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Purification and Characterization of an α-L-Rhamnosidase from *Pichia angusta* X349

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An intracellular α-L-rhamnosidase from *Pichia angusta* X349 was purified to homogeneity through four chromatographic steps. The α-L-rhamnosidase appeared to be a monomeric protein with a molecular mass of 90 kDa. The enzyme had an isoelectric point at 4.9, and was optimally active at pH 6.0 and at around 40°C. The *Ki* for L-rhamnose inhibition was 25 mM. The enzyme was inhibited by Cu^{2+}, Hg^{2+}, and *p*-chloromercuribenzoate. The α-L-rhamnosidase was highly specific for α-L-rhamnopyranoside and liberated rhamnose from naringin, rutin, hesperidin, and 3-quercitrin. The α-L-rhamnosidase was active at the ethanol concentrations of wine. It efficiently released monoterpenols, such as linalool and geraniol, from an aroma precursor extracted from Muscat grape juice.

Key words: α-L-rhamnosidase; *Pichia angusta*; aroma improvement; monoterpenol

L-Rhamnose has been found as a constituent of glycolipids and glycosides such as plant pigments, pectic polysaccharides, gums, or biosurfactants. Some rhamnosides are known to exist as α-L-rhamnopyranosyl residues in grape terpenyl glycosides, the glycosidic precursor of aromatic terpenols, and in citrus bitter-flavor flavonoid compounds such as naringin and hesperidin. α-L-Rhamnosidases (EC 3.2.1.40) are exo-type enzymes that hydrolyze terminal nonreducing α-L-rhamnosyl groups from L-rhamnose-containing polysaccharides and the glycosides. This enzyme has been industrially used in debittering citrus juice and in studies of the carbohydrate research.

It is now well established that certain grape monoterpenols, which are linked to diglycosides, such as 6-*O*-α-L-arabinofuranosyl-, 6-*O*-α-L-rhamnopyranosyl-, 6-*O*-β-apiofuranosyl-β-D-glucosides, contribute significantly to the flavor of wine.[1–4] These glycosidically bound volatile compounds can be released by enzymatic hydrolysis. As α-L-rhamnosidases are involved in the hydrolysis of grape monoterpenyl glycosides, the enzyme have been attracting attention for improving aromas in wine by the hydrolyzing ability of grape monoterpenyl glycosides. α-L-Rhamnosidases derived from *Aspergillus terreus* has been used in the improvement of wine flavor.[5]

α-L-Rhamnosidases are produced by a number of mammalian tissues, plants, bacteria and fungi. Among the α-L-rhamnosidase-producing fungi, the enzyme preparations from *Penicillium decumbens*[6] and *Aspergillus niger*[7] are commercially available. Bacterial α-L-rhamnosidase production has been studied in the plant pathogen, *Corticium rolfsii*,[8] the human intestinal *Bacteroides*,[9] *Sphingomonas* sp.,[10] and *Clostridium stercorarium*.[11] Despite the contribution of microbial α-L-rhamnosidases to the improvement of wine flavor and the debitterness of citrus juice preparation, there is a lack of information about the enzyme activity in yeasts. So far there have been only a few reports on the production of α-L-rhamnosidase activity in yeast. Low levels of α-L-rhamnosidase activity were found in *Saccharomyces cerevisiae* Tokaj 7,[12] *Hansenula anomala*,[12] and *Debaryomyces ploymorphus*.[13] Recently the enzyme production of *Aureobasidium pullulans*, and *Candida guillermondii* were described.[14] Unfortunately, no yeast α-L-rhamnosidase was isolated and fully characterized.

We have screened for the yeasts producing α-L-rhamnosidase and found that *Pichia angusta* X349 was the best producer of the enzyme under the conditions tested.

Here we report results on the production, purification, and properties of the α-L-rhamnosidase produced by *P. angusta* X349.

Materials and Methods

Chemicals. *p*-Nitrophenyl α-L-arabinofuranoside, *p*-nitrophenyl α-L-rhamnopyranoside, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-xylopyranoside, *p*-nitrophenyl β-D-cellobioside, naringin, rutin, hesperidin, and 3-quercitrin were obtained from Sigma (St. Louis, MO, USA). Concanavalin A-Sepharose was purchased from Amersham Pharma-

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cia Biotech. DEAE Bio-Gel A agarose and hydroxyapatite were obtained from Bio-Rad Laboratories (Hercules, Calif.). All other chemicals used were of reagent grade.

Microorganism and cultivation. A total of 386 yeast strains belonging to the genera *Candida*, *Debaryomyces*, *Saccharomyces*, *Kluyveromyces*, *Rhodotorula*, *Pichia*, *Cryptococcus*, *Zygosaccharomyces*, *Torulaspora*, *Trichosporon*, and *Brettanomyces* were obtained from stock cultures maintained in our laboratory and were examined for their ability to produce α-L-rhamnosidase.

The strains were cultivated aerobically at 28°C for 48 h in a medium (pH 4.0) containing 1% rhamnose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract. After cultivation, the culture broths were ground with glass beads (0.45–0.55 mm in diameter) and cellular extracts were obtained by centrifugation of the ground cell suspension. The resulting supernatants were used for the assay.

Enzyme assays. α-L-Rhamnosidase activity was measured by the spectrophotometric method with *p*-nitrophenyl α-L-rhaminopyranoside (Sigma) as the substrate. The assay mixture contained 200 μl of the substrate solution (2 mM *p*-nitrophenyl α-L-rhaminopyranoside in 50 mM sodium phosphate buffer, pH 6.5), and 200 μl of appropriately diluted enzyme solution. After incubation at 30°C for 10 min, the reaction was stopped by the addition of 1.6 ml of 1 M Na_2CO_3. The absorbence at 405 nm due to the release of *p*-nitrophenol in the mixture was measured. One unit of α-L-rhamnosidase activity was defined as the amount of enzyme required to release 1 μmole of *p*-nitrophenol per min in the reaction mixture under these assay conditions. α-L-Arabinofuranosidase, β-D-glucopyranosidase, β-D-xylopyranosidase, and β-D-cellobiohydrolase were measured under the same conditions with the corresponding *p*-nitrophenyl glycosides.

Purification of α-L-rhamnosidase. All purification steps were done at 4°C, unless otherwise stated.

(i) *Ammonium sulfate treatment.* The washed cells isolated from 4 liters of culture broth were suspended in 20 mM Bis/Tris buffer (pH 6.5) and then disrupted with glass beads. After centrifugation, the resulting supernatant (crude extract) was fractionated with ammonium sulfate. The precipitate obtained with 30 to 80% saturation was collected, and dialyzed against 100 mM sodium acetate buffer (pH 6.0) containing 1 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.003 mM NaN_3.

(ii) *Concanavalin A-Sepharose column chromatography.* The dialyzed enzyme solution was put onto a Concanavalin A Sepharose column (2.6×6.5 cm) equilibrated with the same buffer. The active proteins were unabsorbed the column. The enzyme fractions were pooled, concentrated by ultrafiltration with a PM 10 membrane, and dialyzed against 20 mM Bis/Tris, pH 6.5.

(iii) *DEAE Bio-Gel A agarose column chromatography.* The dialyzed solution was put on a DEAE Bio-Gel A agarose column (2.6×6.0 cm) equilibrated with 20 mM Bis/Tris, pH 6.5. The enzyme was eluted with a linear gradient of 0 to 300 mM NaCl in the same buffer. The active fractions were combined and dialyzed against 50 mM sodium acetate buffer, pH 5.0.

(iv) *Rhamnose-Sepharose 6B affinity chromatography.* A Rhamnose-Sepharose 6B affinity matrix was prepared by coupling rhamnose to epoxy-activated Sepharose 6B according to the manufacturer's recommendations. The enzyme obtained after DEAE Bio-Gel A agarose was put on a Rhamnose-Sepharose 6B column (1.5×7.2 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0. The column was washed with this buffer, and the adsorbed proteins were eluted with 1 M NaCl in the same buffer. The active enzyme fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.8).

(v) *Hydroxyapatite chromatography.* The dialyzed enzyme solution was put on a hydroxyapatite column (1.5×2.8 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8). The column was extensively washed with this buffer, and the enzyme was eluted with a linear gradient of 10 to 200 mM sodium phosphate buffer (pH 6.8).

Protein assay. The protein concentrations were estimated with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, Calif.) for the Bradford dye-binding method,[15] with bovine serum albumin (Sigma) as the protein standard.

Polyacrylamide gel electrophoresis (PAGE). Polyacrylamide gel electrophoresis (PAGE) was done with a PhastSystem apparatus (Amersham Pharmacia Biotech). Sodium dodecyl sulfate (SDS)-PAGE and native PAGE were done with 12.5% polyacrylamide gel and 8 to 25% polyacrylamide gel, respectively. Isoelectric focusing (IEF) PAGE was done with PhastGel IEF 3–9 precast gel. The low- and high-molecular mass calibration kits and the broad p*I* calibration kit were purchased from Amersham Pharmacia Biotech. The proteins in the polyacrylamide gels were stained with Coomassie brilliant blue R-250.

N-terminal amino acid sequencing. The amino acid sequence of the N-terminal region of α-L-rhamnosidase was analyzed on a PVDF membrane blotted with the enzyme using a Hewlett Packard G 1005 A protein sequencer. Sequence comparisons were done

with the EMBL/GenBank and SWISS-PROT databases.

Enzymatic hydrolysis of natural flavonoid rhamnoglycosides. We used naringin, rutin, hesperidin, and 3-quercitrin as substrates. The hydrolytic reactions were done at 30°C for 18 h in a reaction mixture consisting of 1 mg of substrate, 0.2 U of purified α-L-rhamnosidase, and 50 mM sodium acetate buffer, pH 6.5 (final volume 1 ml). The reaction temperature (30°C) was selected by the stability of the enzyme protein. The L-rhamnose liberated was analyzed by high-pressure liquid chromatography on Shodex RS pak DC-613 column (Showa Denko K. K. Tokyo, Japan). The column was kept at 60°C, and the products were eluted with CH_3CN:H_2O (70:30) at a flow rate of 1.0 ml/min. Peaks were detected by refractive index and were identified and measured by comparison to retention times of authentic standards (L-rhamnose and D-glucose).

Enzymatic hydrolysis of the glycosides. Grape glycosides were isolated from the juice of Muscat of Alexandria grapes.[16] The hydrolysis was done at 30°C for 2 days in a reaction mixture consisting of 10 mg of the glycoside sample, 0.5 U of α-L-rhamnosidase, and 50 mM sodium phosphate buffer, pH 6.5 (final volume 2 ml). The liberated aglycones were extracted twice with 5 ml of pentane/ether (1:1). The extract was dried over anhydrous sodium sulfate, concentrated *in vacuo*, and analyzed by the GC-MS analysis described later. In the same manner, a blank test without the addition of the enzyme was done.

Gas chromatography-mass spectrometry (GC-MS). The volatile compounds were identified by comparing the retention times of the peaks with those of commercial standards. The identification of peaks were verified by GC-MS using a HP 6890 series apparatus (Hewlett-Packard) as previously described.[16]

Results and Discussion

Screening of α-L-rhamnosidase producing strains

The 386 yeast strains were examined for their production of α-L-rhamnosidase activity using L-rhamnose as the inducer. The screening results showed that α-L-rhamnosidase was generally restricted to the genera *Saccharomyces*, *Cryptococcus*, and *Pichia* (Table 1). Within these three genera, only species corresponding to *S. cerevisiae*, *C. terreus*, *P. angusta*, and *P. capsulata* showed α-L-rhamnosidase activity. No enological yeast, conventionally used for alcoholic fermentation, tested in our study showed detectable enzymatic activity. The genus *Saccharomyces* has been described previously as being able to hydrolyse *p*-nitrophenyl α-L-rhamnopyranoside,[7] but there is no such report for the genera *Cryptococcus* and *Pichia*. One strain, *P. angusta* X349, was sellected as the best producer of the enzyme.

Production of α-L-rhamnosidase and the location of enzyme activity

When grown in the presence of rhamnose or rutinose, *P. angusta* produced an α-L-rhamnosidase, but the enzyme was not produced when glucose, arabinose, or xylan was used as the carbon source. The highest enzyme production (0.85 U/mg-protein) was observed in the rhamnose-grown culture broth. These results suggest that α-L-rhamnosidase is not produced constitutively in *P. angusta* and that its synthesis may be under carbon catabolite repression.

The location of the enzyme activity was studied after cell growth on the medium containing rhamnose. No activity was detected in the culture medium. Almost all activities were found in the disrupted cells, and in the supernatant after an ultracentrifugation at $100{,}000 \times g$ for 90 min. The enzyme was found to be a soluble intracellular enzyme.

Purification of α-L-rhamnosidase

An intracellular α-L-rhamnosidase was purified to be homogeneous from a crude extract of *P. angusta* grown on rhamnose. A summary of the purification procedures is presented in Table 2. The final purifica-

Table 1. Screening of α-L-Rhamnosidase Producing Yeasts

Strain	α-L-Rhamnosidase (mU/ml)
Saccharomyces cerevisiae IAM 4561	13.7
Cryptococcus terreus IFO 0727	6.5
Pichia angusta X349	34.0
Pichia capsulata X91	28.8

Table 2. Summary of Purification of α-L-Rhamnosidase from *Pichia angusta* X349

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Crude extract	384	563	0.7	1.0	100
Ammonium sulfate	232	286	0.8	1.2	60
Concanavalin A-Sepharose	148	73	2.0	3.0	39
DEAE-BioGel A	121	9	13.4	19.2	32
Rhamnose-Sepharose 6B	41	1.7	24.0	34.3	11
Hydroxyapatite	9.5	0.3	33.9	49.7	2.5

tion resulted in a yield of 2.5% of the activity and a 49.7-fold increase in specific activity. The specific activity of the purified α-L-rhamnosidase was 33.9 U/mg of protein. SDS-PAGE analysis of the purified enzyme indicated the presence of a single band when stained with Coomassie brilliant blue (Fig. 1(A)).

Biochemical characterization of α-L-rhamnosidase

The molecular mass of native α-L-rhamnosidase was estimated to be around 90 kDa by native PAGE (Fig. 1(B)) and was 88 kDa by SDS-PAGE (Fig. 1(A)). This enzyme seemed to be a monomeric protein. Molecular masses of α-L-rhamnosidases had been reported to range from 70 to 240 kDa, and several enzymes consisted of two identical subunits. The molecular mass of the enzyme was similar to those of the extracellular α-L-rhamnosidase from *Aspergillus nidulans* (90 kDa),[17] *A. terreus* (90 kDa),[5] *Penicillium decumbens* (90 kDa),[6] *A. niger* (85 kDa),[18] and *A. aculeatus* (87 kDa and 84 kDa).[19] For other α-L-rhamnosidases, values of 70 kDa and 110 kDa have been described in *Fagopyrum esculentum*[20] and *Sphingomonas* sp.,[10] respectively. The isoelectric point of the protein was 4.9. The influence of pH and temperature on the enzyme activities is summarized in Figs. 2(A) and 2(B), respectively. The enzyme showed optimum activity at pH 6.0 and at around 40°C. It was stable from pH 5.0 to 7.0 and below 30°C. Both pH and temperature optimum were similar to those reported for a filamentous fungus, *A. nidulans* α-L-rhamnosidase.[17] In the pH range (3.0–3.8) usually found in winemaking, *P. angusta* α-L-rhamnosidase activity was 10% of its maximum value. With respect to the temperature (20–30°C) in winemaking, the enzyme showed around 70% of its initial activity.

Effects of inhibitors on the enzyme activity

The influence of various cations at 10^{-2} M on the enzyme activity was studied (Table 3). The enzyme

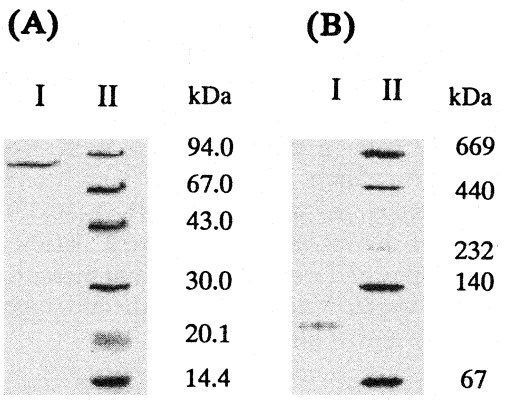

Fig. 1. Sodium Dodecyl Sulfate and Native Polyacrylamide Gel Electrophoresis of the purified α-L-Rhamnosidase.
(A): SDS-PAGE
Lane I, Purified enzyme. Lane II, The molecular weight markers including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20, 1 kDa), and α-lactalubumin (14, 4 kDa).
(B): Native PAGE
Lane I, Purified enzyme. Lane II, The molecular weight markers including thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa).

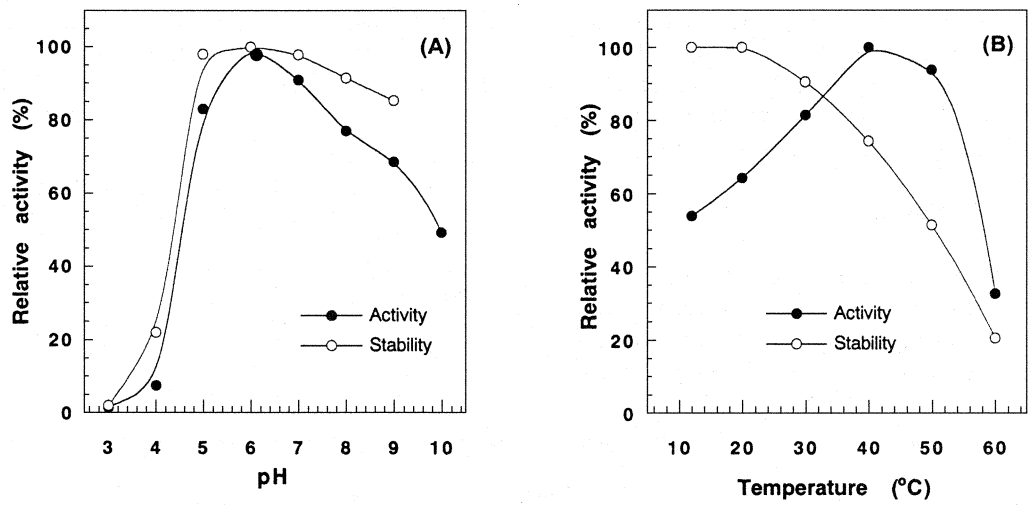

Fig. 2. Effects of pH and Temperature on Stability and Activity of the α-L-Rhamnosidase.
(A): pH stability (○); the enzyme solutions in 50 mM buffer at various pHs were incubated for 30 min at 30°C. The residual activity was assayed by the standard method. pH optimum (●); the enzyme activity was assayed by the standard assay method by changing the buffer to obtain the desired pH. The buffers used were citrate-phosphate (pH 3.0 to 5.0), sodium phosphate (pH 6.0 to 8.0), and glycine-NaOH (pH 9.0 to 10.0).
(B): Thermostability (○); the enzyme solutions in sodium phosphate buffer (50 mM, pH 6.5) was incubated for 30 min at various temperatures, and then the residual enzyme activities were assayed. Optimum temperature (●); the enzyme activity was assayed at various temperatures by the standard assay method.

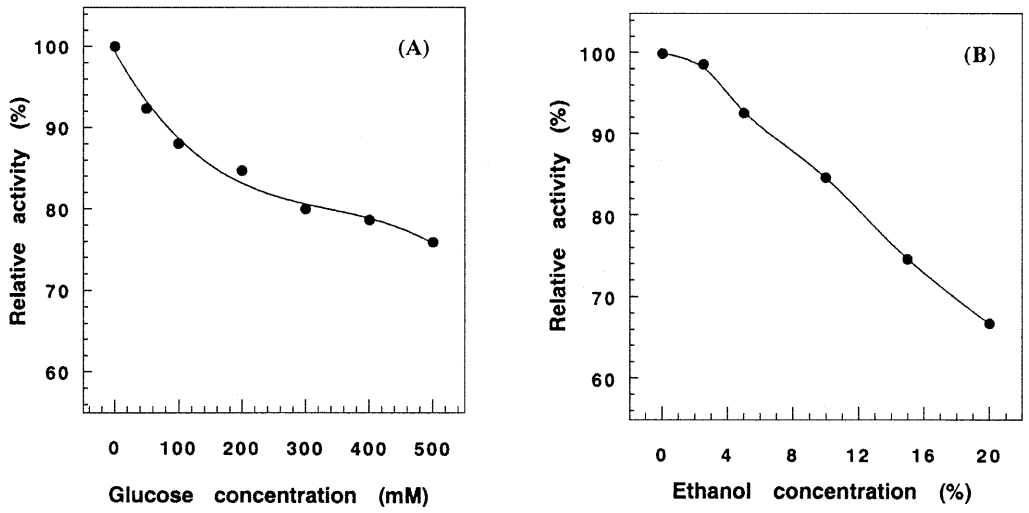

Fig. 3. Effects of Glucose and Ethanol Concentrations on α-L-Rhamnosidase Activity.
Enzyme activities were measured in the presence of various amounts of glucose (A) or ethanol (B) under the standard assay conditions.

Table 3. Effects of Various Metal Ions and Organic Compounds on Activities of α-L-Rhamnosidase from *P. angusta* X349

Compound (1 mM)	Relative activity (%)
No addition	100
$CuCl_2$	11
$CaCl_2$	98
$ZnCl_2$	94
$FeCl_2$	100
$MgCl_2$	99
$CoCl_2$	95
$MnCl_2$	102
$AlCl_3$	103
$HgCl_2$	6
EDTA*	98
2-Mercaptoethanol	98
p-CMB**	2

* EDTA, ethylenediaminetetraacetic acid.
** *p*-CMB, *p*-chloromercuribenzoate.

did not show any requirement for divalent cations for the activity. EDTA, 2-mercaptoethanol, Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, and Al^{3+} had little effect on the activity. The enzyme activity was significantly inhibited by a sulfhydryl reagent (*p*-chloromercuribenzoate) and sulfhydryl oxidant metal (Hg^{2+}), suggesting some importance of a sulfhydryl group for the expression of the enzyme activity.

Inhibition by several compounds of enological relevance and L-rhamnose

A glucose inhibition test was done with *p*-nitrophenyl α-L-rhamnopyranoside as the substrate. The enzyme had 76% of its maximal activity even in the presence of 500 mM glucose (Fig. 3(A)). A similar result has been previously reported for the *A. terreus*[5] and *A. nidulans* α-L-rhamnosidases.[17] As grape juice has about 500 mM glucose concentration, the enzyme will be active in grape juice. The enzyme showed 20% inhibition by ethanol at a concentration of 12% (v/v) that normally found in wine (Fig. 3(B)). This inhibition level was less than those of *A. terreus*[5] and *A. nidulans* α-L-rhamnosidase.[17]

The study of inhibition by L-rhamnose was done with *p*-nitrophenyl α-L-rhamnopyranoside as the substrate. L-Rhamnose acted as a competitive inhibitor of *p*-nitrophenyl α-L-rhamnopyranoside hydrolysis with an inhibition constant (*Ki*) of 25 mM obtained at the intersection of the lines on the Dixon plot analysis (Fig. 4). For other enzymes from the seeds of *F. esculentum*, *A. niger*, and *Penicillium* sp., *Ki* values have been reported to 15 mM, 3.5 mM, and 1.2 mM, respectively.[18,20,21]

Substrate specificity

Relative rates of hydrolysis of various *p*-nitrophenyl glycosides by the enzyme were examined. The activities on *p*-nitrophenyl α-L-arabinofuranoside, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-xylopyranoside, or *p*-nitrophenyl β-D-cellobioside were 6.1, 2.1, 0.2, or 0.1%, respectively (expressed as a percentage of that obtained with *p*-nitrophenyl α-L-rhamnopyranoside). The enzyme cleaved *p*-nitrophenyl α-L-rhamnopyranoside, but had little activity toward a variety of other *p*-nitrophenyl glycosides. It was highly specific to the α-L-rhamnopyranoside configuration.

N-terminal amino acid sequences

The first 17 amino acids of NH_2-terminal sequence of the purified α-L-rhamnosidase were Val-Glu-Val-Arg-Glu-Val-Arg-Leu-Glu-Ser-Asn-Ala-Leu-Gly-Pro-Thr-Asn-. The amino acid sequence showed no significant similarity to another α-L-rhamnosidase gene (ramA) from *Clostridium stercorarium*,[11] and

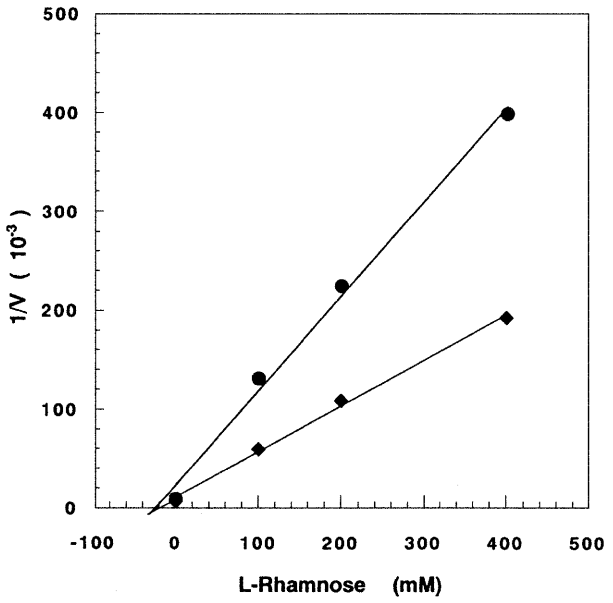

Fig. 4. Dixon Plot of Inhibitory Effect of L-Rhamnose on *p*-Nitrophenyl α-L-Rhamnopyranoside (*p*NPRham) Hydrolysis by α-L-Rhamnosidase.

The reaction time was 15 min at pH 6.0 and 40°C. The enzyme was used at 145 mU/ml. The *p*NPRham concentrations used were 1.0 (●) and 2.0 (◆) mM.

Table 4. Action of α-L-Rhamnosidase on Various Flavonoid Rhamnoglycosides

Substrate	Type of linkage involving rhamnose	Activity (%)[a]
Naringin[b]	α-1,2	100
Rutin[b]	α-1,6	63
Hesperidin[b]	α-1,6	34
3-Quercitrin[b]	α-1	31

[a] To measure L-rhamnose-releasing activity towards the rhamnoglycosides, the L-rhamnose released from each substrate was determined by high-pressure liquid chromatography. Activity using naringin as a substrate was taken as 100%.

[b] Naringin, naringenin-7-(α-L-rhamnosyl)-2-β-glucosides; rutin, quercetin-3-(α-L-rhamnosyl)-6-β-glucosides; hesperidin, hesperetin-7-(α-L-rhamnosyl)-6-β-glucoside; 3-quercitrin, quercetin-3-α-L-rhamnoside.

Table 5. Monoterpenols Released by *P. angusta* X349 α-L-Rhamnosidase from the Muscat Glycoside Extract (1 mg)

Monoterpenol (ng)	*P. angusta* Blank	α-L-Rhamnosidase Treatment
Linalool	0	394
α-Terpineol	0	25
Citronellol	0	21
Nerol	0	72
Geraniol	0	358

two hypothetical protein sequences, ramA-like sequences (CAB53318, CAB55731) from the unfinished chromosomal sequence of *Streptomyces coelicolor*.[22]

Hydrolysis of natural flavonoid rhamnoglycosides and grape monoterpenyl glycosides

A natural substrate for the yeast α-L-rhamnosidase could be the flavonoids of plant origin, such as naringin, rutin, and hesperidin. The relative activity of the enzyme for the natural flavonoid rhamnoglycosides is presented in Table 4. The enzyme released only L-rhamnose from naringin, rutin, and hesperidin, which are rhamnosyl glycosides containing L-rhamnose at the non-reducing terminal, and flavonoid rhamnopyranoside, 3-quercitrin. The best substrate was naringin, followed by rutin, hesperidin, and 3-quercitrin. The L-rhamnose residue is α-1,2-linked to a β-glucosidic residue in naringin, α-1,6-linked in rutin and hesperidin, and directly linked to the aglycon in 3-quercitrin. To the specificity for the type of linkages to the β-D-glucoside, the enzyme seems to be more specific towards α-1,2-linkage to β-D-glucose than α-1,6-linkage. *Sphingomonas* sp. α-L-rhamnosidase with similar substrate specificities has been reported.[10]

The enzyme was added to a sodium phosphate buffer (pH 6.5) containing the glycosides extracted from Muscat juice, and incubated at 30°C for 2 days. The results on the liberation of monoterpenols by the α-L-rhamnosidase are shown in Table 5. The enzyme efficiently liberated monoterpenols, especially linalool and geraniol, from the glycoside extract. Other α-L-rhamnosidases activity from *A. niger*[18] and *P. decumbens*[6] have been described as active towards grape monoterpenyl disaccharide glycosides.

The wide spectrum of activity of this α-L-rhamnosidase toward monoterpenyl rhamnosyl glycosides and flavonoid rhamnosyl glycosides suggests the practical usefulness for aroma improvement of grape juice and derived beverages, and for degradation of bitter flavonoids, naringin and hesperidin of citrus fruits.

With regard to technological applications during juice processing and winemaking, the *P. angusta* α-L-rhamnosidase may be useful due to the high tolerance to glucose and ethanol, their specificity for the aglycone moieties of grape glycosides, and their hydrolytic activities to natural flavonoid rhamnoglycosides.

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