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Purification and partial characterization of *Oenococcus oeni* exoprotease

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Abstract

The exoprotease from *Oenococcus oeni* produced in stress conditions was purified to homogeneity in two steps, a 14-fold increase of specific activity and a 44% recovery of proteinase activity. The molecular mass was estimated to be 33.1 kDa by gel filtration and 17 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). These results suggest that the enzyme is a dimer consisting of two identical subunits. Optimal conditions for activity on grape juice were 25°C and a pH of 4.5. Incubation at 70°C, 15 min, destroyed proteolytic activity. The SDS–PAGE profile shows that the enzyme was able to degrade the grape juice proteins at a significantly high rate. The activity at low pH and pepstatin A inhibition indicate that this enzyme is an aspartic protease. The protease activity increases at acidic pH suggesting that it could be involved in the wine elaboration.

Keywords: Exoprotease; Purification; *Oenococcus oeni*

1. Introduction

Lactic acid bacteria are nutritionally fastidious microorganisms, requiring complex organic media for growth. The lactics have lost their ability to synthesize many specific compounds required for metabolic activity and require certain preformed compounds such as vitamins and amino acids for growth, in addition to their organic compounds [1–3]. In laboratory cultivation, many workers find it necessary to augment standard culture media of yeast extract and protein hydrolysates with fruit or vegetable juices. Thus variations in the susceptibility of wines to lactic acid bacteria are partly due to differences in available nutrients and metabolic intermediates, as well as variations in particular lactic strains [4].

We demonstrated that *Oenococcus oeni* isolated from Argentinean wine, the lactic acid bacteria most commonly reported in inducing the malolactic fermentation in wines, has extracellular proteolytic activity. The studies on the synthesis of exoproteases have mostly been confined to growing cultures, in which exoprotease synthesis occurs in two stages of growth [5–7]. The proteolytic activity of *O. oeni* on the nitrogenous macromolecular fraction of white and red wines was reported [8,9]. When subjected to a total energy and nutrient starvation regime, cells of *O. oeni* showed extracellular proteolytic activity after 2 h of incubation at 30°C [10]. The exoprotease production by starved cells of *O. oeni* suggests that it could be an effective mechanism for survival in the stress conditions encountered during winemaking.

In this paper, we examined some of the physical and biochemical properties of *O. oeni* protease from stress conditions, purified to homogeneity.

2. Materials and methods

2.1. Microorganism

O. oeni X_2L was isolated from red wine [11].

2.2. Growth and starvation conditions

O. oeni X_2L was grown in a basal medium containing: 10 g yeast extract, 1.0 ml Tween 80 and 57 ml grape juice per liter distilled water. The pH was adjusted to 4.8 with

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0.1 mol l^{-1} HCl and the medium was sterilized for 15 min at 121°C. Cells were grown to the exponential phase of growth ($OD_{560} = 0.6$), were harvested by centrifugation at $10\,000 \times g$ for 15 min at 4°C, washed and then suspended in citrate buffer 0.05 mol l^{-1} pH 5 and incubated for 2 h at 20°C.

2.3. Proteolytic activity and its release during starvation

At 2 h of starvation, cells were centrifuged and the collected supernatant was assayed for proteolytic activity. The modified Cd-ninhydrin method [12], with grape juice autoclaved as substrate, was utilized. After 1 h incubation at 30°C, the reaction was stopped by the addition of 0.65 ml 24% trichloroacetic acid (TCA). To a sample of TCA supernatant (0.5 ml), 1.7 ml Cd-ninhydrin reagent was added. A control precipitated with TCA immediately before incubation was conducted in all cases.

2.4. Analysis of protein content

The proteins were determined by the reaction with Coomassie brilliant blue G-250 [13].

2.5. Extracellular protease purification

Step 1: Ammonium sulfate precipitation: solid ammonium sulfate was added to the cell-free supernatant (starvation conditions) to 80% saturation. The mixture was stirred for 1 h at 4°C. Precipitated protein was collected by centrifugation at $30\,000 \times g$ for 30 min and was suspended in 0.05 mol l^{-1} citrate buffer. Then, it was dialyzed overnight at 4°C against three changes of the same buffer 10-fold diluted.

Step 2: Gel filtration chromatography: the dialyzed solution was applied to a column of Sephadex G-100 (1×50 cm), equilibrated with 0.05 mol l^{-1} citrate buffer containing 0.1 mol l^{-1} NaCl plus 0.02% sodium azide. The elution was carried out with the equilibration buffer at a flow rate of 25 ml h^{-1}. Fractions of 3 ml were collected and tested for proteolytic activity towards autoclaved grape juice. Those showing maximal specific activities were pooled and concentrated by ultrafiltration through Centricon-3 concentrators, Amicon.

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the procedure of Laemmli [14], using 11% acrylamide Hoefer SE 250 Mighty Small II (Pharmacia Biotech). Proteins were then visualized by silver staining (Bio-Rad laboratories).

2.7. Molecular mass determination

The molecular mass of the native protease was determined by the method of Andrews [15], using a 1×50-cm Sephadex G-100 column calibrated with known standard protein (Sigma laboratories): alcohol dehydrogenase (141 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). The molecular mass value of the purified protease was estimated by interpolation of the protease elution volume, relative to the elution volumes of the protein standards.

The molecular mass value of the purified protease was also estimated by comparing their migration rates through 11% acrylamide after SDS–PAGE with those of know standard proteins: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-P-dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.2 kDa).

2.8. pH optimum

The pH range for activity of the purified protease was determined using a modified Cd-ninhydrin method. A series of 0.05 mol l^{-1} K_2HPO_4–KH_2PO_4 was used to cover the pH range 6–7.5, and 0.05 mol l^{-1} citrate buffers were used for the pH range 3–6. The substrate was prepared in each of these buffers.

2.9. Temperature optimum

The temperature for enzyme activity of the purified protease was determined using modified Cd-ninhydrin assay, performed between 20 and 60°C.

2.10. Heat stability

Samples of the purified protease were checked for activity after heating at 30, 40, 50, 60 and 70°C for 15 min. The heated samples were immediately cooled by immersion of tubes in ice water. The proteolytic activity was performed on the samples.

2.11. Inhibitors of protease

The activity of the isolated protease was tested in the presence of various know inhibitors: EDTA, *o*-phenanthroline, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, ρ-hydroxymercuribenzoate, iodoacetate and 1,4-dithiothreitol. Protease was incubated with the inhibitor for 30 min at 30°C before addition of substrate and incubation then continued for 60 min.

2.12. Protease activity on grape juice

The purified enzyme, concentrated by filtration (Centricon-3 concentrators, Amicon) was incubated with autoclaved grape juice as substrate. To 1 ml of grape juice, 200 μl of a 0.2-mg ml^{-1} solution of the purified protease

Table 1
Purification of the exoprotease produced by *O. oeni* X_2L

Purification step	Total protein (mg)	Activity (mmol l^{-1})	Specific activity (mmol l^{-1} mg^{-1})	Purification factor	Recovery (%)
Stress supernatant	0.92	0.17	0.18	1	100
Ammonium sulfate (0–80%)	0.25	0.11	0.45	2.50	65
Sephadex G-100	0.03	0.075	2.50	14.00	44

(concentrated fraction from Sephadex G-100 column) was added. The contents of the tubes were mixed and incubated at 30°C. At 0, 30, 60 min, and 3 and 24 h, a portion (50 μl) was removed, added to 50 μl of electrophoresis buffer, and boiled for 5 min. The samples were applied to an 11% SDS–PAGE gel.

3. Results and discussion

Cell-free supernatants of starved cells of *O. oeni* after 2 h of incubation at 30°C in citrate buffer 0.05 mol l^{-1} pH 5 were used for exoprotease purification.

The protease from *O. oeni* was purified to homogeneity in two steps, ammonium sulfate precipitation and Sephadex G-100 gel filtration. Information on protein content, protease activity, specific activity and the efficiency of purification is summarized in Table 1. The enzyme was purified 14-fold, with a yield of 44%. Fig. 1 shows the elution profile from application of the material from sulfate ammonium precipitation onto a Sephadex G-100 column. The peak with protease activity was located between fractions number 20 and 30. The molecular mass corresponds to 33.1 kDa. The purity of the isolated enzyme was confirmed by gel electrophoresis under denaturing conditions. Only one stained protein band with molecular mass of 17 kDa was observed (Fig. 2). These results obtained under native and denaturing conditions indicate that the enzyme is a dimer consisting of two identical subunits.

The pH range for the proteolytic activity was narrow, from 4.0 to 5.5, and the optimum was determined to be 4.5.

Data on enzyme activity tested at various temperatures indicated that the purified protease was active from 15 to 37°C. Maximum activity occurred at 25°C. The thermostability studies showed a residual protease activity of 25% after incubation for 15 min at 60°C. When the protease was heated to 70°C, 15 min, none of the original activity remained.

To determine the type of protease from *O. oeni*, a number of inhibitors were tested (Table 2). The proteolytic activity was completely inhibited by pepstatin A. Activity at low pH and pepstatin A inhibition are characteristics of the typical aspartic protease [16]. The reducing agent, 1,4-dithiothreitol at a 10 mmol l^{-1} concentration, reduced

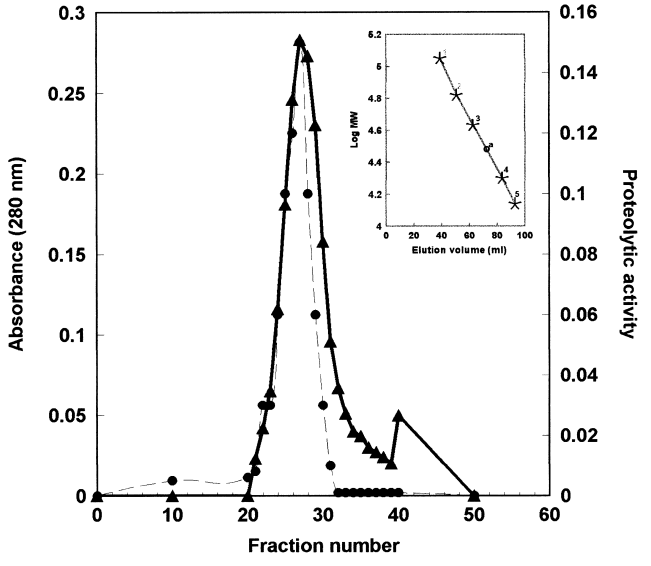

Fig. 1. Elution profile of exoprotease in Sephadex G-100 column chromatography: ▲, absorbance 280 nm; ●, proteolytic activity expressed as mmol l^{-1}. Insert figure: estimation of the molecular mass of exoprotease from *O. oeni* by Sephadex G-100 filtration: (1) alcohol dehydrogenase, (2) bovine serum albumin, (3) ovalbumin, (a) purified exoprotease, (4) chymotrypsinogen and (5) ribonuclease A.

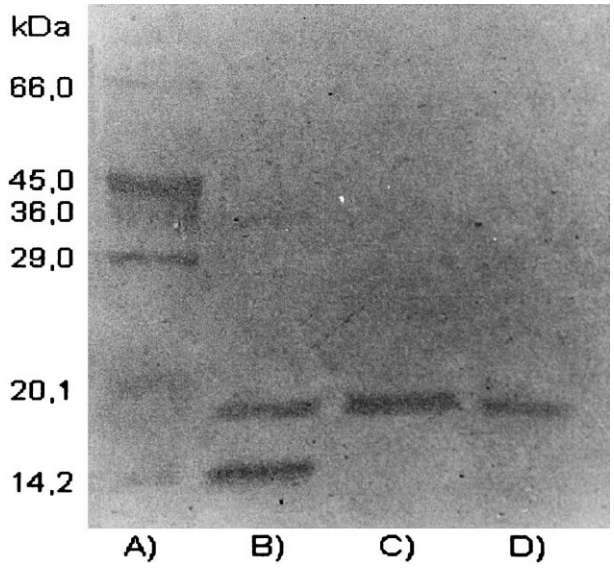

Fig. 2. PAGE patterns of fractions in different steps of the purification procedure of exoprotease from *O. oeni*. Lane A, molecular mass standards: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-P-dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa); lane B, cell-free supernatant; lane C, 0–80% $(NH_4)_2SO_4$ fraction and lane D, peak eluted from Sephadex G-100 column chromatography.

Table 2
Effect of inhibitors on purified protease activity from *O. oeni*

Inhibitor	Concentration (mmol l^{-1})	Class of inhibitor	Relative activity[a] (%)
Control			100
EDTA	10	Metal chelator	94
o-Phenanthroline	10	Metal chelator	89
Iodoacetate	10	Cysteine	103
ρ-Hydroxymercuribenzoate	10	Cysteine	90
PMSF	10	Serine	102
1,4-Dithiothreitol	10	Reducing agent	32
Pepstatin A	1	Aspartic	0

[a]Percentages of relative activities are means of two experiments with duplicate determinations.

activity by almost 70%, an indication that important disulfide bonds were present.

The proteolytic activity of the enzyme from *O. oeni* was measured by the disappearance of grape juice protein. The results observed in SDS–PAGE profile indicate that the enzyme was able to degrade the protein at a significantly high rate, evident after only 15 min of incubation (data not shown). Thus this enzyme behaved as a protease with a significant affinity for a specific substrate.

The malolactic fermentation is a catabolic pathway in which L-malic acid is enzymatically oxidized to L-lactic acid and carbon dioxide. Depending upon the strain of lactic acid bacteria involved, several by-products may be produced that impact the sensory properties in wine. *O. oeni* is the species most commonly reported in inducing the malolactic fermentation, and because the characteristics of wine, this microorganism must have strategies to confront the stress conditions. The exoprotease production by starved cells of *O. oeni* suggests that it could be an effective mechanism for survival in the stress conditions encountered during winemaking.

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