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Purification of α-galactosidase from *Aspergillus niger* for application in the synthesis of complex oligosaccharides

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Abstract

An α-galactosidase capable of α-galactosyl transfer on to a 4-position of sugar acceptors was isolated from *Aspergillus niger* and purified to homogeneity. The enzyme was characterised with respect to its pH and temperature optima and stability, molecular mass and p*I*. Inhibition by compounds relevant to a potential application of the enzyme for oligosaccharide synthesis was investigated.

Keywords: α-Galactosidase; *Aspergillus niger*; Enzymatic glycosylation; Oligosaccharide synthesis

1. Introduction

α-Galactosidases (α-D-galactoside galactohydrolase EC 3.2.1.22) are widely distributed in microorganisms, plants and animals [1]. These enzymes are of particular interest in view of their technological applications, e.g., treatment of molasses to remove raffinose, hydrolysis of galactooligosaccharides in legume based foods to improve their nutritional quality, modifications of guar-gum-based gels [2,3].

Recent advances in glycobiology have highlighted important roles the oligosaccharide epitopes play in living organisms [4]. The oligosaccharides of globo-series bearing terminal αGal(1 → 4)βGal-sequences illustrate a variety and significance of such interactions [5,6]. They act as preferential binding sites for a host of pathogens (*Streptococcus suis*, uropathogenic *E. coli*), toxins (*Shigella, E. coli*), and some viruses. Moreover, globo H antigen is associated with certain types of cancer [7]. This led to the notion of a role for α-galactooligosaccharides as potential inhibitors of microbial adhesion [8,9]. Globotriose residue covalently coupled to an inert support is undergoing Phase 3 clinical trial for the treatment of enteric infections [9]. A production of antibodies against human cancer cells was successfully achieved after immunisation of mice with fully synthetic globo H antigen [10].

Even though there is a large demand for globo-oligosaccharide derivatives, no methods for their industrial production have been reported. Chemical synthesis requires multiple protection and deprotection steps which render it unrealistic for industrial application [11]. Enzymatic synthesis using glycosyltransferases

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could circumvent the drawbacks of the chemical methods. However, the published procedure required cloning at least five different genes to establish also a production system for UDP-Gal, a substrate required by the enzyme [12].

The complexity of these approaches led us to investigate the possibility of utilising the ability of α-galactosidase, though essentially a hydrolytic enzyme in nature, to synthesise oligosaccharides from simple starting materials. However, no glycosidase performing an α-galactosyl transfer on to 4-position of a galactosyl acceptor has been reported so far. After an extensive screening and careful examination of the regioselectivity of the galactosyl transfer performed by individual enzymes we have identified an α-galactosidase from *Aspergillus niger*. We report the purification and characterisation of this novel biocatalyst.

2. Materials and methods

2.1. Source of enzyme and chemicals

A crude preparation of Transglucosidase L was kindly donated by the Amano Pharmaceutical, (Milton Keynes, UK). *p*-Nitrophenyl derivatives of various monosaccharides used as enzyme substrates were obtained from Sigma. Q-Sepharose (Pharmacia) and Macro-Prep ceramic hydroxyapatite (Bio-Rad) chromatography media were employed in protein purification. Relative molecular mass was estimated using precast gel slabs, buffer strips and protein standards on the PhastSystem (Pharmacia). A preparative isoelectric focusing was done with Rotofor instrument and RotoLyte buffer pH range 3.2–4.2 (Bio-Rad). All other chemicals were purchased from commercial sources as reagent grade.

2.2. Assay methods

The enzyme solution (10 μl) was added to McIlvain (phosphate–citrate) buffer (50 mM, 90 μl, pH 5.0) containing *p*-nitrophenyl glycoside (5 mM). The mixture was incubated for 10 min at 30°C. The assay solution was diluted with sodium carbonate solution (0.1 M, 3.9 ml) and the absorbance was read at 400 nm. The *p*-nitrophenolate concentration was determined from a standard curve. One unit of enzyme activity is defined as the quantity of enzyme that hydrolyses 1 μmol of *p*-nitrophenyl glycoside per minute under the conditions given above. The specific activity is expressed as units per mg of protein. Protein concentration was determined according to Bradford [13] with bovine serum albumin as standard.

2.3. General procedures

All enzyme preparations were handled at 4°C, unless otherwise stated in the text. Samples were centrifuged on a Sorvall RC-5B refrigerated superspeed centrifuge ($8500 \times g$, 20 min, 4°C). Proteins were concentrated by membrane ultra filtration (Centriprep, Amicon, 30 kDa cut off) on a Mistral 2000R refrigerated centrifuge ($3500 \times g$, repeated 15-min runs at 4°C). Samples of a small volume were concentrated in Minicon concentration cells (Amicon, 15 kDa cut off). Chromatography columns (XK series, Pharmacia) were packed with commercial chromatography media and elution buffers were supplemented with glycerol (10% w/V). A Waters 650E Advanced Protein Purification System (Millipore, USA) was employed for column development.

2.4. Purification of an α-galactosidase from A. niger

2.4.1. Ammonium sulfate precipitation

Transglucosidase L (50 ml) was diluted with an equal amount of deionised water, and proteins were precipitated by ammonium sulfate. The protein precipitate collected between 40 to 70% saturation was found to contain most of the

α-galactosidase activity. However, as the composition of a crude preparation is highly variable, the ammonium sulfate fractionation step had to be optimised for each individual batch.

2.4.2. Protein chromatography

The proteins were dialysed overnight against potassium phosphate buffer (20 mM, pH 6.5) and separated on a Q-Sepharose column ($L = 30$ cm, i.d. = 2.6 cm) equilibrated with potassium phosphate buffer (20 mM, pH 6.5). The column was washed with the starting buffer and the glycosidases were eluted by increasing salt concentration (0–0.27 M NaCl linear gradient). α-Galactosidase activity was detected in two separate peaks (fraction A and B, respectively). A trial transglycosylation reaction at this stage revealed that only α-galactosidase A yielded an α(1 → 4)-linked transfer product. A large amount of contaminating β-galactosidase activity still present in this preparation was efficiently removed on a hydroxyapatite column ($L = 33$ cm, i.d. = 1.6 cm) equilibrated with potassium phosphate buffer (5 mM; pH 6.5). The column was washed with the starting buffer and α-galactosidase was eluted by increasing phosphate buffer concentration (0.005–0.25 M linear gradient). Such α-galactosidase preparations were free of β-galactosidase, α- and β-mannosidase, α- and β-glucosidase and β-*N*-acetylhexosaminidase activities.

2.5. Relative molecular mass and isoelectric point

The relative molecular mass of the purified enzyme was determined by native and SDS polyacrylamide electrophoresis (PAGE) using precast gel slabs PhastGel Gradient 10–15 (continuous 10 to 15% gradient gel zone, 2% cross-linking) calibrated with protein standards. Preparative isoelectric focusing was carried out with the enzyme preparation from the hydroxyapatite column using RotoLyte buffer pH 3.2–4.2. The pH and enzyme activity of individual fractions were assayed.

2.6. pH dependence

The pH influence on the activity was studied using the pure enzyme. To 90 μl of 5 mM substrate solution in buffer (McIlvain 50 mM) of various pH was added 10 μl of enzyme solution. The enzyme activity assay was performed as described above. Stability at various pH levels was investigated by incubating the enzyme solution with McIlvain buffer (100 mM) of various pH at 30°C. The enzyme activity assay was performed with enzyme solution aliquots under standard conditions.

2.7. Temperature optimum and stability

The substrate solution (5 mM) in McIlvain buffer (50 mM, pH 5.0) was equilibrated at various temperatures, and the reaction was started by adding the enzyme. The aliquots were taken 3 and 5 min after the addition of the enzyme and assayed for the amount of liberated *p*-nitrophenol.

The stability of the enzyme was determined by incubating α-galactosidase in McIlvain buffer (50 mM, pH 5) at various temperatures. At preset time intervals, aliquots were taken and assayed for a residual activity. A sample kept at 30°C was assumed to maintain a 100% activity.

2.8. Inhibitory effect of various substances

A range of substances which might potentially interfere with the activity of the enzyme (e.g., various mono- and disaccharide derivatives, D-galactonic acid γ-lactone, *p*-nitrophenol) were tested for their inhibitory effect on α-galactosidase activity. The enzyme activity was assayed in McIlvain buffer (50 mM, pH 5.0) containing *p*-nitrophenyl α-D-galactopyranoside (5 mM) and the compounds tested at

Table 1
Purification of the α-galactosidase from *A. niger*

Sample	Protein [mg]	Units	α-Gal [U/mg prot]	β-Gal [U/mg prot]	Purif. factor	Recovery [%]
Crude extract	510	43	0.085	1.491	1	100
40–70% AS	248	42	0.169	1.483	2	97
Q-Sepharose	13.6	9.5	0.698	6.379	8	22
Hydroxyapatite	0.5	7.1	14.98	0	176	17

three different concentrations (10, 50, and 100 mM).

3. Results and discussion

As an alternative to traditional cultivation techniques [14–16] commercially available crude enzyme preparations continue to be a convenient source of α-galactosidases [17,18]. It should be noted, however, that the quality and levels of individual activities are highly variable which often precludes the design of a 'standard' purification procedure. We isolated α-galactosidase from Transglucosidase L (Amano) using a simple ammonium sulfate precipitation followed with a chromatography on Q-Sepharose and hydroxyapatite (Table 1). Similar separation techniques have been reported for other α-galactosidases from *A. niger*, albeit often incorporating more chromatographic steps, thereby introducing a complexity to the process and giving lower yields. Partitioning of glycosidases in aqueous two-phase systems could be used as an initial purification step [19].

Two proteins with α-galactosidase activity found in Transglucosidase L preparation separated well on Q-Sepharose column. Both enzymes accepted *p*-nitrophenyl α-D-galactopyranoside as a substrate but differed remarkably in their regioselectivity of α-galactosyl transfer [20]. Only one fraction, α-galactosidase A (a minor component of the total α-galactosidase activity), transferred an α-galactosyl residue on to the 4-position of a galactosyl acceptor, an ability crucial for the synthesis of globotriose-like structures.

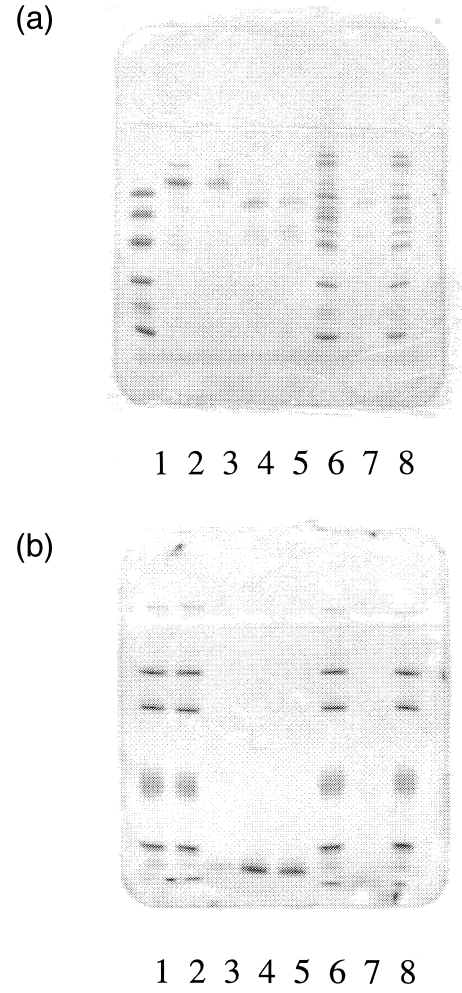

Fig. 1. Purified α-galactosidase from *A. niger*. A. SDS-PAGE, Coomassie blue stain. Molecular mass standards (94, 67, 43, 30, 20.1, 14.4 kDa)—line 1; crude Transglucosidase L—line 2; the α-galactosidase A after purification on Q-Sepharose—line 3; the α-galactosidase A purified on hydroxyapatite—line 4; the final preparation of α-galactosidase A after isoelectric focusing—line 5, 7; molecular mass standards (212, 170, 116, 94, 76, 67, 53, 43, 30, 20.1, 14.4 kDa)—lines 6, 8. B. Native-PAGE, Coomassie blue stain. Molecular mass standards (669, 440, 232, 140, 67 kDa)—lines 1, 2, 6, 8; purified enzyme from different batches—lines 3, 4, 5.

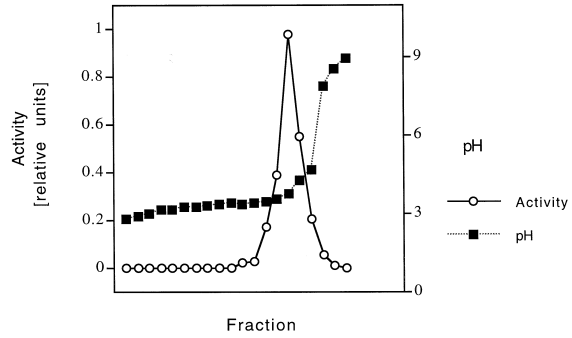

Fig. 2. Preparative isoelectric focusing of the purified α-galactosidase in a shallow gradient pH 3.2–4.2.

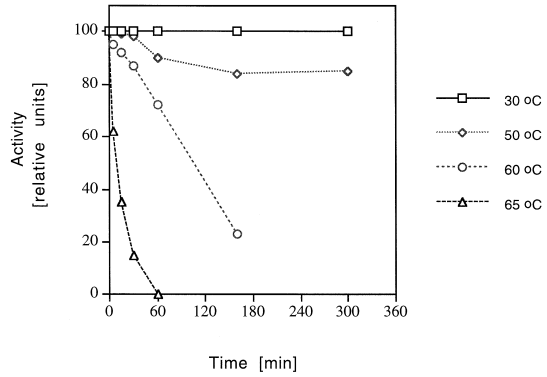

Fig. 4. Thermal stability of α-galactosidase from *A. niger*.

There are several reports in the literature on the occurrence of α-galactosidases from *A. niger* as multiple isoenzymes [15,16,18]. Variations in p*I* and molecular weight have been attributed to a differential glycosylation of the same protein molecule [16]. However, it is entirely possible that several different proteins are actually produced by the fungus that give rise to distinct patterns of substrate hydrolysis or glycosyl transfer specificity. A quite low agreement in the *N*-terminal amino acid sequence of two α-galactosidases from different strains of *A. niger* certainly support this notion [15,16].

The purified α-galactosidase A migrated as a single band on SDS- and native-PAGE (Fig. 1) with a relative molecular mass of 82 kDa. An activity stain performed with both *p*-nitrophenyl and 4-methylumbelliferyl α-D-galactopyranosides in the native gel corresponded to the same protein band as visualised with Coomassie blue stain. The enzyme preparation after chromatography on hydroxyapatite was subject to isoelectric focusing in a shallow pH gradient (Fig. 2) which confirmed the homogeneity of the preparation and its p*I* 3.73. The pH optimum of the α-galactosidase was detected between pH 4–4.5 (Fig. 3). The enzyme retained most of its activity when kept at pH 3.0–4.0. As expected, the activity of the enzyme was higher at elevated temperatures, i.e., increasing four times from 30°C to 60°C. The enzymatic activity was rapidly lost at 65°C. Overall, the α-galactosidase remained remarkably active at temperatures up to 50°C (Fig. 4).

Other known α-galactosidases from *A. niger* share very similar properties (Table 2). They are protein monomers with optima for the hydroly-

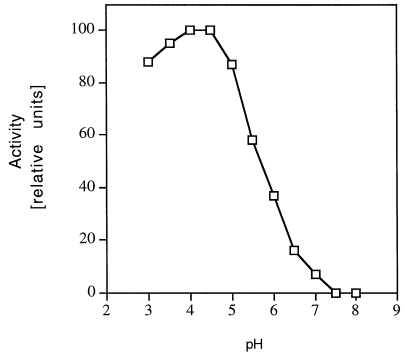

Fig. 3. pH optimum of α-galactosidase from *A. niger*.

Table 2
Comparison of some properties of various α-galactosidases from *A. niger*

Preparation	pH opt	p*I*	MW	Ref.
Transglucosidase L	4.0–4.5	3.73	82 000	
Rhozyme HP-150	3.8–4.2			[17]
Rhozyme HP-150	3.9–4.5	4.1–4.7		[18]
A. niger	4.0–4.5		45 000	[14]
A. niger N406		4.8	82 000	[15]
A niger N400	4.5	4.2–4.6	54 000[a]	[16]
A. niger ATCC-46890		4.2–6.6		[19]

[a]Relative molecular mass after deglycosylation with *N*-glycanase F.

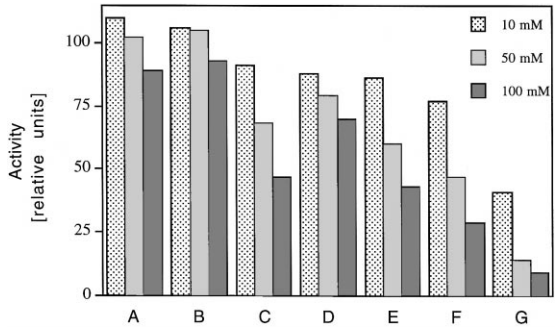

Fig. 5. Inhibitory effect of various saccharide derivatives on α-galactosidase from *A. niger*. Inhibitory effect of *N*-acetyl-galactosamine (A), methyl β-D-galactoside (B), methyl α-D-galactoside (C), D-galactonic acid γ-lactone (D), D-galactose (E), lactose (F) and mellibiose (G) at varied concentrations (10, 50, 100 mM) was investigated.

sis of *p*-nitrophenyl α-D-galactoside at pH 4.0–4.5. Reported p*I* values are slightly higher than observed with our enzyme.

Having in mind the practical use of our enzyme for the synthesis of oligosaccharides we tested several compounds for their potential inhibitory effect on α-galactosidase activity. Lactose, methyl α-D-galactoside and methyl β-D-galactoside were considered as possible galactosyl acceptors, and galactose and *p*-nitrophenol usually develop during the course of a transglycosylation reaction in high quantities. D-Galactonic acid γ-lactone was also tested as it is a good inhibitor of β-galactosidase activity which would allow the use a semi-purified preparation of α-galactosidase still contaminated with β-galactosidase activity in transglycosylation reactions. A strong inhibitory effect of some substances was truly surprising; more than 50% of α-galactosidase activity was lost in the presence of 100 mM lactose, galactose, and methyl α-D-galactoside. Mellibiose in 10 and 100 mM concentrations reduced the α-galactosidase activity to 41% and 9% of its original activity, respectively (Fig. 5). A similar inhibitory effect of mellibiose and galactose was observed with other α-galactosidases from *A. niger*.

4. Conclusion

A crude preparation from *A. niger* was identified as a source of α-galactosidase capable of a glycosyl transfer on to 4-position of saccharide acceptor. A two-step purification procedure resulted in a homogeneous enzyme preparation free of contaminating glycosidase activities. A thorough characterisation of the enzyme provides a base for its possible application in cost effective synthesis of complex oligosaccharides so much needed for advancing biological and medicinal research.

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