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ORIGINAL PAPER

Cross-linked α-L-rhamnosidase aggregates with potential application in food industry

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Abstract α-L-Rhamnosidase (E.C. 3.2.1.40) from *Brevundimonas* sp. Ci19 was immobilized as a cross-linked enzyme aggregate (CLEA) via precipitation with acetone followed by glutaraldehyde cross-linking. The effects of precipitation and cross-linking on CLEA activity were investigated and characterized. Sixty percent acetone solution and 2.0 % glutaraldehyde were used at pH 7.0 for 1-h cross-linking reaction. The yield of rhamnosidase-CLEA was approximately 80 % starting from crude extracts or pure enzyme suggesting non-purification steps are required for extended use. No significant differences in optimum pH and temperature values of the enzyme were recorded after immobilization. The rham-CLEA recycled 4 times showed about 80 % rhamnosidase activity; meanwhile, in the fifth and six recycled time, the enzyme activity was reduced to about 40 and 20 %, respectively.

Keywords Immobilization · α-L-Rhamnosidase · *Brevundimonas* sp. Ci19 · Cross-linked enzyme aggregate (CLEA)

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Introduction

α-L-Rhamnosidase (EC 3.2.1.40) catalyzes hydrolysis of terminal rhamnosyl group from naringin to release prunin and rhamnose. These enzymes are widely distributed in plants and microorganisms including fungi and bacteria and also have been found in animal tissues [1]. In the food processing industry, the rhamnosidases have been applied primarily for debittering of citrus juices. The presence of bitterness is a major limitation in the commercial acceptance of juice. Hydrolysis of the rhamnosyl residue in naringin can reduce the bitterness and improve the quality of the juice [2].

Both free and immobilized α-L-rhamnosidases from different microorganisms have been explored for naringin hydrolysis. Immobilized enzymes are preferred over free enzymes in large-scale applications due to increased enzyme stability and reusability; however, loss of enzyme activity during the immobilization and reaction process, large mass transfer resistance between enzyme and substrates, and the inconvenience of choosing the right carriers are drawbacks of their use [3]. Immobilization of naringinases (enzyme complex of rhamnosidase and other enzymes) and rhamnosidases on the surface of different supports such as bagasse [4], hen egg white [5], Celite [6], ferromagnetic supports [7] were reported. However, surface immobilization is disadvantageous for industrial

purposes since the enzyme is exposed to environmental conditions and the high shear rate generally used in reactor. Other immobilization strategies by enzyme encapsulation–entrapment were developed on PVA, alginate, silica-based sol–gel matrices, and blends [8–10]. The procedures for encapsulation require a food-grade matrix associated to the presence of cross-linker agent. The encapsulation procedures are sometimes tedious and labor intensive. Alternatively, cross-linked enzyme aggregates (CLEAs) can be produced by simple method of enzyme precipitation and cross-linking with bifunctional reagents such as glutaraldehyde or others [11]. Hence, CLEAs are a versatile and reusable system able to convert a biological catalyst into a process biocatalyst showing high mechanical resistance to shear [12]. In a previous work, naringinase from *Penicillium decumbens* was immobilized in CLEA [13].

Brevundimonas sp. Ci19 rhamnosidase has shown naringin hydrolysis capability and was active in the presence of metabisulfite, ethanol, and glucose, environmental conditions commonly encountered in winemaking. The α-L-rhamnosidase from *Brevundimonas* sp. Ci19 could have potential for improving the quality of wine and citrus juice and reducing the bitterness [14]. Based on the enzyme properties and potential applications, the preparation of *Brevundimonas* sp. Ci19 rhamnosidase CLEAs (rham-CLEAs) and a comparison of their properties and reusability with those of the free enzyme are reported in the present work.

Materials and methods

Chemicals

Commercially available rhamnose and 4-nitrophenyl-α-L-rhamnopyranoside (4-NRP) was purchased from Sigma–Aldrich (St. Louis, USA). Ammonium sulfate and glutaraldehyde (50 %) were acquired from Cicarelli (Buenos Aires, Argentina). All other chemicals used were of analytical grade.

Protein determination

Protein content was determined by the Lowry's method as previously reported [14].

α-L-Rhamnosidase

α-L-Rhamnosidase crude extracts and partially purified enzyme were produced from batch cultures of *Brevundimonas* sp. Ci19 as previously reported [14].

α-L-Rhamnosidase activity

Soluble α-L-rhamnosidase activity was assayed on 4-nitrophenyl-α-L-rhamnopyranoside (4-NRP) as previously reported [14]. The activity of α-L-rhamnosidase on CLEA (rham-CLEAs) was assayed in 1.0 ml reaction volume containing 0.10 g CLEA, and 15 μl of substrate (100 mmol l^{-1} of 4-NRP in dimethylformamide) and 1.0 ml of buffer.

One enzyme unit was defined as the amount of enzyme that released 1.0 μmol of 4-NP in at the indicated temperature for minute.

Preparation of CLEA

Cold acetone solution (5 °C) was added up to the final concentration of 60 % (v/v) under agitation in 10 ml crude enzyme solution to precipitate the enzyme. After 30 min of dissolving the precipitate in 50 mM PBS (pH 7.00), glutaraldehyde was added slowly to the final concentration of 2.0 % (v/v), and the mixture was kept at 0 °C under stirring for 1 h. Finally, the resulting CLEA was washed three times with 5.0 ml of 100 mM PBS (pH 7.0) and centrifuged at 10,000×*g* at 4 °C for 10 min. All the steps were performed at 0 °C.

The activity recovery in CLEAs was calculated as follows:

$$\text{Activity recovery (\%)} = (\text{Total activity of CLEA in units/Total crude enzyme activity used for CLEA Production in units}) \times 100$$

Effect of precipitant and cross-linking agent on CLEA preparation

Different ammonium sulfate and acetone solutions (20, 40, 60 %, v/v) and glutaraldehyde concentrations (1.0, 1.2, 2.0, 4.0 %, v/v) were used during CLEA preparation from partially crude soluble rhamnosidase extract. The cross-linking reactions were performed at 0 °C and pH 7.0 (50 mM PBS) for 1 h.

Effect of temperature and pH on activity

To determine the optimum temperature of soluble rhamnosidase and rham-CLEAs, enzyme activities were measured in the temperature range of 8–37 °C and pH 7.00 for 1 h.

The effect of pH on enzyme activity was determined in the pH range of 4.00–8.72 at 30 °C. The optimum pH was determined by incubating each enzyme preparation with 4-NRP substrate in Mc Ilvaine buffers at pH 4.0–6.0 and in PBS at pH 7.0–8.7.

Reusability

The reusability of rham-CLEAs was tested by repeated use of the enzyme in the presence of 4-NRP incubated at 30 °C and pH 7.0 for 1 h each cycle. CLEAs were recovered from the reaction media by centrifugation and washed three times with PBS buffer at pH 7.0.

Statistical analysis

Statistical analysis was performed using Infostat (2008) and Minitab 1.4 statistical experiment design software. Results are presented as the mean ± standard deviation. Statistical significance values for the means were evaluated using one-way analysis of variance (ANOVA), and subsequent comparisons were performed using Tukey's post hoc test. Differences were accepted as significant when $p < 0.05$.

Result and discussion

CLEA preparation

Effect of precipitant concentration

Initially, the effect of acetone and ammonium sulfate on the activity recovery in the precipitated aggregates was analyzed. The protein concentration in the crude extract was 134 mg/ml with a α-L-rhamnosidase specific activity of 9.55 ± 0.11 U mg protein^{-1} assayed with 4-NPR as substrate and 20.20 ± 0.29 U mg protein^{-1} over the naringin substrate [14]. Activity recoveries were calculated after precipitation and subsequent cross-linking with glutaraldehyde. The results are shown in Table 1. Activities values were significantly modified by the precipitant concentration ($p < 0.001$). As ammonium sulfate or acetone concentration was increased, activity recovery in the CLEA also increased and no significant differences in the precipitation step with the addition of acetone or ammonium sulfate at 60 % ($p = 0.2561$) were found. Precipitation increases the stability of proteins, since it decreases the surface area that is in contact with solvent [15]. Moreover, precipitation conditions may result in a more active conformation of proteins [16]. SEM images of CLEAs have shown that CLEAs were coarse-grained and less structured at low ammonium sulfate concentration, whereas at high ammonium sulfate concentration, they were fine-grained and more structured with many cavities [17]. Considering the potential use of α-L-rhamnosidase in food processing, acetone was selected as precipitating agent because after recovering the enzyme pellet the solvent can be easily removed by evaporation.

Table 1 Effect of precipitation and cross-linking on α-L-rhamnosidase activity

Precipitant agent	Concentration (%)	α-L-Rhamnosidase activity (%)	
		after precipitation	after cross-linking
Ammonium sulfate	20	33.1 ± 0.3 a	ND
	40	62.0 ± 0.1 b	ND
	60	98.2 ± 0.2 c	ND
Acetone	20	6.7 ± 0.5 d	ND
	40	55.8 ± 0.3 b	35.4 ± 1.0 a
	60	95.4 ± 2.5 c	81.5 ± 8.5 e

Data are presented as mean value ± standard deviation calculated from at least three independent experiments. Values with different letters (a–d) are significantly different ($p < 0.05$)

ND no detected

By the addition of glutaraldehyde as cross-linking agent, the orderly arranged proteins are cross-linked and their conformation is stabilized [11]. Therefore, CLEAs retained nearly all of the free enzyme activity at higher precipitant concentrations. Nevertheless, 81.5 % activity recovery was achieved with 60 % acetone as precipitant agent after cross-linking in the present study. This value was higher than those reported for other CLEAs activity recovery prepared from *Candida rugosa* lipase [18].

Effect of glutaraldehyde concentration

Glutaraldehyde is generally the cross-linking agent of choice as it is inexpensive and readily available in commercial quantities [19]. However, not all enzymes give optimum results with glutaraldehyde as cross-linking agent [19, 20]. Glutaraldehyde is a small, reactive molecule which could penetrate the internal structure of the protein and react with amino residues that are crucial for enzyme catalytic activity [19]. Optimization of the CLEA procedure also involves optimization of the glutaraldehyde/enzyme ratio. If the amount of the cross-linker is too little, the enzyme molecule may still be too flexible, while too much cross-linker can result in a loss of the minimum flexibility needed for the activity of enzyme [19]. The influence of the amount of glutaraldehyde on the activity recovery is shown in Fig. 1. Activities values were significantly modified by the cross-linker concentration ($p < 0.001$). At 2.0 % (v/v) glutaraldehyde, maximum activity recovery of rham-CLEAs from the partially purified α-L-rhamnosidase extract was observed. This optimal concentration of glutaraldehyde for the α-L-rhamnosidase from *Brevundimonas* sp. Ci19 is lower compared to that previously used for naringinase [13].

The effect of pH and temperature on α-L-rhamnosidase activity

The pH effect on both free α-L-rhamnosidase and rham-CLEAs activities were determined in the pH range of

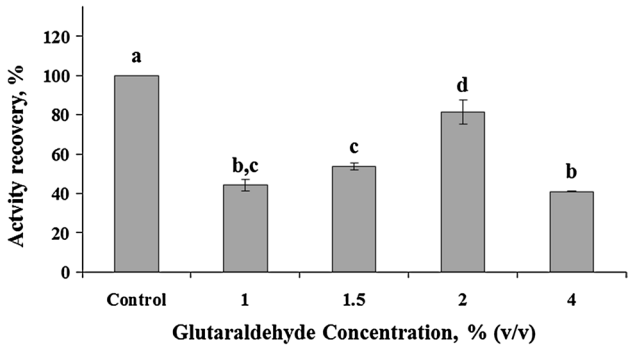

Fig. 1 Effect of glutaraldehyde concentration on CLEAs activity recovery. In each case, 10 mg of protein extract containing 9.55 U/mg^{-1} of rhamnosidase and 60 % acetone solution were utilized. The cross-linking procedures were performed at pH 7.0 (50 mM PBS) at 0 °C for 1 h. *Bars* with different letters (*a–d*) are significantly different ($p < 0.05$)

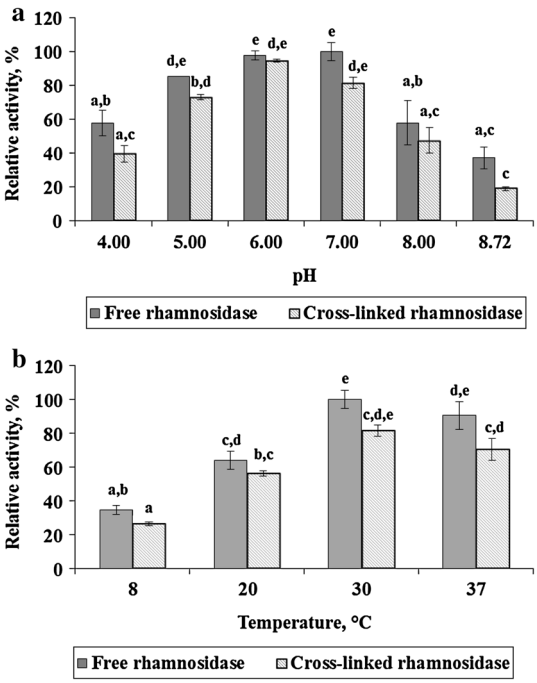

Fig. 2 Effect of pH and temperature on free and rham-CLEAs. **a** pHs at 30 °C; **b** temperatures at pH 7.0. *Bars* with different letters (*a–e*) are siginificantly different ($p < 0.05$)

4.0–8.7 at 30 °C. Free α-L-rhamnosidase activity showed a sharp optimum between pH 6.0 and 7.0 (Fig. 2a), but maximum activity value was detected at pH 6.0 in rham-CLEAs activity (94.7 %). On the other hand, in the alkaline pH range, the enzyme activity decreased. Also, rham-CLEAs showed 40 % approximately its recovery activity at pH 4.0, with no significant differences observed between free enzyme and rham-CLEAs activity ($p = 0.1044$). However, at pH 5.00, activities are

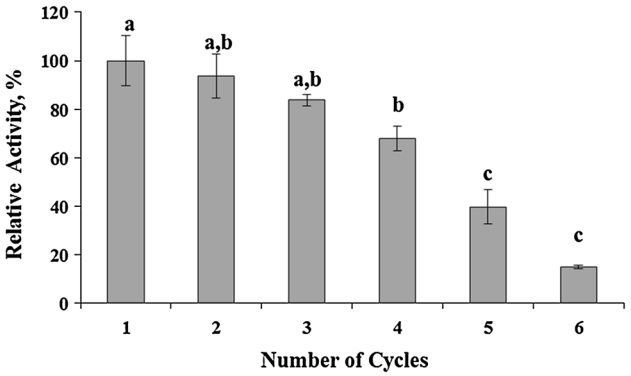

Fig. 3 Reusability of rham-CLEAs on pNPR hydrolysis at 30 °C and pH 7.0 incubated for 1 h per cycle. *Bars* with different letters (*a–c*) are significantly different ($p < 0.05$)

significantly different ($p = 0.006$) between free enzyme (85.3 %) and rham-CLEAS (73 %).

Regarding the effect of temperature at pH 7.0 (Fig. 2b), maximum activity values were observed in the range of 30–37 °C (Fig. 2b), with no significant differences observed between free enzyme and rham-CLEAs activity (at 30 °C, $p = 0.053$; at 37 °C, $p = 0.1102$). At 20 °C, the soluble enzyme and rham-CLEAS retained 63.8 and 56.1 % of their activity, respectively.

Reusability

The operational stability of rham-CLEAs produced was evaluated with 4-NRP at 30 °C, pH 7.0. Results of recycle experiments are shown in Fig. 3. The activity of the freshly prepared matrices in the first run was defined as 100 %. The activity remained constant until three recycles with no significant differences ($p = 0.2689$) but then it decreased. A reduction to approximately 30 % of enzymatic activity was observed after three reutilization cycles. Similar results were previously reported [13].

These results indicate that the rham-CLEAs can be reused without appreciable loss of activity. The lower hydrolysis obtained in cycles 5 and 6 could be due to mechanical losses of CLEAs during the washing and centrifugation procedures.

Conclusion

In this work, rham-CLEAs were produced with a high enzymatic activity using acetone as precipitating agent and glutaraldehyde (2.0 %) as cross-linking agent, at pH 7. The immobilization of *Brevundimonas* sp. Ci19 α-L-rhamnosidase in CLEAs is a simple and inexpensive process and offers the highest degree of efficiency and

stability. The rham-CLEAs can be synthesized from crude extracts avoiding expensive purifications steps, time, and labor. Also, the rham-CLEAs have similar profile of optimum pH and temperature than soluble enzyme which suggest no change in wine and citrus current main processes to be used. The rham-CLEA technique offers great potential for significant improvements in rhamnosidase application in the citrus-processing industry and in winemaking with reduction in costs by enzyme reuse.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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