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FEMS Microbiology Letters 174 (1999) 41–47

FEMS MICROBIOLOGY LETTERS

A proteolytic effect of *Oenococcus oeni* on the nitrogenous macromolecular fraction of red wine

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Received 18 December 1998; received in revised form 25 February 1999; accepted 1 March 1999

Abstract

The proteolytic activity of *Oenococcus oeni* protease on the release of individual amino acids and peptides from a red wine macromolecular nitrogenous fraction was evaluated. 148.7 mg l^{-1} of individual amino acids were released and a difference of 109.5 mg l^{-1} with respect to the amino acids liberation from white wine was observed. Stimulatory amino acids for *O. oeni* growth, glutamic acid and proline, were the most important amino acids obtained by the protease activity. In the HPLC and spectral analysis of peptides before and after the protease activity of *O. oeni*, several changes have been observed. Three new peaks, six higher and one lower have been found in the chromatogram obtained after the enzyme action. The specific activity of *O. oeni* protease is higher on the red than on the white wine proteins and this is important considering the lower protein concentration in red wine.

Keywords: *Leuconostoc oenos*; *Oenococcus oeni*; Proteolytic activity; Red wine; Peptide; Amino acid

1. Introduction

Lactic populations in wine may reach levels equivalent to yeast populations (10^6–10^8 cells ml^{-1}). Aside from their primary transformation, it might be expected that by-products of their metabolism would have a continuing influence on the wine long after the bacterial cells were gone. It is believed that esterases, lipases and proteases of bacterial origin may play an important sensory role in wine undergoing malolactic fermentation [1]. *Oenococcus oeni (Leuconostoc oenos)* [2] is generally the microorganism involved in malolactic fermentation during wine elaboration. Malolactic fermentation by *O. oeni* takes place in the wine after the alcoholic fermentation, when the medium is depleted in assimilable nitrogenous compounds [3]. Amino acids are important for the growth of *O. oeni* strains, both as nitrogen and carbon sources [4–7]. The nutritional amino acids requirements by four strains of *O. oeni* were reported [7]. The amounts of proteins in wines reported in the

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0378-1097/99/$20.00
PII: S0378-1097(99)00119-6

literature varies in a great range (from 1 to 250 mg l^{-1}) depending on the variety of grape and the analytical method used for their quantification, but in general, red wines have less proteins than white wines. The predominant source are the grapes. Precipitation of unstable proteins in wine is responsible for haze formation in red wines and a lacquer-like deposit on inside surfaces of bottles of red wine [8,9]. Charpentier and Feuillat [10] reported that wine proteins and peptides come from the grape proteins and from the hydrolysis through the proteolytic action of extracellular yeast protease and from the release of proteins and peptides in the autolysis of the yeast. The production of two extracellular proteases by each of the four strains of *O. oeni* isolated from wine, the characterization of the enzymes of *O. oeni* X_2L strain and the influence of nutritional factors on its production were reported [11–13].

In a previous paper, we determined the proteolytic effect of *O. oeni* protease on the white wine proteins and polypeptides [14]. The protease action permits to find two new peptide peaks and an increase of another two peptide peaks. Arginine, that has a stimulatory effect on *O. oeni* growth, was quantitatively the most important amino acid obtained by the protease activity.

The aim of this work was to study the proteolytic effect of *O. oeni* on the proteins and polypeptides contents of red wine. A comparative analysis with the previous results obtained from the white wine nitrogenous macromolecular fraction was realized.

2. Materials and methods

2.1. Microorganism

O. oeni X_2L (formerly *L. oenos* X_2L), was isolated from Argentinean wine [15].

2.2. Medium and culture conditions

The basal medium contained per l: yeast extract, 10 g; glucose, 5.0 g; Tween 80, 1.0 ml and grape juice, 170 ml. Any alterations of these concentrations are noted in the text. The pH was adjusted to pH 4.5 with 0.1 M HCl before sterilization by autoclaving for 15 min at 121°C.

2.3. Isolation of the wine polypeptide and protein fraction

A commercial Cabernet Sauvignon red wine was used. To obtain the macromolecular fraction, the wine was dialyzed against tap water, for 48 h, using membranes with a pore size of 3500 Da (Spectrum Medical Industries, Los Angeles, CA, USA). The retentate was lyophilized.

2.4. Determination of the proteolytic activity

The assay mixtures contained: cell-free culture supernatants (enzyme solution), substrate (red wine dialyzed and lyophilized) and 0.05 M citrate buffer pH 5.0 to a 1.0-ml final volume. After 1 h incubation at 30°C, the reaction was stopped by the addition of 0.65 ml 24% trichloroacetic acid (TCA). A control precipitated with TCA immediately before incubation was carried out in all cases. To a sample of TCA supernatant (20–100 μl), depending on the concentration of amino acids expected, was 1.7 ml Cd-ninhydrin reagent (0.8 g ninhydrin was dissolved in a mixture of 80 ml 99.5% ethanol and 10 ml acetic acid, followed by the addition of 1 g $CdCl_2$ dissolved in 1 ml of distilled water) added. The mixture was heated at 84°C for 5 min, cooled and the absorbance at 507 nm was determined [16].

2.5. Total protein

The proteins of the dialyzed samples were determined by the reaction with Coomassie brilliant blue G-250 [17]. Calibration was carried out using bovine serum albumin (Sigma Chemical Company).

2.6. Amino acid analysis

Amino acid analysis was carried out by reverse phase HPLC (RP-HPLC) using a Waters liquid chromatograph controlled by the Maxima 820 Program. Samples were submitted to an automatic pre-column double derivatization with *o*-phtaldialdehyde (OPA) to determine primary amino acids and with 9-fluorenylmethylchloroformate (FMOC) to determine secondary amino acids [18]. All separations were performed on a Waters Nova-Pak C18 column (150×3.9 mm i.d., 60 Å, 4 μm). The detection was

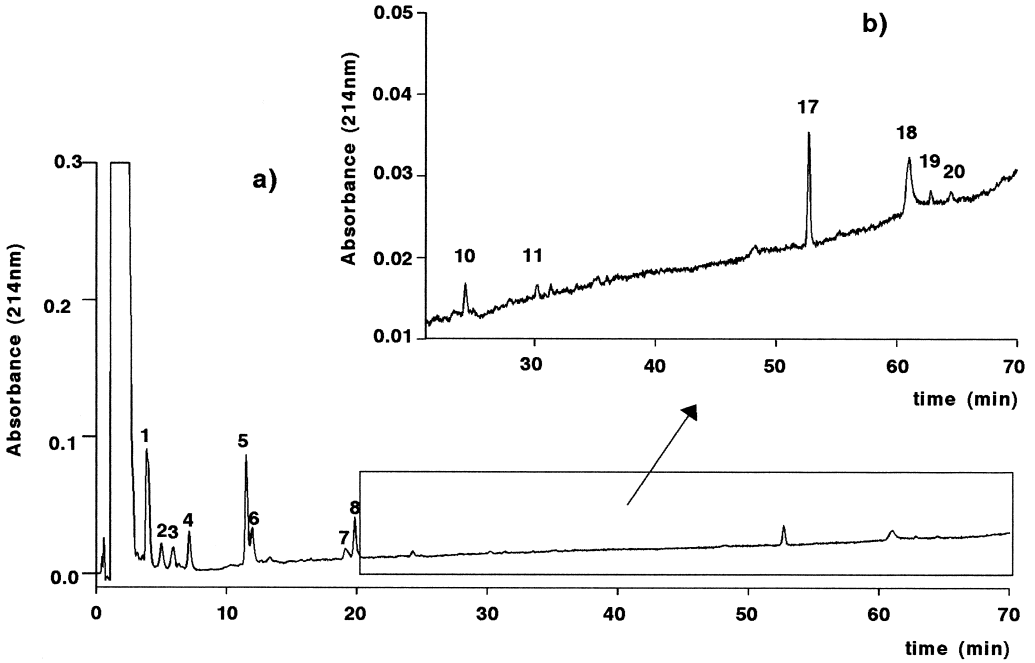

Fig. 1. HPLC chromatograms of peptides before the proteolytic activity of *O. oeni* X_2L. (a) Full scale 0.3 UAFS. (b) Amplified section, full scale 0.05 UAFS.

by fluorescence using wavelengths of excitation and emission at 340 and 425 nm, respectively, for OPA derivatives. For FMOC derivatives, the excitation and emission wavelengths were 250 and 335 nm, respectively. Samples were injected in duplicate onto the column after being filtered through a 0.22 μm filter. Prior to RP-HPLC analysis, all samples were diluted with 0.4 M borate buffer, pH 10.

2.7. Peptide analysis

Peptides were separated by RP-HPLC following the method described by Moreno-Arribas et al. [19]. A liquid chromatograph was used consisting of two Beckman M116 pumps, a Beckman System Organizer, a Waters M717 Plus automatic injector and a Beckman M168 Diode Array detector. Equipment control, obtention and processing of data were carried out using the Beckman Gold System program.

All separations were performed on a Waters Nova-Pak C18 column (150×3.9 mm i.d., 60 Å, 4 μm). Eluent A was 0.1% trifluoroacetic acid in

Table 1
Free amino acids (mg l^{-1}) before (A) and after (B) the proteolytic activity of *L. oenos*

Amino acids	Control (A)	After proteolytic activity (B)	(B)−(A)
Asp	24.9	31.2	6.3
Glu	74.2	100.1	25.9
Asn	16.9	22.0	5.1
Ser	24.4	32.8	8.4
Gln	0.0	3.0	3.0
His	12.0	13.1	1.1
Gly	18.5	24.3	5.8
Thr	23.7	30.0	6.3
Arg	27.3	35.2	7.9
Ala	50.4	64.9	14.5
Gaba	3.1	4.0	0.9
Tyr	11.8	13.6	1.8
Met	8.2	9.7	1.5
Val	36.6	45.9	9.3
Trp	6.2	6.7	0.5
Phe	29.2	35.3	6.1
Ile	30.1	37.5	7.4
Leu	47.2	59.3	12.1
Orn	4.0	5.3	1.3
Lys	32.8	40.7	7.9
Pro	33.1	48.7	15.6
Total	514.6	663.3	148.7

Gaba: γ-amino butyric acid.

water, eluent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient of B increased from 0 to 40% over 70 min. The flow rate was 1 ml min^{-1}. 50 μl of samples, previously filtered through a 0.45-μm membrane filter, were injected.

2.8. Spectral analysis

A spectral analysis has been done for the assessment of the peptidic nature of the chromatographic peaks and identification of the aromatic amino acid residues (tyrosine, tryptophan and phenylalanine) contained in the peptides. The methodology reported by Bartolomé et al. [20] has been used. Spectral data have been obtained by the photodiode array detector in the 190–390 nm range. The spectral parameters determined were wavelengths of the spectrum maxima, wavelengths of the second derivative spectrum maxima, both given by the software of the photodiode array detector and the convexity interval (the distance between the inflection points before and after the maximum in the original spectrum).

3. Results and discussion

The activity of *O. oeni* X_2L protease, obtained after 77 h incubation at 30°C in basal medium, was determined using the lyophilized red wine proteins as a substrate. The specific activity shows that 9.1 μmol of amino acids was liberate by the action of 1 mg of protein after 1 h incubation at 30°C. Taking into account the difference between the protein concentrations before and after the protease action, 2.66% of the macromolecules were hydrolyzed. Manca de Nadra et al. [14] reported that by the protease action of *O. oeni*, 2.25% of the nitrogenous macromolecular fraction of white wine was hydrolyzed.

The effect of the protease action on the release of individual amino acids from red wine proteins was evaluated by comparing samples taken before and after proteolysis (Table 1). From these results, 148.7 mg l^{-1} of individual amino acids has been released. Glutamic acid and proline, quantitatively the most important amino acids obtained by the protease activity (25.9 and 15.6 mg l^{-1}, respectively), and arginine (7.9 mg l^{-1}) have a stimulatory effect

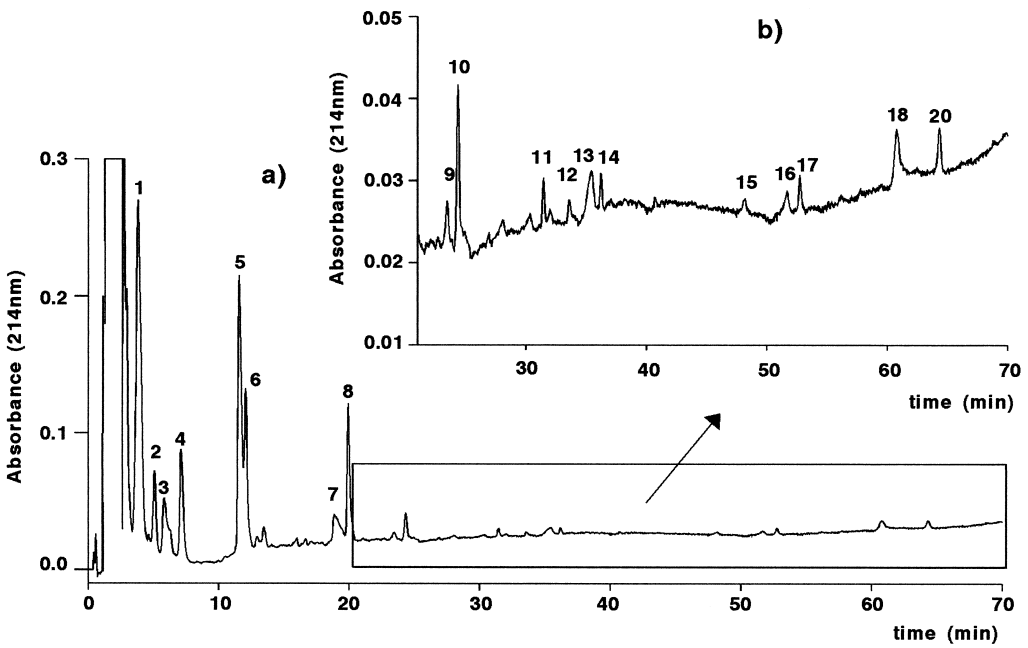

Fig. 2. HPLC chromatograms of peptides after the proteolytic activity of *O. oeni* X_2L. (a) Full scale 0.3 AUFS. (b) Amplified section, full scale 0.05 AUFS.

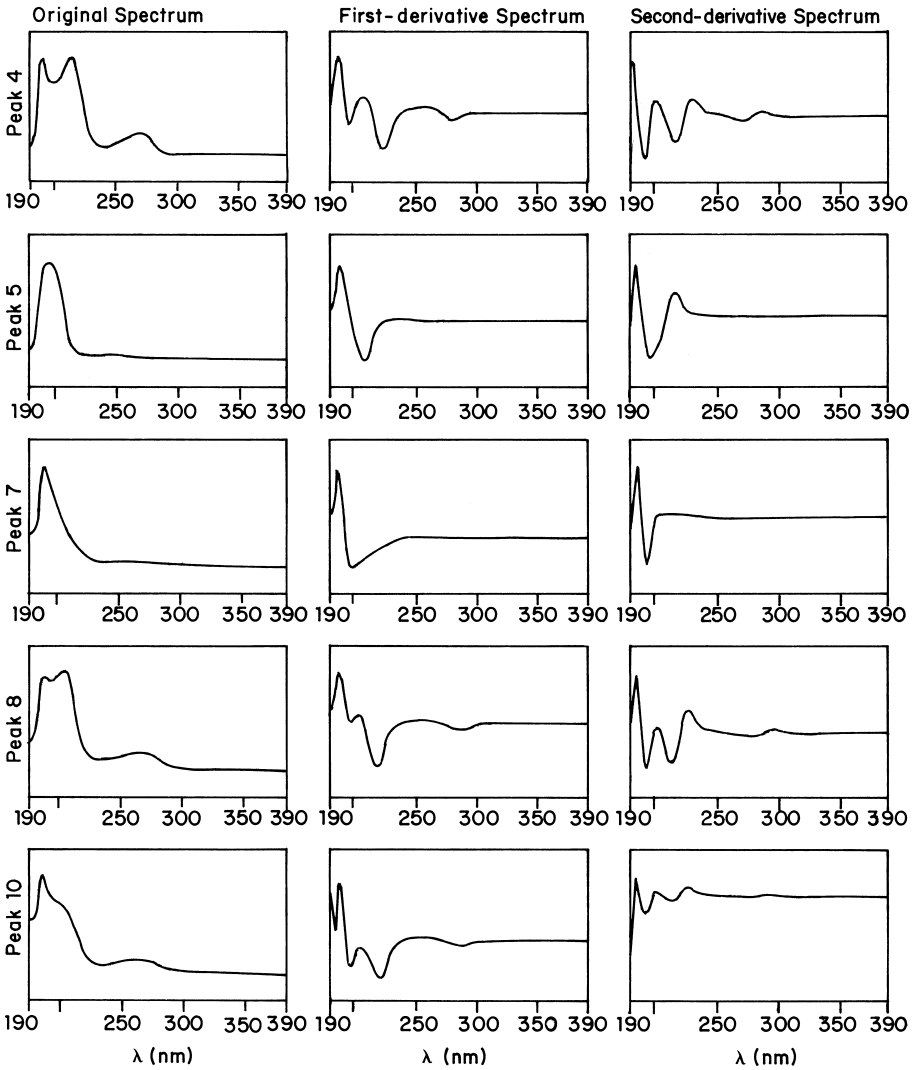

Fig. 3. UV original and first and second derivative spectra of peptide 4 (with tyrosine), 5 (with pheylalanine), 7 (without aromatic amino acids), 8 (with tryptophan plus phenylalanine) and 10 (with tryptophan plus tyrosine).

on the growth of *O. oeni* X_2L in synthetic medium added with L-malic and citric acids, as reported by Amoroso et al. [7]. On the other hand, leucine (12.1 mg l^{-1}) and alanine (14.5 mg l^{-1}) were reported as essential growth factors in the same conditions. The 39.2 mg l^{-1} of free amino acids released for the protease of *O. oeni* X_2L on the white wine proteins [14] shows a difference of 109.5 mg l^{-1} with respect to the amino acids liberation in red wine. Nevertheless, the lower protein concentrations in red wine, the action of proteolytic enzyme of *O. oeni* X_2L was more effective on red wine proteins.

Figs. 1 and 2 show the HPLC chromatograms obtained in the analysis of peptides before and after the protease activity of *O. oeni*. Peaks identified with the same number in Figs. 1 and 2 correspond to compounds with identical retention times and spectral parameters. In the first minutes, near the dead volume, amino acids and other not retained compounds elute [21]. Peptides elute with retention times

higher than 3 min. A total of 14 peaks have been detected in the analysis of peptides before the action of the protease (Fig. 1) and 19 peaks after the action of the protease (Fig. 2). The peaks have been submitted to spectral analysis. Peaks 2, 3 and 6 showed non-peptide type spectra. The spectrum maxima and convexity interval values of peak 4 are proper of peptides containing tyrosine (Fig. 3). Peaks 5, 8 and 10 show spectra proper of peptides containing phenylalanine, tryptophan plus phenylalanine and tryptophan plus tyrosine, respectively. Peaks 7, 9 and 11–20 show spectra proper of peptides not containing aromatic amino acids. A higher number of peptides has been found after the action of the protease. Peptides 9 and 12–16, present in the chromatogram showed in Fig. 2, were not present before the proteolytic activity of *L. oenos* (Fig. 1). Peptides 5, 7, 8, 10, 11 and 20 are in a higher concentration and peptide 17 in lower concentration after the protease action. No significant changes have been observed in the quantification of peptide 18. The last peptide peak after proteolytic enzyme of *O. oeni* on proteins and polypeptides of white wine appeared at a retention time of 40 min [14]. On the nitrogenous macromolecular fraction of red wine can differences be observed between the peaks before and after the enzyme action in the retention times from 40 to 70 min (Figs. 1 and 2), indicating differences in the profile of peptides not containing aromatic amino acids. The results obtained confirm that the protease enzyme of *O. oeni* X_2L has an activity on the nitrogenous macromolecular fraction of red and white wine. Taking into account that the variations in the susceptibility of wines to lactic acid bacteria are partly due to differences in available nutrients and metabolic intermediates, the presence of assimilable nitrogen sources has a significant impact on the potential for microbial growth. The higher specific activity of *O. oeni* protease on the red wine proteins is important in the essential amino acids liberation, considering the lower proteins concentration with respect to white wine proteins.

Acknowledgments

This work was carried out in the framework of the Iberoamerican Cooperation Program from the Spanish Ministerio de Educación y Cultura. The authors thank the financial support given by CIUNT and CONICET, Argentina and the Comisión Interministerial de Ciencia y Tecnología (Project ALI97-0630) España.

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