Purification and properties of two lactose hydrolases from *Trichosporon cutaneum*

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Two enzymes that hydrolysed lactose were purified essentially to homogeneity from cell extracts of the oleaginous yeast *Trichosporon cutaneum*. One enzyme of M_r 120 000 had properties typical of a β -galactosidase (EC 3.2.1.23). It hydrolysed lactose, lactulose and nitrophenyl- β -D-galactosides. The enzyme required K^+ or Rb^+ for activity, and other monovalent cations tested were not effective. Enzyme activity was abolished by EDTA and stimulated by Mg^{2+} , Mn^{2+} and Ca^{2+} . The β -galactosidase was induced by lactose, galactose, lactulose and lactobionic acid. The other enzyme, a β -glycosidase (EC 3.2.1.21) of M_r 52 000 showed no ionic requirements and it hydrolysed lactose, nitrophenyl- β -D-galactosides, 4-nitrophenyl- β -D-glucoside, cellobiose, laminaribiose, laminaritrise and sophorose, but not gentiobiose, 4-nitrophenyl- β -D-mannoside or sucrose. This enzyme was induced by lactose, galactose and lactulose, and also by cellobiose.

Introduction

Lactose hydrolysis in most micro-organisms is attributed to a β -galactosidase which hydrolyses lactose and other β -galactosides, but not β -glucosides. Typical β -galactosidases have a multi-subunit structure and metal ion requirements similar to the enzyme from *Escherichia coli* (Wallenfels & Weil, 1972). Few yeasts grow on lactose, and Barnett (1981) concluded that of 439 yeasts surveyed, only 51 could utilize the disaccharide. Lactose hydrolases have been purified from several yeasts, including *Kluyveromyces lactis* (Dickson *et al.*, 1979) and *Saccharomyces fragilis* (Uwajima *et al.*, 1972), and these are similar to the *E. coli* β -galactosidase. In this paper we report the purification and properties of two lactose-hydrolysing enzymes from the oleaginous yeast *Trichosporon cutaneum*.

Methods

Organism, growth conditions and media. *Trichosporon cutaneum* was originally isolated from dairy factory floor drains by Moon *et al.* (1978). Our isolate, obtained from the American Type Culture Collection as *Apiotrichum curvatum* ATCC 20509, was provided by J. R. Davies, Industrial Processing Division, DSIR, Petone, New Zealand. It was subsequently identified by the National Collection of Yeast Cultures,

Norwich, UK as a strain of *T. cutaneum*. The organism was maintained on 3.4% (w/v) malt extract agar slopes (Difco). Synthetic medium contained (w/v) 5% lactose, 0.1% casein acid hydrolysate, 0.15% yeast extract, trace elements and salts except $(NH_4)_2SO_4$ (Wickerham, 1946). Synthetic medium (10 l), in a 10 l New Brunswick Microgen fermenter was inoculated with 100 ml of an 18 h culture, and grown at 28 °C with 10 l min⁻¹ aeration and 400 r.p.m. agitation to an OD₆₀₀ of approximately 5.0. Cells were harvested in a Sharples continuous flow centrifuge.

Enzyme purification. The cell paste (150–300 g wet weight) was resuspended to a cream consistency in extraction buffer (8 mM-sodium phosphate, pH 7, containing 1 mM-EDTA, 1 mM-dithiothreitol and 10% v/v, glycerol), and passed once through a French Press at 20 000 p.s.i. (= 138 Pa). To reduce viscosity, the homogenate was sonicated (10 × 2 s) with a Branson Sonifier. This suspension was diluted to 600 ml with extraction buffer, and centrifuged at 35 000 *g* for 60 min. It was not possible to recover all of the cell-free extract from the loose cell debris. The cell-free extract was mixed with 140 ml (bed volume) of DEAE-Sepharose CL-6B (Pharmacia) equilibrated in extraction buffer, and the suspension was stirred for 1–2 h. The resin was recovered by filtration, resuspended in an equal volume of buffer, and stirred for a further 1–2 h. Finally, the resin was washed for 30 min with extraction buffer containing 0.1 M-NaCl, filtered, resuspended in the buffered NaCl and transferred to a 30 × 2.5 cm column. This was washed with extraction buffer plus 0.1 M-NaCl until the A_{280} was zero. Protein was then eluted with 360 ml of a linear 0.1–0.25 M-NaCl gradient in extraction buffer at 15 ml h⁻¹. Two peaks which hydrolysed 4-nitrophenyl β -D-galactopyranoside (PNPGal) were separately pooled. The first, a β -glycosidase was concentrated by ultrafiltration with a 50 000 M_r cut-off membrane, and the second a β -galactosidase was concentrated with a 100 000 M_r cut-off membrane. The concentrates were diluted fivefold in buffer A (8 mM-sodium phosphate, pH 7, containing 1 mM-EDTA, 2 mM-dithiothreitol and 5% v/v, glycerol), and concentrated again by ultrafiltration. Each enzyme preparation

Abbreviations: ONPGal, 2-nitrophenyl β -D-galactopyranoside; PNPGal, 4-nitrophenyl β -D-galactopyranoside; PNPGlu, 4-nitrophenyl β -D-glucopyranoside.

was loaded onto a Fast-Flow DEAE-Sephacrose (Pharmacia) column (300 mm $\times$ 10 mm) equilibrated in buffer A containing 0.1 M-NaCl. The β -glycosidase was eluted with a linear gradient (180 ml) of 0.1–0.4 M-NaCl in buffer A and the β -galactosidase was eluted with a linear gradient (180 ml) of 0.1–0.5 M-NaCl in buffer A. Each enzyme fraction was again concentrated by ultrafiltration. The β -glycosidase preparation was applied to a Mono Q (HR 5/5) column (Pharmacia) equilibrated with buffer A plus 0.1 M-NaCl. It was eluted with a linear gradient (26 ml) of 0.1–0.4 M-NaCl in buffer A. The β -galactosidase was treated in the same way. $(\text{NH}_4)_2\text{SO}_4$ (3.9 M) was added to the pooled fractions of each enzyme to a final concentration of 1.4 M. After centrifuging (2700 *g* for 5 min) each preparation was applied to a Phenyl Superose (HR 5/5) column (Pharmacia) equilibrated with 1.4 M- $(\text{NH}_4)_2\text{SO}_4$ in buffer A, and the column was washed until the A_{280} returned to zero. For each enzyme preparation, protein was then eluted with a reverse linear gradient (16 ml) of 1.4–0 M- $(\text{NH}_4)_2\text{SO}_4$ in buffer A and the active fractions were pooled. Finally, each enzyme preparation was applied to and eluted from a 600 mm $\times$ 3.7 mm column of Superose 12 (Pharmacia) in buffer A at 0.3 ml min⁻¹. The purified enzymes were stored at 4 °C in buffer A.

Enzyme assays. β -Galactosidase (EC 3.2.1.23) was assayed at 37 °C in a total volume of 1.2 ml containing 20 mM-potassium phosphate, pH 7.0, 0.1 mM-MgSO₄ and 2.2 mM-PNPGal. The reaction rate was constant and time allowed for colour to develop varied from 0.5 to 20 min. The reaction was stopped by the addition of 0.5 ml 1.0 M-Na₂CO₃, and absorbance was read at 420 nm. β -Glycosidase (EC 3.2.1.21) activity was measured in the same way but the reaction mixture contained 20 mM-potassium phosphate, pH 7.0, and 2.2 mM-4-nitrophenyl β -D-glucopyranoside (PNPGlu). One unit of each enzyme catalyses the production of 1 μ mol 4-nitrophenol min⁻¹ (4-nitrophenol ϵ = 16000 l mol⁻¹ cm⁻¹). For substrates other than the nitrophenyl derivatives, the assay conditions were as described above except that the total assay volume was 0.5 ml, the reactions were stopped by heating at 100 °C for 1 min, and glucose and fructose were measured as described below. Assays were done in duplicate with a reliability of $\pm$ 5%.

Analytical methods. Protein was measured by the Lowry method after precipitation with trichloroacetic acid (Peterson, 1977), using bovine serum albumin as a standard. SDS-PAGE was done with 10% separating gels and 5% stacking gels (Laemmli & Favre, 1973) in a mini-gel apparatus (Matsudaira & Burgess, 1978). Samples (1–20 μ l) were loaded and the gels were run at constant voltage (150 V) for approximately 90 min. Carbonic anhydrase (*M_r* 29000), ovalbumin (45000), bovine serum albumin (66000), phosphorylase (97400) and β -galactosidase (116000) were used as *M_r* markers. Gels were fixed for 2 h in 25% (v/v) propan-2-ol containing 10% (v/v) acetic acid. After washing in deionized water for at least 2 h, the gels were silver-stained (Morrisey, 1981), and the staining reaction was stopped with 2 M-acetic acid. Glucose was assayed as described by Brüss & Black (1978) using glucose oxidase (Sigma type V-S) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) as the chromogen. Fructose was measured using the procedure of Sturgeon (1980).

Chemicals. The nitrophenyl substrates and protein standards were from Sigma. Laminari-biose, -triose, -tetraose and -pentose were from Seikagaku Kogyo (Tokyo).

Results

Cellular location of the activity

Preliminary experiments were done to determine the cellular location of the lactose hydrolase activity. No

Fig. 1. DEAE-Sephacrose chromatography. Cell-free extract was treated batchwise with DEAE-Sephacrose, washed and the resin transferred to a column as described in Methods. Protein was eluted with a NaCl gradient (----). The eluate was monitored at A_{280} (—). Fractions of 10 ml were collected and enzyme activity measured with PNPGal (○) and PNPGlu (●).

activity was detected in the culture medium. Lactose-grown cells were treated with Novozyme 234, a cell wall lyticase, as described by Mörtberg & Neujahr (1986). No activity was released, and the activity of spheroplast extracts was the same as that of cell-free extracts. From these observations we concluded that the lactose hydrolase activity was intracellular.

Purification of the two enzymes

Cells for the enzyme purification were harvested at an OD₆₀₀ of 5.0 because of the relatively high enzyme activity and the low lipid content. Even so, it was necessary to decant the cell-free extract carefully to reduce the carry-over of the floating lipid and the soft pellet of cell debris. Preliminary experiments showed that the enzyme activity was bound tightly to DEAE-Sephacrose, and that the initial purification was most effective when the absorption was done in a batchwise method. The washed resin was transferred to a column, and the subsequent application of a shallow NaCl gradient resulted in the elution of two enzymes. The first, a β -glycosidase, hydrolysed both PNPGal and PNPGlu; the second, β -galactosidase, only PNPGal (Fig. 1). These two enzyme fractions were purified separately by ion exchange, hydrophobic and gel filtration chromatography. The pooled enzyme fractions were concentrated by ultrafiltration, the recoveries ranging from 50 to 100%. The recovery of the β -glycosidase after the Mono Q step was usually greater than 50%. Recoveries from Phenyl Superose chromatography [including the

Table 1. Purification of β -glycosidase and β -galactosidase

Treatment	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units mg ⁻¹)
<i>β-Glycosidase</i>				
Crude preparation	262	260	1600	0.16
After DEAE-Sepharose	52	134	68	2.0
After Fast-Flow DEAE-Sepharose	21	124	25	5.0
After Mono Q	4.3	20	3.5	5.7
After Phenyl Superose	1.9	12	0.7	17
After gel filtration	6.4	11	0.45	24
<i>β-Galactosidase</i>				
Crude preparation	262	260	1600	0.16
After DEAE-Sepharose	41.7	210	46	4.6
After Fast-Flow DEAE-Sepharose	22.0	155	13	12
After Mono Q	5.2	72	2.9	25
After Phenyl Superose	2.6	41	0.44	93
After gel filtration	7.7	44	0.34	130

addition of $(\text{NH}_4)_2\text{SO}_4$ and centrifugation] ranged from 30 to 60% for both the β -galactosidase and β -glycosidase. Each enzyme eluted from this column was associated with one symmetrical peak. The overall purifications were 150- and 810-fold for the β -glycosidase and the β -galactosidase, respectively (Table 1).

M_r values of the two enzymes

Both enzyme preparations were at least 90% pure as judged by SDS-PAGE and silver staining. The M_r values for the β -galactosidase and the β -glycosidase were 120000 and 52000, respectively. The β -glycosidase eluted from the Superose 12 gel filtration column (in 100 mM-sodium phosphate buffer, pH 7, containing DTT, EDTA and glycerol at 2 and 1 mM and 10%, v/v, respectively) had an apparent M_r of 59000. With the same buffer system the β -galactosidase had an apparent M_r of 180000, but with 8 mM buffer or 8 mM buffer containing 20 mM-NaCl the apparent M_r was 400000. These data showed that the native β -glycosidase exists in the monomeric form, whereas the β -galactosidase is an oligomer. We suggest that the β -galactosidase species identified by gel filtration are dimeric and tetrameric forms. The native *E. coli* β -galactosidase is a tetramer with an apparent M_r of 540000 (Wallenfels & Weil, 1972).

Stability

The enzyme activity in crude cell-free extracts prepared in 8 mM-sodium phosphate buffer was not stable (95% of the activity was lost after 11 d storage at 4 °C) unless DTT, EDTA and glycerol were added to the preparations (Table 2). These reagents were therefore

included in the buffer solutions used throughout the purifications. When the purified enzymes were stored under similar conditions, more than 90% of the activity was retained after 1 month.

The stability of each enzyme was also measured in dilute solutions stored for 7 d at 4 °C (Table 2). The β -galactosidase was most stable in potassium phosphate, pH 6 (16% loss of activity), and the β -glycosidase was most stable in the range pH 7 to 9 in potassium phosphate or triethanolamine ($\leq 16\%$ loss of activity). At 37 °C dilute solutions of the β -galactosidase specifically required K^+ for stability (Table 2).

Properties of the two enzymes

Both enzymes were optimally active at 37 °C when tested over the range 25 to 45 °C. The β -glycosidase exhibited a broad pH optimum from pH 6 to 7, and the β -galactosidase had maximum activity in the pH range 6.5 to 7.

Both enzymes were assayed at pH 7.0 in the following range of 50 mM-buffers: sodium phosphate, triethanolamine/HCl, TES/ NH_4OH and Tris/HCl, supplemented with 200 mM-KCl and 10 mM- MgSO_4 for the β -galactosidase. Compared with the activity under the standard assay conditions, the β -galactosidase was not markedly affected by any of the buffers. The β -glycosidase, however, was inhibited strongly by Tris (88% inhibition) and to a lesser extent by triethanolamine (35% inhibition).

The β -glycosidase did not exhibit any requirement for metal ions, and it was fully active in 5 mM-EDTA. The β -galactosidase had an absolute requirement for a monovalent ion, and of those tested, only K^+ and Rb^+ activated the enzyme (Table 3). The enzyme also required a divalent cation. No activity was observed

Table 2. *Stability of the two enzymes*

Expt 1. Samples of crude extract [9.6 μmol PNPGal hydrolysed min^{-1} (ml enzyme) $^{-1}$] in 8 mM-sodium phosphate buffer, pH 7.0, were incubated as indicated at 4 °C for 11 d and assayed for activity with PNPGal. Expt 2. Purified β -galactosidase (130 units mg^{-1} , 4.4 μg ml^{-1}) and β -glycosidase (24 units mg^{-1} , 7 μg ml^{-1}) were stored for 7 d at 4 °C in 90 mM buffers as indicated. Expt 3. Purified β -galactosidase (130 units mg^{-1} , 0.37 μg ml^{-1}) was incubated for 10 min at 37 °C as indicated. Results are expressed as percentage of activity remaining after the incubations.

Expt 1 Addition to crude extract	Total β -galactosidase activity (%)	
None	5	
5 mM-DTT	18	
1 mM-EDTA	25	
10% (v/v) glycerol	11	
DTT, EDTA and glycerol	70	
Expt 2 Conditions	β -Galactosidase activity (%)	β -Glycosidase activity (%)
Sodium acetate, pH 5.0	10	0
Sodium acetate, pH 6.0	64	50
Sodium phosphate, pH 6.0	84	75
Sodium phosphate, pH 7.0	69	91
Sodium phosphate, pH 8.0	31	91
Triethanolamine/HCl, pH 8.0	9	94
Triethanolamine/HCl, pH 9.0	0	84
Expt 3 Conditions	β -Galactosidase activity (%)	β -Glycosidase activity (%)
17 mM-potassium phosphate, pH 7.0	90	ND
17 mM-sodium phosphate, pH 7.0	5	ND
17 mM-sodium phosphate, pH 7.0, plus 17 mM-KCl	50	ND

ND, Not determined.

when 5 mM-EDTA was included in the enzyme assay. When Mg^{2+} was omitted from the standard assay system, the enzyme showed 30–60% of the activity under the standard conditions. This may have been due to varying amounts of divalent ions in the preparations. Two other divalent cations, Mn^{2+} and Ca^{2+} , also stimulated activity (Table 3), but to a lesser extent than Mg^{2+} . β -Mercaptoethanol (300 mM), increased the β -galactosidase activity by 50%. Wallenfels & Weil (1972) suggested that a similar result with the *E. coli* β -galactosidase was due to the thiol reagent being a more efficient acceptor of the galactosyl moiety. β -Mercaptoethanol had no effect on the β -glycosidase activity.

Table 4 summarizes the kinetic constants k_{cat} and K_{m} for the enzymes with lactose, PNPGal and 2-nitrophenyl β -D-galactopyranoside (ONPGal). Although the k_{cat} of the β -galactosidase with lactose is eight times greater than that of the β -glycosidase (400 s^{-1} and 50 s^{-1} ,

Table 3. *Effect of monovalent and divalent ions on β -galactosidase activity*

The activity is expressed relative to the standard assays using (a) 0.0085 units and (b) 0.052 units of β -galactosidase. The results are means of duplicate determinations. For testing monovalent ions each assay contained 20 mM-triethanolamine/HCl buffer, pH 7.0, 0.1 mM- MgSO_4 , and 20 mM monovalent cation (the chloride salt except for lithium which was the acetate salt). For testing divalent ions each assay contained 17 mM-potassium phosphate, pH 7.0, and 0.01 mM divalent cation (the chloride salt except for Mg^{2+} and Cu^{2+} which were the sulphate salts).

(a) Monovalent ion	β -Galactosidase activity (%)	(b) Divalent ion	β -Galactosidase activity (%)
Standard assay	100	Mg^{2+}	100
No additions	0	No additions	36
Li^+	0	Mn^{2+}	89
Na^+	0	Ca^{2+}	61
K^+	44	Ba^{2+}	36
Cs^+	0	Zn^{2+}	0
Rb^+	15	Cu^{2+}	0
NH_4^+	0		
Tris/HCl	0		

Table 4. *Kinetic constants for the β -galactosidase and the β -glycosidase*

Substrate . . .	β -Galactosidase			β -Glycosidase	
	Lactose	PNPGal	ONPGal	Lactose	PNPGal
K_{m} (mM)	20	0.75	0.75	5.1	2.3
k_{cat} (s^{-1})	400	340	400	50	30

respectively) the β -glycosidase has a fourfold higher affinity for this substrate.

The β -glycosidase exhibited Michaelis–Menten kinetics with PNPGal, but the double reciprocal plot with PNPGlu was non-linear, and hence no kinetic constants were obtained with this substrate.

Substrate specificities of the two enzymes

The β -galactosidase was highly specific for lactose (Table 5). Lactulose (4-O- β -D-galactopyranosyl-D-fructofuranose) was hydrolysed at a much slower rate. Of the nitrophenyl substrates, only ONPGal and PNPGal were hydrolysed by this enzyme (Table 5).

The β -glycosidase had a much wider substrate specificity. It hydrolysed lactose efficiently, and all the disaccharides of the formula n -O-(β -D-glucopyranosyl)-D-glucopyranoside, where $n = 2, 3, 4, 6$ in order of decreasing reactivity. Indeed, at the same molar concentrations, sophorose, the β -1,2 disaccharide, was a much better substrate than lactose. The enzyme also hydro-

Table 5. Substrate specificities of the two enzymes

β -Galactosidase and the β -glycosidase were measured with the substrates listed under standard conditions. Activities are expressed as μmol (ONP, PNP, glucose or fructose) $\text{min}^{-1} \text{mg}^{-1}$ (2-nitrophenol $\epsilon = 4500 \text{ l mol}^{-1} \text{cm}^{-1}$). No activity was detected with PNP- β -D-mannopyranoside, PNP- α -D-mannopyranoside, PNP- α -D-glucopyranoside or PNP- α -D-galactopyranoside (both enzymes), sucrose or maltose (β -glycosidase), and ONP-1-thiogalactopyranoside (β -galactosidase). The results are the means of at least duplicate determinations.

Substrate	Substrate concn (mM)	β -Galactosidase activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	β -Glycosidase activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
PNPGal	2.2	118	17
PNPGlu	2.2	0	24
ONPGal	2.2	157	17
Lactose	50	125	44
Lactose	0.5	ND	4.7
Lactulose	80	16	3.1
Cellobiose	10	0	31
Laminaribiose	0.5	ND	34
Laminaritriose	0.5	ND	17
Lamaritetraose	0.5	ND	2.3
Lamaripentaose	0.5	ND	0.9
Gentiobiose	4	ND	0.7
Sophorose	0.5	ND	36

ND, Not determined.

lysed oligomers of the β -1,3 series, and the reactivity decreased as the chain was increased from biose to pentaose. Of the nitrophenyl β -glycosides tested, only ONPGal, PNPGal and PNPGlu were hydrolysed.

Expression of β -galactosidase and β -glycosidase

Experiments were done to determine the relative activities of the two enzymes during growth in different media, and to identify specific inducers for each enzyme. Two simple assay systems were used specifically to measure each enzyme in cell-free extracts. The combined activity of both enzymes was measured with PNPGal in the standard β -galactosidase assay. The β -glycosidase was measured with PNPGal in an assay system without MgSO_4 but containing 5 mM-EDTA to abolish the β -galactosidase activity. β -Galactosidase activity was calculated as the difference between the two assays. Fig. 2 shows the expression of the two enzymes during growth in lactose medium. The enzyme activities increased in parallel from the onset of growth. The β -galactosidase activity persisted into the late exponential phase, but the β -glycosidase activity decreased rapidly after the mid-exponential phase. The maximum activity for each enzyme coincided with the maximum growth rate.

Cell-free extracts were prepared from cells grown in a medium containing 0.15% yeast extract plus 0.1% casein

Fig. 2. Production of β -galactosidase and β -glycosidase during growth. A culture (400 ml) containing 0.1% casein acid hydrolysate, 0.15% yeast extract and 5% lactose was measured for growth at 600 nm ($\square$). Cell extracts were prepared at various times and assayed for β -glycosidase ($\bullet$) and β -galactosidase ($\circ$) as described in the text. Enzyme activity is expressed as units (ml culture) $^{-1}$.

Table 6. Induction of β -galactosidase and β -glycosidase

Cells grown in a medium containing 0.1% casein acid hydrolysate, 0.15% yeast extract and 2% peptone were harvested at 18 h and used to inoculate flasks containing nitrogen-free Wickerham's salts with trace elements (Wickerham, 1946) and sugar at 20 mM, except for lactose plus glucose, where the concentrations were 20 and 10 mM, respectively. After 4 h the cells were harvested and cell-free extracts were assayed for activity towards PNPGal (2.2 mM) in 20 mM-potassium phosphate, pH 7, containing 1.1 mM- MgSO_4 or 20 mM-potassium phosphate, pH 7, containing 5 mM-EDTA. Activity was calculated as described in the text. The extracts were also assayed with PNPGlu. The β -galactosidase, β -glycosidase and PNPGlu activities in cell-free extracts from uninduced cells were 0.5–0.9, <0.1 and 0.8–1.1 μmol (ml culture) $^{-1}$ (OD_{600} unit) $^{-1}$, respectively. Cultures were grown in duplicate on each sugar, and the cell-free extracts from each culture were assayed in duplicate.

Sugar	Enzyme activity [$\mu\text{mol ml}^{-1}$ (OD_{600} unit) $^{-1}$]		
	β -Galactosidase	β -Glycosidase	PNPGlu
Lactose	4.8	7.5	14
Lactose plus glucose	1.6	3.3	6.4
Lactulose	4.8	3.8	ND
Cellobiose	0.2	2.4	5.6
Galactose	4.1	4.0	ND
Lactobionic acid	2.3	0.2	ND
IPTG	1.1	0.0	0.2

ND, Not determined.

hydrolysate and a carbon source (5%, w/v). These media gave rapid growth, but there was a 2 to 3 h lag phase before the enzyme activities increased. A trace of β -galactosidase activity was detected in cells grown with glucose. There was significant activity in the cells grown on glycerol, sorbitol and peptone [1.2 to 1.9 μmol

Table 7. *Properties of lactose hydrolases from micro-organisms*

	<i>E. coli</i> *	<i>K. lactis</i> †	<i>T. cutaneum</i>		<i>S. singularis</i> ‡
			(1)	(2)	
M_r	116000	135000	120000	52000	140000
Substrates	Lactose	Lactose	Lactose	Lactose and cellobiose	Lactose and cellobiose
Type of enzyme	β -Galactosidase	β -Galactosidase	β -Galactosidase	β -Glycosidase	β -Glycosidase
Monovalent metal ion requirement	+	+	+	None	ND
Divalent metal ion requirement	+	+	+	None	None
pH optimum	7.0	7.25	6.5–7.0	6.0–7.0	3.9–4.0

ND, Not determined.

* Wallenfels & Weil (1972); Cohn & Monod (1951).

† Dickson *et al.* (1979).

‡ Blakely & MacKenzie (1969).

(ml culture)⁻¹ (OD₆₀₀ unit)⁻¹], and a sixfold greater activity in lactose-grown cells. In contrast, only a trace of β -glycosidase activity was present in cells grown on each of these carbon sources except lactose [2.9 μ mol (ml culture)⁻¹ (OD₆₀₀ unit)⁻¹].

Enzyme production was measured in cells incubated in a minimal medium consisting of nitrogen-free Wickerham's salts and various carbon sources. Growth was limited, but there was no lag phase in the enzyme production. As shown in Table 6, the two enzyme activities increased four- to sevenfold with lactose, lactulose and galactose with respect to the starter culture. When 10 mM-glucose was included in the medium with lactose, the β -galactosidase and the β -glycosidase activities were decreased to 33% and 44% respectively of those observed with lactose alone. Lactobionic acid (4-O- β -D-galactopyranosyl D-gluconic acid) produced a significant increase in the β -galactosidase activity, but not the β -glycosidase, whereas cellobiose produced a significant increase in the β -glycosidase alone. IPTG had no effect on the activities of either the β -galactosidase or the β -glycosidase.

Discussion

The conversion of whey-lactose to lipid by *T. cutaneum* is of current interest because it could be the basis of an industrial process (Verwoert *et al.*, 1989). The metabolism underlying the production of lipid by *T. cutaneum* has been studied in detail (Holdsworth *et al.*, 1988) but the initial steps in lactose metabolism have not been investigated. The present study has shown that *T. cutaneum* has two lactose hydrolases, a β -galactosidase (M_r 120000) and a β -glycosidase (M_r 52000). N-terminal amino acid sequence analyses (30 residues) of the two enzymes were quite different (data not shown) and these showed no homologies with any published sequences.

The sequence analyses confirmed that each preparation consisted of one polypeptide.

The properties of several lactose hydrolases from micro-organisms are summarized in Table 7. The *T. cutaneum* β -galactosidase is similar to the β -galactosidases from *E. coli* and *K. lactis*, but it requires K⁺ or Rb⁺ for activity and stability, whereas the enzymes from the other two organisms are activated by either Na⁺ or K⁺. The enzyme from *Kluyveromyces fragilis* also specifically requires K⁺ for stability (Mahoney & Whitaker, 1977).

The β -glycosidase has a wider substrate specificity than the β -galactosidase and it hydrolyses glucosides including cellobiose, and galactosides, but not PNP- β -D-mannopyranoside, gentiobiose or sucrose. Sucrose is invariably hydrolysed by highly specific β -D-fructofuranosidases (Goldstein & Lampen, 1975). Cellobiose hydrolysis is usually ascribed to β -glucosidases (Sanyal *et al.*, 1988; Bronnenmeier & Staudenbauer, 1988), but as the name suggests, these enzymes are specific for substrates in which glucose is joined by a β -link to another sugar or a nitrophenyl group. Both enzymes from *T. cutaneum* exhibited Michaelis-Menten kinetics with β -galactosides but the double reciprocal plot for the β -glycosidase with PNPGlu curved downwards. This type of plot is usually indicative of cooperativity or substrate activation, but other complexities also generate non-linear plots (Dixon & Webb, 1979). The kinetics of the β -glycosidase require a more detailed study.

By exploiting the differing metal ion requirements and substrate specificities of the β -galactosidase and the β -glycosidase, each enzyme activity can be separately measured in crude extracts. When cultures were grown in lactose, both PNPGal- and PNPGlu-hydrolysing activities were expressed. With EDTA added to the assay system to eliminate the β -galactosidase activity, the ratio of PNPGal/PNPGlu activity in extracts from cells grown on different carbon sources ranged from

1:1.8 (lactose) to 1:2.3 (cellobiose), whereas the ratio for the purified β -glycosidase was 1:1.4. This suggests that other enzymes in crude extracts hydrolyse PNPGlu. β -Glycosidase activity in cell extracts was therefore measured with PNPGal in the presence of EDTA.

Many of the β -glucosidases in bacteria and fungi are part of a cellulose-degrading system. Few yeasts, however, are able to hydrolyse cellulose, but many still possess β -glucosidases. These enzymes may be present to hydrolyse cellobiose that has been produced by the actions of other organisms (Leclerc *et al.*, 1987), but they are undoubtedly able to hydrolyse other β -glucosides. *T. cutaneum* grows well on cellobiose, and the β -glycosidase is induced by this substrate. Our data strongly suggest that this enzyme also contributes to lactose hydrolysis, along with the β -galactosidase. The presence of two distinct lactose-hydrolysing enzymes in the same organism is unusual, and presumably confers some advantage.

Mörtberg & Neujahr (1986) have carried out extensive studies with a yeast isolated from soil near a paper mill, and identified as *T. cutaneum*. This organism is, however, clearly different from the *T. cutaneum* used in the present study; the yeast studied by Mörtberg & Neujahr (1986) produced an exocellular β -galactosidase, but both of the enzymes from our strain of *T. cutaneum* are intracellular.

β -Glucosidases have been purified from several yeasts including *Saccharomyces cerevisiae* (Inamdar & Kaplan, 1966), *Candida molischiana* (Gondé *et al.*, 1985) and *Candida pelliculosa* (Kohchi *et al.*, 1985). These enzymes characteristically have native M_r values in the range 80 000 to 500 000, and are oligomeric. Most are active in the presence of EDTA, have pH optima ranging from 4.5 to 7, and hydrolyse cellobiose and PNPGlu, but not lactose (Leclerc *et al.*, 1987). The *T. cutaneum* β -glycosidase shares several of these properties (pH optimum, activity in the presence of EDTA, and activity towards PNPGlu and cellobiose), but it has a lower M_r . It is also capable of hydrolysing lactose. Hydrolases with activity towards cellobiose and lactose have been purified from snail gut extract (Got & Marnay, 1968) and bacteria (Patchett *et al.*, 1987; Takase & Horikoshi, 1988). We have found only one report of an enzyme with this specificity in yeast. Blakely & MacKenzie (1969) reported the characterization of a β -glucosidase from *Sporobolomyces singularis* that hydrolysed lactose and cellobiose. This enzyme has similar ionic requirements to the β -glycosidase characterized in this study, but the M_r (140 000) and the pH optimum (4.0) were different. It would be of interest to investigate the uncharacterized lactose hydrolases in other yeasts and fungi that grow on this disaccharide.

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