Purification and Properties of *Streptococcus lactis* β -Galactosidase¹

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β -Galactosidase of *Streptococcus lactis* 7962 was partially purified, and its properties were studied. Enzyme from only this strain of numerous lactic streptococci tested was stable in cell exudates prepared by various means. Cell-free extracts of the 7962 strain were prepared by sonic treatment of washed cells previously grown in presence of lactose to fully induce enzyme synthesis. Protamine sulfate precipitation of the nucleic acids and ammonium sulfate precipitation of protein were used for partial purification of the enzyme. The resulting enzyme, when resuspended in cold (5 C) phosphate buffer, was extremely labile. However, ammonium sulfate in high concentrations (0.85 M) stabilized and stimulated β -galactosidase activity. Sephadex G-200 gel filtration was used to achieve further purification and to monitor homogeneity of the enzyme. Separation of the β -galactosidase in buffer at 5 C yielded an enzyme elution pattern showing two peaks of activity. However, addition of the enzyme solution in 0.85 M ammonium sulfate to the column equilibrated with the same salt concentration yielded only one peak of enzyme activity. The data suggested that the native enzyme was dissociating into active subunits which were stabilized in the presence of the ammonium sulfate.

β -Galactosidase (E. C. 3.2.1.23 β -D-galactoside galactohydrolase) is an enzyme of prime importance in the metabolism of lactic streptococci, since their initiation of growth in milk, their natural habitat, is dependent on its function. A survey of about 50 strains of streptococci of the lactic group for this enzyme has shown that only one strain produces enzyme of sufficient stability in cell-free extracts to allow purification. This strain is *Streptococcus lactis* 7962. The organism produces acid slowly in milk at 22 C, requiring at least 24 hr of incubation to cause coagulation; accepted taxonomic tests (15), however, have confirmed that the bacterium is indeed a strain of *S. lactis*. In an earlier paper (4), preliminary information regarding the β -galactosidase from this organism was published. This report presents data on the partial purification and some of the unusual properties of the β -galactosidase from this *S. lactis* strain.

MATERIALS AND METHODS

Microorganisms and media. *S. lactis* 7962 obtained from the American Type Culture Collection, Washington, D.C., was used. The culture was maintained in sterile nonfat milk medium. However, a lactose broth was used to grow the cells required in each

experiment with incubation at 32 C. The lactose broth consisted of the following: lactose, 10 g; tryptone, 20 g; yeast extract, 5 g; gelatin, 2.5 g; NaCl, 4.0 g; L-ascorbic acid, 0.5 g; and water to 1 liter. This medium was autoclaved for 15 min at 121 C.

Buffer solutions. Sodium phosphate buffer (0.1 M at pH 7.0) was used to wash the cells during enzyme extraction and purification and in the enzyme assay substrate solution. Ammonium sulfate (0.85 M) was used in the buffer solution during some experiments as indicated.

Enzyme extraction and preliminary purification. Cells grown in the lactose broth for 12 hr from a 1% inoculum were harvested in a refrigerated centrifuge (0 C) at 4,000 $\times$ g. These growth conditions allowed maximal enzyme production as indicated by Citti et al. (4). Cells grown for 12 hr at 32 C in 1 liter of medium were washed three times with cold 0.1 M phosphate buffer, resuspended to a volume of 50 ml, and then treated for 1 hr in a Raytheon 10-kc sonic oscillator. This crude extract was centrifuged at 3,000 $\times$ g to remove the cellular debris. The resulting cell-free extract was stored at 5 C. Various other extraction procedures, such as grinding with various abrasives and use of a pressure extrusion cell, were tested but were unsuitable as they resulted in the destruction of enzymatic activity.

Protamine sulfate was used to precipitate the nucleic acids from the crude enzyme extract. Solid reagent was added such that the ratio of protamine sulfate to protein (milligrams per milliliter) minimized the ratio of absorbancy at 260 m μ to that at

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280 $m\mu$ after the solution was held at 20 C for 15 min and was centrifuged at $3,000 \times g$.

Ammonium sulfate fractionation was used to accomplish further enzyme purification and concentration. Solid salt was added to the extract which was allowed to stand for 12 hr at 5 C after each fractionation; the mixture then was centrifuged at $12,000 \times g$ for 10 min. The active enzyme was precipitated under these conditions by an ammonium sulfate concentration of 1.3 to 2.5 M. Upon resuspension of this fraction in cold phosphate buffer, only 50% of the original enzyme activity in the crude extract was recovered. Enzyme activity, as well as protein concentration, was followed through the above stages. Disc electrophoresis was performed on samples taken at various times during the above procedure and also after the gel filtration.

Assay of β -galactosidase. Hydrolysis of the chromogenic substrate *o*-nitrophenyl- β -D-galactopyranoside (ONPG) was used as a measure of enzyme activity (10). Solutions of ONPG at 5×10^{-3} M were prepared in the phosphate buffer and used as substrate solution in the enzyme assay reaction. Enzyme solution (0.5 ml) was added to 2.0 ml of the ONPG solution. The reaction, which took place at 37 C and lasted 2 to 5 min, was terminated by the addition of 2.5 ml of 0.5 M sodium carbonate. The absorbancy at 420 $m\mu$ was measured and converted to molar concentration of *o*-nitrophenol by multiplying by the appropriate molar extinction ($1/E_{cm}^{\mu M} = 0.238 \mu\text{mole}^{-1} \text{cm}^{-1}$) determined from an *o*-nitrophenol standard curve. A unit of enzyme activity was defined as the number of micromoles of *o*-nitrophenol liberated from ONPG per minute of reaction time. This could be calculated directly by use of the molar extinction coefficient as described above. The method of Lowry et al. (11) was used to determine the protein concentration in enzyme preparations; data were expressed as milligrams per milliliter.

Gel filtration procedure. Sephadex G-200 was used to achieve further purification of the enzyme. A 2.5 by 38 cm column was prepared in the usual manner, then used and stored at 5 C. A flow rate of 0.11 ml per cm^2 per min was maintained during the separations. Fractions (50 drops) were collected in a refrigerated fraction collector at 5 C. Blue dextran was used to determine the void volume. The elution data were expressed as fraction number after the void volume. The enzyme units and protein concentrations were determined immediately after the collection of each fraction to minimize postseparation inactivation. Columns were also equilibrated with buffer containing ammonium sulfate to provide a final concentration of 0.85 M. These columns were used in the same way as those not equilibrated with salt in an effort to separate enzyme activity.

RESULTS

Purification procedures. The choice of extraction procedure was made on the basis of which technique gave the least amount of inactivation. Most procedures gave complete or nearly complete enzyme inactivation. Sonic treatment of the

FIG. 1. Effect of sonic-treatment time on protein and β -galactosidase release from *Streptococcus lactis* 7962. A 1:10 dilution of the cell suspension prior to sonic treatment provided an absorbancy of 0.45 at 420 $m\mu$.

cell suspension yielded reasonable amounts of enzyme activity. For example, Fig. 1 shows the effect of sonic-treatment time on enzyme and protein release from a suspension of washed cells in buffer at 20 C. The assays were done after the cells were removed from the sonic treatment at various time intervals and centrifuged at 0 C for 10 min at $12,000 \times g$. Protein concentration in solution reached a maximal level in 30 min and then remained constant; the enzyme activity in solution, however, reached a maximum in 80 min and then decreased rapidly. The difference between the time required to achieve maximal protein concentration and the enzyme activity suggested that the enzyme was particulate, possibly bound to ribosomes. In view of this, the method of Zipser (20) was followed to detect ribosomally bound enzyme, but results of several experiments were negative. Centrifugation studies on the enzyme also failed to give evidence that the enzyme was bound to cellular particles.

Protamine sulfate treatment was carried out prior to the ammonium sulfate fractionation because the protamine was precipitated when the first salt addition was made. A 1:1 ratio of protamine to protein (milligrams per milliliter) gave the maximal decrease in the ratio of absorbancy at 260 $m\mu$ to that at 280 $m\mu$.

Table 1 shows typical results obtained from the partial purification of the enzyme. Only 13% of

TABLE 1. Typical data obtained before and after the partial purification of the β -galactosidase of *Streptococcus lactis* 7962

Determination	Crude extract	After $(\text{NH}_4)_2\text{SO}_4$ fractionation
Protein ($\mu\text{g}/\text{ml}$)	2.15	1.40
Enzyme (units per ml)	0.290	0.766
Specific activity	0.135	0.547
Volume (ml)	100	20
Total protein (μg)	215	28
Per cent original protein	100	13
Total enzyme units (units per ml $\times$ ml)	29	14.3
Per cent total original enzyme units (units per ml $\times$ ml)	100	49
Per cent original enzyme (units per ml)	100	264
Per cent original specific activity	100	406

the protein was recovered in the enzyme solution after the salt fractionation and nearly half of the enzyme in the crude extract was lost during this relatively gentle procedure, emphasizing the lability of the enzyme. The increase in enzyme activity and specific activity was modest, 2.5- and 4-fold respectively, again reflecting the lability of the enzyme.

Stability of the partially purified enzyme. Upon resuspension of the precipitate obtained after partial purification, the enzyme was quite labile, especially at low protein concentration (Fig. 2). When resuspended in 0.1 M buffer at 5 C, the more concentrated preparation revealed a much higher stability than the dilute solution. Even in concentrated form the activity decreased exponentially, with an initial inactivation rate of 5% per day. In the more dilute form, the initial rate of decrease in enzymatic activity was 1% per hour. The enzyme was very sensitive to freezing, lyophilization, dialysis, and to various inorganic ions and organic solvents. This made further purification difficult and also made storage of the enzyme a problem.

Enzyme stabilization. In an effort to stabilize the enzyme and possibly learn more about the inactivation process, various compounds were added to the enzyme solution. Protamine sulfate did afford protection by retarding the inactivation rate slightly; however, ammonium sulfate provided dramatic protection.

Figure 3 shows the effect of 0.85 M ammonium sulfate on a partially purified enzyme solution in 0.1 M phosphate buffer at 5 C. Upon addition of the salt, there was an immediate decrease in the enzyme activity, then a gradual increase reaching

FIG. 2. Effect of storage at 5 C on enzyme activity of partially purified β -galactosidase from *Streptococcus lactis* 7962. The protein concentrations were 0.1 mg/ml (●) with the time measured in minutes, and 0.5 mg/ml (■) with the time measured in hours.

a plateau which represented 90% of the original activity. The activity in the salt-free preparation decreased rapidly as also seen in Fig. 2.

The effect of various concentrations and times of exposure to ammonium sulfate was examined next, and an interesting stimulatory effect was noted (Fig. 4). After 15 min, the enzyme samples at the various salt concentrations were assayed, and peak activity occurred at about 1.25 M. However, after 18 hr at 5 C, the samples were assayed again, and a marked peak of stimulation was seen. The activity of the control sample that contained no salt dropped from 13 to 2 units as seen before, but at 1.0 M salt concentration a very definite stimulation was noted that produced activity greater than the original control and eight times greater than that of the 18-hr control. The decrease in activity after addition of 1.3 M salt can be explained by the previous observation that the enzyme is precipitated in salt solutions stronger than 1.3 M. This same procedure was attempted with the β -galactosidase of *Escherichia coli* prepared as a crude extract, and only a slight stimulatory peak was seen at 0.85 M. Combinations of ammonium and sulfate ions with various other ions were tested in an attempt to duplicate this salt effect; all such compounds failed to stabilize or stimulate the enzyme.

FIG. 3. Effect of addition of 0.85 M ammonium sulfate to enzyme on stability of β -galactosidase of *Streptococcus lactis* 7962 at 5 C. Symbols: ■, with $(\text{NH}_4)_2\text{SO}_4$; ●, without $(\text{NH}_4)_2\text{SO}_4$.

Gel filtration of partially purified enzyme. Sephadex gel filtration was carried out on a 2.5 by 38 cm column to achieve further purification of the enzyme. Fractions (50-drop) were collected at 5 C, and then both enzyme and protein were assayed from each fraction as described. An enzyme sample partially purified as described above and containing 96 units of enzyme was added to the column and eluted with 0.1 M buffer; only 8.5 units of enzyme was recovered, which represented 9% of the enzyme added to the column. Virtually all of the protein added to the column was accounted for by the time fraction no. 40 was collected. The elution pattern seen in Fig. 5 resulted from such an experiment. The majority of the protein was in the first peak that was partially or wholly excluded. The enzyme curve shows an early peak at fraction no. 8 and a rather broad peak from fractions 16 to 26. On the basis of the enzyme-recovery data, it appeared that significant breakdown of the enzyme had occurred on the column, which seemed reasonable because of the sensitivity of the enzyme to dialysis. Dissociation of the enzyme into enzymatically active subunits was a reasonable explanation for this elution pattern. Consequently, it was felt that the first peak was the native enzyme, and the second, the enzymatically active subunits. Since ammonium sulfate produced a more stable enzyme, it was felt that column purification could be more readily accomplished in the presence of this salt. Therefore, enzyme previously stabilized for 30 hr in 0.85 M ammonium sulfate and then centrifuged to insure the absence of insoluble particles was run through a G-200 column exactly as before, except the column was equilibrated with buffer containing 0.85 M ammonium sulfate. Figure 6 shows such a separation. The enzyme sample that was added to the column contained 131.7 units of enzyme, and 54.9 units (41%) were

FIG. 4. Effect of various concentrations of ammonium sulfate on β -galactosidase activity of *Streptococcus lactis* 7962 upon storage for 15 min (●) and 18 hr (■) at 5 C.

FIG. 5. Sephadex G-200 gel filtration of partially purified β -galactosidase from *Streptococcus lactis* 7962 using a 2.5 by 38 cm column at 5 C eluted with 0.1 M sodium phosphate buffer, pH 7.0. Each fraction contained 50 drops.

FIG. 6. Sephadex G-200 gel filtration of partially purified β -galactosidase from *Streptococcus lactis* 7962 using a 2.5 by 38 cm column equilibrated with 0.1 M sodium phosphate buffer (pH 7.0). The buffer also was made 0.85 M with ammonium sulfate, and elution was carried out with the same buffer. Each fraction contained 50 drops.

recovered in the eluant. The protein curve was much the same as seen in Fig. 5, except it lacked the small peak corresponding to the presumed subunit peak on the enzyme curve. The enzyme curve in Fig. 6 is radically different, showing the native enzyme peak much larger relative to the protein peak than that shown in Fig. 5. It also may be noted that the shoulder on the native peak seen in Fig. 6 at fractions 16 to 19 corresponds in position to the subunit peak in Fig. 5. Finally, it should be re-emphasized that 41% of the enzyme activity added to the salt-equilibrated column was recovered upon separation, whereas only 9% was recovered from the unstabilized preparation.

DISCUSSION

The lability of the β -galactosidase of *S. lactis* 7962 is clearly demonstrated by the purification data. Other enzymes have been found to be unstable in buffer but stable in high concentrations of ammonium sulfate (12). Reasons for the inability of salts other than ammonium sulfate to stabilize the enzyme are not clear. It was expected that other salt combinations of the ammonium and sulfate ions would afford some protection; however, this was found not to be the case. The effects of various neutral salts on macromolecular

conformation have been studied by Von Hippel and Wong (17), and the ammonium and sulfate ions both ranked quite high in order of affording stability. Other workers have reported a stabilizing effect of ammonium sulfate on enzymes other than β -galactosidase (7, 13). However, additional ions also provided protection, which is in contrast to the present findings with the β -galactosidase of *S. lactis* 7962. Rickenberg (14) found that the β -galactosidase of *E. coli* was sensitive to dialysis against water and that manganese, magnesium, and sodium ions were required to stabilize activity. Furthermore, he observed that these ions would reactivate the enzyme. However, in the present study, the activity of an inactivated enzyme solution of *S. lactis* 7962 was not reactivated under these or any conditions.

One type of change that may take place as inactivation of *S. lactis* β -galactosidase occurs is the dissociation of the native enzyme into smaller subunits. The elution pattern of the salt-protected enzyme seen in Fig. 6, in contrast to that seen in Fig. 5 where the enzyme is not protected by salt and where more than five times more activity is lost, indicates that while ammonium sulfate protects the enzyme it also prevents dissociation into subunits. After dissociation of the native polymeric enzyme into monomeric subunits, further changes must occur that result in the total loss of enzyme activity; the subunits may undergo further dissociation into polypeptide chains lacking enzyme activity as was demonstrated by Wallenfels et al. (18) for the β -galactosidase of *E. coli*. Additional evidence for this mechanism of inactivation of *S. lactis* 7962 β -galactosidase will be published in a subsequent paper. The broad shape of the subunit peak seen in Fig. 5 might indicate that the subunits are not identical, unlike the β -galactosidase of *E. coli* K-12 (16, 18, 19). The β -galactosidase of *S. lactis* 7962, while in the native form, may find its active center protected, but once it dissociates into smaller units the active center may no longer be protected.

Recently, Cowman and Swaisgood (5) reported that a proteinase of *S. lactis* undergoes in vitro a temperature-dependent reversible dissociation into components of lower molecular weight. Also, it has been suggested by Appel et al. (1) that this process actually occurs in vivo with the β -galactosidase of *E. coli*. Goodman and Pickett (6) made the same point in a recent paper stating that different molecular forms of β -galactosidase may exist, some being unstable and dissociating easily because of a tertiary structure alteration. It seems likely, therefore, that this type of dissociation is occurring with the *S. lactis* 7962 enzyme and that the reverse reaction is lacking owing to the possible absence of some unknown

factor or ion. Such an occurrence has been reported for amylase where zinc ions are required for the reassociation of two monomers into the dimer (8). The reversal of the dissociation reaction in lactic and malic dehydrogenases has been studied by Chilson et al. (3), who found that halides inhibit, whereas citrate favors, reactivation.

Since electric current destroyed the enzyme activity of the samples separated by disc electrophoresis, it was not possible to determine whether electrophoretically separable species of the enzyme of *S. lactis* 7962 exist. It is well known, however, that such multiple forms of enzymes (isozymes) do exist, and, for example, Chesbro et al. (2) have separated, by gel filtration, staphylococcal nuclease into several active forms. In the present study, two forms of the β -galactosidase of *S. lactis* 7962 have been shown to be separable by gel filtration. The data suggest, however, that these are both the same enzyme species in two forms, the native (or polymer) and the subunit (or monomer). If the subunits are nonidentical, as suggested by the shape of the peak on the chromatogram in Fig. 5, and if the dissociation-association reaction is reversible, several enzyme forms could be realized from combinations of the various subunits. In this regard, evidence will be presented in a subsequent paper to show that the two enzyme forms seen here are the same, but in two states of dissociation-association. This general idea is discussed by Kaplan (9), who maintains that multiple enzyme forms must be shown to be dissimilar by critical analysis; the criteria of molecular weight and mobility in electrophoresis are not sufficient to define separate molecular species of a given enzyme.

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