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ORIGINAL RESEARCH PAPER

Dose-dependent significance of monosaccharides on intracellular α-L-rhamnosidase activity from *Pseudoalteromonas* sp.

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Abstract Intracellular α-L-rhamnosidase (EC 3.2.1.40) from the psychrotolerant *Pseudoalteromonas* sp. 005NJ showed a dose-dependent inhibition for L-rhamnose (IC_{50} = 20 mM) and D-ribose (IC_{50} = 95 mM), whereas D-glucose and L-fucose presented a lower inhibition, with IC_{50} values as high as >0.5 and >0.2 M, respectively. On the other hand, D-fructose enhanced enzyme activity threefold, reaching a plateau of maximum specific activity between 0.2 and 0.4 M of this monosaccharide. Both effects, low inhibition and stimulation, caused by key fruit sugars (glucose and fructose), make this biocatalyst an interesting system in terms of its potential application for debittering fruit juices.

Keywords Fructose · *Pseudoalteromonas* · Psychrotolerant · Rhamnose · Rhamnosidase · Ribose

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Introduction

α-L-Rhamnosidases, together with α-L-arabinofuranosidases and β-D-glucosidases are used as debittering agents for citrus fruit in food and beverage industries, acting through the release of rhamnose from the plant glycoside naringin (Cui et al. 2007). Glycoside hydrolases are usually inhibited by simple sugars (Birgisson et al. 2004). Thereby, biocatalysts with a better kinetic performance in presence of high sugar concentrations are required for fruit juices treatment (Camerlingo et al. 2007).

Additionally, low temperatures are frequently convenient for biotransformations of food and volatile organic compounds. Cold-active enzymes carry structural features that increase their catalytic efficiency at low temperatures in comparison with their mesophilic and thermophilic counterparts. This explains the technological interest of microorganisms physiologically adapted to cold-environments (Lang et al. 2005).

Bacteria belonging to *Pseudoalteromonas* genus, frequently reported as producers of bioactive compounds, are usually isolated from marine environments. *Pseudoalteromonas* sp. 005NJ, isolated from the sub-Antarctic sea, was selected for its cold-active α-L-rhamnosidase activity. Although this strain grows at low temperatures, its α-L-rhamnosidase displayed considerable activity (50%) at 25°C (Orrillo et al. 2007). This study deals with the effect of monosaccharides on production and activity of intracellular α-L-rhamnosidase from this psychrotolerant marine bacteria.

Materials and methods

Microbial source and cultivation

Pseudoalteromonas sp. 005NJ was grown at 25°C in a medium containing (g l^{-1}): milk peptone 10, yeast extract 5 and NaCl 35, in an orbital shaker (140 rpm). After 12 h, different carbon sources at 5 g l^{-1} (see Table 1) were added and the incubation was continued for 6 and 24 h before cells harvesting. Duplicate cultures for each carbon source were performed. Samples (20 ml) were collected by centrifugation (12,000*g* for 10 min), washed twice with 9 g NaCl l^{-1} and suspended in 5 ml 0.1 M buffer Tris/HCl pH 7.8. After that, they were disrupted once through a French press at 25,000 psig and finally centrifuged (12,000*g* for 10 min) to eliminate cell debris. Supernatants were frozen at −20°C until processing.

Analytical assays

Intracellular protein extract samples were dialysed against 20 mM Tris/HCl pH 7.8 during 6 h (changing dialysis buffer after 3 h) to remove remaining reducing sugars. For α-L-rhamnosidase quantification, each reaction contained 28.5 µl substrate [70 mM *p*-nitrophenyl-α-L-rhamnopyranoside (*p*-NPR) in dimethylformamide], 870 µl buffer (0.1 M Tris/HCl pH 7.8) and 100 µl enzyme sample. Reactions were performed during 1 h at 40°C and stopped by adding 100 µl 0.1 M NaOH. One unit of activity was defined as the amount of enzyme that released 1 µmol *p*-NPR in 1 h at 40°C.

Protein concentration was determined according to Bradford, using bovine serum albumin as standard. The four parameter sigmoid model [a + (b − a)/(1 + 10^(x − c))] was applied for calculation of IC_{50} (concentration at which activity is 50% inhibited). It was performed by using the BioDataFit software (Chang Bioscience Inc. http://www.changbioscience.com).

Table 1 Carbon sources (5 g l^{-1}) effect on the intracellular α-L-rhamnosidase specific activity of *Pseudoalteromonas* sp. 005NJ

Monosaccharide	α-L-rhamnosidase specific activity (units mg^{-1})	
	6 h	24 h
Control (without sugar addition)	20 ± 2	16 ± 1
L-arabinose	13 ± 1	20 ± 1
D-fructose	9 ± 1	63 ± 4
L-fucose	22 ± 1	Nd
D-galactose	11 ± 1	Nd
D-glucose	8 ± 1	8 ± 1
L-rhamnose	967 ± 79	534 ± 36
L-xylose	26 ± 1	75 ± 9
L-rhamnose + D-glucose	10 ± 1	Nd
L-rhamnose + D-galactose	814 ± 88	Nd

The activity measurements were performed at 6 and 24 h after adding the carbon source using *p*-NPR as substrate. Before measuring activity the samples were dialysed to avoid the influence of unconsumed sugars. Nd = not determined

Results and discussion

Effect of the carbon source on α-L-rhamnosidase production

With the aim of studying α-L-rhamnosidase production from *Pseudoalteromonas* sp. 005NJ, several monosaccharides as carbon sources were assayed, individually or combined, and evaluated after 6 and 24 h of cultivation (Table 1). The highest enzymatic specific activity was reached with L-rhamnose after 6 h, while D-glucose decreased the activity values to levels below to those found in the control medium (without the addition of a monosaccharide as carbon source). When both monosaccharides were added together, the low production detected with glucose was not reverted by the presence of rhamnose. Furthermore, D-galactose showed a similar effect as glucose, although the addition of rhamnose induced high levels of enzyme activity. These results are in good agreement with that previously observed in other bacteria and fungi (Orejas et al. 1999; Birgisson et al. 2004).

The other monosaccharides assayed did not induce considerable amounts of enzyme; in comparison with rhamnose. Nevertheless, L-xylose and D-fructose after 24 h showed significant activity values, while rhamnose resulted in a decrease of 24% at the same time (Table 1).

Effect of monosaccharides on the α-L-rhamnosidase activity

The remarkable low inhibition displayed by glucose ($IC_{50} > 0.5$ M) over 005NJ α-L-rhamnosidase follows

a similar profile to that reported for the same enzyme of *Pichia angusta* X349 (Yanai and Sato 2000). This is in contrast to the strong inhibition of this monosaccharide upon *Aspergillus niger* rhamnosidase (Spagna et al. 2000). Also, L-fucose exhibited a low inhibition with increasing concentrations, although the IC_{50} value was slightly lower (>0.2 M) (Fig. 1).

On the other hand, rhamnose showed the highest inhibition, reaching 15% of the initial activity at 0.2 M (IC_{50} of 20 mM), while D-ribose conserved 40% of the activity at the same concentration (IC_{50} of 95 mM) (Fig. 1). Recently, the binding sites of rhamnose in the structure of crystallized α-L-rhamnosidase from *Bacillus* sp. were identified, explaining the effect of the end product on enzyme activity (Cui et al. 2007).

Tree fruits, like apples, contain fructose at between 40 and 70 g l^{-1}, usually in combination with sucrose and glucose (Camerlingo et al. 2007). While α-L-rhamnosidase from *Aspergillus niger* was strongly inhibited by fructose in a comparable manner to that found with rhamnose (Spagna et al. 2000), our findings showed that α-L-rhamnosidase from *Pseudoalteromonas* sp. 005NJ was stimulated threefold in presence of 18 g fructose l^{-1}, and remained at the same level up to 70 g l^{-1} (Fig. 1). Phosphorylated fructoses at 0.03–2 mM have allosteric effects,

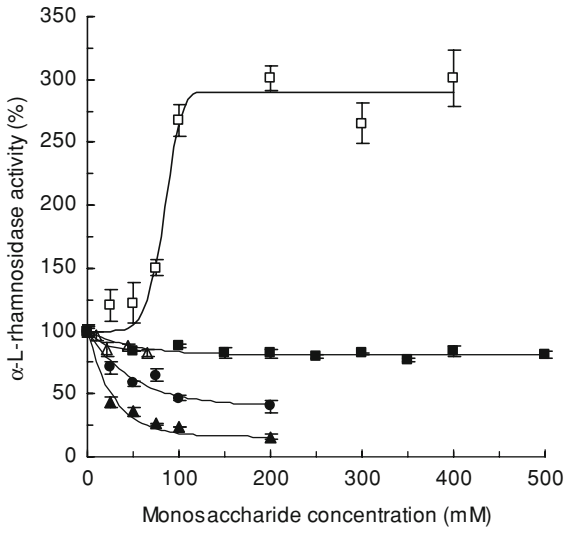

Fig 1 Dose-dependent effect of monosaccharides concentration on the α-L-rhamnosidase activity of *Pseudoalteromonas* sp. 005NJ. ■ D-glucose, Δ L-fucose, ▲ L-rhamnose, ● D-ribose and □ D-fructose. The 100% of the activity corresponded to 351 U ml^{-1}

activating several enzymes of sugar metabolism; while few enzymes of vegetal, mammalian and microbial origin are stimulated by fructose itself (Boehlein et al. 2005). So far, there are no reports describing fructose stimulation of glycosyl hydrolases of biotechnological interest, however there is some evidence that bacterial levansucrase can be slightly stimulated by fructose as well as by fructose 1,6-biphosphate (Euzenat et al. 1998; Lyness and Doelle 1983).

Despite the psychrotolerant character of *Pseudoalteromonas* sp. 005NJ, its rhamnosidase displayed only 6% of the activity at 4°C. This feature implies that food biotransformations could be carried out at moderate, although not cold, temperatures. However, the thermal instability of the biocatalyst could be favourable for its removal or inactivation process at relative low temperatures (Orrillo et al. 2007). The feasibility to carry out both, biotransformation and biocatalyst removal at moderated temperatures, could contribute to natural flavour conservation. At present, one limiting factor to overcome for 005NJ α-L-rhamnosidase applications in food and beverage areas is its low activity in acidic media. The improvement of enzyme activity at low pH is an important subject for further research.

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