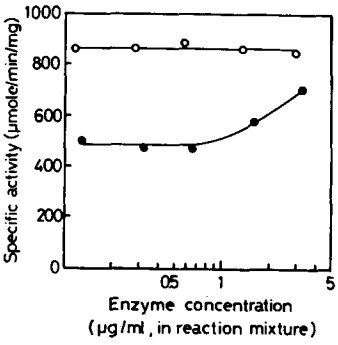
Methods

β-Amylase activity was assayed by the same method as described previously[15]. The enzyme solution was mixed with an equal volume of the reagent mixture and incubated at 37° for 10 min. The resultant reductive capacity was measured by Somogyi–Nelson's method[16]. The enzyme concentration was measured by spectrophotometry, using $E^{1\%}_{1\,cm} = 17.7$ at 280 nm (ref. 15). The sulfhydryl groups of the enzyme were determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), as described by Ellman[17].

RESULTS

Effect of Triton X-100

On specific activity. Dilution of the crude extract of β-amylase from sweet potato and a crystalline suspension of the enzyme with 0.04% Triton X-100 in 50 mM acetate buffer, pH 4.8, resulted in an approx. 1.6-fold higher activity than with the buffer in the absence of the detergent. The specific activity of the purified enzyme, assayed by the procedure described in the previous paper[15], was appreciably lower than that obtained by the method of Balls *et al.*[2], but an equally high specific activity could be obtained by the addition of 0.02% Triton X-100 into our assay system[15], while the detergent had no effect on the method of Balls *et al.*[2]. Our assay method uses approx. 300-fold more diluted enzyme solution and the enzyme concentration is 40-fold more diluted in our assay conditions than in those of the method of Balls *et al.*[2]. These facts suggest that inactivation of the enzyme occurs in our procedure and that this inactivation may be reversed or inhibited by the detergent. This was confirmed by the following results. As shown in Fig. 1, the specific activity of the enzyme was dependent on concentration and it decreased upon dilution in the absence of Triton X-100, while it was higher and constant in the presence of the detergent. Thus, the effect of the detergent on the activity is remarkable at higher dilutions. Below a concentration of 0.6 μg/ml, however, the specific activity no longer



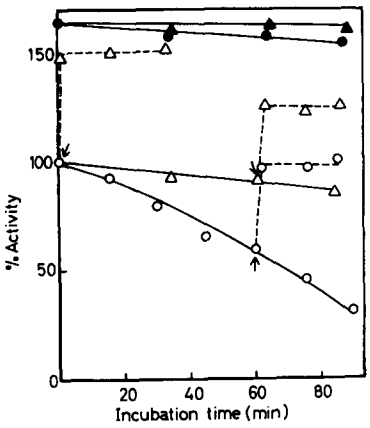


Fig. 1. Relation between specific activity and enzyme concentration with and without Triton X-100. The enzyme was diluted with 50 mM acetate buffer, pH 4.8, (●—●) or the buffer containing 0.04% Triton X-100 (○—○). Activity was assayed at 20° in 3% soluble starch and other conditions were the same as in the text. The activity in the absence of Triton X-100 varied somewhat in each determination and the plots are the mean values of 3 determinations.

Fig. 2. Effect of Triton X-100 on the inactivated enzyme upon dilution. A stock enzyme solution (50 mg/ml) was diluted to 0.26 μg/ml with ice-cold 50 mM acetate buffer, pH 4.8, (○,△) or 0.4% Triton X-100 in this buffer (●,▲) and the diluted enzyme solution was incubated at 0° (△,▲) and/or 37° (○,●). At the time indicated by the arrows, Triton X-100 (final concn., 0.04%) was added and the activity was followed, as shown (— — —).

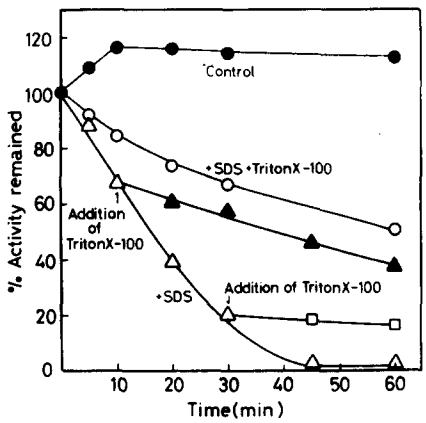
decreased and the specific activity was approx. 1.7-fold higher in the presence of Triton X-100. The suitable enzyme concentration for our assay procedure is approx. 0.03–0.15 μg/ml in the incubation mixture.

On inactivation. The enzyme activity decreased by 39% on dilution under the conditions indicated in Fig. 2, but 77% of the lost activity was restored immediately by the addition of Triton X-100 after the dilution. Therefore, the inactivation upon dilution was mainly reversible but a small portion of its was irreversible. The enzyme maintained the initial (zero time) activity at 0° in the presence or absence of Triton X-100, but was inactivated rapidly at 37° in the absence of Triton X-100 and this inactivation was prevented effectively by the detergent. Upon incubation without Triton X-100, the irreversible part of the enzyme activity increased. Thus, it is clear that Triton X-100 is capable of reactivating the inactivated enzyme on dilution and of protecting the enzyme against the inactivation by heat. Therefore, it is concluded that the activation caused by Triton X-100 in our assay procedure was not true activation of the enzyme.

The enzyme was inactivated on incubation with sodium dodecyl sulfate and the inactivation velocity was greatly reduced by the addition of Triton X-100, as shown in Fig. 3. The inactivation, however, could not be reversed in this case.

On pH–activity. The pH–activity curves in the presence or absence of Triton X-100 are shown in Fig. 4. In both cases, the pH–activity profiles were quite similar and the activity optima were at the same pH of 5.5. The activity was increased approx. 1.6-fold by the addition of Triton X-100 in a pH range measured.

On kinetic constants. The Michaelis constant for soluble starch was the same in the presence or absence of Triton X-100 but the v was 1.5-fold higher in the presence



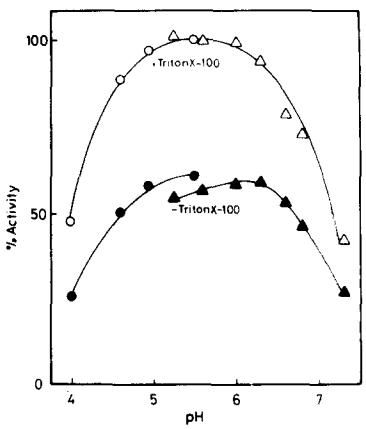
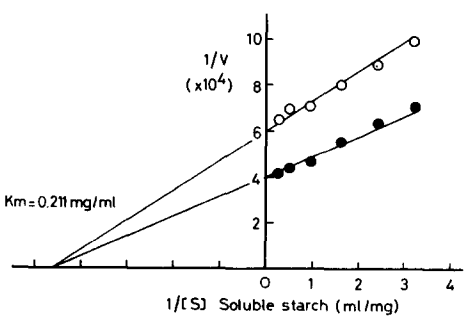


Fig. 3. Effect of Triton X-100 on the denaturation of the enzyme by sodium dodecyl sulfate. The enzyme in 40 mM phosphate buffer, pH 8.0, was incubated at 25° with and without 0.48% sodium dodecyl sulfate. At the time intervals indicated, Triton X-100 (final concn., 0.08%) was added to the incubation mixture. The incubation was terminated by 1500-fold dilution with ice-cold 50 mM acetate buffer, pH 4.8. SDS represents sodium dodecyl sulfate.

Fig. 4. pH–activity curves of the enzyme in the presence and absence of Triton X-100. Activity was assayed as described in the text, but 0.1 M acetate buffer (○,●) and 50 mM phosphate buffer (△,▲) were used. The activity at pH 5.5 in the presence of 0.02% Triton X-100 is expressed as 100%.

of the detergent (Fig. 5). This fact is keeping with the thought that the detergent protects the enzyme from partial inactivation and recovers the active form in highly diluted conditions.

On sulfhydryl groups. The effect of Triton X-100 on the conformation of the protein has been examined by the behavior of the sulfhydryl groups of the enzyme. Sulfhydryl groups have been titrated with DTNB, since it is known to react with sulfhydryl groups without causing denaturation of protein. As shown in Fig. 6, sulfhydryl groups in the native enzyme were not modified by DTNB in the presence or absence of Triton X-100, but in the presence of sodium dodecyl sulfate, 6 sulfhydryl groups per monomer of the enzyme (mol. wt. 57 200 (ref. 8)) were modified. This value



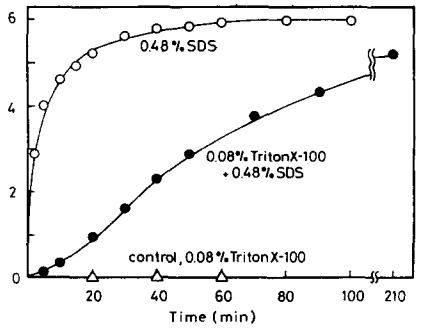


Fig. 5. Effect of Triton X-100 on the kinetic constants of the enzyme. The stock enzyme solution was diluted with 50 mM acetate buffer, pH 4.8, (○) and 0.04% Triton X-100 in this buffer (●).

Fig. 6. Titration of sulfhydryl groups of the enzyme with DTNB. Titration was carried out at pH 8.0 and at 23°. SDS represents sodium dodecyl sulfate.

agreed well with that of half cystine, obtained by an amino acid analysis[18]. Therefore, all the sulfhydryl groups in the enzyme are thought to be in the reduced form and are buried in the enzyme molecule. This coincides with the result reported recently by Uehara *et al.*[7]. The titration rate of the sulfhydryl group with DTNB in the presence of sodium dodecyl sulfate was retarded appreciably by the addition of Triton X-100, suggesting the counteraction of Triton X-100 and sodium dodecyl sulfate. Incidentally, Triton X-100 did not interfere with the titration of cysteine with DTNB.

On chromatographic nature. When the highly diluted enzyme solution with the buffer was applied onto the column of Sephadex G-25 in the absence of Triton X-100, only a small portion of the activity was found in the effluent and a large portion of the activity was retained in the column. The retained activity was recovered by eluting the column with Triton X-100 solution. On the other hand, when the diluted enzyme solution with the buffer containing Triton X-100 was applied onto the column, which

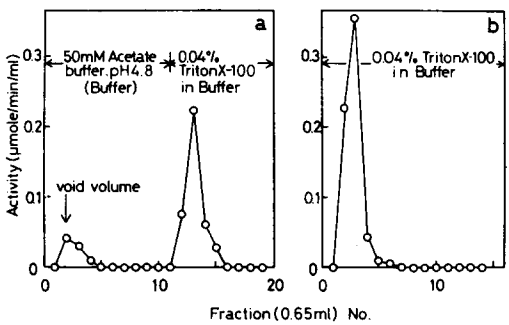


Fig. 7. Effect of Triton X-100 on the dextran gel chromatography of the enzyme. (a) A stock enzyme solution was diluted with 50 mM acetate buffer pH 4.8, and was applied onto a Sephadex G-25 (coarse) column (0.7 cm × 3.7 cm) which was prepared in this buffer. The column was eluted as indicated. (b) A stock enzyme solution was diluted with 0.04% Triton X-100 in the buffer and was applied onto the column, equilibrated with the buffer containing 0.04% Triton X-100. In both cases, 1 ml of the diluted enzyme solution (0.28 μg/ml) was applied on the column and the recovery of the activity was approx. 63%. All the procedures were carried out at 0° except the assay of the activity.

was equilibrated with the buffer containing Triton X-100, most of the enzyme activity was found in the effluent (Fig. 7). These facts suggest that the partially inactivated enzyme on dilution is bound to Sephadex, possibly by the negative charges scattered on the resin, while the active or reactivated enzyme by the detergent fails to bind to the resin.

Substances having similar effects as Triton X-100

Some possible substances which may act like Triton X-100 to prevent the enzyme from the inactivation upon dilution have been examined. As listed in Table I, many substances, such as proteins, polysaccharides, detergents and synthetic polymers are found to be effective protectors. Among them, albumins, gelatin and Tween 20 were potent protectors, similar to Triton X-100. Sulfhydryl groups of the enzyme have been presumed to have an important role in the enzyme activity, but some sulfhydryl compounds tested and EDTA showed the least effect. This suggests that

TABLE I

EFFECT OF SOME SUBSTANCES ON INACTIVATION OF THE ENZYME UPON DILUTION

The enzyme solution was diluted with the indicated solution and the activity was assayed immediatly after dilution.

*Dilution with 0.04% solution**		*Activity (%)*
None		59.2
Triton X-100		100
Tween 20		101
Ethylene glycol		59.8
Polyethylene glycol	200**	60.9
	600**	77.5
	1000**	83.5
	2000**	84.0
Polyvinyl alcohol	500**	88.2
	1500**	85.8
Agar-agar		82.8
Carboxymethyl cellulose		66.8
Egg albumin		99.5
Gelatin		98.8
Bovine serum albumin		101
Bovine serum albumin *plus* Triton X-100		101
EDTA	(1 mM)	66.8
Cysteine	(1 mM)	65.1
2-Mercaptoethanol	(1 mM)	66.3

* In 50 mM acetate buffer, pH 4.8.
** Average degree of polymerization.

the inactivation of the enzyme has little connection with the oxidation of sulfhydryl groups.

Bovine albumin and Tween 20, as well as Triton X-100, also protected the enzyme from inactivation at high temperature, as listed in Table II.

DISCUSSION

It is well recognized that some enzymes, such as β-glucuronidase[19,20], testicular

TABLE II

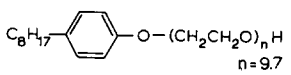
EFFECT OF SOME SUBSTANCES ON HEAT DENATURATION OF THE ENZYME

The enzyme was diluted with the indicated solution (0.36 μg/ml) and incubated at 37° for 1 h.

*Incubation with 0.04% solution**	*Activity (μmole/min per ml)*		*Activity remained (%)*
	Before incubation	*After incubation*	
None	0.305	0.138	45.3
Triton X-100	0.508	0.465	91.5
Tween 20	0.518	0.483	93.3
Polyethylene glycol 1000	0.416	0.205	49.3
Polyvinyl alcohol 1500	0.416	0.207	49.8
Bovine albumin	0.536	0.538	100

* In 0.05 M acetate buffer, pH 4.8.

hyaluronidase[21], aldolase, lactate dehydrogenase, α-amylase as well as β-amylase[13], are inactivated easily when the enzyme solution is diluted beyond a certain level. Bernfeld *et al.*[13,21] demonstrated that this inactivation could be reversed by the addition of polycations and they explained the phenomenon by reversible dissociation of the enzymes into inactive products. They emphasized the cationic nature of the polycations in relation to their reactivation and stabilization effects. In the present study, various kinds of substances, such as proteins, polysaccharides, synthetic polymers and neutral detergents have been shown to stabilize the enzyme to various extents. Ethylene glycol was ineffective but its polymerized products were effective and the effect was enhanced with increasing the molecular weight up to a certain level (Table I). From these results, we may point out that they have a high molecular weight as a common feature.



Triton X-100

Triton X-100* (non-ionic detergent, polyoxyethylated octylphenol (ethylene oxide: 9.7 units)) is not a high molecular material (approx. mol. wt. 635), but it forms micelles above a certain concentration and behaves as a high molecular material. According to Hsiao *et al.*[22], the critical micelle concentration of polyoxyethylated octylphenol (ethylene oxide: 8.5 units) is 0.013%. This concentration agrees reasonably with the minimum concentration showing the highest protective effect, as

TABLE III

TRITON X-100 CONCENTRATION DEPENDENCE OF THE ENZYME ACTIVITY ON DILUTION

The enzyme solution was diluted with the buffer containing Triton X-100, at the indicated concentrations, and the activity was assayed immediately after dilution. The activity diluted with the 0.04% solution is expressed arbitrarily as 100%.

Concentration of Triton X-100 (%)	*Activity (%)*
0	62.5
0.00004	68.8
0.0004	79.4
0.004	93.8
0.04	100
0.2	101
0.4	102

indicated in Table III. The molecular weight of the micelle, measured by ultracentrifugation analysis, has been reported to be 63 000 (ref. 23). These facts suggest that Triton X-100 is effective as a macromolecule.

It has been well demonstrated by the present study that the effect of Triton X-100 in bringing about a higher specific activity in the highly diluted state is due to the reactivation of the inactivated enzyme on dilution and to the protection of the enzyme from the inactivation.

* Purchased from Wako Pure Chemical Industries (Osaka, Japan), a product of Rohm and Haas Co. (Philadelphia, Pa., U.S.A.)

The detergent protected the enzyme from denaturation by heat (Fig. 2) and sodium dodecyl sulfate (Fig. 3) and retarded the titration rate of the buried sulfhydryl groups of the enzyme in the presence of sodium dodecyl sulfate (Fig. 6). These facts imply that the detergent restores and stabilizes the native folded structure of the enzyme. It is of interest that a reverse effect of the detergent has been reported on the Na^+,K^+-activated ATPase of rat brain[24], *i.e.* the detergent discloses some sulfhydryl groups of the enzyme.

Although it is uncertain whether the partly inactivated enzyme on dilution is a mixture of the fully active and inactive form or a partly active form, the kinetic properties (Fig. 5) and pH–activity curves (Fig. 4) seem to suggest that the enzyme is a mixture of the active and inactive form. The inactive form (or partially active form) should be differentiated from the denatured form, which is not activated by the detergent. Therefore, we may write the inactivation process of the enzyme as follows:

$$\text{Active form} \rightleftharpoons \text{inactive form (or partially active form)} \dashrightarrow \text{denatured form}$$

Here, the active and the inactive forms are in equilibrium below a certain concentration and Triton X-100 inhibits the inactivation and facilitates the activation. The inactive form is assumed to be unfolded slightly more than the active form or might be less oligomeric, since the enzyme has been suggested to be composed of 3 or 4 polypeptide chains[6–8]. This equilibrium mechanism may be supported by the chromatographic behavior of the enzyme, as shown in Fig. 7. The active molecule, by its compact conformation, is presumed to carry insufficient positive charges on the surface for retention by the negative charges scattered on the resin. On the other hand, the inactive form has possibly more available surface and reveals more positive charges than the active form and is retained on the resin. When the diluted enzyme solution without Triton X-100 is applied on the column, a large portion of the enzyme may be retained on the resin in the inactive form of the equilibrium, being eluted with Triton X-100 by restoration of the active form. While, when the diluted enzyme

TABLE IV

PROTECTIVE EFFECT OF TRITON X-100 ON SOME ENZYMES

Enzyme	*Protein concentration in the assay mixture (μg/ml)*	*Ratio of activity diluted with and without Triton X-100 (0.04%)*
Sweet potato β-amylase (crude extract)*	0.67	1.60
Soy bean β-amylase (crude extract)**	12.5	1.54
Glucoamylase (*Rhizopus delemar*, crystalline)	7.90	1.40
α-Amylase (*Bacillus subtilis*, crystalline)	0.25	1.57

* Crude extract was prepared as described previously[15].

** Soy bean was steeped in water overnight and homogenized with a 9-fold volume (v/w) of distilled water for 1.5 min using a blender. The homogenate was centrifuged for 10 min at 10 000 × *g* and the resulting supernatant was used.

solution with Triton X-100 is put on the column, a large portion of the enzyme is already in the active form and may pass through the column unchanged.

It is of interest whether the present effect of Triton X-100 is common to other enzymes. It has been observed that Triton X-100 is capable of protecting the β-amylase of soybean, the α-amylase of *Bacillus subtilis* and the glucoamylase of *Rhizopus delemar*, to a greater or lesser extent, from the inactivation upon dilution (Table IV).

Triton X-100 is a very useful stabilizer of β-amylase, since it is more effective than sulfhydryl compounds and not a reducing agent, which interferes with the determination of reducing sugars. In fact, it does not interfere with the assay of maltose by Somogyi–Nelson's method at the concentration which is optimal in stabilization of β-amylase.

REFERENCES

1 M. Nakamura, K. Yamazaki and B. Maruo, *Nippon Nōgei Kagaku Kaishi*, 24 (1950) 197.
2 A. K. Balls, M. K. Walden and R. R. Thompson, *J. Biol. Chem.*, 173 (1948) 9.
3 A. Piguet and E. H. Fischer, *Helv. Chim. Acta*, 35 (1952) 257.
4 K. H. Meyer, P. F. Spahr and E. H. Fischer, *Helv. Chim. Acta*, 36 (1953) 1924.
5 J. Fukumoto and Y. Tsujisaka, *Kagaku to Kōgyo*, 28 (1954) 282.
6 J. Spradlin and J. A. Thoma, *J. Biol. Chem.*, 245 (1970) 117.
7 K. Uehara, T. Mizoguchi and S. Mannen, *J. Biochem.*, 68 (1970) 359.
8 Y. Takeda, S. Hizukuri and T. Murakami, *Agric. Biol. Chem.*, 35 (1971) 778.
9 S. Englard and T. P. Singer, *J. Biol. Chem.*, 187 (1950) 213.
10 A personal communication from M. Burr and D. Yphantis to J. A. Thoma, see J. A. Thoma, D. E. Koshland, Jr., J. Ruscia and R. Baldwine, *Biochem. Biophys. Res. Commun.*, 12 (1963) 184.
11 Y. Takeda and S. Hizukuri, *Annu. Meet. Denpun Kōgyo Gakkai, Japan*, abstr. in Japanese, 1969, p. 25.
12 G. J. Walker and W. J. Whelan, *Biochem. J.*, 76 (1960) 264.
13 P. Bernfeld, B. J. Berkeley and R. E. Bieber, *Arch. Biochem. Biophys.*, 111 (1965) 31.
14 W. Banks and C. T. Greenwood, *Stärke*, 21 (1969) 177.
15 Y. Takeda and S. Hizukuri, *Biochim. Biophys. Acta*, 185 (1969) 469.
16 J. E. Hodge and B. T. Horfeiter, *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, p. 386.
17 G. L. Ellman, *Arch. Biochem. Biophys.*, 82 (1959) 70.
18 J. A. Thoma, D. E. Koshland, Jr., R. Shinke and J. Ruscica, *Biochemistry*, 4 (1965) 714.
19 P. Bernfeld, H. C. Bernfeld, J. S. Nisselbaum and W. H. Fishman, *J. Am. Chem. Soc.*, 76 (1954) 4872.
20 P. Bernfeld, S. Jacobson and H. C. Bernfeld, *Arch. Biochem. Biophys.*, 69 (1957) 198.
21 P. Bernfeld, L. P. Tuttle and R. W. Hubbard, *Arch. Biochem. Biophys.*, 92 (1961) 232.
22 L. Hsiao, H. N. Dunning and P. B. Lorenz, *J. Phys. Chem.*, 60 (1956) 657.
23 C. W. Dwiggins, Jr., R. J. Bolen and H. N. Dunning, *J. Physical Chem.*, 64 (1960) 1175.
24 J. Somogyi and J. G. Gaál, *Experientia*, XXI (1965) 573.