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Purification and partial characterization of β-galactosidase from *Tritrichomonas foetus*

Marion Vella and Pamela Greenwell

School of Biological and Health Sciences, University of Westminster, 115, New Cavendish Street, London W1M 8JS, U.K.

The work presented in this paper describes the purification and properties of a β-galactosidase from the protozoan *Tritrichomonas foetus*. An inexpensive and straightforward method for extraction of the enzyme involving ammonium sulphate precipitation, ion exchange and affinity chromatography resulted in a high level of purification. After purification β-*N*-acetylglucosaminidase was the only enzyme present as a contaminant at a significant level. The β-galactosidase isolated had a pH optimum of 5.8. The K_m determined at pH 5.8 was found to be 2.2 mM. Interesting results were obtained when studies were carried out to determine the effect of various metal ions on enzyme activity. Of the metal ions used in this study only manganese ions were found to activate the enzyme. This seems to be a characteristic of trichomonad enzymes, as *N*-acetyl-β-glucosaminidase, α-galactosidase and *N*-acetyl-α-galactosaminidase are also activated by manganese ions. The strongest inhibition was recorded with lead and to a lesser extent by zinc. The result with lead is not unexpected as the heavy metal is known to cause irreversible inhibition by binding to the amino-acid backbone of the enzyme. The result with zinc is interesting as high levels of zinc are present and trichomonads are known to be apathogenic in semen. The purified β-galactosidase was found to have the capacity to hydrolyse lactose (Gal β1-4 Glc), lacto-*N*-biose 1 (Gal β1-3 GlcNAc) and *N*-acetyllactosamine (Gal β1-4 GlcNAc). When the enzyme was applied to a non-denaturing polyacrylamide gel a single band was observed when stained with Coomassie brilliant blue. This band coincided with that obtained when the gel was stained with p-nitrophenyl β-galactopyranoside. When the same gel was incubated with p-nitrophenyl *N*-acetyl β-glucopyranoside a band was detected which did not coincide with that of β-galactosidase. Since the β-*N*-acetyl-glucosaminidase enzyme does not move to the same position on a non-denaturing gel as the β-galactosidase, we will use this technique to isolate the latter enzyme and determine the N-terminal sequence as a prelude to cloning and further study of the gene.

Keywords*: β-galactosidase, *Tritrichomonas foetus

Introduction

Tritrichomonas foetus is a sexually transmitted pathogen that infects cow uteri, causing inflammation and spontaneous abortion of the developing foetus [1, 2]. The economic costs for the dairy farmer are considerable and estimates from just one Californian herd show losses of up to $665 can be incurred per infected cow [3].

Little is known about the exact mode of pathogenicity of these protozoa. A number of enzymes including proteases, neuraminidases and glycosidases are secreted by the protozoan [4–10]. The hydrolytic actions of these enzymes contribute to the failure of foetal development, mucilaginous lesions and spontaneous miscarriage seen in bovine trichomoniasis although the exact mechanism is still not understood [11]. Since *T. foetus* is a mucilaginous parasite, the major step in pathogenicity must be to overcome the protective mucin barrier. This should take place by systematic hydrolysis of sugar linkages particularly those between the sugars *N*-acetylglucosamine and galactose. Unfortunately the early work carried out by Watkins on these glycosidase enzymes [12] concentrated mainly on their ability to hydrolyse blood group substances. It is our intention to study the types of linkages hydrolysed by this enzyme as a means of understanding the pathogenetic mechanisms of this organism. Before this could be done, it was necessary to develop a simple rapid method for purification of the enzyme. In this paper we present this method together with some of the characteristics of the β-galactosidase enzyme.

Methods

Enzyme assay

Hydrolytic activity was determined in the presence of the substrate p-nitrophenyl β-galactoside (pNP-Gal), 4.0 mM and Tris buffer (pH 6.5), 15 mM at 37 °C. One ml of 0.2 M sodium carbonate was used to stop the

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reaction. The extent of the coloured chromogen formed was read on a spectrophotometer at 420 nm. The specific activity of β-galactosidase is defined as the amount of enzyme required to produce 1 μmol of the chromogen p-nitrophenol per minute per mg of protein at 37 °C and pH 5.8.

Protein assay

The method of Bradford [13] with BSA as a standard was used to measure protein.

Separation and purification of β-galactosidase

T. foetus Belfast strain was obtained from stocks which had been lysed by freeze-thawing then stored at –70 °C. Aliquots were thawed and centrifuged to remove cell debris. The supernatant was subjected to ammonium sulphate fractionation. The precipitate collected over a 40–60% range was then dialysed overnight against 0.01 M phosphate buffer pH 5.8.

Following dialysis the sample was filtered aseptically through a 0.22 μm filter and 0.8 ml of sample was applied to a DEAE MemSep cartridge (Amicon UK) which had been equilibrated with 40 ml of 0.01 M phosphate buffer pH 5.8 (buffer A). Seven ml of equilibration buffer was used to remove loosely bound material. This was followed by 1 ml additions of 0.01 M phosphate buffer pH 7.0 containing increasing concentrations of NaCl to a final concentration of 1 M (buffer B). The eluant was collected in 1 ml fractions and assayed for enzyme activity and protein content.

Fractions containing high enzyme activity and lowest protein contamination were pooled and applied to an affinity column consisting of the carbohydrate resin p-aminobenzyl-1-thio-β-D-galactopyranoside linked to agarose. The resin was equilibrated with 30 ml of 0.05 M Tris-HCl pH 7.3, then rinsed with the same buffer to remove loosely bound extraneous material. The enzyme was eluted with the same buffer containing 1 M lactose. Again 1 ml fractions were collected and assayed for protein and enzyme activity.

Samples containing high levels of enzyme and low protein were pooled and concentrated using a Centricon 100 (Amicon, UK). The purified β-galactosidase was then characterized.

Determination of enzyme characteristics

Effect of inorganic ions on enzyme activity

The effect of different cations (2.5 mM) on hydrolytic activity was assessed in a standard assay mixture containing 4 mM pNP-Gal, pH 5.8 are 37 °C.

Determination of pH optimum

The optimum pH for hydrolysis of pNP-Gal was determined in a standard assay in the presence of a range of buffers (pH from 2.0–10.0) at 37 °C.

Determination of K_m and K_i

pNP-Gal (0.005 M–0.01 M) and lactose (0.005 M–0.3 M) in 0.1 M phosphate buffer (pH 5.8) were used as substrate and inhibitor respectively. The kinetic constants were calculated using the Lineweaver-Burk plot.

Hydrolysis of low molecular weight substrates

Enzyme samples were dialysed against 15 mM Tris-HCI pH 6.5 to remove traces of glucose. The hydrolytic activity of the enzyme was assessed using 50 mM *N*-acetyllactosamine (Gal β1-4 GlcNAc), 50 mM lacto-*N*-biose 1(Gal β1-3 GlcNAc) and 30 mM lactose (Gal β1-4 Glc) as substrates. The samples were incubated for 2 h at 37 °C, loaded onto Whatman No. 40 chromatography paper, developed overnight in ethyl actetate : pyridine : water (10 : 4 : 3, by vol) and stained with alkaline silver nitrate.

Determination of the presence of other glycosidase enzymes

The extent of hydrolysis of a range of p-nitrophenyl glycopyranosides, 2.0 mM, (refer to Table 2) was determined in the presence of 125 mM phosphate buffer (pH 5.8) at 37 °C.

Results

Enzyme purification

Only one species of β-galactosidase was identifiable. Typical elution profiles from ion exchange and affinity chromatography are shown in Figures 1 and 2. Table 1 summarizes the purification procedure. With this method, an overall yield of 3.6% was achieved, whilst the enzyme was purified 270-fold.

Effect of inorganic ions on enzyme activity

The purified β-galactosidase was activated ($\sim$ 10%) by the presence of manganese ions. Addition of other metal ions or EDTA decreased the activity of the enzyme.

Determination of pH optimum

The optimum pH for the hydrolysis of p-NP-Gal was found to be 5.8.

Kinetic constants

At pH 5.8 the K_m value for the purified enzyme was found to be 2.2 mM. The V_{max} was 12.0 μmol min^{-1}. In the presence of inhibitor the K_i was 9.0 mM and the V_{max} was 8.5 μmol min^{-1}.

Hydrolysis of different substrates

The enzyme hydrolysed lactose (Gal β1-4 Glc) with concomitant release of glucose and galactose. The enzyme also

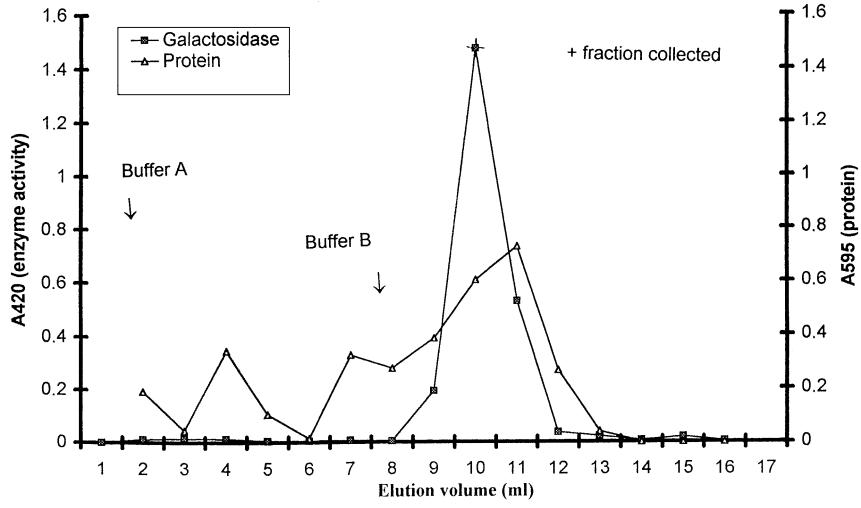

Figure 1. Elution of β-galactosidase from a DEAE Memsep cartridge.

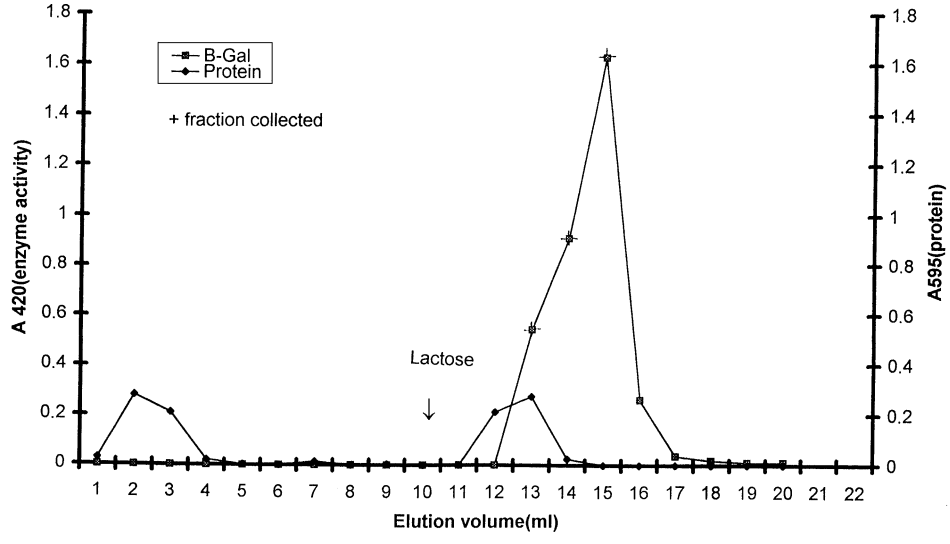

Figure 2. Elution of β-galactosidase from the affinity column.

Table 1. Summary of enzyme purification.

Preparation step	*Total volume (ml)*	*Protein content (mg ml^{-1})*	*Specific activity (μ mol min^{-1} mg^{-1})*	*Purification fold*
Crude filtrate	50	15	1.85	–
Ammonium sulphate fractionation 40–60%,	1	13	1.92	1
Ion exchange	2	< 0.5	37.8	20
Ligand affinity	1	< 0.1	> 500	> 270

hydrolysed the low molecular *N*-acetyllactosamine (Gal β1-4 GlcNAc).

Presence of other glycosidase enzymes in the purified enzyme extract is shown (Table 2).

Discussion

We have developed an inexpensive and straightforward method for extraction of β-galactosidase from *T. foetus*.

Table 2. Hydrolysis of nitrophenyl sugars by the enzyme eluted from the affinity column.

Pnp-substrate	*Concentration (mM)*	*Pnp released (μmol)*	*Enzyme activity (μmol min^{-1} ml^{-1} enzyme)*
α-galactopyranoside	2.0	3.5	1
α-glucopyranoside	2.0	0	0
α-mannopyranoside*	2.0	0	0
β-galactopyranoside	2.0	66	22
β-glucopyranoside	4.0	0	0
β-mannopyranoside	4.0	0	0
β-GlcNAcpyranoside	2.0	60	22
β-GalNAcpyranoside	2.0	1.5	0.5
β-xylopyranoside[a]	4.0	0.5	0.15

[a] Ortho-nitrophenyl substrate used

A high level of purification is attained which is satisfying considering the range of glycosidase enzymes present in the cell lysate prior to purification. After purification *β*-*N*-acetylglucosaminidase is the only enzyme present as a contaminant at a significant level. The method presented here can be modified fairly easily to isolate most glycosidase enzymes found in trichomonads.

Harrap and Watkins [9] identified two forms of the *β*-galactosidase enzyme using gel filtration chromatography on Sephadex G-200. One isoform of the enzyme, *β*-galactosidase 1, was activated by the cations Mn^{2+} and Co^{2+} and displayed a pH optimum of 5.8–6.0. The second enzyme had a broad pH optimum between 4.5–6.0 and was not activated by metal ions. The enzyme isolated in this study had a pH optimum of 5.8 and is probably that previously identified as *β*-galactosidase 1. *β*-galactosidase 2 was not identified in the eluate from the DEAE cartridge.

The K_m was determined at pH 5.8 and was found to be 2.2 mM. This corresponded to that found earlier by Harrap and Watkins [9] where the K_m value for *β*-galactosidase 1 was found to be 2.0 mM. The slight variation may be accounted for as the ortho-nitrophenyl substrate was used in the earlier study.

Interesting results were obtained when studies were carried out to determine the effect of various metal ions on enzyme activity. Of the metal ions used in this study only manganese ions were found to activate the enzyme and this corresponded to the results published earlier [9]. This seems to be a characteristic of trichomonad enzymes as *N*-acetyl-*β*-glucosaminidase, α-galactosidase and *N*-acetyl-α-galactosaminidase are also activated by manganese ions [9]. Unfortunately there is no information which describes the levels of manganese ions found in cow uteri. The strongest inhibition was recorded with Pb^{2+} and to a lesser extent by Zn^{2+}. The result with Pb^{2+} is not unexpected as the heavy metal is known to cause irreversible inhibition by binding to the amino-acid backbone of the enzyme. The result with zinc is interesting as semen is high in zinc and trichomonads are known to be apathogenic is semen.

The purified *β*-galactosidase was found to have the capacity to hydrolyse lactose (Gal *β*1-4 Glc). It also appeared to cleave the galactose from lacto-*N*-biose 1 (Gal *β*1-3 GlcNAc) and *N*-acetyllactosamine (Gal *β*1-4 GlcNAc). Finally, when the enzyme was applied to a non-denaturing polyacrylamide gel a single band was observed when stained with Coomassie brilliant blue. This band coincided with that obtained when the gel was stained with p-nitrophenyl *β*-galactopyranoside. When the same gel was incubated with p-nitrophenyl *N*-acetyl-*β*-glucosamine the bands seen did not coincide with that of *β*-galactosidase. On a denaturing gel, sharp bands with molecular weights of 200 000, 120 000, 75 000, approximately 70 000 and 40 000 were observed when stained with either Coomassie Brillant Blue or Silver stain. We are unable to determine which bands correspond to the enzyme on the denaturing polyacrylamide gel as there is no antibody which identifies the enzyme. Therefore, from the denaturing polyacrylamide gel alone it is difficult to estimate the native molecular weight of the enzyme.

Further work

Since the *β*-*N*-acetylglucosaminidase enzyme does not separate at the same position on a non-denaturing gel as the *β*-galactosidase, we will use this technique to isolate the latter enzyme and determine the N-terminal sequence as a prelude to cloning and further study of the gene. In addition we intend to carry out further studies to identify preferential hydrolytic capacity of the *β*-galactosidase enzyme using a technique such as capillary electrophoresis.

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