Purification and characterization of a heterogeneous glycosylated invertase from the rumen holotrich ciliate *Isotricha prostoma*

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The invertase (β -fructofuranosidase, EC 3.2.1.26) of the rumen holotrich ciliate *Isotricha prostoma* has been purified. This is the first report of an enzyme purification from a known species of rumen protozoon. Cells were disrupted by ultrasonic treatment and the enzyme was purified from the cell-free extract by three successive liquid column chromatographies (Sephacrose CL4B/octyl-Sepharose CL4B, DE52 DEAE-cellulose and concanavalin A-Sepharose 4B). This resulted in a 160-fold purification and a 15% yield. The major form of the purified enzyme was a tetramer with M_r about 350 000 that was readily dissociated by electrophoresis. The invertase was heterogeneous, as five types of monomers were shown by SDS/polyacrylamide-gel electrophoresis after denaturation. Part of this heterogeneity was due to different glycosylated forms of one of the polypeptides present in the purified enzyme. *Isotricha prostoma* invertase exhibited maximum activity at pH 5.5–6.0 and 50 °C. The kinetic properties of the purified enzyme were very similar to those of invertases from other sources such as yeast or plants (substrate and product inhibition, transferase activity).

INTRODUCTION

Micro-organisms living in the stomach of ruminants are dependent on the host for their food supply. Some have developed specialized pathways for the assimilation of particular substrates and possess, for example, an array of carbohydrases to degrade and utilize the plant structural polysaccharides. Others, such as the holotrich ciliates (Family Isotrichidae), depend more specifically on readily available substrates. Although these ciliates can depolymerize both pectic and hemicellulosic polysaccharides, they are only capable of metabolizing simple substrates such as the soluble sugars D-glucose, D-fructose or sucrose (for a review see ref. [1]). The rumen holotrich protozoa show chemotaxis to these sugars [2]. A diet rich in soluble carbohydrates also increases the number of holotrich ciliates found in the rumen [3].

Many carbohydrase activities have been reported in rumen ciliates [4]. However, no detailed studies have hitherto been carried out on the structure and properties of these enzymes owing to the difficulty in obtaining pure cultures of rumen ciliates. Therefore their exact role in rumen metabolism is still unclear.

Sucrose, for instance, is an easily fermentable sugar, and hence there is the possibility of severe competition for this sugar in the rumen. The metabolism of sucrose generally commences by its hydrolysis into D-glucose and D-fructose by invertase (β -fructofuranosidase, EC 3.2.1.26). The presence of a very active invertase in rumen holotrich ciliates has been known for many years [5,6], and in addition some exocellular activity has also been reported [7].

Although numerous reports have been published on invertases from bacterial, fungal or vegetable origin (for a review see ref. [8]), hitherto no protozoal invertase has been characterized in detail.

In the present paper we now describe the purification and characterization of the invertase from an isolated species of holotrich ciliate, *Isotricha prostoma*.

MATERIALS AND METHODS

Isolation of ciliates

The cells were obtained from the rumen fluid of a sheep mono-contaminated with the holotrich ciliate *Isotricha prostoma*. The rumen, for this purpose, was defaunated with Mannoxol OT (BDH Chemicals) according to the method of Demeyer & Van Nevel [9]. Then 100 cells of *Isotricha prostoma*, selected microscopically, were re-introduced through the permanent fistula subsequent to injection of 500 ml of normal rumen fluid freed from living ciliates by freezing in liquid N₂. The *Isotricha prostoma* cells were washed by repeated decantations in Hungate's salt solution [3] at 40 °C. Cells were frozen in liquid N₂ and stored at –70 °C until use.

Invertase purification

All purification steps were performed at 0–4 °C.

Step 1: preparation of the cell-free extract. Frozen cells were thawed in buffer A (20 mM-potassium phosphate buffer, pH 6.5, containing 0.25 M-mannitol and 1 mM-EDTA) and sonicated for 3 × 1 min (Virsonic Cell Disrupter model 16-850; The VirTis Co.). Large cell debris and organelles were removed by two successive centrifugations (10³ and 4 × 10⁵ g · min) in respectively Sorvall GLC2 and Beckman J21 centrifuges. The final supernatant was sonicated for 10 × 1 min and centrifuged (6 × 10⁶ g · min) in a Beckman L8-70 centrifuge to provide a supernatant termed the cell-free extract.

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Step 2: chromatography on Sepharose CL4B/octyl-Sepharose CL4B. A 90 cm × 3 cm column was poured up to a height of 74 cm with Sepharose CL4B (Pharmacia) and further completed with octyl-Sepharose CL4B (Pharmacia), both equilibrated in buffer A without mannitol but containing 1 M-(NH₄)₂SO₄. The column was loaded with the cell-free extract adjusted to 1 M-(NH₄)₂SO₄ and then eluted with the equilibration buffer (22 ml/h). Fractions (4.4 ml) were collected for assay for invertase activity and measurement of the absorbance at 280 nm. Active fractions were pooled and dialysed overnight against buffer B (0.1 M-sodium acetate buffer, pH 6.0).

Step 3: chromatography on DE52 DEAE-cellulose. The dialysed sample from step 2 was applied to a 5.7 cm × 1.4 cm column containing DE52 DEAE-cellulose (Whatman) and equilibrated in buffer B (10 ml/h). After the column had been washed with 10 ml of the equilibration buffer, the bound proteins were eluted with a 0–1 M-NaCl linear gradient in buffer B (40 ml). Fractions (1 ml) were analysed as in step 2.

Step 4: chromatography on concanavalin A-Sepharose 4B. The pooled active fractions from step 3 were adjusted to 1 M-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂ and 1 mM-MnCl₂. They were applied (2 ml/h) to a 6 cm × 0.4 cm column of concanavalin A-Sepharose 4B (Pharmacia) (approx. 10 mg/ml of gel) and equilibrated in buffer C (0.1 M sodium acetate buffer, pH 6.0, containing 1 M-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂ and 1 mM-MnCl₂). The column was washed with 3 ml of buffer C and the invertase was then eluted with a 0–0.25 M-methyl α -mannoside linear gradient in buffer C. Fractions (0.3 ml) were analysed as in step 2.

The purified enzyme was either dialysed extensively against 50 mM-sodium acetate buffer, pH 5.5, or desalted by passage through a Sephadex G-25 (medium grade) (Pharmacia) column and stored at –70 °C.

Enzyme assay

Invertase activity was assayed by measuring the production of reducing sugars by the 2,4-dinitrosalicylic acid method [10]. Typical assay was performed in 125 mM-sodium acetate buffer, pH 5.5, at 40 °C in the presence of 40 mM-sucrose. In the presence of excess D-glucose or D-fructose, the activity was assayed either by the sorbitol dehydrogenase reaction [11] or by the glucose oxidase–peroxidase method [12].

One unit of enzyme activity was defined as the amount of enzyme that produces 1 μ mol of reducing sugars/min, with D-fructose or D-glucose as standard.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed according to the technique of Laemmli [13]. Invertase activity was observed by staining as follows. The gels were washed overnight in 50 mM-sodium acetate buffer, pH 5.5, and incubated for 30–60 min at 40 °C in the same buffer supplemented with 40 mM-sucrose. The gels were briefly washed in distilled water and incubated in 1 M-NaOH containing 0.1% 2,3,5-triphenyltetrazolium chloride. The reaction was stopped by immersing the gels in 1% (v/v) acetic acid. The transfer of proteins on to nitrocellulose membrane [14] and the detection of

glycosylated polypeptides by concanavalin A binding [15] were performed as described in the references cited.

Centrifugation on a glycerol gradient

Linear 10–30% (v/v) glycerol gradients were prepared in 50 mM-sodium acetate buffer, pH 5.5. The gradients were centrifuged [24 000 rev./min (76 000 g_{av}) for 36 h at 4 °C] in the SW27 rotor of the Beckman L8-70 centrifuge. Fractions (0.5 ml) were collected and assayed for invertase activity. The refractive index of each fraction was measured to allow the comparison between separate gradients.

Other techniques

Measurement of protein [16], determination of the carbohydrate composition of the invertase [17], chemical deglycosylation with trifluoromethanesulphonic acid [18], enzymic deglycosylation with endoglycosidase H [19] or glycopeptidase F [20] and t.l.c. [21] were performed as described in the references cited.

RESULTS

Purification of the invertase

Starting with a cell-free supernatant obtained after ultrasonic disruption of *Isoetria prostoma* cells, the invertase was purified by three successive chromatographic steps. The first step took advantage of the high M_r of the enzyme and of its low hydrophobicity under the conditions used [1 M-(NH₄)₂SO₄, pH 4.5]. The Sepharose CL4B/octyl-Sepharose CL4B column was unable to retain the invertase and allowed a purification factor of approx. 11.5-fold for this step with a 76% yield (Fig. 1a and Table 1).

After dialysis of the active fractions, most of the contaminating proteins remained unbound to the ion-exchange DE52 DEAE-cellulose column (Fig. 1b, fractions 10–60) whereas the invertase activity was eluted as a major peak by the NaCl gradient.

Finally, all invertase activity was retained on the concanavalin A-Sepharose 4B affinity column. The enzyme was eluted with a methyl α -mannoside gradient as a single, slightly asymmetric, peak (Fig. 1c).

The final purification factor was approx. 160-fold with a 15% yield. The specific activity of the purified enzyme was approx. 3000 units/mg (Table 1).

Purity and M_r

The analysis by SDS/polyacrylamide-gel electrophoresis of the purified invertase revealed several major bands displaying enzymic activity (Fig. 2, lanes A and C). The major form had a mobility corresponding to an M_r of about 170 000–200 000. The M_r values of the subunits were estimated to be between 65 000 and 80 000 after the sample had been boiled for 2 min in SDS before electrophoresis (Fig. 2, lane B).

The native enzyme appeared to be a tetramer with an M_r of about 350 000 that was slightly dissociated into dimeric forms of M_r about 170 000 during centrifugation on the glycerol gradient (Fig. 3). The same results were obtained by gel filtration on a Sepharose 6B column in acetate buffer (results not shown).

Carbohydrate composition

The sugars associated with the purified enzyme were N-acetylglucosamine (3.1%), mannose (2.9%), glucose

Fig. 1. Purification of *Isotricha prostoma* invertase

The Figure shows the elution patterns of protein (A_{280} , ■) and invertase (A_{575} , ●) after (a) Sepharose CL4B/octyl-Sepharose CL4B chromatography, (b) DE52 DEAE-cellulose chromatography and (c) concanavalin A-Sepharose 4B chromatography. Conditions of chromatography are given in the Materials and methods section. Horizontal bars represent the fractions pooled for further purification (a and b) or characterization (c).

(1.4%) and galactose (0.9%). The percentages are expressed as weight of sugar per weight of protein and are only indicative owing to the heterogeneity of invertase.

Glycosylation and peptide heterogeneity

The presence of glycosylated polypeptides in the purified invertase fraction was demonstrated by reaction with concanavalin A after SDS/polyacrylamide-gel

electrophoresis and electroblotting on nitrocellulose membrane. Only the forms corresponding to the higher M_r reacted significantly with the concanavalin A (Fig. 4, lane B).

The unglycosylated polypeptides were those that were resistant to treatment with trifluoromethanesulphonic acid. Two bands (or two groups of bands) appeared after SDS/polyacrylamide-gel electrophoresis (Fig. 4, lane C),

Table 1. Summary of the purification of invertase from *Isotricha prostoma* cells

For experimental details see the Materials and methods section.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	767	14649	19.1	—	100
Cell-free supernatant	320	11112	34.7	1.8	76
Sepharose CL4B/octyl-Sepharose CL4B chromatography	10.6	4188	395	20.7	29
DE52 DEAE-cellulose chromatography	1.7	3792	2204	115.4	26
Concanavalin A-Sepharose 4B chromatography	0.74	2204	2978	156.0	15

Fig. 2. SDS/polyacrylamide-gel electrophoresis of purified *Isotricha prostoma* invertase

Lane A, purified enzyme (10 μ g); lane B, same as lane A but enzyme denaturated by heating for 2 min at 100 °C before electrophoresis; lane C, purified invertase stained for activity. The standard proteins whose positions are indicated on the left-hand side were ferritin (M_r 220000), phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000) and carbonic anhydrase (M_r 30000). The electrophoresis was performed in a 5–15% polyacrylamide gel as described in the Materials and methods section.

and correspond to those that showed little or no reactivity with concanavalin A. Enzymic deglycosylation of the invertase with glycopeptidase F also decreased the M_r of the higher- M_r forms (Fig. 4, lane D). However, endoglycosidase H was inactive on the invertase, even after preliminary denaturation of the enzyme with SDS (Fig. 4, lane E).

Optimum pH and temperature

The effect of pH on the enzyme activity was determined by using sodium acetate (pH 3.5–6.0) and phosphate

(pH 5.0–8.0) buffers. The enzyme was most active at pH 5.5–6.0. The activity decreased rapidly at more acidic and alkaline pH values.

The effect of temperature on the enzyme activity was assayed in the temperature range between 20 °C and 65 °C. The enzyme showed highest activity at 50 °C under the conditions used.

Substrate-specificity

The invertase was fully active on sucrose. Raffinose displayed 30% of the activity observed on sucrose. There was slight activity on inulin (1%) but none on maltose, melezitose, melibiose, lactose and cellobiose.

Kinetic properties

The effects of substrate and products on the invertase activity were tested at 40 °C.

At low concentrations of sucrose (less than 30 mM) the enzyme obeys the Michaelis–Menten equation (Fig. 5). The K_m value at these concentrations was found to be 3.64 mM. Invertase activity was maximal at 25–45 mM-sucrose but rapidly decreased with increasing substrate concentration to reach a plateau at about 500 mM. This effect is typical of a partial substrate inhibition.

The inhibitory effects of D-fructose and D-glucose were characterized at sucrose concentrations for which substrate inhibition was negligible.

With D-fructose inhibition, the reciprocal plot of the activities obtained for various initial D-fructose concentrations resulted in straight lines showing a common intercept with the $1/v$ axis (Fig. 6). The corresponding values of the apparent Michaelis constant ($K_{m(app.)}$) showed a linear relationship with the initial D-fructose concentrations (Fig. 6 inset). The D-fructose acted as a competitive inhibitor of the invertase. The inhibition constant was calculated to be 47 mM.

Linear plots were also obtained with the reciprocal representation for D-glucose inhibition (Fig. 7). The slope increased with the glucose concentration. There was a common intercept with the $1/[S]$ axis except for the plot obtained in the absence of D-glucose. The inverse of the values for the apparent initial reaction rate were linearly related to the initial D-glucose concentrations (except for the data without D-glucose) (Fig. 7 inset). D-Glucose first induced a modification of the K_m of invertase and then acted as a non-competitive inhibitor. The inhibition constant was calculated to be 136 mM.

During the hydrolysis of a 0.6 M-sucrose solution by

Fig. 3. Centrifugation on a glycerol gradient of purified *Isotricha prostoma* invertase

Fractions were assayed for both refractive index (n_D , ■) and invertase activity (A_{575} , ●). Inset: gradients were calibrated by centrifugation of standard proteins thyroglobulin (M_r 669 000), catalase (M_r 232 000) and fructose-bisphosphate aldolase (M_r 158 000).

Fig. 4. Glycosylation of invertase from *Isotricha prostoma*

Lane A, SDS/polyacrylamide-gel electrophoresis of purified invertase. Lane B, concanavalin A binding and staining after SDS/polyacrylamide-gel electrophoresis and electroblotting of purified invertase. Lanes C–E, SDS/polyacrylamide-gel electrophoresis of purified invertase after treatment with trifluoromethanesulphonic acid (lane C), glycopeptidase F (lane D) and endoglycosidase H (lane E). Samples were denatured by heating for 2 min at 100 °C before electrophoresis. Standard proteins were the same as in Fig. 2.

Fig. 5. Effect of various substrate concentrations on *Isotricha prostoma* invertase activity

The enzymic activity was assayed at 40 °C to avoid thermal inactivation.

the *Isotricha prostoma* invertase several products supplementary to D-glucose and D-fructose were observed after t.l.c. The transferase activity of the invertase led mainly to the synthesis of trisaccharides with a small proportion of tetrasaccharides. This activity was quantified by the addition of [U- 14 C]sucrose during the assay and subsequent measurement of the distribution of the radioactivity in the different spots of the t.l.c. plates. About 15% of the radioactivity was incorporated into compounds migrating more slowly than the sucrose. There was no transferase activity at 5 mM-sucrose, whereas only 1–2% of transferase products were detected at 40 mM-substrate concentration.

DISCUSSION

We have purified the invertase of *Isotricha prostoma* from a homogenate of cells collected from a mono-

contaminated sheep. To our knowledge, only one carbohydrase, a hemicellulase, had been previously purified from an extract of total goat rumen ciliates [22]. The invertase of *Isotricha prostoma* is thus the first enzyme to be purified from a known species of rumen ciliate and the first invertase to be purified from a protozoan. Despite its intrinsic heterogeneity, the enzyme has been purified to homogeneity as shown by SDS/polyacrylamide-gel electrophoresis (Fig. 2). The high final specific activity (3000 units/mg) combined with the relatively low purification factor (160-fold) indicates that the invertase appears to be abundant in *Isotricha prostoma* compared with, for example, the invertase of the yeast *Saccharomyces cerevisiae* [23].

The purified enzyme is a tetramer that dissociates into dimers or monomers during centrifugation on a glycerol gradient or during electrophoresis. There are at least five distinct types of monomeric forms. This heterogeneity

Fig. 6. Inhibition of *Isotricha prostoma* invertase by D-fructose

Lineweaver-Burk plots of sucrose hydrolysis in the presence of D-fructose: ○, 0 mM; ●, 50 mM; □, 100 mM; ■, 200 mM. Inset: effect of initial D-fructose concentration on the $K_{m(app.)}$ value.

Fig. 7. Inhibition of *Isotricha prostoma* invertase by D-glucose

Lineweaver-Burk plots of sucrose hydrolysis in the presence of D-glucose: ○, 0 mM; ●, 50 mM; □, 100 mM; ■, 200 mM. Inset: effect of initial D-glucose concentration on the inverse of the value for the apparent initial reaction rate.

has two different origins. Firstly, at least two distinct polypeptide backbones can be identified after chemical deglycosylation. There are no data currently available to suggest or exclude a common origin for these two polypeptides. Secondly, the comparable intensities of the lower bands in lanes A and D of Fig. 4 show that the longest of these polypeptides displays different states of glycosylation. We cannot exclude the possibility of some proteolysis or carbohydrate hydrolysis during the purification of the enzyme. However, we have never observed any variation in the activity patterns on SDS/polyacrylamide-gel electrophoresis or during the different steps of purification in different experiments (results not shown).

The complete binding of the tetrameric enzyme to the concanavalin A-Sepharose 4B column indicates that the

tetramers are composed simultaneously of glycosylated and unglycosylated subunits. One possibility could be that higher aggregates, composed of homogeneous glycosylated and unglycosylated tetramers, first bind to the lectin and then dissociate. However, this is unlikely, since the invertase peak eluted from the first purification column does not correspond to such aggregates (Fig. 1a).

We can only speculate on the reasons for the diversity of the monomers. It is perhaps a way of exerting control on invertase activity, as the synthesis of invertase appears to be constitutive in *Isotricha prostoma*. It could also provide some particular properties to the enzyme in order to make efficient use of the sucrose available in the rumen. However, the properties studied in this work do not seem to be uncommon as compared with the properties of invertases from other sources. Most of the invertases have an acid optimum pH and display some activity with raffinose as substrate. The transferase properties of the *Isotricha prostoma* invertase are similar to those of the yeast *Saccharomyces cerevisiae* [24]. Yeast and plant (*Ricinus communis*, *Saccharum officinarum*) invertases are also inhibited by an excess of substrate or products [25–27]. However, it is difficult to imagine that very high concentrations of sugars can be obtained in the rumen. Nevertheless, in this case, the *Isotricha prostoma* cells may have developed a system that allows access of invertase to locally concentrated sugars.

The *Isotricha prostoma* invertase, as mentioned above, is glycosylated and shows similarity to other eukaryotic invertases characterized so far. The binding of invertase to concanavalin A-Sepharose 4B, its sensitivity to glycopeptidase F, its insensitivity to endoglycosidase H and its carbohydrate composition suggest that the glycosylation is linked to the asparagine of the polypeptides and is of the complex type.

Some properties had been reported for invertase in cell homogenates of holotrich ciliates [5–7, 28–31]. The present data obtained on the purified enzyme are in good agreement with these reports and describe new characteristics of the enzyme, especially with respect to its kinetic and structural properties. Since none of these properties provides a qualitative advantage for the use of sucrose in the rumen environment of *Isotricha prostoma*, one has to conclude that it is either the abundance of invertase or some undetected characteristic (for example, particular organization or subcellular localization) that confers efficient sucrose utilization to this ciliate.

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