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Taro α-galactosidase: A new gene product for blood conversion

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ABSTRACT

The N-terminal sequence and internal sequences of the purified taro α-galactosidase (α-Gal) were determined using tandem mass spectrometry. By using reverse transcriptase PCR (RT-PCR), 5′ and 3′ rapid amplification of cDNA ends (RACE) with designed, degenerate primers, a novel cDNA sequence was obtained. The recombinant taro α-Gal not only hydrolyzes α1→4 linked galactosyl residues, which are accumulated in the tissues from patients with Fabry disease, but also hydrolyzes the α1→3 linked galactoside of B red blood cells (RBC). The recombinant taro α-Gal provides an ideal enzyme source for biomedical systems.

1. Introduction

α-Galactosidase (α-Gal) (EC3.2.1.21) has been isolated from various sources, such as *plants*: coffee beans (Haparaz et al., 1974; Zhu and Goldstein, 1994; Zhu et al., 1995; Zhu et al., 1996), figs (Li and Li, 1972), bean sprouts (Chien, 1985), coconuts (Balasubramaniam and Mathew, 1986), taro tubers (Chien and Lin-Chu, 1991), melon fruit (Gao and Schaffer, 1999), grape flesh (Kang and Lee, 2001), rice culture (Kim et al., 2002), and papayas (Soh et al., 2006); *seeds*: soybeans (Harpaz et al., 1977), pinto beans (Davis et al., 1977), and tomato seeds (Feutado et al., 2001); *bacteria*: *Clostridium sporogens* (Dybus and Aminoff, 1981) and *Bacillus fragilis* (Liu et al., 2007); and *fungi*: *Calvatia cyathiformis* (Shetlar and Li, 1964), *Mortierella vinacea* (Suzuki et al., 1970), *Pycnoporus cinnabarinus* (Ohtakara et al., 1984), and *Penicillium purpurogenum* (Shibuya et al., 1998).

In 1991, taro α-Gal was isolated by Chien and Lin-Chu (1991) and was shown to have hydrolyzing activity toward raffinose family oligosaccharides and also the terminal α1→3 linked galactosyl residue of the blood group B to the O antigen. The availability of "converted O cells" is critical in Taiwan because there is a continual shortage of group O red blood cells (RBC) for transfusion services. Previously, we have shown that taro α-Gal has a higher blood converting activity than green coffee beans. To fulfill this blood conversion research, a large quantity of recombinant enzyme is desirable. We started to clone the α-Gal encoding cDNA. An electrospray ionization–tandem mass spectrometry (ESI-MS-MS) technique (Lodge et al., 2007) was first adopted to obtain partial amino acid sequences of the purified taro α-Gal protein. Then, from the total RNA and designed primers, which was then followed by reverse transcriptase PCR (RT-PCR) technique, the specific cDNA sequence was obtained. Subsequently, the open reading frame sequence was cloned, and the active enzyme was expressed in yeast. To the best of our knowledge, this is the first report to provide the full-length coded sequence for taro α-Gal.

2. Materials and methods

2.1. Materials

Fresh taro tuber was purchased from a local market. *Pichia pastoris* SMD 1168 (protease-deficient strain) and GS115, Geneticin (G418) and pPIC9K vector with α-mating factor were purchased from Invitrogen (Taipei, Taiwan). Phusion® High-fidelity DNA polymerase was purchased from Finnzymes (Espoo, Finland). A yeast nitrogen base without amino acid, peptone and yeast extract was purchased from BD (Becton, Dickinson & Company, Sparks, MD, USA). Green coffee bean α-Gal (G8507-5UN), lyticase (L4025-50KU) and *p*-nitrophenyl-α-D-galactopyranoside (*p*NP-α-Gal) were purchased from Sigma Chemical Co. (St. Louis, USA). Taq DNA polymerase was purchased from Gene-Mark Technology (Tainan, Taiwan). ImProm-II™ reverse transcriptase (Catalog # A3802), RNasin® plus RNase inhibitor and T4 DNA ligase were purchased from Promega (Madison, WI, USA).

Abbreviations: α-Gal, α-galactosidase; RT, Reverse transcriptase; RACE, Rapid amplification of cDNA ends; RBC, Red blood cells; ESI-MS-MS, Electrospray ionization-tandem mass spectrometry

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A RNA extraction kit was purchased from Macherey-Nagel (MN) (Düren, Germany). RBC were provided by Mackay Hospital in Taiwan. Anti B was purchased from BCA Cooper Biomedical Co. (Malvern, PA, USA). Anti H lectin (*Ulex europaeus*) was purchased from Immucor Gamma® (Atlanta, GA, USA).

2.2. Methods

2.2.1. Isolation of α-gal from taro tuber

Taro tuber was homogenized, and α-Gal was extracted and centrifuged. The supernatant was concentrated by ultrafiltration. The concentrate was purified by Sephadex G-100 that was followed by DEAE Sephadex A-50 column chromatography. The α-Gal was collected and concentrated. The sample was further purified on an ÄKTA FPLC (DEAE Sepharose Fast Flow). α-Gal was obtained in a linear gradient at 1% NaCl in 0.02 M sodium phosphate buffer, pH 8.0. The sample was run on polyacrylamide gel electrophoresis (PAGE) with a molecular weight standard (Hames, 1990). The gel was then transferred to a PVDF membrane, according to a method developed by Towbin et al. (1992).

2.2.2. Protein sequence determination

The Edman degradation method was used to determine the N-terminal sequence. The sample was obtained from the transferred PVDF membrane, which was lightly stained with Amido Schwartz 10 B (amido black). The stained zone was cut and analyzed. For the internal sequence, the sample was embedded in a gel and digested with trypsin, purified, and analyzed by a MS/MS ion search (Mission Biotech Company, Taiwan).

2.2.3. RT-PCR for full-length DNA sequencing

The total RNA was prepared by homogenizing the taro tuber in liquid nitrogen and extracted with a commercial kit, NucleoSpin ® RNA II, which was purchased from Macherey-Nagel (MN) in Germany. RT was used to obtain the cDNA (Frohman et al., 1988). The degenerate primers were designed according to the corresponding amino acid sequences previously determined for the taro α-Gal protein. The forward and reverse primers and the PCR conditions are shown in Tables 1–3. The cDNA of the taro tuber RNA was prepared according the Promega protocol. The PCR product was cloned into a pOptima®-T Cloning Vector (Strong Biotech, Taipei, Taiwan) and transformed into DH5α. After the correct plasmid DNA sequence was confirmed, specific primers were designed for 3′ and 5′ RACE. Primers with terminal restriction sites Tgal(F)EcoRI and Tgal(R)NotI were used to prime the full length for cloning into the pPIC9K vector, which was then transformed into *Pichia pastoris* SMD1168 and GS115 strains.

2.2.4. Gene expression

Gene expression was conducted according to the procedure described in Chien et al. (2008) and Chien (2010). A single colony was cultured in 2 ml of BYPG medium (buffered yeast extract peptone glycerol) and grown at 30°C overnight. The second overnight culture was prepared with 1 ml of the previous culture in 100 ml of the same medium. Furthermore, 700 ml of the same medium was added to this culture, and it was then cultured until the OD_{600} reached 40–60. Cells were centrifuged and suspended in 100 ml of BMM (buffered minimal methanol) containing 2% glycerol and cultured to OD_{600} 40–60, which was followed by induction with 1.5% methanol and incubated at 23 °C. Cells were harvested after 24 h and suspended in 0.01 M sodium phosphate buffer, pH 6.0. The cell suspension was broken with lyticase (8 mg/40 ml) that contained 0.1 M EDTA in 0.01 M citrate-phosphate buffer, pH 5.5. The enzyme activity was obtained from the supernatant and concentrated. The sample was further purified by Sephadex G-100, Q Sepharose anion exchange, DEAE Sephadex A-50 and Superose 12 column chromatographies.

Table 1
Degenerate primers and the PCR for the 680 bp fragment.

Degenerate primers
Forward degenerate primer IA (dFP IA):
5′–TGGAAYCAYTTYAGYTGYAAYAT-3′
Reverse degenerate primer 2A (dRP 2A)-17 mer:
5′–GCYTTNACYAANGCCCA-3′
PCR (cycles)
(1) 94 °C, 5′
(1) 50 °C, 3′
(35) 72 °C, 1′; 94 °C, 45”; 50 °C, 45”
(1) 72 °C, 7′

Table 2
The 3′ RACE primers and the PCR condition.

Gene specific forward primers
Forward primer X (FP X):
5′–CATCAATATAGATGACTGC-3′
Adapter primers
Adapter primer:
5′-GACTCGAGTCGACATCGA-3′
Adapter oligo dT primer:
5′–GACTCGAGTCGACATCGA$(T)_{16}$V-3′
PCR (cycles)
(1) 98 °C, 1′
(1) 50 °C, 40″
(35) 72 °C, 40″; 94 °C, 15″; 50 °C, 30″
(1) 72 °C, 7′

Table 3
N terminal primers and the PCR condition.

Primers
N-Terminal degenerate forward primer IIC (dN IIC):
5′–TTRGARAAYGGNTTRGGNCAYAC-3′
Gene specific reverse primer (RP 2):
5′–ATGAGGCAAAAGTTTTCGCGTC-3′
PCR (cycles)
(1) 94 °C, 5′
(1) 50 °C, 3′
(35) 72 °C, 1′; 94 °C, 45″; 50 °C, 45″
(1) 72 °C, 7′

2.2.5. Enzymatic blood conversion

RBC conversion from B to O was carried out according to the procedure described in Chien et al. (2008). The experiment was carried out at 37 °C in 50 μl of mixture, containing 1 unit of the recombinant taro α-Gal and B RBC in PBS, pH 5.5. A parallel experiment was carried out for coffee bean α-Gal; hemagglutination tests were performed at different times for up to 3 h. The reaction mixture was prepared by adding 10 μl of anti-B, which was diluted twenty-fold with PBS, or 10 μl anti-H lectin, from Immucor Gamma® to 5 μl of the treated RBC on the glass plate. After mixing, the cell agglutination was observed in 15 min. Hydrolysis of $GbOse_3Cer$ was carried out and analyzed according to a method described by Chien and Lin-Chu (1991).

3. Results and discussion

3.1. Taro α-gal isolation and characterization

Taro α-Gal was extracted and purified by gel filtration, DEAE anion exchange and DEAE Fast Flow on an ÄKTA FPLC (GE).

As shown in Fig. S1, SDS-PAGE revealed a single band with a molecular weight of approximately 40 KDa. The specific band in the native gel showed enzyme activity with the *p*NP-α-Gal substrate.

3.2. Amino acid sequencing for N-terminal and internal peptide sequences for α-gal protein

The partial sequences from N-terminal sequence and ESI-MS-MS were aligned with the known amino acid sequences of α-Gal from various species. The N-terminal sequence of the α-Gal enzyme protein is LENGLGHTPPMGWNSWNHFSCNI. The alignment of internal sequences showed similarity to that of the α-Gal from tomatoes (*Solanum lycopersicum*), which was documented in NCBI GenBank (accession no. **GI: 10312171**, or **AAG16693.1**) of nominal mass (Mr) 41841 and 380 amino acids as determined by Mascot Search Results (Matrix Science Database). The homology sequences of the three trypsinized fragments were indicated by bold characters in the amino acid sequence of *Solanum lycopersicum* (Sl) as shown in Fig. 1. These three internal sequences are: TFASWGVDYLK, residues 163–173, SHFSIWALVKAPLIIGCDLR, residues 277–296; and VAVVLWNR, residues 344–351. The similarities of the amino acid sequences in these regions were also found in the following species: *Oryza sativa*, Genbank accession no. **BAB12570.1**; *Phaselus vulgaris*, Genbank accession no. **AAA7396.1**; *Coffea arabica*, CAI Genbank accession no. **47559.1**; *Carica papaya*, Genbank accession no. **AAP04002.1**; *Glycine max*, Genbank accession no. **AA73963.1**; and *Solanum lycopersicum*, Genbank accession no. **AAG16693.1**. The N-terminal sequence of the α-Gal enzyme protein is LENGLGHTPPMGWNSWNHFSCNI.

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  1 MSSTSPLLLW CCLCLSLATV YARLQPRNLI VNSNLSVNEF NRRNLLGNGL
 51 GQTPQMGWSS WNHFGCNIDE NIIKGTADAM VHTGLASLGY EYINIDDCWA
101 EANRDSQGNM VAKGSTFPSG IKALADYVHG KGLKLGVYSD AGTQTCSKQM
151 PGSLGHEEQD AKTFASWGVD YLKYDNCNNE DRSPRERYPI MSNALQNSGR
201 AIFYSMCEWG DDNPATWASS VGNSWRTTGD ITDDWNSMTS RADLNDQWAS
251 YAGPGGWNDP DMLEVGNGGM SFGEYRSHFS IWALVKAPLI IGCDLRSMDN
301 TAHDILSNPE VIAVNQDKLG VQGKKVKQYG DLEVWAGPLS GKRVAVVLWN
351 RGSYKADITA YWSDIGLDYS TLVDARDLWA
```

Fig. 1. Homology of the amino acid sequences of α-Gal between tomato (*Solanum lycopersicum*) and taro. Trypsin digestion of taro α-Gal protein resulted in three peptides, which were detected by ESI-MS-MS, and are shown in the sequences with bold characters.

3.3. Primer design and RT-PCR results

Because the DNA sequence for taro α-Gal has never been reported previously, we derived the degenerate primers for RT-PCR, which was carried out with the degenerate primers dFP 1A and dRP 2A (Table 1). The product is indicated by an arrow in lane 1, Fig. S2. The correct size of PCR product was verified using an agarose gel. The proper DNA fragment was recovered and cloned. The resulting sequence was shown to have the predicted length of 680 bp, bases 1–680 in Fig. S3.

3.4. The 3′ RACE

The forward primer (FP X), adapter primers (oligo dT) and the PCR condition used are listed in Table 2, where the primers were used for PCR with the proper cDNA template. The resulting PCR product was cloned. The taro α-Gal DNA sequence was obtained from two of the clones: RACEX-351 and RACEX-352. The overlapping sequence with the 680 bp fragment containing clones, taro 680-1 and 680-2, as described in section 3.3, was found in clones RACEX-351 and RACEX-352, bases 96–680 in Fig. S3. The resulting sequence of the 3′ end, including the poly A tail, is shown in Fig. S3.

3.5. N-terminal sequence

The PCR process for 5′ RACE was carried out with dN IIC and RP 2 primers (Table 3). From the N-terminal end, a DNA fragment with 365 bp was obtained and cloned. These clones are 4, 8, 11,

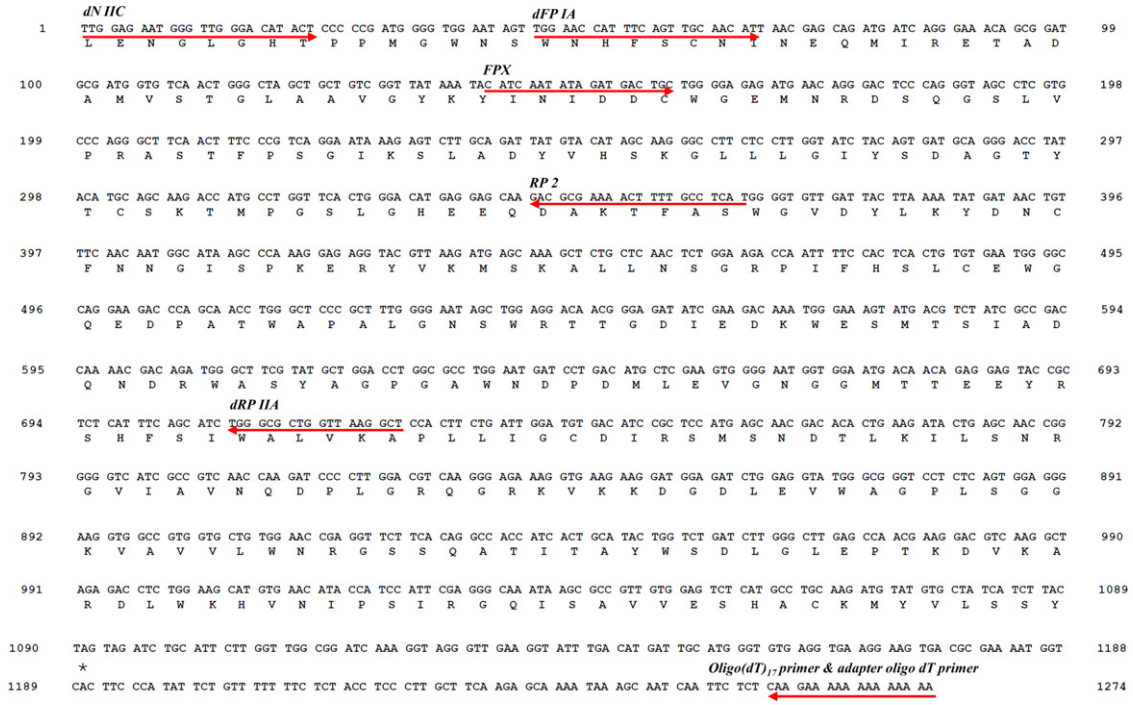

Fig. 2. Full-length open reading frame DNA sequence and the corresponding amino acids of α-Gal are shown. The designed primers: dN IIC, dPF IA, FP X, RP 2, dRP 2A, oligo $(dT)_{17}$ and the adapter oligo dT primers are indicated by underlined arrows.

14, 17, 18, and 19, respectively, as listed in Fig. S4. The complete open reading frame of DNA sequence for α-Gal was successfully obtained and is listed in the first line of Fig. S5. A comparison of the amino acid sequence of taro α-Gal with that from other sources is listed in Fig. S6. *Colocasia esculenta* is abbreviated as Ce in both Figs. S5 and S6.

3.6. Taro α-gal cDNA cloning and expression

Primers containing restriction sites (underlined) are the forward primer, Tgal(F)EcoRI, 5′–TTTTTTGAATTCTTGGAGAATGGGTTGGGACA-3′, and the reverse primer, Tgal(R)NotI, 5′–TTTTTTGCGGCCGCGTAAGATGATAGCACATAC-3′. They were designed from the open reading frame sequence, bases 1–20 and 1071–1089 (Fig. 2). With the proper cDNA used as the template, PCR was carried out according to the conditions listed in Table 2. The PCR product was verified and cloned into an expression vector, pPIC9K, and was further transformed into yeast cells, SMD1168 and GS115. Enzyme activity was found in both strains.

3.7. Recombinant enzyme activity and blood group conversion

Most of the enzyme activity was found inside the cell. Approximately 2500 units of active enzyme from one liter of cell culture could be produced. One unit of enzyme activity was defined as the amount of enzyme that could convert 1 μmol of *p*NP-α-Gal substrate into product per minute. Both GS115 and SMD1168 yeast strains were shown to have the same expression capacity. Following purification by Q Sepharose, DEAE Sephadex and Superose 12 on a FPLC™ system, the enzyme was used for blood conversion. The conversion from blood type B to O was clearly observed after 15 min. Fig. 3(a) is the RBC in PBS only. Fig. 3(b) showed the cells that were treated after 1 h. The converted O blood cell was agglutinated by anti-H lectin; Fig. 3(c) showed the remaining B RBC that were agglutinated by anti-B. The complete conversion was found in 3 h. As shown in Fig. 3(d), no agglutination occurred when the anti-B was added to the enzyme-treated cells. Therefore, cells were agglutinated by anti-H, as shown in Fig. 3(e). When a parallel experiment with the same amount of the commercial α-Gal coffee bean was conducted, the taro enzyme surprisingly showed stronger conversion activity than that from coffee bean by at least 3-fold. The recombinant taro α-Gal has characteristics of high specificity and efficiency in the cleavage of the B antigen. This reaction condition is suitable to maintain the integrity and function of RBC. The electrophoretic mobility in native gel electrophoresis showed that this enzyme has a slightly basic character. This might facilitate the cell washing from the negatively charged RBC after conversion. The ability of hydrolysis of α1→3 linked substrate CTH (Ceramide trihexoside or $GbOse_3Cer$) is comparable to the hydrolysis by coffee bean α-Gal. This ability indicates that the taro enzyme may play a potential role in the treatment of Fabry disease. The availability of this enzyme could lead to the development of an efficient process for producing a universal RBC (or converted O) in transfusion medicine.

4. Conclusion

In conclusion, the open reading frame sequence for the taro α-Gal is reported here for the first time. The recombinant taro α-Gal showed significant superiority to the commercially available enzyme from coffee bean in the conversion from the B to O blood group substance of red cells. Our ultimate goal is to produce a large quantity of the taro α-Gal for biomedical applications. This experiment was performed with a minimal amount of full-length cDNA, which led to our difficulty in verifying the 5′ end sequence; moreover, the signal peptide portion of mRNA remains to be determined. However, we have obtained the entire length of the open reading frame sequence for taro α-Gal and constructed the proper restriction site for each end to clone into the expression vector. Despite the minor difference in DNA sequence at nucleotides 6, 10, 13, 19, 22 and 138 from the N terminus (Fig. S4), the differences in these DNA bases will not change the corresponding amino acid residues in the protein. Further research, such as DNA

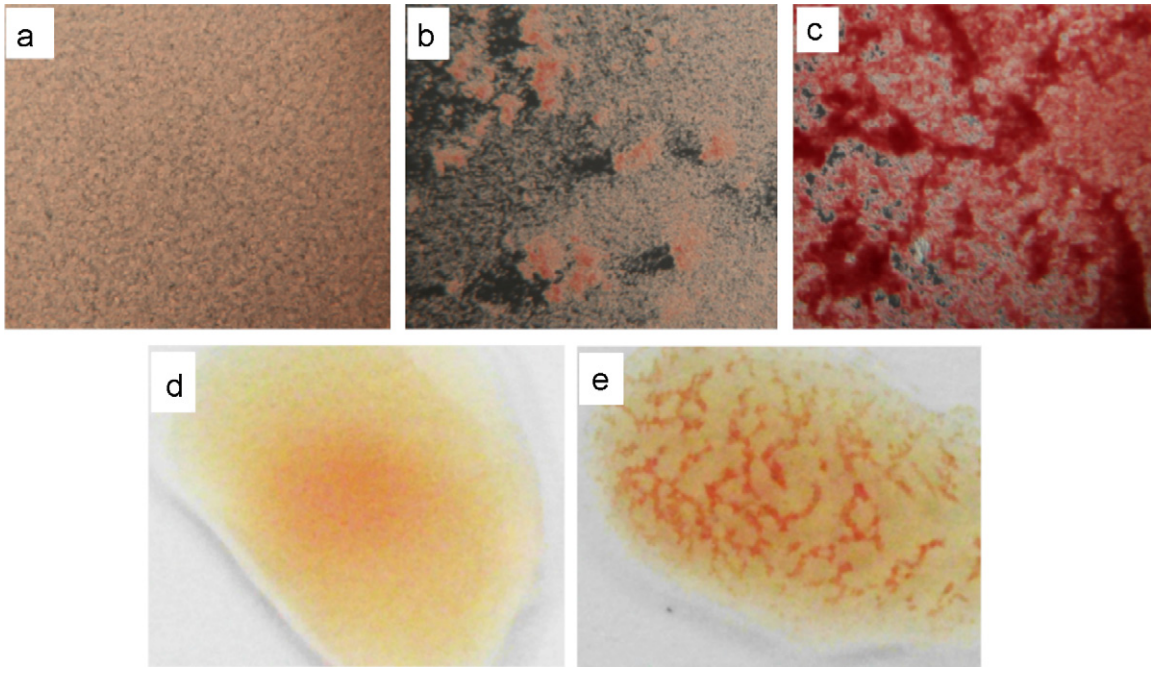

Fig. 3. Results of blood conversion determined by hemagglutination. B RBC in PBS as control (a); recombinant α-Gal treated B RBC with anti-H (b) and with anti-B (c), respectively, after the cells were treated for 1 h at 37 °C. The treated cells were not agglutinated with anti-B in (d) and were agglutinated with anti-H in (e), respectively, after 3 h.

walking, which uses longer primers ranging from 40–50 bp, should be undertaken to verify the minor difference in the 5′ end. In the flask culture, we were able to produce 2500∼3000 units/L in 24 h. In a large-scale culture, our previous result has shown seven-folds of expression Chien et al., 2008).

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Appendix A. Supporting materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bcab.2011.09.004.

References

Balasubramaniam, K., Mathew, C.D., 1986. Purification of α-D-galactosidase from coconut. Phytochemistry 25, 1819–1821.
Chien, S.F., 1985. Isolation and characterization of glycosidase from bean sprouts (*Vigna radiate*). J. Chin. Biochem. Soc. 14, 39–51.
Chien, S.F., Lin-Chu, M., 1991. The conversion of group B red blood cells into group O by an α-galactosidase.from taro (*Colocasia esculenta*). Carbohydr. Res. 217, 191–200.
Chien, S.F., Chen, S.H., Chien, M.Y., 2008. Cloning, expression, and characterization of rice α-galactosidase. Plant. Mol. Biol. Rep. 26, 213–224.
Chien, S.F., 2010. Cloning and expression of bioactive human granulocyte colony stimulating factor in *Pichia pastoris*. J. Chin. Chem. Soc. 57, 850–856.
Davis, M.O., Hata, D.J., Johnson, S.A., Jones, D.E., Harmata, M.A., Evans, M.L., Smith, D.S., 1977. Cloning, sequence, and expression of a blood group B active recombinant alpha-D-galactosidase from pinto bean (*Phaseolus vulgaris*). Biochem. Mol. Bio. Int. 42, 453–467.
Dybus, S., Aminoff, D., 1981. Action of α-galactosidase from *Clostridium sporogenes* and coffee beans on blood group B antigen and erythrocytes. Transfus 21, 244–247.
Feutado, J.A., Banik, M., Bewley, J.D., 2001. The cloning & characterization of α-galactosidase present during and following germination of tomato (*Lycopersicon esculentum* Mill.) seed. J. Exp. Bot. 52, 1239–1249.
Frohman, M.A., Dush, M.K., Martin, G.R., 1988. Rapid production of full-length cDNA from rare transcripts: Amplification using a single specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85, 8998–9002.
Gao, Z., Schaffer, A.A., 1999. Novel alkaline α-galactosidase from melon fruit with a substrate preference for raffinose. Plant Physiol. 119, 979–987.
Haparaz, N., Flowers, H.M., Sharon, N., 1974. Purification of coffee Bean α-galactosidase by affinity chromatography. Biochim. Biophys. Acta 341, 213–221.
Harpaz, N., Flowers, H.M., Sharon, N., 1977. Alpha-D-galactosidase from soybean destroying blood-group B antigens. Eur. J. Biochem. 77, 419–426.
Hames, B.D., 1990. One-dimensional polyacrylamide gel electrophoresis. In: Hames, B.D., Richard, D. (Eds.), Gel Electrophoresis of Protein-A Practical Approach, 2nd ed. IRL Press, pp. 1–49.
Kang, H.C., Lee, S.H., 2001. Characteristics of an α-galactosidase.associated with grape flesh. Phytochemistry 58, 213–219.
Kim, W.D., Kobayashi, O., Kaneko, S., Sakakibar, Y., Park, G.G., Kusakabe, I., 2002. Alpha-galactosidase from cultured rice (*Oryza sativa* L. var Nipponbare) cells. Phytochemistry 61, 621–630.
Li, S.C., Li, Y.T., 1972. Alpha-galactosidase from figs. Methods Enzymol. 28, 714–720.
Liu, Q.P., Sulzenbacher, G., Yuan, H., Bennett, E.P., Pietz, G., Saunders, K., Spence, J., Nudelman, E., Levery, S.B., White, T., Neveu, J.M., Lane, W.S., Bourne, Y., Olsson, M.L., Henrissat, B., Clausen, H., 2007. Bacterial glycosidases for the production of universal red blood cells. Nat. Biotechnol. 25, 454–464.
Lodge, J., Lund, P., Minchin, S., 2007. The analysis of the regulation of gene expression, Gene Cloning-Principles and Applications. Taylor & Francis Group., pp. 359–361 (Chapter 11).
Ohtakara, A., Mitsutomi, M., Uchida, Y., 1984. Purification and enzymatic properties of α-galactosidase from *Pycnoporus cinnabarinus*. Agric. Biol. Chem. 48, 1319–1327.
Soh, C.P., Ali, Z.M., Lazan, H., 2006. Characterization of an alpha-galactosidase with potential relevance to ripening related texture changes. Phytochemistry 67, 242–254.
Shetlar, M.R., Li, Y.T., 1964. Occurrence of α-galactosidase in higher fungi: Isolation of α-galactosidase from *Calvatia cyathiformis*. Arch. Biochem. Biophys. 10, 523–536.
Suzuki, H., Li, S.C., Li, Y.T., 1970. Alpha-galactosidase from *Mortierella vinacea*. Crystalization and properties. J. Biol. Chem. 245, 781–786.
Shibuya, H., Nagasaki, H., Kaneko, S., Yoshida, S., Park, G.G., Kusakabe, I., 1998. Cloning and high level expression of α-galactosidase cDNA from *Penicillium purpurogenum*. Appl. Environ. Microbiol. 64, 4489–4494.
Towbin, H., Stachelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.
Zhu, A., Goldstein, J., 1994. Cloning and functional expression of a cDNA encoding coffee bean α-galactosidase. Gene 140, 227–231.
Zhu, A., Leng, L., Monahan, C., Zhang, Z., Hurst, R., Lenny, L., Goldstein, J., 1995. High level expression and purification of coffee bean α-galactosidase produced in the yeast *Pichia pastoris*. Arch. Biochem. Biophys. 324, 65–70.
Zhu, A., Leng, L., Monahan, C., Zhang, Z., Hurst, R., Lenny, L., Goldstein, J., 1996. Characteriation of recombinant alpha-galactosidase for use in seroconversion from blood group B to O of human erythrocytes. Arch. Biochem. Biophys. 327, 324–329.