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New source of α-D-galactosidase: Germinating coffee beans

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ABSTRACT

Enzyme activities of α-Gal from dormant and germinating coffee beans (*Coffea arabica*) were studied and compared to develop one new source of α-D-galactosidase (α-Gal). During the germination, enzyme activity showed a continuous improvement: it increased slowly within 25 days and then rapidly increased. At the beginning of the germination, enzyme activity was lower than that from dormant coffee beans (DCB). It became higher than the latter around the 30th day, and rose to a maximum at the 35th day. The partially purified enzymes from germinating coffee beans (GCB) and DCB were obtained through ammonium sulphate precipitation, acetone precipitation and DEAE Sepharose ion exchange chromatography. The results showed that enzyme activity of purified α-Gal from GCB was 1.73 times greater than that from DCB. It was most stable for six weeks at its optimal pH (4.8) during the storage. GCB could become a new source of α-Gal instead of DCB.

1. Introduction

α-D-Galactosidase (α-Gal; EC 3.2.1.22) has been reported to occur widely in a variety of sources: plants, animal tissues and micro-organisms (Barham, Dey, Griffiths, & Pridham, 1971; Carchon & Bruyne, 1975; Dey & Pridham, 1972; Sklenářova & Tichá, 1991). This enzyme catalyses cleavage of the terminal galactosyl residues from a wide range of substrates, including linear and branched oligosaccharides, polysaccharides and various synthetic substrates, such as *p*-nitrophenyl-α-D-galactopyranoside (PNPG) (Shabalin, Kulminskaya, Savel'ev, Shishlyannikov, & Neustroev, 2002; Simerská et al., 2006). It is important for the germinating of the seeds, and the germination is restrained by deficiency of α-Gal (Mathew & Balasubramaniam, 1986). In the human body, the deficiency of α-Gal leads to Anderson–Fabry's disease (Peters, Vermeulen, & Kho, 2001; Takena, Qin, & Brady, 1999). Dormant coffee beans (DCB) became a focus of research for many years, because α-Gal from DCB had two functions: the blood conversion (B type cell to O type cell) (Lenny & Goldstein, 1991; Lenny, Hurst, & Goldstein, 1991), and the galactosylation of cyclodextrins (Kitahata, Hara, Fujita, Kuwahara, & Koizumi, 1992). The effects of this α-Gal were better than that from all other sources on these functions.

Commercial α-Gal comes from two sources: green coffee beans (namely DCB) and *Aspergillus niger*. It is difficult to remove the impurities (β-*N*-acetylglucosaminidase, β-xylosidase and β-galactosidase) during the extraction of α-Gal from *A. niger*. Therefore the main material used to prepare α-Gal is DCB. There have been many studies on applications of α-Gal from DCB, covering wide fields, but the enzyme activity and the yield could not meet the requirements of application. Koizumi et al. (1995) reported studies on branched cyclomalto-oligosaccharides (cyclodextrins) produced by transgalactosylation with raw α-Gal from DCB, and enzyme activity of raw α-Gal was low. These restricted the application of α-Gal, and led to α-Gal being expensive in the international market (Carchon & Bruyne, 1975; Harpaz, Flowers, & Sharon, 1974). For obtaining a high yield, Zhu and Goldstein (1994) studied cloning and functional expression of a cDNA encoding coffee bean α-Gal. Zhu et al. (1995) reported high-level expression in the yeast, *Pichia pastoris*, and purification of α-Gal.

During the germination of coffee beans, the activity of α-Gal was different from that in DCB. Moreover, it was higher than the latter in some germinating periods (Marraccini et al., 2005). Therefore, in this study, the aim was to develop a new source of α-Gal: germinating coffee beans (GCB). The preparation and purification of α-Gal from GCB and DCB were studied. Enzyme activities were compared in the different germinating periods, and their stabilities were also studied on storage.

Abbreviations: α-Gal, α-D-galactosidase; DCB, dormant coffee beans; GCB, germinating coffee beans; DIC, days in culture; WOS, weeks on storage; MFM, microfiltration membrane; EDTA, ethylene diamine tetraacetic acid; PNPG, *p*-nitrophenyl-α-D-galactopyranoside; PVPP, polyvinylpolypyrrolidone; BSA, bovine serum albumin

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2. Materials and methods

2.1. Materials

Coffee beans (*C. arabica*) came from Hainan Fushan in China. DEAE Sepharose was purchased from Pharmacia Corporation. *p*-Nitrophenyl-α-D-galactopyranoside (PNPG) was the product of Sigma–Aldrich (Shanghai) Trading Co., Ltd. All other regents were commercial preparations of analytical grade.

2.2. Germination of coffee beans

Grains of DCB were sterilized by stirring in the solution of sodium hypochlorite (10% w/v) for 30 min, rinsed five times in sterile H_2O, and put individually into sterile plugged tubes with gaseous exchange and a 2 cm bed of agar (0.7% w/v) at 26 °C, 70% humidity. The whole germinating culture was performed in the constant temperature and humidity incubator. The germinating period was expressed as days in culture (DIC) (Marraccini et al., 2005).

2.3. Preparation of raw α-Gal

Grains (20 g) of DCB were soaked in 30 °C water for 12 h, and then they were ground in extraction (sodium chloride, 0.9% w/v, polyvinylpolypyrrolidone (PVPP), 0.9% w/v, ethylene diamine tetraacetic acid (EDTA), 0.1 mM, pH 4.8). The mixture was stirred at 4 °C for 30 min, centrifuged at 4 °C for 15 min at 21,500*g*, filtered and stored at −40 °C. Grains (20 g) of GCB were ground without soak in water, and the other processes were the same as those for DCB.

2.4. Ammonium sulphate precipitation

The raw extract was made 75% saturated (0 °C) by slow addition of solid ammonium sulphate. The precipitate was isolated by centrifugation (15 min, 10,000*g*), and then suspended in distilled water for 24 h. Insoluble material was isolated by centrifugation (10 min, 10,000*g*), and discarded (Carchon et al., 1975).

2.5. Acetone precipitation

Acetone (200 ml) was slowly added to 200 ml of the above enzyme solution, with constant stirring. The precipitate was isolated by centrifugation (15 min, 10,000*g*), and then suspended in distilled water for 24 h. Insoluble material was isolated by centrifugation (10 min, 10,000*g*), and discarded (Carchon et al., 1975).

2.6. DEAE Sepharose chromatography of α-Gal

After acetone precipitation, the solution was dialyzed against phosphate buffer solution (PBS pH 7.0) for 48 h. Then the solution was filtered by a microfiltration membrane (MFM 0.45 μm). Equilibration of a column of DEAE Sepharose was performed with 20 mM PBS buffer (pH 7.0). The elution were performed with 20 mM PBS buffer (pH 7.0) and 0–1 M sodium chloride solution.

2.7. Storage of α-Gal

The enzyme solution after passing through the column of DEAE Sepharose, was dialyzed against 50 mM McIlvain's buffer (at different pH) for 24 h at 4 °C. Then the solution was stored at 4 °C in plastic containers containing bovine serum albumin (BSA) (0.5 mg/ml).

2.8. Enzyme activity assay of α-Gal

Enzyme activity of α-Gal was determined by the hydrolyzing of PNPG as substrate. Enzyme solution (50 μl) were incubated with 50 μl of 10 mM PNPG in 150 μl McIlvain's buffer (pH 6.5) at 26 °C for 10 min. The reaction was stopped by adding 1 ml of 100 mM Na_2CO_3–$NaHCO_3$ solution (pH 10.2); *p*-nitrophenol released from the substrate was measured with a spectrophotometer at 405 nm (Hara et al., 1994; Marraccini et al., 2005; Shabalin et al., 2002). Total protein was determined by using the method of Bradford (Bradford, 1976). All assays were performed in triplicate and results were recorded as the means.

3. Results and discussion

3.1. Enzyme assay of α-Gal during the germination

Enzyme activities of the raw extract from GCB were measured at different germinating periods (Fig. 1). The activity of α-Gal increased slowly before the 25th DIC (from 0.16 nkat/mg to 0.36 nkat/mg) and rapidly rose between the 25th DIC and the 35th DIC (from 0.36 nkat/mg to 2.31 nkat/mg). The maximum of enzyme activity at 35 DIC was 14.4 times higher than the minimum at the beginning of the germination. The enzyme activity of the raw extraction from DCB measured by the same methods was 1.10 nkat/mg. The enzyme activity of the raw extract from GCB at 35 DIC was 2.1 times greater than that from DCB. GCB could become a source to prepare and purify the α-Gal because of the high enzyme activity in the raw extract. Radicle length of the coffee beans gradually increased during the germination (Fig. 1), up to the maximum of 3.48 cm in the 35th DIC. At that time, a long radicle appeared in GCB and there were great changes in the endosperm for providing the nutrition for the germinating grains. There were more complicated components in grains of GCB, which were not suitable for preparing and purifying α-Gal. The measurement of enzyme activity was stopped after 35 DIC.

The activity of α-Gal increased during the germination. This is a general phenomenon in nature: during the germination of most plant seeds, enzymes become different from those during dormancy. The reasons for such changes in coffee bean seeds were not clarified in previous reports. Muller and Jacks (1983) and Plant and Moore (1983) reported that the α-Gal was synthesized and targeted to protein bodies, and substrates were accumulated in the cytosol during seed development. The activity of α-Gal had a compact relationship with the degradation of the substrates: the raffinose family of oligosaccharides and galactosyl cyclitols (Obendorf, 1997).

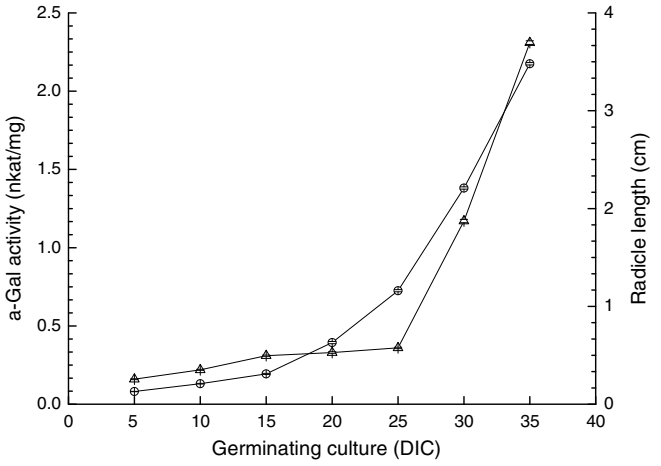

Fig. 1. Activity of α-Gal during the germination. Enzyme activity (Δ) and radicle length (○) are indicated in this figure at the different germinating times.

The activity of α-Gal was lower when extracted from GCB at the beginning of the germination (0.16 nkat/mg to 0.36 nkat/mg before the 25th DIC) than when extracted from DCB (1.10 nkat/mg), and exceeded the latter after the 25th DIC (1.17 nkat/mg to 2.31 nkat/mg). This was an abnormal phenomenon. Marraccini et al. (2005) reported that it was difficult to explain such a difference during germination. It could be attributed to problems of shipments and storage of the samples. When matured or dormant coffee bean seeds became the germinating seeds, there were great changes of external environmental and intrinsic factors. The activity of α-Gal from GCB sharply decreased in the prophase of the germination and was lower than that from DCB. With the germination of the coffee beans, these changes became useful for the enzyme activity in the anaphase of the germination. The activity of α-Gal increased to a high level and was higher than that from DCB at the end of the germination. It was difficult to understand the reasons for this change in the coffee bean during germination. On the contrary, there were no changes of external and intrinsic factors in DCB, and enzyme activity from DCB remained constant.

3.2. Preparation and purification of α-Gal

The α-Gal was prepared and purified from two sources: GCB (at 35 DIC) (Table 1) and DCB (Table 2) based on α-Gal activity of the raw extract. Enzyme activities from GCB and DCB were increased by three methods: ammonium sulphate precipitation, acetone precipitation and DEAE Sepharose chromatography. Grains of coffee beans were ground for extraction and the mixture gradually became brown. It was difficult to remove this brown colour by using ammonium sulphate precipitation and it affected the next purification. This brown extract gradually deteriorated on storage, and caused contamination of DEAE Sepharose in the next process. Plant polyphenol in coffee bean seeds was the main reason for this brown colour (Takena, Qin, & Brady, 1999). It was oxidized by polyphenol oxidase and became brown. It could also combine with protein, including α-Gal, and became the precipitate containing the inactivated α-Gal. The α-Gal activity rapidly degraded with the brown becoming dark. Therefore, plant polyphenol became the major factor for the stabilization of the enzyme preparation.

PVPP was insoluble in the raw extract and plant polyphenol preferentially combined with it (compared with the protein) (Wang & Vodkin, 1994). PVPP could remove some plant polyphenol from the raw extract and protect the α-Gal. It was necessary to add PVPP to the raw extract to remove the plant polyphenol. But enzyme activity was also low in the raw extract after adding the PVPP to it. This showed that PVPP could not remove plant polyphenol completely. Therefore acetone precipitation was used to remove plant polyphenol, for getting more α-Gal after the ammonium sulphate precipitation. Acetone, as an organic solution, could destroy the combination of enzyme and plant polyphenol. At the same tine, it could not destroy the enzyme, which retained a high activity. The α-Gal precipitate with high enzyme activity could be obtained in acetone solution (50% v/v), and plant polyphenol was dissolved in this solution. Enzyme and plant polyphenol could be separated in this solution by centrifugation. After acetone precipitation, most of the plant polyphenol was removed and the purification folds of α-Gal were rapidly increased when extracted from DCB and GCB (Tables 1 and 2). They were 16.2 from DCB and 15.4 from GCB. Carchon et al. (1975) also reported that acetone solution (50% v/v) could be used to obtain α-Gal from coffee beans in purification of this enzyme.

Grains from GCB and DCB were the same weight (20 g) in this experiment. Total enzyme, total protein and specific activity of GCB were higher than those of DCB in all the purifying procedures (Tables 1 and 2) because of more α-Gal in GCB. It was more difficult to purify α-Gal from GCB than from DCB because of the more complicated components in GCB. Also, the purification fold of α-Gal was lower when extracted from GCB (15.4) than from DCB (16.2). The difference showed in that the total yields of α-Gal from GCB (19%) and DCB (23%) were close. After the same purification, the percent of total protein of α-Gal from GCB was 1.62 times greater than that from DCB; the total activity was 2.79 times, and the specific activity was 1.73 times because of the high enzyme activity in the raw extract of GCB. It was not difficult to get GCB in laboratories or in plants. Based on these results, GCB was a better source than DCB for preparing and purifying α-Gal.

3.3. Stability of α-Gal on storage

The raw extracts and purified enzymes from DCB and GCB were in 50 mM McIlvain's buffer (pH 5.0). They were stored at 4 °C for several weeks and the results are shown in Fig. 2 (DCB) and Fig. 3 (GCB) for the different storage times. All enzyme activities determined by using the same method before the storage were regarded as 100%, respectively. After six weeks of storage, the α-Gal in raw extract from DCB was 92.4%; purified α-Gal from DCB was

Table 1
Purification of α-Gal from DCB

Fraction	Enzyme (nkat)	Protein (mg)	Activity (nkat/mg)	Yield (%)	Purification (-fold)
Raw extract	38.4	34.9	1.10	100	1
$(NH_4)_2SO_4$ fraction	27.4	20.2	1.35	71	1.23
Acetone fraction	19.6	1.10	17.8	51	16.2
DEAE Sepharose	8.83	0.13	67.9	23	61.7

Results are means of three determinations.

Table 2
Purification of α-Gal from GCB

Fraction	Enzyme (nkat)	Protein (mg)	Activity (nkat/mg)	Yield (%)	Purification (-fold)
Raw extract	129	55.9	2.31	100	1
$(NH_4)_2SO_4$ fraction	84.1	30.5	2.76	65	1.19
Acetone fraction	59.4	1.67	35.5	46	15.4
DEAE Sepharose	24.7	0.21	117	19	50.9

Results are means of three determinations.

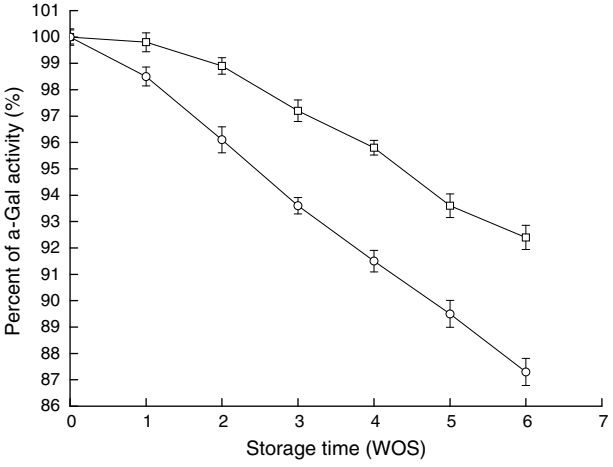

Fig. 2. Percent of activity of α-Gal from DCB on storage. Two activities of original enzymes from DCB, including raw extract (□) and purified α-Gal (○) were determined and were regarded as 100%, respectively. The activities of α-Gal at the different storage times were compared with two corresponding original activities and shown as percent.

87.3%; the α-Gal in raw extract from GCB was 87.2%; purified α-Gal from GCB was 81.1%. Two raw extracts, from DCB and GCB, became less stable after purification. Carchon et al. (1975) reported the same phenomenon. The inactivating speed of purified α-Gal was faster than the raw extracts on storage. Purified α-Gal from GCB was less stable than other types because of the complicated components during the germination.

pH was the important factor for the stabilization of α-Gal from DCB and GCB on storage. Some experiments were performed to find the influence of pH. The results shown in Fig. 4 (DCB) and Fig. 5 (GCB) for different pH values and different storage times. Purified α-Gal from DCB was in 50 mM McIlvain's buffer of different pH (4.9, 5.0, 5.1, 5.2, 5.3), and purified α-Gal from GCB, also, was in 50 mM McIlvain's buffer of different pH (4.6, 4.7, 4.8, 4.9, 5.0). All enzyme activities determined by using the same method before the storage were regarded as 100%, respectively. After six weeks of storage, the optimal remaining activity of purified α-Gal

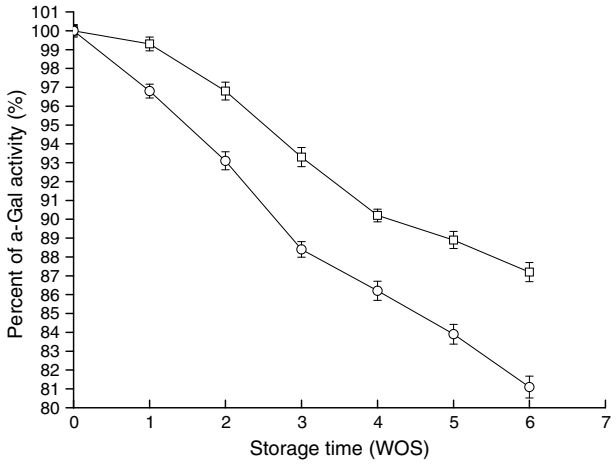

Fig. 3. Percent of activity of α-Gal from GCB on storage. Two activities of original enzymes from GCB, including raw extract (□) and purified α-Gal (○), were determined and were regarded as 100%, respectively. The activities of α-Gal at the different storage times were compared with two corresponding original activities and shown as percent.

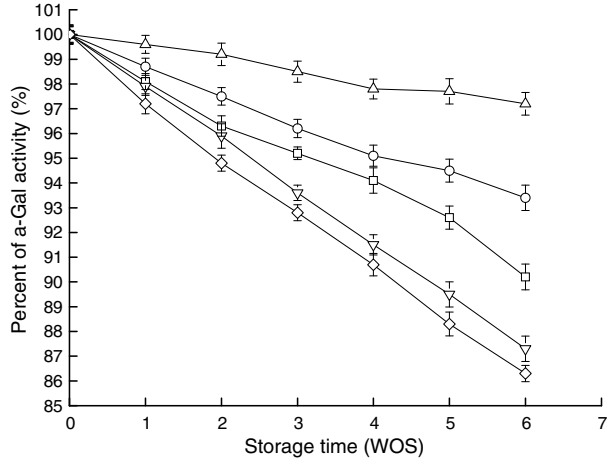

Fig. 4. Percent of activity of purified α-Gal from DCB at different pH (5.1 (△), 5.2 (○), 5.3 (□), 5.0 (▽), 4.9 (◇)) on storage. The original enzyme activities were regarded as 100%, respectively. The activities of α-Gal at the different storage time were compared with corresponding original activities and shown as percent.

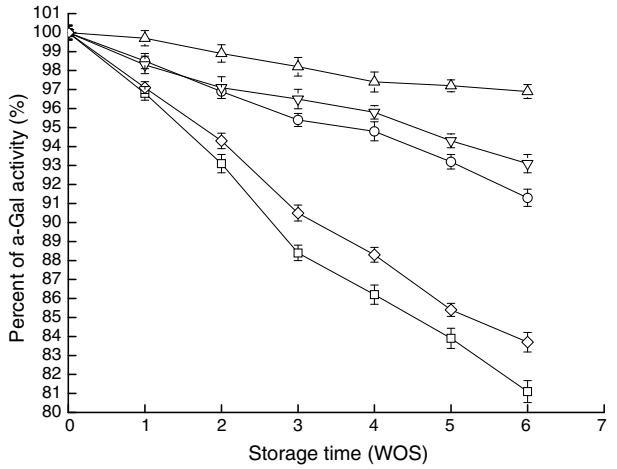

Fig. 5. Percent of activity of purified α-Gal from GCB at different pH (4.8 (△), 4.7 (▽), 4.9 (○), 4.6 (◇), 5.0 (□)) on storage. The original enzyme activities were regarded as 100%, respectively. The activities of α-Gal at the different storage times were compared with corresponding original activities and shown as percent.

from DCB was 97.2% at pH 5.1 and it was 96.9% at pH 4.8 from GCB. Therefore, the purified enzyme from DCB was most stable at pH 5.1, and the purified enzyme from GCB was also most stable at pH 4.8. Enzymes could be stored at their optimal pH for six weeks without loss of activity. But above or below their optimal pH, inactivation of purified α-Gal, from DCB and GCB, occurred rapidly.

The activity of purified α-Gal from GCB was higher than that from DCB and it could be stable at its optimal pH on storage.

4. Conclusion

The activity of α-Gal from Coffee beans continuously increased during germination, up to the maximum at 35 DIC. The activity of α-Gal from GCB was 1.73 times greater than that from DCB after ammonium sulphate precipitation, acetone precipitation and DEAE Sepharose chromatography. This purified enzyme was stable for six weeks at its optimal pH on storage. It was not difficult to obtain the GCB and purify α-Gal from it. The results indicated that GCB could become the new material for preparing α-Gal with high enzyme activity. In the next plan, enzymology properties and an application of the transglycosylation of cyclodextrins will be studied.

Acknowledgements

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