Chapter V
PURIFICATION AND CHARACTERIZATION OF MULTIPLE THERMOSTABLE $\alpha$-GALACTOSIDASES

5.1. INTRODUCTION

Purification and characterization of any enzyme are of paramount importance in revealing the novel biochemical and catalytic properties suitable for its excellent industrial application. The enzyme recovery process is considered to begin once the fermentation has achieved peak yield. In purification of enzymes there is often a dramatic reduction in the overall yield, which follows as a direct consequence of the number of steps involved in the recovery process. Generally it may be assumed that the higher the product specification then the more numerous and sophisticated are the stages involved in its preparation. Therefore, to achieve maximum yield and minimize production costs the recovery process must be capable of attaining the desired specification by the most direct route.

Purification and characterization of $\alpha$-galactosidases from thermophilic (Gote et al. 2006) and hyperthermophilic micro-organisms (Miller et al. 2001; King et al. 1998) have been reported recently. Production and characterization of $\alpha$-galactosidases from *Streptomyces olivaceus* (Suzuki et al. 1966) and *S. erythrus* (Elshafei et al. 2001) are reported. Reports are available in the literature documenting cloning and expression of the gene encoding $\alpha$-galactosidase in *S. coelicolor* A3(2) (Kondoh et al. 2005). Crystal structure of $\alpha$-galactosidases from *Trichoderma reesei* (Golubev et al. 2004) and rice (Fujimoto et al. 2003) has been elucidated. Competitive inhibition by galactose is a common characteristic of most $\alpha$-galactosidases (Ademark et al. 2001; Gote et al. 2004). This chapter describes the purification and characterization of multiple $\alpha$-galactosidases from *S. griseoalbus* and the presence of a novel galactose-tolerant $\alpha$-galactosidase.
5.2. MATERIALS AND METHODS

5.2.1. Production and extraction of α-galactosidases

α-Galactosidase production by S. griseoflavus was carried out under solid-state fermentation conditions as described in Chapter IV. Enzyme extraction was carried out by mixing the fermented matter with 50 mM sodium phosphate buffer (pH 7.0; 1:5, w/v) on a rotary shaker at 200 rev/min for 1 h. The thoroughly agitated fermented matter was then filtered through muslin cloth and the filtrate obtained was centrifuged at 10,000 rpm for 20 min. The resultant supernatant was used as the enzyme preparation for further purification.

5.2.2. Purification of α-galactosidases

The steps involved in the purification of α-galactosidases are shown as a flow chart in Fig.5.1. The crude enzyme preparation was concentrated five times at 4 °C by an Amicon Ultrafiltration unit using a 10 kDa cut-off membrane and was then applied to Phenyl Sepharose CL 4B hydrophobic interaction chromatographic column (10 cm x 2.8 cm) previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 1.25 M ammonium sulphate. The enzyme was eluted at a flow rate of 0.5 mL/min with 50 mM sodium phosphate buffer (pH 7.0) with a linear decreasing gradient of ammonium sulphate (1.25-0 M). The fractions collected were screened for protein content, α-galactosidase activity and conductivity. The α-galactosidase active fractions were pooled, concentrated by ultrafiltration as described above and desalted by dialyzing against 50 mM sodium phosphate buffer (pH 7.0). The resultant enzyme sample was then applied to Sephadex G-100 (30 cm x 3 cm) previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The proteins were eluted with the same buffer at a flow rate of 0.5 mL/min. Three peaks with α-galactosidase activity were pooled separately, concentrated by ultrafiltration and stored at 4°C until further use.
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Crude enzyme extract

- 10000 rpm, 20 min, 4 °C

Ultra-filtration (Amicon ultra-filtration unit, 10 kDa cut off membrane)

Phenyl Sepharose CL 4B
(Hydrophobic interaction chromatographic column, 10 x 2.8 cm)

- Reverse gradient of (NH₄)₂SO₄ (1.25 - 0 M) in 50 mM sodium phosphate buffer pH 7.0, Flow rate: 0.5 mL/min

Sephadex G100 (Gel filtration chromatographic column, 30 x 3 cm)

- 50 mM sodium phosphate buffer pH 7.0, Flow rate: 0.5 mL/min

Concentration by ultra-filtration

Fig. 5.1. The steps involved in the purification of α-galactosidases from S. griseoloabrus. The curved arrows indicate the elution of proteins.

5.2.3. α-Galactosidase assay

The activity of α-galactosidase was routinely determined using p-nitrophenyl-α-D-galactopyranoside (pNPG) as reported earlier in Section 2.2.4 of Chapter II. When oligosaccharides like raffinose and stachyose and polysaccharides like locust bean gum and guar gum were used as substrates, the amount of galactose liberated was
estimated as reducing sugar by the method of Nelson (1944). When melibiose was used as substrate, the amount of galactose liberated was determined by galactose oxidase method using the galactose assay kit (Biovision Research Products, USA) following the manufacturer’s directions. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated one µmol of the product (p-nitrophenol or galactose) per min under the assay conditions.

5.2.4. Protein estimation

The total soluble protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. All chromatographic fractions were monitored for protein by absorbance at 280 nm.

5.2.5. Relative molecular mass (Mr) and zymogram analysis

The native Mr of the purified α-galactosidases was determined by native PAGE (10%, w/v) in a vertical slab gel apparatus. For calibration of the gel standard molecular mass markers (catalase, 240 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; trypsin soybean inhibitor, 20.1 kDa and lactoglobulin, 18.4 kDa; Genei™, Bangalore) were also electrophoresed along with the purified α-galactosidases. The half of the gel was stained with Coomassie Brilliant Blue R-250 (0.25%, w/v) and destained with methanol: acetic acid: water (1:1:8, v/v) to visualize the protein bands. For zymogram analysis, α-galactosidase activity in the other half of the gel was visualized as fluorescent band under UV transilluminator after incubating the gel at 55 °C for 10 min in 0.1 M citrate phosphate buffer (pH 7.0) containing the fluorescent substrate, 4-methyl-umbeliferyl-α-D-galactopyranoside (MU-α-gal, 1 mM).

The Mr of the subunits of purified α-galactosidases was calculated based on the relative mobility of standard molecular mass markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; soyabean trypsin inhibitor, 20.1 kDa and lysozyme, 14.3 kDa; Genei™, Bangalore) in SDS PAGE (12%, w/v), which was done in a vertical slab gel apparatus according to
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the method of Laemmli (1970). The protein bands were visualized by silver staining (0.2%, w/v) (http://www.proteinchemist.com/tutorial/silverst.html).

The relative mobility of molecular mass markers and purified α-galactosidases in native PAGE and SDS PAGE was calculated according to the equation given below.

\[
\text{Relative mobility} = \frac{\text{Distance of protein migration} \times \text{Length of gel before staining}}{\text{Distance of dye migration} \times \text{Length of gel after staining}} \quad \text{Eq. (1)}
\]

The molecular mass of the α-galactosidase was determined by interpolation from a linear semi logarithmic plot of log molecular mass of standard markers versus the relative mobility.

5.2.6. N-terminal sequencing

The region of gel corresponding to the main α-galactosidase protein band was cut from an unstained SDS PAGE gel and thoroughly mixed with 1 ml Tris buffer (pH 7.6). It was then kept overnight at -20 °C, centrifuged and the supernatant containing dissolved protein was dialyzed (12 h) against distilled water which was frequently replaced with fresh distilled water to ensure complete removal of Tris. The first 10 amino acid residues at the N-terminus of the enzyme were sequenced using Shimadzu Protein Sequencer at Rajiv Gandhi Centre for Biotechnology, Trivandrum, India and its sequence homology with α-galactosidases from other sources was determined using the BLASTP search of the Non-redundant Peptide Sequence Database at the National Centre for Biotechnology Information via the World Wide Web Interface (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

5.2.7. Glycoprotein analysis

The total carbohydrate content of purified α-galactosidases was determined by the method described by Dubois et al. (1956).
5.2.8. Isoelectric focusing

Isoelectric pH of the purified α-galactosidases was determined using rotofor (Biorad) according to manufacturer's instructions. The ampholyte used was in the pH range of 3.0-10.0. Focusing was carried out at a constant power of 12 W for 3 hrs. The pH and enzyme activity of individual fractions were assayed.

5.2.9. Effect of pH and temperature on enzyme activity and stability

The optimum pH for α-galactosidase activity was determined in the assay mixture over a pH range of 3.0-9.0, using different buffers (0.1 M) such as citrate phosphate buffer (pH 3.0-7.0), sodium phosphate buffer (pH 6.0-8.0) and Tris-HCl buffer (pH 7.5-9.0). For pH stability determination, enzyme preparations were incubated at pH 3.0-9.0 for 2 h at optimum temperature and residual activity was determined at regular time intervals of 30 min.

The optimum temperature for enzyme activity was determined by incubating the assay mixture at 40-75 °C. Thermostability of α-galactosidases was determined by studying the time-dependent thermal inactivation of enzyme at temperatures in the range of 50-75 °C for 2 h at optimum pH.

5.2.10. Substrate specificities and kinetic parameters

The relative substrate specificities of the three α-galactosidases towards various synthetic and natural substrates were determined under standard assay conditions. For kinetic studies the initial rate of hydrolysis of various glycosides at different concentrations (0.5-5 mM) was measured under standard assay conditions and the kinetic constants $K_m$ and $V_{max}$ were determined from Lineweaver-Burk plot using the Enzyme Kinetics module of Sigmaplot (Systat software Inc. Version 1.2.0.0).

5.2.11. Hydrolysis of polymeric galactomannans

The polymeric galactomannan, locust bean gum (0.1%; w/v) was incubated with 0.2 U of each α-galactosidase at its optimum pH and temperature for 2 h. Aliquots (100 µL) were taken at regular time intervals of 1 h, boiled to stop the reaction and 5 µL was applied on silica gel G plates (20 x 20 cm) and developed by ascending thin layer
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chromatography using n-propanol: ethyl acetate: water (6:1:3, v/v) as the solvent system (Tanaka et al. 1975). The hydrolysis products were located by keeping the plates in an oven at 140 °C for 5 min after spraying with 1% α-naphthol in absolute ethanol containing 10% of ortho-phosphoric acid (Albon & Gross 1950).

5.2.12. Stereochemical analysis of hydrolysis products by ¹H NMR

The stereochemistry of hydrolysis by α-galactosidases was determined by ¹H NMR spectroscopy using pNPG as substrate. Prior to analysis by NMR, the reaction mixture was concentrated to dryness using Eppendorf concentrator 5301 and then dissolved in ²H₂O. The ¹H NMR spectra of the hydrolysis products were recorded using Bruker Avance DPX Spectrometer operating at 300 MHz. The anomeric resonances of α- and β-anomers were determined relative to the chemical shift δ of HO²H at 4.79 ppm.

5.2.13. Effect of various metal ions, sugars and inhibitor reagents

The enzyme was incubated with various metal ions, sugars and inhibitor reagents at room temperature for 10 min and enzyme assay was carried out under standard conditions. Relative activity was calculated as the percentage of activity obtained in the absence of these chemicals.

5.2.14. Kinetics of enzyme inhibition

To determine the type of inhibition, kinetic constants Kᵣ and Vₘₐₓ of hydrolysis of pNPG was determined under standard assay conditions after incubating the enzyme in the absence and presence of different concentrations of inhibitor (10-100 mM). For the determination of inhibition constant (Kᵢ) for competitive inhibitors enzyme assay was carried out under standard assay conditions at two different substrate concentrations after incubating the enzyme in different concentrations of inhibitor (10-100 mM). The Kᵢ was determined by Dixon method using the Enzyme Kinetics module of Sigmaplot (Systat software Inc. Version 1.2.0.0).
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5.2.15. Galactose tolerance studies

The galactose tolerance of the enzyme was investigated by determining relative activity under standard assay conditions after incubating the enzyme with different concentrations of galactose (10-100 mM) for 30 min at room temperature.

5.2.16. Effect of amino acid group specific reagents

The residual activity of enzyme samples was determined by standard assay method after incubating (30 min at room temperature) the purified α-galactosidases with chemical reagents (N-ethyl maleimide (NEM), p-chloromercuribenzoate (PCMB), 2,2-dithiobisnitrobenzoic acid (DTNB), diethylpyrocarbonate (DEPC), N-acetylimidazole (NAI), 2,4,6-trinitrobenzenesulfonic acid (TNBS), N-bromosuccinimide (NBS), phenylmethyl-sulfonylfluoride (PMSF), 2,3-butanedione, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and Woodward's reagent K (WRK); Sigma) specific to various amino acid functional groups at specific concentration.

5.2.17. Statistical analysis

The data presented here are the average of triplicate determinations and the standard deviation for all the values were <± 5%.

5.3. RESULTS AND DISCUSSION

5.3.1. Purification and biochemical properties of α-galactosidases

The crude enzyme extract obtained after solid-state fermentation was subjected to ultra-filtration, hydrophobic interaction chromatography and gel filtration chromatography. Preliminary zymogram analysis of the crude enzyme extract with MU-α-gal indicated the presence of three proteins with α-galactosidase activity (Fig. 5.2). But all the three α-galactosidases co-eluted during hydrophobic interaction chromatography (Fig. 5.3a) with a reverse gradient of ammonium sulphate (0.8-0.45 M) in 50 mM sodium phosphate buffer (pH 7.0). Complete purification of the three α-galactosidases was achieved by gel filtration chromatography in which they were resolved into three peaks (Fig. 5.3b). The three purified α-galactosidases were
designated α-Gal I, α-Gal II and α-Gal III. α-Gal I which showed the highest enzyme activity and specific activity was identified as the main α-galactosidase. The summary of purification steps is shown in Table 5.1.

![Image of native PAGE and zymogram analysis](image)

Fig. 5.2. (A) Native PAGE and (B) zymogram analysis for α-galactosidase activity of the crude enzyme extract from *S. griseololabus*. α-Galactosidase activity was visualized by incubating gel at 55 °C for 10 min in 0.1 M citrate phosphate buffer (pH 7.0) containing fluorescent substrate, MU-α-gal (1 mM)

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<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification fold</th>
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<td>2</td>
<td>176.9</td>
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<td>61.8</td>
<td>17.7</td>
<td>79.2</td>
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Table 5.1. Purification of α-galactosidases from *S. griseololabus*
Fig. 5.3. Elution profile of proteins (---) and α-galactosidases (-----) in (a) hydrophobic interaction chromatography with Phenyl Sepharose CL 4B and (b) gel filtration chromatography with Sephadex G 100.
\(\alpha\)-Gal I, \(\alpha\)-Gal II and \(\alpha\)-Gal III were purified to homogeneity with gel filtration chromatography as indicated by the single protein bands obtained with each sample in native PAGE, zymogram and SDS PAGE (Fig. 5.4a, b, c). The native \(M_r\) determined by native PAGE (Fig. 5.4a) showed a molecular mass of 141 kDa for \(\alpha\)-Gal I, 113 kDa for \(\alpha\)-Gal II and 89 kDa for \(\alpha\)-Gal III. The zymogram analysis with MU-\(\alpha\)-gal (Fig. 5.4b) confirmed the \(\alpha\)-galactosidase activity of the three purified proteins. The \(M_r\) determined by SDS PAGE (Fig. 5.4c) showed a molecular mass of 72 kDa, 57 kDa and 35 kDa respectively for \(\alpha\)-Gal I, \(\alpha\)-Gal II and \(\alpha\)-Gal III. Thus it could be possible that \textit{S. griseolaloabus} \(\alpha\)-galactosidases are dimeric proteins with monomers of approximately same molecular masses.

![Fig. 5.4. Electrophoretic patterns of \textit{S. griseolaloabus} \(\alpha\)-galactosidases in (a) Native PAGE after each chromatographic step of purification, Lane 1: Fraction from hydrophobic interaction chromatography, Lanes 2, 3 and 4: \(\alpha\)-Gal I, \(\alpha\)-Gal II and \(\alpha\)-Gal III from gel filtration chromatography (b) Zymogram analysis of (Lane 1) \(\alpha\)-Gal I, (Lane 2) \(\alpha\)-Gal II and (Lane 3) \(\alpha\)-Gal III from gel filtration chromatography (c) SDS PAGE of the fractions from gel filtration chromatography, Lane 1: Molecular mass markers, Lanes 2, 3 and 4: \(\alpha\)-Gal I, \(\alpha\)-Gal II and \(\alpha\)-Gal III respectively.](image-url)
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The N-terminal sequence of first 10 amino acid residues of the purified α-Gal I, the main α-galactosidase active protein, was MAHLPMRAPR. The BLASTP search of the Non-redundant Peptide Sequence Database using the confirmed 10 N-terminal amino acid sequence of α-Gal I showed that it was 80% homologous (Fig. 5.5) to the N-terminus of α-galactosidase from S. coelicolor A3(2), accession number CAB54169 (Bentley et al. 2002) belonging to family 27 of glycosyl hydrolases (GH) (http://www.cazy.org/fam/GH27.html). This sequence similarity suggested that α-Gal I from S. griseoaloalbus could also be classified as a member of GH27 family.

> ref_NM_619611... G probable secreted alpha-galactosidase [Streptomyces coelicolor A3(2)]
> esa_00001265... G probable secreted alpha-galactosidase [Streptomyces coelicolor A3(2)]
Length=692

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<th>Expect</th>
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<td>5/10 (50%)</td>
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<td>Gaps</td>
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</table>

Query 1 MAHLPMRAPR 10
Subject 1 MAHLPMRAFR 10

Fig. 5.5. BLASTP search result for the confirmed 10 N-terminal amino acid sequence of S. griseoaloalbus α-Gal I

The total carbohydrate contents of the three α-galactosidases were 15.3%, 19.1% and 8% respectively. The pI of α-Gal I, α-Gal II and α-Gal III were 4.41, 5.6 and 6.13 respectively indicating that they are acidic in nature. The isoelectric point of the α-galactosidases reported here are in well agreement with the values reported for other α-galactosidases (Hashimoto et al. 1993; Puchart et al. 2000; Gote et al. 2006).

5.3.2. Effect of pH and temperature on enzyme activity and stability

The optimum pH for highest enzyme activity was 5.0, 6.5 and 5.5 respectively for α-Gal I, α-Gal II and α-Gal III (Fig. 5.6a). All the three enzymes showed more activity towards acidic range than towards alkaline range where only negligible activity was detected. α-Gal I was active over a wider range of pH (3.0-9.0) than α-Gal II (pH 4.0-
8.0) and α-Gal III (pH 4.0-9.0). The pH stability studies also gave similar results (Fig. 5.6b), with α-Gal I having stability over a wider pH range (5.0 to 7.0) for 2 h than α-Gal II and α-Gal III which remained stable only in a narrow range of pH (5.5-7.0). Moreover, α-Gal I could retain more than 85% of its original activity at pH 4.0 and 8.0 after 2 h. Most bacterial α-galactosidases show narrow pH optima and are stable over an alkaline pH range (Gote et al. 2004). S. coelicolor A3(2) family 36 α-galactosidase is reported to be most active at pH 7.0 and is stable between pH 7.0 and 9.5 for 1 h (Kondoh et al. 2005). S. griseoloalbus α-Gal I is unusual in this respect with broad pH activity and stability profile. This is an appreciable character for diverse industrial applications since it eliminates the need for pH adjustment for enzyme action.

The optimum temperature for maximum activity of α-Gal I, α-Gal II and α-Gal III were 65, 50 and 55 °C respectively (Fig. 5.7a). Thermostability of α-galactosidases was determined by studying the time-dependent thermal inactivation of enzymes as shown in Fig 5.6b, c and d. α-Gal I was completely stable at 50-65 °C for 2 h. The half-life of
inactivation \( t_{1/2} \) of \( \alpha \)-Gal I at 70 °C was 30 min. \( \alpha \)-Gal II and \( \alpha \)-Gal III behaved similarly with respect to thermal stability. Both \( \alpha \)-galactosidases were completely stable up to 55 °C for 2 h and the \( t_{1/2} \) at 60 °C was 30 min. \( S. \) coelicolor A3(2) family 36 \( \alpha \)-galactosidase is reported to be most active at 40 °C and stable only up to 30 °C for 1 h (Kondoh et al. 2005).

Fig. 5.7. (a) Effect of temperature on activity of purified \( \alpha \)-galactosidases. The enzyme assay was carried out at different temperature (40-75 °C) and optimum pH and the relative activity was determined. (b) Time-dependent thermal inactivation of \( \alpha \)-Gal I at 50-75 °C, (c) \( \alpha \)-Gal II and (d) \( \alpha \)-Gal III at 50-65 °C. The enzyme was incubated at the above mentioned temperatures and optimum pH for 2 h and the residual activity was determined at regular time intervals of 30 min.
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The thermostability of the enzyme is advantageous for industrial application, since most of the industrial processes are carried out at high temperatures which lead to denaturation of thermolabile enzymes. \( \alpha \)-Galactosidase of hyperthermophilic bacteria *Thermotoga maritima* (t\(_{1/2} 6\) h at 85 °C) (Miller et al. 2001) and *Thermotoga neapolitana* (75% activity after 4 h at 85 °C) (King et al. 1998) are reported to have activity and prolonged stability above 75 °C.

5.3.3. Substrate specificities and kinetic parameters

Table 5.2 summarizes the relative substrate specificities of the three \( \alpha \)-galactosidases and the kinetics of hydrolysis of the different synthetic and natural \( \alpha \)-galactosides. The Lineweaver-Burk plots for the hydrolysis of various synthetic and natural \( \alpha \)-galactosides by \( \alpha \)-Gal I, \( \alpha \)-Gal II and \( \alpha \)-Gal III are shown respectively in Figs. 5.8, 5.9 and 5.10. The best substrate for all the three \( \alpha \)-galactosidases appeared to be the aryl glycoside pNPG for which the enzymes showed lowest \( K_m \) values and highest \( V_{\text{max}} \). The \( K_m \) values are not absolute constants but depend on the temperature, substrate and source of enzyme. Most microbial \( \alpha \)-galactosidases have in common the fact that they can hydrolyze the synthetic substrates more extensively than the natural \( \alpha \)-galactosides (Varbanets et al. 2001). None of the \( \alpha \)-galactosidases was active on mNPG which could be due to steric hindrance.

Table 5.2. Kinetics of hydrolysis of different synthetic and natural \( \alpha \)-galactosides by purified \( \alpha \)-galactosidases from *S. griseoloalbus*

<table>
<thead>
<tr>
<th>Substrates</th>
<th>( K_m ) (mM)</th>
<th>( V_{\text{max}} ) (( \mu )mol min(^{-1}) mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \alpha )-Gal I</td>
<td>( \alpha )-Gal II</td>
</tr>
<tr>
<td>( p )-nitrophenyl-( \alpha )-D-galactopyranoside</td>
<td>0.79</td>
<td>1</td>
</tr>
<tr>
<td>( \alpha )-nitrophenyl-( \alpha )-D-galactopyranoside</td>
<td>0.96</td>
<td>1.9</td>
</tr>
<tr>
<td>Melibiose</td>
<td>3.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Raffinose</td>
<td>5.8</td>
<td>3.7</td>
</tr>
<tr>
<td>Stachyose</td>
<td>6</td>
<td>2.5</td>
</tr>
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</table>

The *S. griseoloalbus* \( \alpha \)-galactosidases were also active on naturally occurring \( \alpha \)-1-6 linked galactooligosaccharides like melibiose, raffinose and stachyose. The
specificities for the hydrolysis of these galactosides were in the order melibiose > raffinose > stachyose for α-Gal I (Table 5.2 and Figs. 5.8c, d, e). For α-Gal II (Table 5.2 and Figs. 5.9c, d, e) and α-Gal III (Table 5.2 and Figs. 5.10c, d, e) the substrate specificities were in the order stachyose > melibiose > raffinose.

Fig. 5.8. Lineweaver-Burk plots for the hydrolysis of (a) pNPG, (b) oNPG, (c) melibiose, (d) raffinose and (e) stachyose by α-Gal I
Fig. 5.9. Lineweaver-Burk plots for the hydrolysis of (a) pNPG, (b) oNPG, (c) melibiose, (d) raffinose and (e) stachyose by α-Gal II
Fig. 5.10. Lineweaver-Burk plots for the hydrolysis of (a) pNPG, (b) oNPG, (c) mellibiose, (d) raffinose and (e) stachyose by α-Gal III
S. griseoloalbus \(\alpha\)-galactosidases showed remarkable differences from S. coelicolor A3(2) family 36 \(\alpha\)-galactosidase with respect to substrate preference also. S. coelicolor A3(2) family 36 \(\alpha\)-galactosidase is reported to hydrolyze raffinose and stachyose, but not melibiose (Kondoh et al. 2005). On the contrary, S. griseoloalbus \(\alpha\)-galactosidases were also active on melibiose.

5.3.4. Hydrolysis of polymeric galactomannans

The hydrolysis of LBG as visually assayed by TLC (Fig. 5.11a) indicated that S. griseoloalbus \(\alpha\)-Gal I was active on polymeric galactomannans. The other two \(\alpha\)-galactosidases were not active on polymeric galactomannans (Fig. 5.11b).

\[\text{Fig. 5.11 (a) Thin layer chromatograph showing the galactose released from LBG by the hydrolytic action of S. griseoloalbus }\]
\[\alpha\text{-Gal I at regular time intervals of 1 h. Gal: Authentic galactose. (b) Thin layer chromatograph showing that }\]
\[\alpha\text{-Gal II and }\alpha\text{-Gal III are not active on polymeric galactomannans, as there was no release of galactose.}\]

\(\alpha\)-Galactosidases can be classified into two groups on the basis of their hydrolytic properties (Dey & Pridham 1972) - one group specific for low molecular weight \(\alpha\)-galactosides such as alkyl- and aryl galactosides, melibiose and the raffinose
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family oligosaccharides, and the other type of enzymes specific for polymeric galactomannans, however they are also able to hydrolyze low molecular weight substrates to various extents. In general bacterial and fungal α-galactosidases of GH27 family are active on oligosaccharides and intact polymeric galactomannans, whereas α-galactosidases of GH36 family are more specific towards oligosaccharides and have little or no activity on polymeric substrates. The results obtained in the present investigation were confirmative of the fact that \textit{S. griseoloalbus} α-Gal I belonged to GH27 family. Since the other two α-galactosidases, α-Gal II and α-Gal III, were not active on polymeric galactomannans, they could be members of GH36 family. There are reports available in the literature documenting the presence of both types of α-galactosidases from the same organism, for e.g. \textit{Trichoderma reesei} (Margolles-Clark et al. 1996) and \textit{A. niger} (Ademark et al. 2001).

5.3.5. Stereochemical analysis of hydrolysis products by $^1H$ NMR

The $^1H$ NMR spectrum of pNPG hydrolysis by α-Gal I, α-Gal II and α-Gal III (Fig. 5.12) clearly suggested a retentive, double displacement mechanism of hydrolysis for α-Gal I, α-Gal II and α-Gal III, though they belonged to two different families of glycosyl hydrolases. The α-anomer of β-galactose was formed at the initial stages of hydrolysis followed by its slow mutarotation to β-anomer at later stages. Recently Comfort et al. (2007) reported the mechanistic commonality of GH27 and GH36 α-galactosidases. The double displacement mechanism involves two catalytic residues, one responsible for the protonation of the glycosidic oxygen and the other stabilization of a carbocationic intermediate. The glycosyl-enzyme intermediate is decomposed by transfer of glycosyl moiety to an acceptor molecule, which in case of hydrolysis is water, but hydroxyl groups of sugar may also act as the glycosyl acceptor, leading to transglycosylation (Henrissat 1991).
5.3.6. Effect of metal ions, sugars and inhibitor reagents

The effects of various metal ions, sugars and inhibitors on *S. griseoalbus* α-galactosidases are summarized in Table 5.3. Among the metal ions tested Hg²⁺, Cu²⁺ and Ag⁺ completely inhibited all the three α-galactosidases. Similar results were reported previously for other thermostable α-galactosidases from *Thermus brockianus* (Fridjonsson et al. 1999) and *Baciullus stearothermophilus* (Gote et al. 2006). This inhibition usually suggests reaction with thiol groups and/or carboxyl, amino and imidazolium group of histidine in the active site (Dey & Pridham 1972).

Other metal cations like Na⁺, K⁺, Ca²⁺, Co²⁺, Mn²⁺ and Fe³⁺ did not influence the activity of *S. griseoalbus* α-galactosidases. Mg²⁺ did significantly stimulate enzyme activity of α-Gal I. However, the metal chelating agent, EDTA did not inhibit the activity of *S. griseoalbus* α-galactosidases, indicating that they are not metalloenzymes. These results suggested that metal cations are not involved in the catalytic site of the enzyme and activation by the specific cation Mg²⁺ could be due to its possible role in modulating enzyme activity according to environmental conditions. Mg²⁺ is known to...
Purification and Characterization of $\alpha$-Galactosidases

play a role in activating $\alpha$-galactosidase from *Thermomyces lanuginosus* (Rezessy-Szabó et al. 2007).

Table 5.3. Effect of metal ions, sugars and inhibitor reagents on the activity of purified $\alpha$-galactosidases from *S. griseolusibus*

<table>
<thead>
<tr>
<th>Cation/Sugar/Inhibitor</th>
<th>Relative activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$\alpha$-Gal I</th>
<th>$\alpha$-Gal II</th>
<th>$\alpha$-Gal III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>100</td>
<td>97.1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>124</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>98.2</td>
<td>95.7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ag&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>97.8</td>
<td>99.5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Co&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>99.9</td>
<td>98.6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>98.6</td>
<td>99.7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>99.4</td>
<td>98.2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SDS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$\beta$-mercaptoethanol</td>
<td>96.9</td>
<td>99.4</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>100</td>
<td>74.9</td>
<td>71.3</td>
<td></td>
</tr>
<tr>
<td>Melibiose</td>
<td>76.3</td>
<td>60.2</td>
<td>77.8</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The concentration of cations and $\beta$-mercaptoethanol were 1 mM, EDTA 10 mM, urea 1 M and sugars 10 mM.

<sup>b</sup> Relative activity was calculated as the percentage of activity obtained in the absence of cations/sugars/inhibitors.

Among the various sugars tested melibiose was found to be inhibitory to all the three $\alpha$-galactosidases when pNPG was used as the assay substrate. $\alpha$-Gal I was not inhibited by galactose whereas the other two enzymes were inhibited by galactose.

5.3.7. Kinetics of enzyme inhibition

Melibiose and galactose (whenever inhibitory) acted as competitive inhibitors since the $K_m$ for pNPG increased and $V_{max}$ remained constant (Fig. 5.13 and 1.14). The
inhibition constant, \( K_i \) of galactose for \( \alpha\)-Gal II and \( \alpha\)-Gal III as determined from Dixon plot (Fig. 5.15) were 23.4 and 13.1 respectively. The \( K_i \) of melibiose (Fig. 5.16) were 13.3, 12.8 and 14.9 respectively for \( \alpha\)-Gal I, \( \alpha\)-Gal II and \( \alpha\)-Gal III.

Fig. 5.13. Lineweaver-Burk plots showing competitive inhibition by galactose on (a) \( \alpha\)-Gal II and (b) \( \alpha\)-Gal III. To determine the type of inhibition, kinetic constants \( K_m \) and \( V_{max} \) of hydrolysis of \( p\)NPG was determined under standard assay conditions after incubating the enzyme in the absence and presence of different concentrations (10-100 mM) of galactose.

Fig. 5.14. Lineweaver-Burk plots showing competitive inhibition by melibiose on (a) \( \alpha\)-Gal I, (b) \( \alpha\)-Gal II and (c) \( \alpha\)-Gal III. To determine the type of inhibition, kinetic constants \( K_m \) and \( V_{max} \) of hydrolysis of \( p\)NPG was determined under standard assay conditions after incubating the enzyme in the absence and presence of different concentrations (10-100 mM) of melibiose.
Fig. 5.15. Dixon plots for the inhibition of (a) α-Gal II and (b) α-Gal III by galactose. α-Galactosidase activity was determined under standard assay conditions at two different concentrations of pNPG after incubating the enzyme for 10 min with different concentrations of galactose (10-100 mM).

Fig. 5.16. Dixon plots for the inhibition of (a) α-Gal I, (b) α-Gal II and (c) α-Gal III by melibiose. α-Galactosidase activity was determined under standard assay conditions at two different concentrations of pNPG (S2 > S1) after incubating the enzyme for 10 min with different concentrations of melibiose (10-100 mM).
5.3.8. Galactose tolerance studies

It was interesting to find that α-Gal I was tolerant to even concentrations of galactose as high as 100 mM. The relative activity was 100% and the kinetic constants $K_m$ (0.79 mM) and $V_{max}$ (693.4 μmol min$^{-1}$ mg$^{-1}$) of hydrolysis of pNPG remained constant despite the increase in galactose concentration. Galactose is reported to be a powerful competitive inhibitor of α-galactosidases from *Aspergillus niger* (Ademark et al. 2001), *Bacillus stearothermophilus* (Gote et al. 2006). But Suzuki et al. (1970) reported a mixed type of inhibition by β-galactose on α-galactosidase from *Mortierella vinacea* suggesting its competitive and non-competitive binding on the enzyme. Luonteri et al. (1998a) reported three α-galactosidases from *Penicillium simplicissimum* of which AGL II showed more resistance to product inhibition by galactose than the other two enzymes, AGL I and AGL III. α-Gal I from *S. griseolalbatus* is novel in this respect and is important from industrial point of view since galactose tolerance is an appreciable character which improves the efficiency of α-galactosidases in liberating galactose residues from oligomeric and polymeric α-galactosides. The exact reason behind this unique galactose tolerance is unclear and can be of interest for another detailed investigation.

5.3.9. Effect of amino acid group specific reagents

The role of amino acid functional groups on activity of the three α-galactosidases was studied using amino acid group specific reagents and the results are summarized in Table 5.4. NAI, TNBS, PMSF and 2,3-butanedione did not affect the activity of α-Gal I, α-Gal II and α-Gal III indicating that tyrosine, lysine, serine and arginine do not play any role in the catalytic action. Chemical modification of sulphydryl groups by NEM and DTNB did not influence the activity of *S. griseolalbatus* α-galactosidases, whereas PCMB almost inactivated the enzymes. This could be attributed to the non-competitive nature of inhibition by Hg$^{2+}$ in PCMB rather than due to the modification of sulphydryl groups. Sulphydryl groups are reported to play an important role in maintaining the active conformation of the protein molecule rather than
Purification and Characterization of α-Galactosidases taking part in catalysis (Malanchuk et al. 2001). α-Gal I, α-Gal II and α-Gal III were inhibited by DEPC, NBS, EDAC and WRK indicating the presence of histidine, tryptophan and carboxylate residues at or near the active site. Although DEPC is used for the specific chemical modification of histidine, it also reacts with cysteine, tyrosine and lysine residues. Since NEM, DTNB, NAI and TNBS did not inhibit enzyme activity in any way, the possible role of these amino acids could be excluded. Hence loss of enzyme activity with DEPC could be correlated to the chemical modification of histidine.

Table 5.4. Effect of amino acid group specific reagents on the activity of purified α-galactosidases from S. griseolosibus

<table>
<thead>
<tr>
<th>Amino acid group specific reagent</th>
<th>Possible amino acid modified</th>
<th>Concentration</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-Gal I</td>
</tr>
<tr>
<td>NEM</td>
<td>Cys</td>
<td>10 mM</td>
<td>100</td>
</tr>
<tr>
<td>DTNB</td>
<td>Cys</td>
<td>1 mM</td>
<td>99.9</td>
</tr>
<tr>
<td>PCMB</td>
<td>Cys</td>
<td>1 mM</td>
<td>2.9</td>
</tr>
<tr>
<td>DEPC</td>
<td>His</td>
<td>2 mM</td>
<td>0</td>
</tr>
<tr>
<td>NAI</td>
<td>Tyr</td>
<td>1 mM</td>
<td>100</td>
</tr>
<tr>
<td>TNBS</td>
<td>Lys</td>
<td>1 mM</td>
<td>99.9</td>
</tr>
<tr>
<td>NBS</td>
<td>Trp</td>
<td>2 mM</td>
<td>0</td>
</tr>
<tr>
<td>PMSF</td>
<td>Ser</td>
<td>2 mM</td>
<td>100</td>
</tr>
<tr>
<td>2,3-Butanedione</td>
<td>Arg</td>
<td>10 mM</td>
<td>100</td>
</tr>
<tr>
<td>EDAC</td>
<td>Asx/Glx</td>
<td>10 mM</td>
<td>19.1</td>
</tr>
<tr>
<td>WRK</td>
<td>Asx/Glx</td>
<td>10 mM</td>
<td>17.9</td>
</tr>
</tbody>
</table>

Tryptophan is reported to play a significant role in the catalytic site as well as in the thermal stabilization of active site conformation of the enzyme molecule at high temperature (Gote et al. 2007). It is also known that hydrophobic interactions are often important contributors to the overall stability of proteins (Bund & Singhal 2002). The tryptophan residue at position 16 of coffee bean α-galactosidase has previously been shown to be essential for enzyme activity (Zhu et al. 1996). Involvement of carboxyl groups in the catalysis of α-galactosidases from Humicola sp. (Kotwal et al. 2000), B. stearothermophilus (NCIM 5146) (Gote et al. 2007) and coconut kernel (Mathew &
Chapter V

Balasubramaniam 1986) has already been reported. α-Galactosidase from Vicia faba also shows the presence of a single carboxylate and a histidine residue in catalysis (Dey & Pridham 1969a).

5.4. CONCLUSION

The multiple α-galactosidases- α-Gal I, α-Gal II and α-Gal III, produced by S. griseoloalbus were purified to homogeneity by a two-step chromatographic process. The purification protocol employed was rapid and provided a consistently pure source of α-galactosidases. The molecular masses and pi of the three enzymes were 72, 57 and 35 kDa, and 4.41, 5.6 and 6.13 respectively. α-Gal I showed N-terminal sequence homology to S. coelicolor A3(2) family 27 α-galactosidase. The optimum pH and temperature of the three α-galactosidases were 5.0, 6.5 and 5.5 and 65 °C, 50 °C and 55 °C respectively. α-Gal I was stable up to 65 °C and α-Gal II and α-Gal III up to 55 °C for 2 h. Based on the hydrolytic properties α-Gal I could be classified as a member of GH27 family and α-Gal II and α-Gal III as members of GH36 family with a retaining mechanism of hydrolysis. Metal cations like Hg$^{2+}$, Ag$^{2+}$ and Cu$^{2+}$ inhibited enzyme activity while Mg$^{2+}$ enhanced the activity of α-Gal I. α-Gal I showed unusual tolerance to even higher concentrations of galactose, unlike the other two α-galactosidases which were competitively inhibited by galactose. Melibiose was a competitive inhibitor of all the three enzymes. Histidine, tryptophan and carboxylic residues were essential for catalytic action of the three α-galactosidases.