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A Crosslinked Preparation of *E. coli* β-D-Galactosidase

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ABSTRACT

β-D-Galactosidase from *E. Coli* was crosslinked using glutaraldehyde and two bisimidoesters. With glutaraldehyde and dimethyl adipimidate (DMA), it is possible to obtain preparations having higher activity than the native enzyme. Glutaraldehyde and DMA gave preparations showing enhanced thermal stability. The preparation crosslinked with DMA, when used for continuous hydrolysis of lactose in milk, was found to be significantly better than the native enzyme.

Index Entries: *E. coli* β-D-Galactosidase; lactose intolerance; crosslinking; bifunctional reagents.

INTRODUCTION

β-Galactosidase (EC.3.2.1.23) is an important enzyme in carbohydrate metabolism. Deficiency of β-galactosidase is known to lead to lactose intolerance (*1*). Thus pretreatment of milk (*2*), in order to obtain low lactose milk and meaningful utilization of the whey, (*3*) require a stable lactase preparation (*4*). Not surprisingly, this has resulted in a large number of studies on immobilization of the enzyme from various sources on a variety of matrices (*5*). It has been suggested (*6*) that immobilization preceded by crosslinking (i.e., combination of two techniques: preparing soluble intramolecularly crosslinked enzyme and immobilization on solid support), may be a useful approach for obtaining stable and reusable enzyme preparations. With this ultimate goal, we have started studies on

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crosslinking of *E. coli* β-galactosidase. Three different crosslinking reagents, such as glutaraldehyde, dimethyl adipimidate, and dimethyl suberimidate, were used. The preparation obtained with dimethyl adipimidate showed enhanced thermal stability and was investigated further for its application to hydrolysis of milk lactose.

MATERIALS AND METHODS

Materials

E. coli β-D-galactosidase, dimethyl adipimidate hydrochloride (DMA), dimethyl suberimidate hydrochloride (DMS), dimethyl 3-3′-dithiobis propionimidate hydrochloride (DTBP), dithiothreitol (DTT), and glucose oxidase-peroxidase (PGO) kit (for glucose estimation) were obtained from Sigma Chemical Co., USA. *o*-Nitro phenyl β-D-galactopyranoside (ONGP) was obtained from the CSIR Centre for Biochemicals, Delhi, India. Lactose was obtained from SRL Laboratories, India. Commercial glutaraldehyde (25%) was a product of Riedel-Dehaena AG Seetze, Hannover.

METHODS

Assay of β-Galactosidase Activity

Enzyme activity toward ONGP was determined following the method described by Craven et al. (*7*).

The enzyme was also assayed using lactose as its substrate following the PGO enzymatic method (Sigma Technical Bulletin No. 510) (*8*).

The activity of β-galactosidase was also measured using milk (*4*). The milk was skimmed by centrifuging at 10,000 rpm for 10 min at 4°C. The enzyme was dissolved in sodium phosphate buffer (0.3*M*; pH 8.0, containing 0.003*M* $MgCl_2$) to make a final concentration of 100 μg/mL. Two mL of skimmed milk was incubated with 250 μL of the enzyme solution. After 30 min of incubation, 1 mL perchloric acid (1*M*) was added, the reaction mixture was centrifuged, and glucose was estimated in the supernatant with the glucose oxidase-peroxidase system.

Crosslinking of β-Galactosidase with Glutaraldehyde

Solutions of β-galactosidase and glutaraldehyde were prepared in sodium phosphate buffer (0.3*M*; pH 8.0) containing 0.003*M* $MgCl_2$. β-Galactosidase (0.1 mg/mL) was incubated at 25°C with different concentrations of glutaraldehyde. The reaction was stopped by the addition of 100 μL lysine (5.75 mg/mL) after 30 min of incubation.

The time of crosslinking was also varied. β-Galactosidase samples (each containing 0.1 mg/mL enzyme) were incubated at 25°C with 0.25

m*M* glutaraldehyde. The reaction was terminated by adding lysine after different time periods of incubation.

In another set of experiments, varying concentrations of β-galactosidase were incubated at 25°C with 0.25 m*M* glutaraldehyde. The crosslinking was terminated after 60 min of incubation.

Crosslinking of β-Galactosidase with Bisimidoesters

Solutions of β-galactosidase, DMA, DMS, and DTBP were prepared in sodium phosphate buffer (0.3*M*; pH 8.0) containing 0.003*M* $MgCl_2$. However, the bimidoesters were neutralized by adding an equivalent amount of alkali before making the solutions, because these were supplied in the form of hydrochloride derivatives. β-Galactosidase (0.1 mg/mL) was incubated at 25°C for 1 h with varying concentrations of DMA and DMS in different sets of experiments.

In another set of experiments, varying concentrations of β-galactosidase were incubated at 25°C for 1 h with 0.1 mg/mL DMA in one case and 0.02 mg/mL DMS in another.

Crosslinking with the cleavable bifunctional reagent DTBP was carried out by incubating the enzyme (0.35 mg/mL) and the reagent (0.1 mg/mL) together for 1 h at 25°C; the buffer again was sodium phosphate (0.3*M*; pH 8.0) containing 0.003*M* $MgCl_2$. The products were cleaved using DTT (*9*).

In each set of experiments, a control was run simultaneously in which a native β-galactosidase was subjected to similar treatment, except that an equivalent volume of buffer was added instead of the crosslinking reagent.

Heat Treatment

Native and crosslinked preparations in each experiment were subjected to heat treatment of 55°C for 30 min and were assayed for their activity before and after heat treatment.

K_m Determination

K_m of the native as well as crosslinked products were determined using ONGP as the substrate.

Hydrolysis of the Lactose Present in Milk

Two mL skimmed milk (2 mL) was incubated with 25 μg enzyme at 50°C. The hydrolysis of lactose was followed by determining the amount of glucose liberated in the reaction mixture at various time intervals. Glucose was estimated as described earlier in this section using the glucose oxidase peroxidase system (*8*). The initial lactose content of the skimmed milk was determined by using the Folin Wu micro method (*10*).

SDS-PAGE of β-Galactosidase

Both native and DMA crosslinked β-galactosidase samples were subjected to SDS-polyacrylamide gel electrophoresis, essentially following the method described by Fairbank et al. (*11*).

Estimation of Amino Group Modification in β-Galactosidase

Amino groups were estimated in native and crosslinked β-galactosidase following the method described by Habeeb (*12*). The protein samples (140 μg) were incubated with 0.25 mL $NaHCO_3$ (0.4%) and 0.25 mL TNBS (1%) at 40°C. After 2 h incubation, 0.25 mL SDS (10%) and 0.25 mL HCl (1*N*) were added. The absorbance was recorded at 340 nm.

Extent of amino group modification was calculated by comparing the final absorbance values obtained with the native and crosslinked enzyme.

RESULTS AND DISCUSSION

E. coli β-galactosidase is one of the well characterized β-galactosidases (*13*). This also is one of the few galactosidases which has received maximum attention from the workers using an immobilized lactase for hydrolysis of milk lactose (*14*). This is largely because it has an optimum pH near neutrality and is not inhibited by excess substrate or product (*15*).

However, immobilization of the *E. coli* enzyme on solid matrices by various workers has not yielded an enzyme preparation that could be the basis of a commercially viable bioreactor design (*16*). The work of Wondolowski et al. (*17*) indicated that although there was no loss of enzyme activity on immobilization of *E. coli* enzyme, it led to a loss in stability both at higher and lower temperatures. In view of this, crosslinking of the *E. coli* enzyme before immobilizing it seems to be an attractive approach.

When β-galactosidase is treated with various concentrations of DMA, DMS, and glutaraldehyde, the changes in biological activity are shown in Fig. 1.

Since one of the objectives was to obtain a more heat stable enzyme preparation, these various crosslinked preparations were subjected to heat treatment at 55°C. The residual activity is also shown in Fig. 1.

In the case of DMA, beyond 6 μg/reaction volume concentration, the derivatives obtained are more heat stable as compared to native molecule. The concentrations corresponding to 8–10 μg range seem to be best in this respect. In the case of DMS, the crosslinking leads to loss in enzyme activity. The derivatives also were less heat stable than the native enzyme. However, for further optimization (i.e., varying enzyme concentration during crosslinking reaction) the condition corresponding to DMS 2 concentration of 2 μg seems the only one, if any, worth studying.

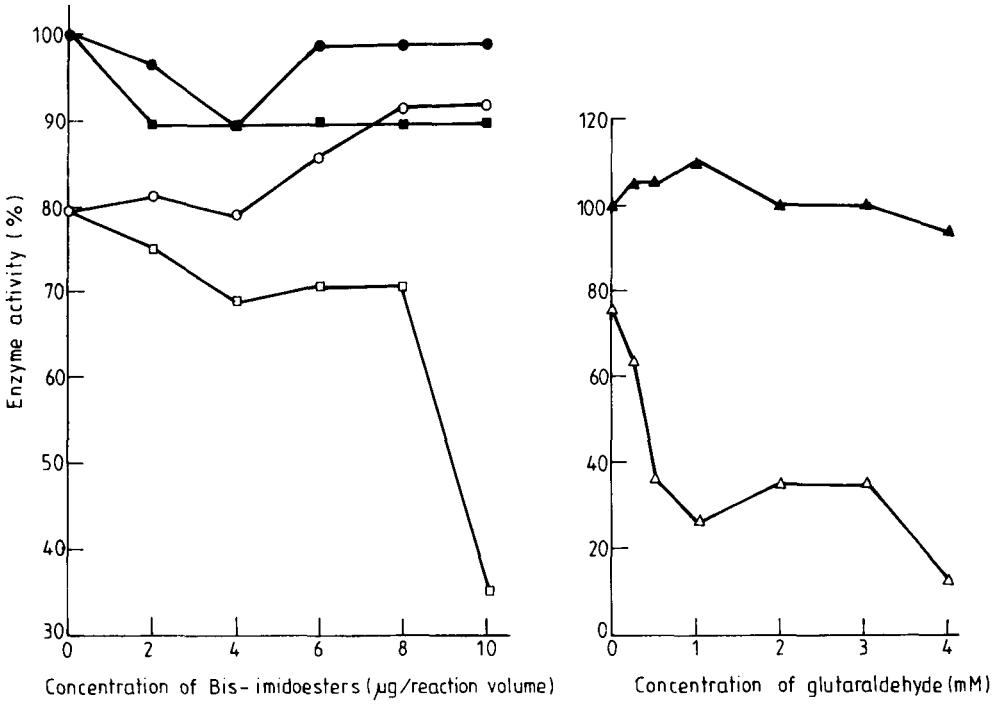

Fig. 1. (a) Crosslinking of β-galactosidase utilizing various concentrations of the bisimidoesters. β-Galactosidase (10 μg) was dissolved in 100 μL sodium phosphate buffer (0.3*M*, pH 8.0), containing 0.001*M* $MgCl_2$; 100 μL of the bisimidoester was added to it and the enzyme activity was assayed after 1 h using ONGP as the substrate. The concentration of the bisimidoesters was varied. For determining the heat stability, the enzyme preparations were incubated at 55°C for 30 min before assaying the activity. ●, DMA product before heat treatment; ○, DMA product after heat treatment; ■, DMS product before heat treatment; □, DMS product after heat treatment. (b) Crosslinking of β-galactosidase using various concentrations of glutaraldehyde. Aliquots of 10 μg of the enzyme in 100 μL of sodium phosphate buffer (0.3*M*, pH 8.0) containing .001*M* $MgCl_2$ were treated with glutaraldehyde of various concentrations in the total reaction volume of 600 μL. The reaction was terminated by adding 100 μL lysine (5.75 mg/mL). The enzyme activities of the product, before and after heat treatment, were measured as described in the case of the bisimidoesters. ▲, Glutaraldehyde product before heat treatment; △, glutaraldehyde product after heat treatment.

With glutaraldehyde as the crosslinking reagent, lower concentration resulted in slight enhancement in enzyme activity. However, at all concentrations, the resultant derivatives were less heat stable compared to the native enzyme. Again for the sake of further attempts at optimization (i.e., varying enzyme concentrations during crosslinking reaction), 0.25 m*M* glutaraldehyde concentration was thought to be worth exploring further, since corresponding to this concentration, the derivative was only a little more heat labile compared to the native enzyme.

Since bisimidoesters are believed to have short half-lives of a few minutes (*18*), and reactions with them are supposed to be fairly fast, a

fixed reaction time of 1 h with no termination step has been used before assaying the activity. The results obtained in our laboratory confirm that 1 h is an adequate time period for carrying out crosslinking of proteins with bisimidoesters and no further reaction occurs after 1 h (*19*). In the case of glutaraldehyde, the influence of varying the reaction time is shown in Fig. 2.

It was seen that in all cases the derivatives had higher enzyme activity. However, the derivatives obtained corresponding to 45–60 min reaction time retained as much activity as native enzymes after being subjected to heat treatment. In all other cases, the derivatives after heat treatment retained less activity compared to the native enzyme. Thus, for checking the effect of varying enzyme concentrations, the reaction time of 60 min seemed appropriate.

Finally, the enzyme concentration was varied, whereas other conditions were chosen as stated and discussed (Fig. 3). The various preparations also were subjected to heat treatment as before (Fig. 3).

From this data, it is obvious that β-galactosidase concentrations of 35 μg and 30 μg were the best choices in case of DMA and glutaraldehyde, respectively. In the case of DMS, even varying enzyme concentrations did not seem to yield a potentially useful product.

The conditions for obtaining the best possible preparations in the case of DMA and glutaraldehyde are summarized in Table 1.

The K_m values of these preparations with ONGP as the substrate were determined and are given in Table 2. A slight increase in the K_m value was observed in the case of both crosslinked preparations. These perhaps represent the changes in enzyme structure that decrease the binding of the substrate or decrease the accessibility of the active site to the substrate.

However, the increase in K_m values was much less compared to the one that occurred on immobilization of the enzyme on solid support (*16,20*).

The derivative obtained with DMA had shown little loss in activity when incubated at 55°C for 30 min. This derivative was chosen for further study.

Bisimidoesters are well known crosslinking reagents that specifically react with amino groups (*6*). Nevertheless, some initial studies directed toward determining the nature of chemical modification were carried out. The TNBS procedure showed that 38% amino acid residues were modified as a result of the reaction with DMA. The SDS-PAGE analysis (Fig. 4) showed that neither inter-subunit nor intermolecular crosslinks have been formed. By a process of elimination, either simple chemical modification (analogous to reaction with a monofunctional reagent) or intra-subunit crosslink formation can be postulated. To decide between these two possibilities, a cleavable bisimidoester analog, namely dimethyl 2,2′-dithiobispropionimidate (DTBP) (*21*) was used. The results

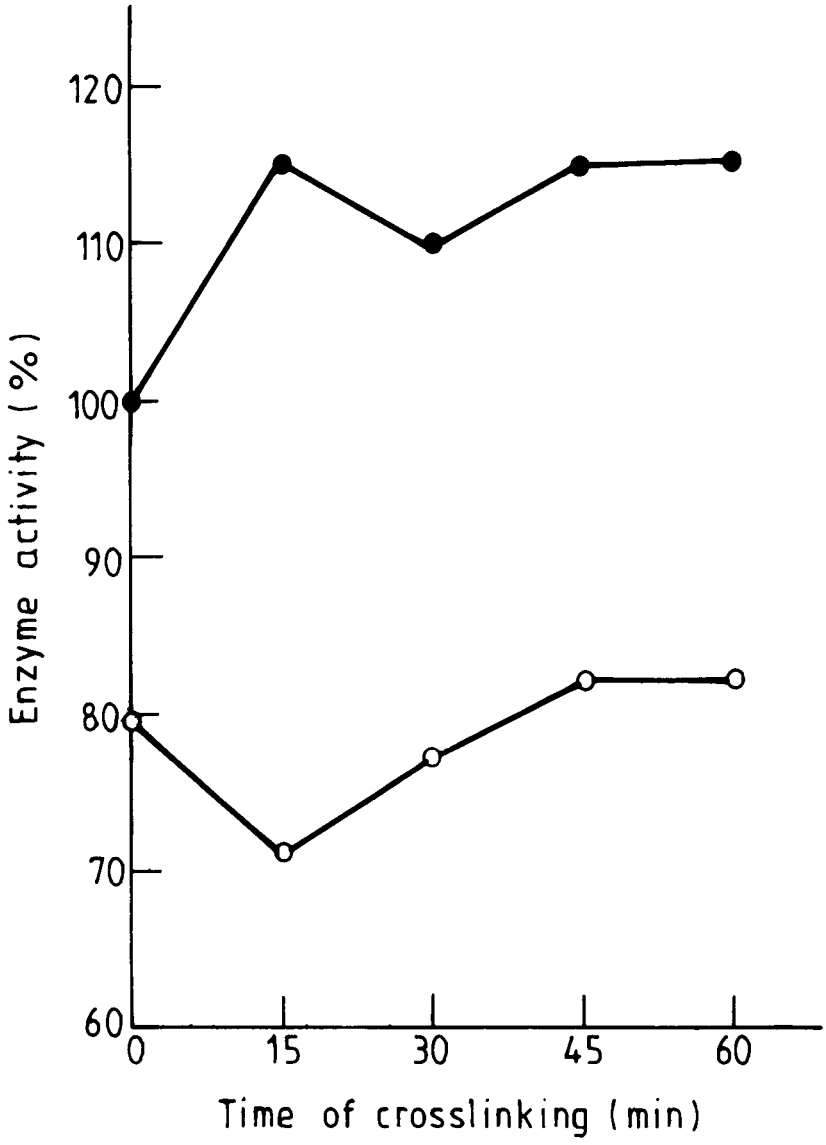

Fig. 2. Time course of crosslinking β-Galactosidase with glutaraldehyde. Stock solutions of β-galactosidase (0.1 mg/mL) and lysine (5.75 mg/mL) were prepared in sodium phosphate buffer (0.3*M*; pH 8.0; containing 0.001*M* $MgCl_2$). The commercially available glutaraldehyde (25%) was also diluted to 0.003% in the above-described sodium phosphate buffer. 100 μL of the β-galactosidase solution was added to the diluted glutaraldehyde solution so that final concentration of glutaraldehyde was 0.25 m*M* in the reaction volume. This reaction mixture was incubated at 25°C. The reactions in different samples were terminated at various time intervals by adding 100 μL of lysine solution. Crosslinked products were subjected to 55°C for 30 min. Enzyme activity was measured before and after heat treatment using ONGP as substrate. ●, β-Galactosidase activity before heat treatment; ○, β-Galactosidase activity after heat treatment.

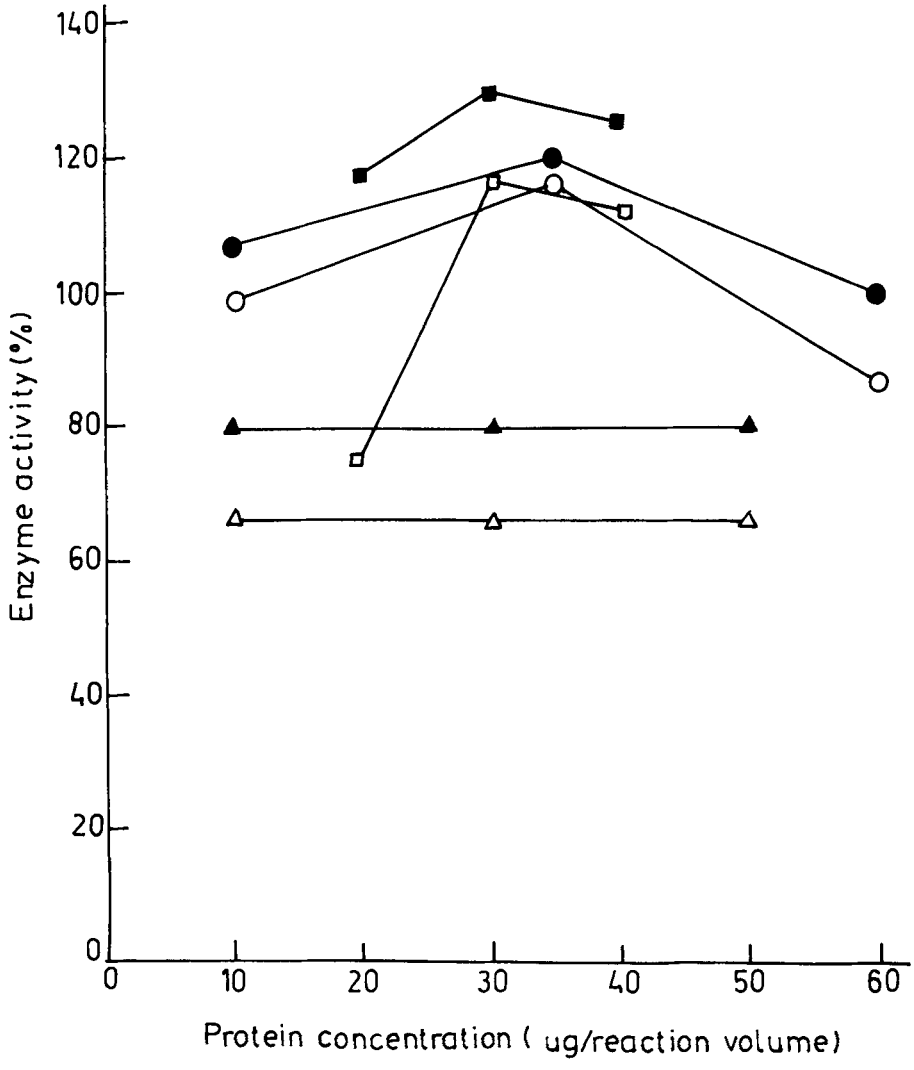

Fig. 3. Crosslinking of β-galactosidase using various concentrations of the enzyme. The enzyme was treated with the reagent (DMA: 10 μg; DMS: 2 μg, glutaraldehyde 0.25 m*M*), the reaction volumes being 200 μL, 200 μL, and 600 μL of sodium phosphate buffer (0.3*M*, pH 8.0, containing 0.001*M* $MgCl_2$), respectively. Other details were as in Fig. 1. The activity values are expressed in terms of percentage activity, assuming the activity of the unmodified enzyme to be 100. The unmodified enzyme lost 20% activity after the heat treatment. ●, DMA product before heat treatment; ○, DMA product after heat treatment; △, DMS product before heat treatment; ▲, DMS product after heat treatment; ■, glutaraldehyde product before heat treatment; □, glutaraldehyde product after heat treatment.

Table 1
Optimum Conditions for Crosslinking of β-Galactosidase

	Conditions for crosslinking				
Bifunctional reagent	Reagent concentration, mg/mL	Protein concentration, mg/mL	Time of cross-linking, min	Temp., °C	pH
Glutaraldehyde	0.025	0.05	60	25	8.0
DMA	0.05	0.17	60	25	8.0

Table 2
K_m Values of Native
and Crosslinked β-Galactosidase Preparations[a]

Sample	K_m, *M*
Native	3.0×10^{-4}
Crosslinked with glutaraldehyde	3.3×10^{-4}
Crosslinked with DMA	4.4×10^{-4}

[a]K_m values were determined from the Lineweaver-Burk plot under the following experimental conditions: ONGP as a substrate in sod. phosphate buffer (0.3*M*) containing 0.003*M* Mg^{++}, pH 7.5, 25°C.

Table 3
Crosslinking with the Cleavable Reagent DTBP[a]

	Percent enzyme activity as compared to native enzyme activity	
Sample	Before addition of DTT	After addition of DTT
Native enzyme	100	95
Enzyme crosslinked with DTBP	126	84

[a]Crosslinking was performed as described in the text and activities were determined using ONGP as the substrate.

summarized in Table 3 indicate that the crosslinked preparation, when subjected to dithiothreitol (DTT), resulted in loss of activity.

Whereas even in the case of native enzyme, DTT treatment has resulted in some loss of activity, the crosslinked enzyme lost slightly more activity. In any case, these data seem to indicate that treatment with the crosslinking reagent led to the formation of crosslinks rather than simple chemical modification of some amino acid residues.

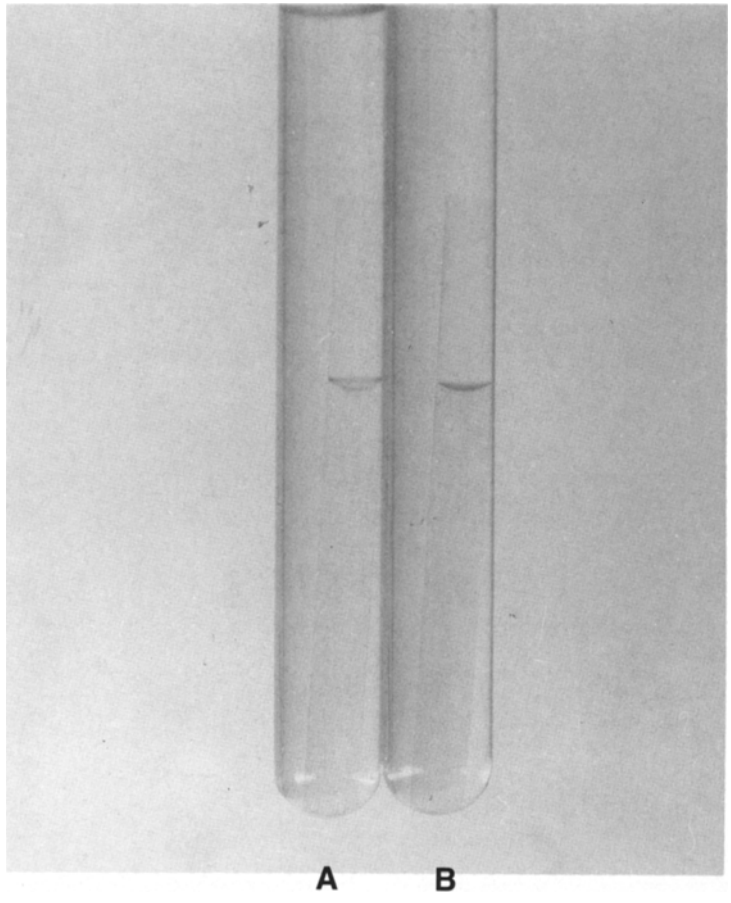

Fig. 4. *SDS-PAGE pattern of β-galactosidase*. A. Native β-galactosidase protein stain; B. DMA crosslinked β-galactosidase protein stain.

The crosslinked preparation obtained with DMA was checked for activity toward lactose and milk. Here, too, most of the activity was found to be retained, and the preparation showed enhanced stability compared to native when subjected to usual heat treatment (Table 4).

Both the native enzyme and the crosslinked enzyme were tested for thermal stability utilizing a longer time period of incubation. It was found that at 50°C, the crosslinked enzyme showed enhanced thermal stability and retained about 35% enzyme activity (compared to 15%) toward ONGP at the end of 20 h incubation at 50°C (Fig. 5).

The above data encouraged us to use the DMA derivative for continuous hydrolysis of milk lactose at 50°C. We were aware that the stability of the enzyme in phosphate buffer and milk may be different at 50°C. However, the stability in milk is always expected to be higher, since milk protein would stabilize to the enzyme against both the heat and dilution factors (*22,23*). Hence, a derivative showing enhanced thermal stability

Table 4
Enzyme Activity of Native and DMA Crosslinked β-Galactosidase Using Lactose and Milk as Substrate[a]

Enzyme	Percent enzyme activity retained as compared to activity before heat treatment, lactose as substrate	Percent enzyme activity as compared to activity before heat treatment, milk as a substrate
Crosslinked enzyme after heat treatment	94	50
Native enzyme after heat treatment	83	20

[a]Before and after incubation at 55°C for 30 min.

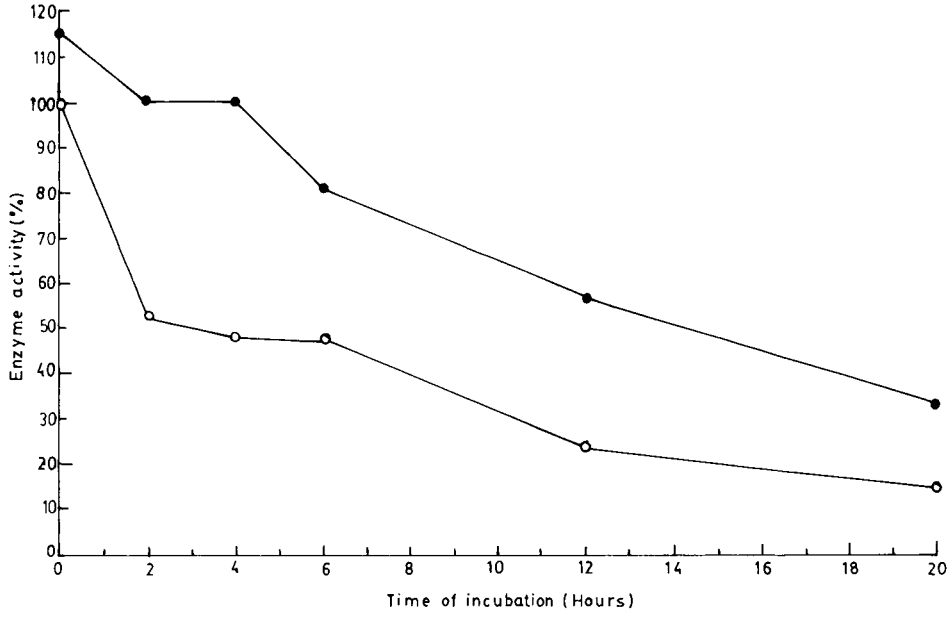

Fig. 5. Effect of temperature on the activity of β-galactosidase. β-Galactosidase preparations (25 μg in 2 mL sodium phosphate buffer, pH 7.0 containing .003*M* Mg^{++}) were incubated at 50°C. Their activities were measured toward ONGP at various time intervals of incubation. ○, Native β-galactosidase; ●, β-galactosidase crosslinked with DMA.

in simple buffer seemed to be a promising derivative to be tried for catalyzing milk lactose hydrolysis at 50°C.

Continuous hydrolysis of milk lactose at 50°C was monitored by using both native and crosslinked enzyme preparations (Fig. 6). Whereas the native enzyme hydrolyzed 40% milk lactose, the crosslinked preparation hydrolyzed 55% milk lactose during 12 h. Pastore and Morisi (24) have described the results of their pilot plant experiment on obtaining

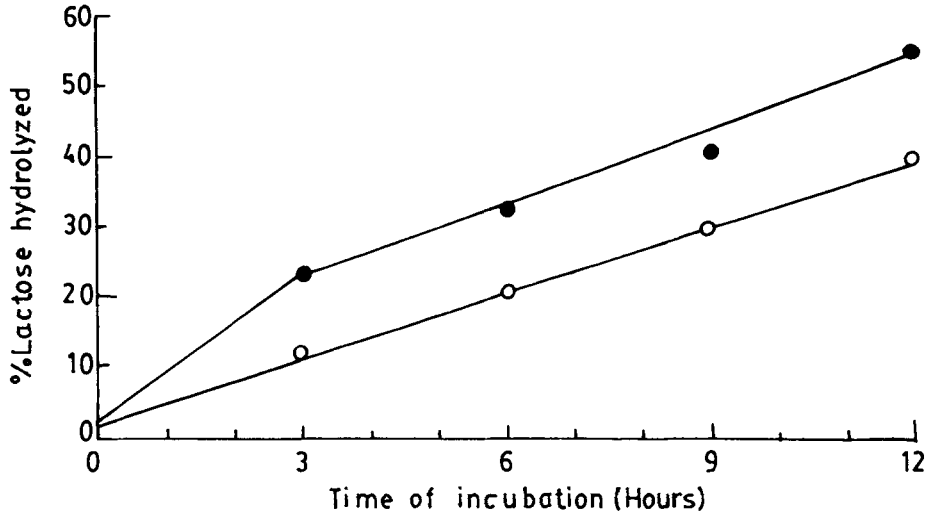

Fig. 6. Continuous lactose hydrolysis of the milk by β-galactosidase. The skimmed milk (2 mL) was incubated with 25 μg enzyme at 50°C. The amount of glucose liberated in the reaction mixture was determined at various time intervals using glucose oxidase peroxidase system. ○, Native β-galactosidase; ●, β-galactosidase crosslinked with DMA.

low lactose milk by fiber entrapped yeast enzyme. In 12 h time, about 56% of the milk lactose is converted to glucose and galactose. That kind of conversion rate has been considered by them to be sufficient for obtaining a dairy product to be used by those suffering from lactose intolerance.

Analysis of the data in Fig. 5 indicates that the crosslinked preparation hydrolyzes greater lactose only in first 3 h. For the remaining time, the rates of hydrolysis were nearly identical. Thus, although the crosslinked preparation hydrolyzed more lactose in 12 h, this advantage would not remain if the crosslinked enzyme preparations were reused. However, in order to make this crosslinked enzyme preparation reusable, an additional step such as immobilization on solid surface or entrapment would be needed.

In order to obtain a reusable enzyme from this crosslinked β-galactosidase, two promising approaches exist. One, of course, is immobilization or entrapment. A second possibility is the formation of a conjugate with a lectin and the use of a selectin column to recover the enzyme from the reaction mixture (25). Both approaches may also lead to greater enhancement in stability. In this context we may add that a ConA-β-galactosidase conjugate when bound to Sephadex retains total enzyme activity up to 12 h at 50°C (26). Also, our preliminary experiments show that the entrapped DMA crosslinked derivative hydrolyzed milk lactose faster than the entrapped native enzyme when the hydrolysis was monitored over a time period of 6 h (26).

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REFERENCES

1. Linko, P. (1982), in *Nutritive Sweeteners* (Birch, G., ed.), pp. 109–131, Appl. Sci. Publ., London.
2. Roland, J. F., Wargel, R. J., Aim, W. L., Kiang, S. P., and Bliss, F. M. (1984), *Appl. Biochem. Biotechnol.* **9,** 15–26.
3. Kosaric, N. and Asher, Y. J. (1985), *in Adv. Biochem. Eng.* (Fiechter, A., ed.), vol. 19, pp. 25–60, Springer-Verlag, New York.
4. Shukla, T. P. (1975), *CRC Crit. Rev. Food Technol.* **5,** 325.
5. Richmond, M. L., Gray, J. I., and Stine, C. M. (1981), *J. Dairy Sci.* **64,** 1759–1771.
6. Uy, R., and Wold, F. (1977), in *Biomedical Applications of immobilized enzymes and proteins* (Chang, T. M. S., ed.), vol. 1, pp. 15–24, Plenum, New York.
7. Craven, G. R., Steers, E., Jr., and Anfinsen, C. B. (1965), *J. Biol. Chem.* **240,** 2468–2477.
8. Sigma Chemical Co. USA, Tech. Bulletin No. 510.
9. Ring, D. (1979), *J. Biol. Chem.* **254,** 11688–11695.
10. Folin, O. and Wu, J. (1971), in *Hawk's Physiological Chemistry* (Oser, B. L., ed.), pp. 392–393.
11. Fairbank, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochem.* **10,** 2606–2617.
12. Habeeb, A. F. S. A. (1966), *Anal. Biochem.* **14,** 328–336.
13. Wellenfels, K., and Weil, R. (1972), in *The Enzyme* (Boyer, P. D., ed.), vol. 7, pp. 617–640, Academic, New York.
14. Finocchairo, T., Olson, N. F., and Richardson, T. (1980), *Adv. Biochem. Eng.* **15,** 71–88.
15. Hinberg, I., Korus, R., and O'Driscoll, K. F. (1974), *Biotechnol. Bioeng.* **16,** 943–963.
16. Morisi, F., Pastore, M., and Viglia, A. (1973), *J. Dairy Sci.* **56,** 1123–1127.
17. Wondolowski, M. V., and Woychik, J. H. (1974), *Biotechnol. Bioeng.* **16,** 1633–1644.
18. Ji, T. H. (1974), *Proc. Natl. Acad. Sci. USA* **71,** 93–95.
19. Khare, S. K., and Gupta, M. N. Unpublished results.
20. Sharp, A. K., Kay, G., and Lilly, M. D. (1969), *Biotechnol. Bioeng.* **11,** 363–380.
21. Wang, K., and Richards, F. M. (1974), *J. Biol. Chem.* **249,** 8005–8018.
22. Asp, N. G., and Dahlqvist, A. (1968), *Biochem. J.* **110,** 143–150.
23. Mahoney, R. R., and Adamchuk, C. (1980), *J. Food Sci.* **45,** 962–968.
24. Pastore, M., and Morisi, F. (1976), in *Methods Enzymol.* (Mosbach, K., ed.), vol. 64, pp. 822–830, Academic, New York.
25. Shier, W. T. (1985), *in Methods Enzymol.* (Widder, K. J., ed.), vol. 112, pp. 248–258, Academic, New York.
26. Khare, S. K., and Gupta, M. N. (1988), *J. Biosci.* in press.