

## Lactose hydrolysis by free and fibre-entrapped $\beta$ -galactosidase from *Streptococcus thermophilus*

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To study lactose hydrolysis by  $\beta$ -galactosidase, this enzyme was produced from *Streptococcus thermophilus* strain 11F and partially purified by acetone and ammonium sulphate fractionation, and ion exchange chromatography on a Q Sepharose FF column. Lactose hydrolysis by the enzyme was affected by lactose concentrations, sugars and milk proteins. The maximum extent of lactose hydrolysis in buffer was obtained with a 15% lactose concentration. Addition of 2% of lactose, glucose, galactose or sucrose in milk inhibited the enzymatic hydrolysis. The enzyme was activated by bovine serum albumin and a combination of  $\alpha$ s-casein and  $\beta$ -casein. Of the casein fractions, the principal fraction,  $\alpha$ s-casein, was less effective than  $\beta$ -casein and  $\kappa$ -casein. The fibre-entrapped enzyme had a temperature optimum of 57°C, and a pH optimum from 7.5 to at least 9.0 with O-nitrophenyl- $\beta$ -D-galactopyranoside as substrate. By recycling with whey and skim milk through a jacketed glass column (1.6 cm x 30 cm) loaded with fibre-entrapped enzyme at 55°C, a lactose hydrolysis of 49.5% and 47.9% was achieved in 11 h and 7 h respectively.

Key words: lactose hydrolysis, *Streptococcus thermophilus*,  $\beta$ -galactosidase, fibre-entrapped enzyme

### Introduction

Hydrolysis of lactose in milk and milk products by  $\beta$ -galactosidase has been regarded as a potential solution to lactose maldigestion, which occurs in the majority of adults all over the world. Manufacture of dairy products with lactose-hydrolyzed milk or whey has many advantages, including enhanced sweetness, improved carbohydrate solubility and digestibility, more readily fermentable sugars, prevention of lactose crystallization etc. Enzymatic processes of lactose hydrolysis by both free and immobilised  $\beta$ -galactosidase are already commer-

cially available (MAHONEY 1985, HARJU 1987). The utilization of  $\beta$ -galactosidase available from yeast and mould, however, is relatively limited, either due to the low temperature optimum or the low pH optimum of the enzyme. Attention has recently been shifted to finding a heat-stable, neutral-pH  $\beta$ -galactosidase that would be suitable for the hydrolysis of lactose in milk and facilitate the enzyme reactors with high operating temperatures to prevent microbial contamination. A thermo-stable  $\beta$ -galactosidase from *Streptococcus thermophilus* (*Str. thermophilus*) is considered such an enzyme and appears promising in this respect. The

purification and characterization of the enzyme has been the subject of several recent studies (RAMANA and DUTTA 1981, GREENBERG and MAHONEY 1982, GREENBERG et al. 1985, SMART et al. 1985, CHANG and MAHONEY 1989a, b). However, the research on the immobilization of the enzyme is still lacking and certain factors influencing the enzymatic hydrolysis of lactose need to be determined in more detail.

The present study was carried out to assay the hydrolysis of lactose in milk and buffer by the  $\beta$ -galactosidase from *Str. thermophilus*, and to evaluate the enzyme immobilized with cellulose triacetate.

## Material and methods

*Str. thermophilus* strain 11F was donated by the Research and Development Centre of Valio Ltd., Helsinki. Cellulose triacetate was purchased from Fluka Chemie AG, Switzerland, O-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) from Sigma Chemical Company, USA, and Q Sepharose FF and the glass column were from Pharmacia Fine Chemicals, Sweden. The chemicals and reagents used were of analytical grade. Double-distilled water was used to make the buffers. Raw milk and whey from edam cheese manufacture were obtained from the dairy of the Food Research Institute, Jokioinen, Finland.

## Enzyme preparation

The procedures of bacterial cultivation and enzyme purification by acetone and ammonium sulphate precipitation basically followed an earlier method described by CHANG and MAHONEY (1989a).

The enzyme extract obtained was further partially purified by ion exchange chromatography with Q Sepharose FF in a glass column (1.6 cm x 30 cm). The elution was carried out at 5 ml/min with a linear increasing gradient of 0.5 M NaCl in buffer A (CHANG and MAHONEY 1989a) from 30% to 80%. The fractions (10 ml) containing enzyme activity were pooled, the protein was precipitated by

adding ammonium sulphate to 65% saturation, and the pellet was dissolved in about 10 ml buffer A and dialyzed at 4°C against buffer A with several changes of the buffer. The enzyme was stored at -25°C. The above procedures of enzyme preparation were carried out and repeated five times. The final enzyme products were used for the following assays of lactose hydrolysis and enzyme immobilization.

## Assay of $\beta$ -galactosidase activity and protein concentration

Enzymatic activity was spectrophotometrically measured at 420 nm as described by GREENBERG et al. (1985). One unit of  $\beta$ -galactosidase activity is defined as the amount of enzyme which will liberate 1  $\mu$ mol of o-nitrophenol (ONP) per minute under the assay conditions.

Protein concentration was determined by the method described by LOWRY et al. (1951). Bovine serum albumin was used as standard protein.

## Immobilization of the enzyme

The partially purified enzyme preparations were entrapped in cellulose triacetate fibres (thickness 0.2-0.3 mm) according to the procedures reported by MORISI et al. (1973).

## Determination of the optimum temperature and pH of the fibre-entrapped enzyme

The activity of the fibre-entrapped enzyme during the assays was examined as follows: 0.15 g fibres were added to 2 ml 2.2 mM ONPG in buffer at 55°C in a water bath. The absorbance of the solution at 420 nm was measured after 2 minutes. The pH optimum was determined with 2 ml 2.2 mM ONPG in 0.01 M potassium phosphate buffer adjusted to the pH of the assay with phosphoric acid. The temperature optimum was determined with 2 ml 2.2 mM ONPG in buffer A at the assay temperatures.

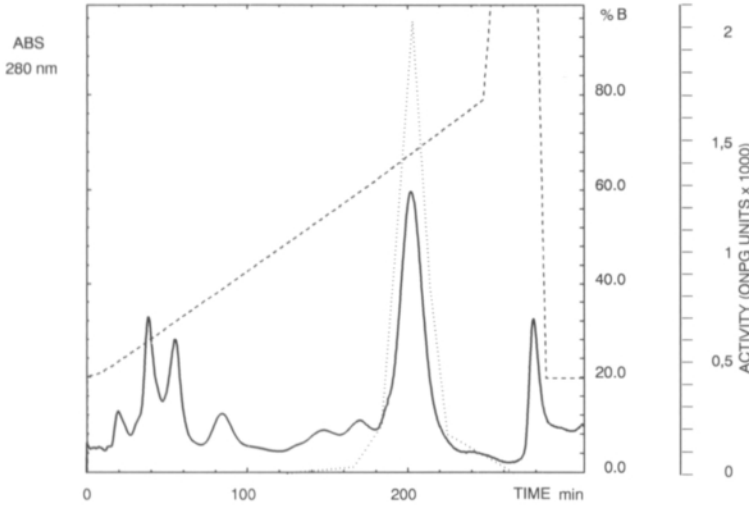


Fig. 1. Purification of the enzyme by ion exchange chromatography on a Q Sepharose FF column. (—) absorbance, (---) NaCl gradient, (····) enzyme activity

### Assay of lactose hydrolysis

Lactose hydrolysis was assayed with a rapid method based on the measurement of freezing point depressing of the solution as described by RAMET et al. (1979). 2 ml of sample (or diluted sample) was taken, and the freezing point determined with a cryoscope.

## Results and discussion

### Enzyme purification

Elution of the enzyme extract by ion exchange chromatography on a Q Sepharose FF column is shown in Figure 1. The dialysate from the ammonium sulphate step had to be centrifuged because of its thickness, and only the supernatant (about 58% of the total activity) was applied to the column. The precipitate, with a specific activity of 16 units/mg protein, could be directly freeze-dried as a crude enzyme. Elution of the enzyme started shortly after the specified gradient was introduced. One main peak of protein coincident with the enzyme activity was eluted. The pooled fractions from the main peak, however, accounted for 86% of the total activity recovered, with a four-fold increase in spe-

cific activity compared with the applied enzyme sample. Thus, further purification of the enzyme was not carried out. The final partially purified enzyme had a specific activity of 117 units/mg protein.

### Lactose hydrolysis in milk and buffer containing carbohydrates or milk proteins

#### *Effect of lactose concentrations*

As shown in Figure 2, the process of lactose hydrolysis by the enzyme was examined at different concentrations of lactose in 0.025 M potassium phosphate buffer, pH 6.8, containing 0.8 mM  $MgCl_2 \cdot 6H_2O$ , 0.125 mM dithioerythritol, 0.005 %  $NaN_3$  and 10% glycerol (buffer B).

Maximum hydrolysis was found in a 15% lactose solution, in which 100% of lactose was hydrolyzed in 4 hours. With lactose concentrations of 5%, 10% and 25%, the degrees of hydrolysis after 4 hours were about 70%, 85% and 50%, respectively. Similar results were observed by using *Saccharomyces fragilis* lactase to hydrolyze reconstituted whey and milk, where the highest degree of lactose hydrolysis was achieved at 10-15% lactose concentrations (WENDORFF et al. 1971). It seems that optimal in-

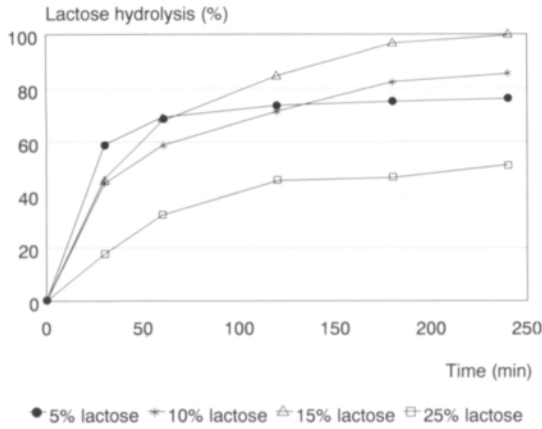


Fig. 2. Hydrolysis of lactose at 55°C in buffer B. 10 ONPG units of enzyme activity were added per ml of buffer B.

teraction between the enzyme and lactose occurs when lactose concentration was about 15%.

*Effect of sugars*

Figure 3 shows that lactose hydrolysis was remarkably reduced when 2% of lactose, glucose, galactose or sucrose was added to the milk. Lactose gave the slightest inhibition to the hydrolysis, whereas there was no evident difference between inhibition caused by the other sugars.

The results in Figure 2 indicate that lactose hydrolysis increased along with increasing lactose concentration in buffer (except 25%). Adding lactose to milk, however, decreased the lactose hydrolysis. Lactose, galactose and glucose have been found to stabilize  $\beta$ -galactosidase from *Str. thermophilus* (CHANG and MAHONEY 1989b), but the lactose hydrolysis in milk by the enzyme may be reversely affected by these sugars.

*Effect of milk proteins*

The effect of several casein fractions and bovine serum albumin on lactose hydrolysis in buffer B containing 15% lactose is shown in Figure 4. The concentrations of  $\alpha$ s-casein,  $\kappa$ -casein and  $\beta$ -casein in buffer B were similar to their corresponding concentrations in milk. After 3 hours, the hydroly-

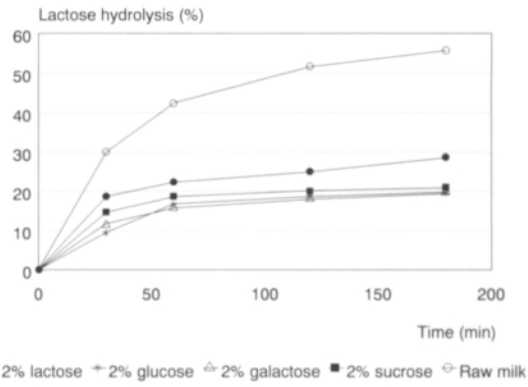


Fig. 3. Hydrolysis of lactose at 55°C in milk with added sugars. 8 ONPG units of enzyme activity were added per ml of milk.

sis degrees in buffer B containing  $\alpha$ s-casein and  $\beta$ -casein, or bovine serum albumin, reached 95%, almost the same as achieved in 15% lactose solution within the same time but using a higher enzyme concentration (shown in Figure 2). This suggests that the enzyme was activated by bovine serum albumin and by the combination of  $\alpha$ s-casein and  $\beta$ -casein. Previous work (MAHONEY and ADAMCHUK 1980) has also shown that  $\beta$ -galactosidase from *S. fragilis* is strongly activated by bovine serum albumin. Casein has been found to activate  $\beta$ -galactosidase from *S. fragilis* and stabilize  $\beta$ -galactosidase from *Str. thermophilus* (CHANG and MAHONEY 1989b). However, the results in Figure 4 indicate that the individual effects of casein fractions on the enzyme were different.  $\alpha$ s-casein was less effective than  $\beta$ -casein and  $\kappa$ -casein, although it is the principal fraction of casein.  $\kappa$ -casein, comprising only about 13% of the total casein, produced the greatest effect.

**Immobilization of the enzyme**

*pH optimum*

The results (Figure 5) indicate a wide pH optimum range from 7.5 to at least 9.0 with a sharp decrease of enzyme activity below pH 7.5. The optimum pH

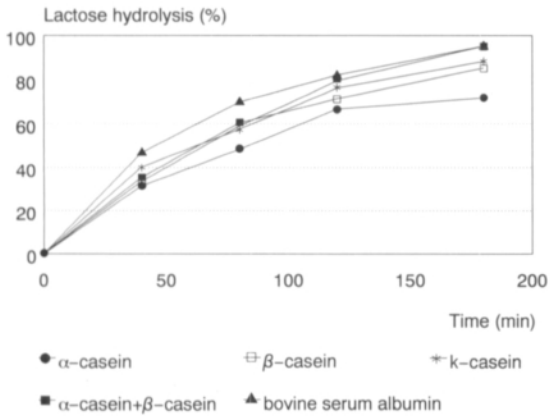


Fig. 4. Hydrolysis of lactose at 55°C in buffer B with added milk proteins. 7 ONPG units of enzyme activity were added per ml of buffer B.

for this free enzyme has been reported to be between 7.0 and 7.5 (SMART et al. 1985). The broader pH optimum of the fibre-entrapped enzyme was probably due to the rate limiting of the substrate diffusion. In addition, since the enzyme preparation was not entirely pure, some protein impurities entrapped in the fibres could also have contributed to the broader pH optimum. MORISI et al. (1973) reported a similar wider range of pH optimum for  $\beta$ -galactosidase from *Escherichia coli*. Immobilized crude pepsin has also been found to have a wider pH optimum range than another more pure immobilized pepsin preparation (HUSTAD et al. 1973).

#### Temperature optimum

The effect of different temperatures on the activity of the fibre-entrapped enzyme is shown in Figure 6. Of the temperatures studied, the optimum appeared to be at about 57°C. This was an upward shift of about 2°C compared with the corresponding free enzyme, for which the optimum temperature has been reported at 55°C (SMART et al. 1985). Although the enzyme was actually entrapped in soluble form within the microcavities of the fibres, it may be that the outer layer of the fibres provided heat protection for the entrapped enzyme, thus resulting in a higher temperature optimum.

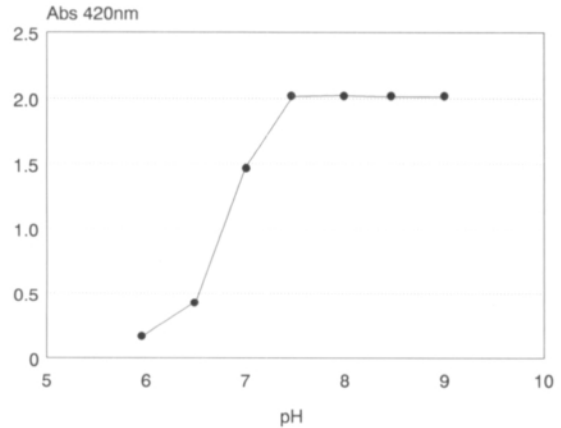


Fig. 5. pH profile of fibre-entrapped  $\beta$ -galactosidase with ONPG as substrate.

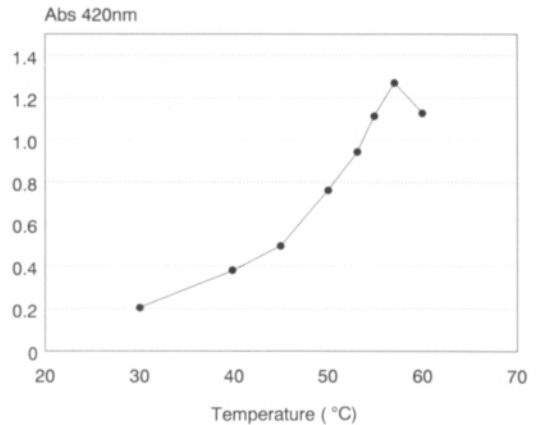


Fig. 6. Temperature profile of fibre-entrapped  $\beta$ -galactosidase with ONPG as substrate.

#### Lactose hydrolysis in milk products by the fibre-entrapped enzyme

Treating 15 ml skim milk, whole milk or whey with 1.0 g of fibre-entrapped enzyme for 3 hours at 50°C or 53°C resulted in lactose hydrolysis of about 70%, 65% and 27%, respectively (Figure 7). Skim milk and whole milk appeared to be more suitable than whey for lactose hydrolysis by the immobilized enzyme. The low extent of lactose hydrolysis in whey may be mainly due to the low pH of the

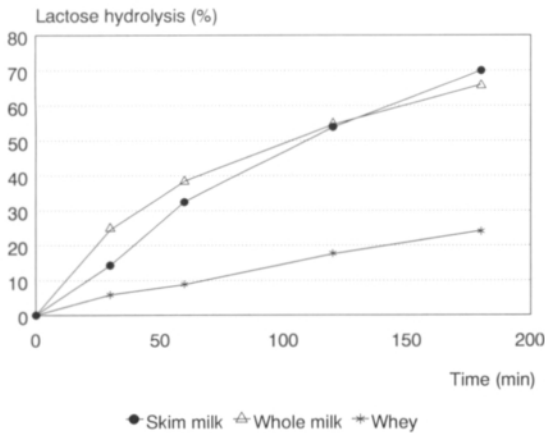


Fig. 7. Lactose hydrolysis in milk products by fibre-entrapped  $\beta$ -galactosidase (1.0 g) (skim milk 50°C, whole milk 50°C, whey 53°C).

whey (about pH 6.3). As indicated in Figure 5, the activity of the immobilized enzyme was strongly affected by pH values below 7.5.

#### Column operation

Trials were made to assay the performance of the enzyme entrapped in the fibres which were parallel-

packed in the jacketed glass column (1.6 cm x 30 cm). The first assay indicated that lactose hydrolysis of only 8.4% in whole milk was obtained with the column containing 9 g fibres at 45°C and at a flow rate of 38 ml/h. The substrate was then changed by feeding whey at a lower flow rate, but no improvement was observed. The column was packed again using more fibres (13 g). After running with whey, skim milk and lactose solution in buffer B at above 50°C and at even lower flow rates, higher degrees of lactose hydrolysis were achieved, but they were still less than 30%. When whole milk or skim milk was passed through the column at flow rates less than 20 ml/h, column plugging by fat and protein became easier. The substrates were, therefore, recycled through the column at 55°C and at much higher flow rates (about 70 ml/h), and lactose hydrolysis of 49.5%, 47.9% was reached with whey (90 ml) and skim milk (90 ml) after 11 and 7 hours respectively.

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## SELOSTUS

### Laktoosin hydrolyysi vapaalla ja kuituun sidotulla *Streptococcus thermophilus*- $\beta$ -galaktosidaasilla

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*Streptococcus thermophilus* 11F kannalla tuotettiin  $\beta$ -galaktosidaasia, joka osittain puhdistettiin asetoni- ja ammoniumsulfaattifraktioinnilla sekä ioninvaihtokromatografialla Q Sepharose FF-pylväässä. Entsyymien aiheuttamaan laktoosin pilkkoutumiseen vaikuttivat laktoosin pitoisuus, eri sokerit ja maidon valkuaisaineet. Korkein hydrolyysiaste puskuriliuoksessa saavutettiin 15 % laktoosipitoisuudessa. Lisättäessä maitoon 2 % joko laktoosia, glukoosia, galaktoosia tai sakkaroosia laktoosin hydrolyysi heikkeni. Seerumin albumiini

sekä  $\alpha$ s- ja  $\beta$ -kaseiinien yhdistelmä aktivoi entsyymiä. Maidon kaseiinien pääkomponentin,  $\alpha$ s-kaseiinin vaikutus hydrolyysiin oli vähäisempi kuin  $\beta$ - tai  $\kappa$ -kaseiinin. Kuituun sidotun entsyymien lämpötilaoptimi oli 57°C ja pH optimi välillä 7,5-9,0 käytettäessä O-nitrofenyyli- $\beta$ -D-galactopyranosiidia substraattina. Kierrätettäessä kuorittua maitoa tai heraa kuituun sidotulla entsyymillä täytetyssä lasikolonnissa saavutettiin 55°C lämpötilassa 7 tunnissa 47,9 % ja 11 tunnissa 49,5 % laktoosin hydrolyysiaste.