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Characterization of two extracellular proteases from *Leuconostoc oenos*

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Two extracellular proteolytic activities were characterized from *Leuconostoc oenos* isolated from Argentinian wines. Both activities were maximal with autoclaved grape juice as substrate. The temperature and pH optima for the two proteolytic activities were different (30 and 40°C, and pH 4.0 and 5.5, respectively). Both enzymes were thermostable and their activities were unaltered by heating at 70°C for 15 min. Metal ions were not required for the activities. Neither enzyme appeared to be a serine protease but both were strongly inhibited by cysteine and β-mercaptoethanol, indicating the involvement of disulphide bridges.

Key words: *Leuconostoc oenos*, proteases.

Wine is a poor medium for bacterial growth because of its limited content of nutrients. We have already reported the production of two extracellular proteases by each of four strains of *Leuconostoc oenos* (X_2L, L_2, m and ST) isolated from Argentinian wines (Rollán *et al.* 1993). One enzyme appears in the early growth phase and the other is maximal at the end of growth. The pH and temperature optima for the production of the two proteases from each strain differ and the effects of divalent metal ions differ within and between strains.

The proteolytic activities of *Leuconostoc oenos* X_2L on different substrates, the effects of inhibitors, pH and temperature on them and their thermal stability are the subjects of the present study.

Materials and Methods

Microorganism and Growth

Leuconostoc oenos strain X_2L (see Rollán *et al.* 1993) was grown in a basal medium containing 10 g yeast extract, 5 g glucose, 1.0 ml Tween 80 and 170 ml grape juice/ l distilled water. The pH was adjusted to 4.5 with 0.1 M HCl and the medium was sterilized for 15 min at 121°C.

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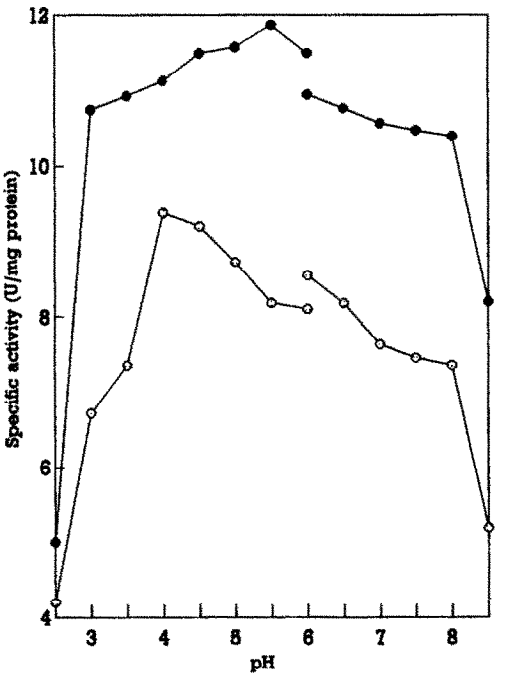

Figure 1. Effect of pH on the proteolytic specific activities produced by *Leuconostoc oenos* X_2L using autoclaved grape juice as substrate. ○—Protease I at 30°C; ●—protease II at 40°C. 0.05 M citrate buffers were used for pH 2.5 to 6.0 and 0.05 M phosphate buffers for pH 5.8 to 8.5.

Determination of Proteolytic Activity

The method of Hull (1947), modified by Citti *et al.* (1963), was

Table 1. Substrate specificity of proteases I and II from *Leuconostoc oenos* X_2L.

Substrate	Activity (nmol tyrosine released/mg. min) of:*	
	Protease I	Protease II
Grape juice†	7.6	12.0
Grape juice‡	3.3	9.0
Basal medium‡	4.8	7.2
Albumin§	1.3	9.6
Gelatin§	4.2	9.0
Casein§	0.6	9.0

*Cells were cultured in basal medium at 30°C. Protease I was assayed in the culture supernatant taken after 26 h growth and protease II was assayed after 77 h growth. The values are the means of duplicate experiments.
†Autoclaved.
‡Precipitated with $(NH_4)_2SO_4$ at 80% saturation and the pellet suspended in 0.2 M phosphate buffer, pH 7.0, and dialysed overnight against the same buffer.
§Filtered.

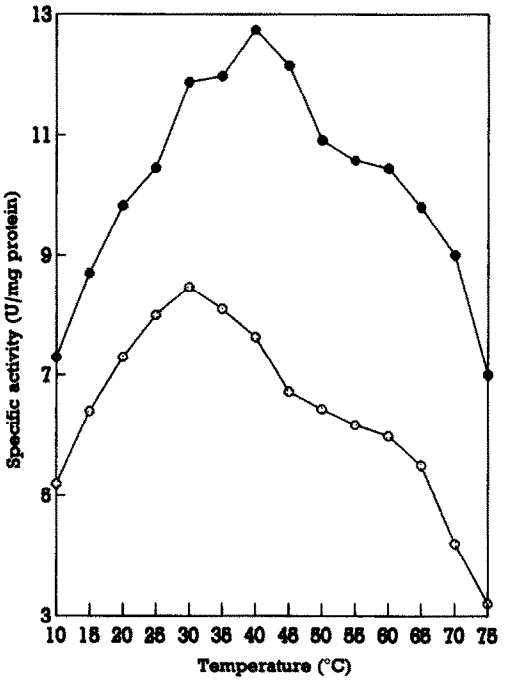

Figure 2. Effect of temperature on the proteolytic specific activities produced by *Leuconostoc oenos* X_2L using autoclaved grape juice as substrate. ○—Protease I at pH 4.0; ●—protease II at pH 5.5.

used to evaluate the two proteolytic activities in culture supernatants. Autoclaved grape juice was used as substrate. The reaction was developed at 30°C, pH 5, for 60 min, then stopped by adding 0.72 M trichloroacetic acid. The tyrosine released was determined with the phenol reagent, reading at 650 nm. One unit of protease activity (U) was defined as the amount releasing 1 nmol tyrosine per min under the assay conditions.

Results and Discussion

Leuconostoc oenos X_2L produces two extracellular proteolytic activities during growth at 30°C: protease I occurs in the early growth phase, optimally after 26 h, and protease II optimally after about 77 h, at the end of the growth (Rollán *et al.* 1993). Both proteases, taken at their optimal time of production, were maximal with autoclaved grape juice as substrate (Table 1). With this substrate, proteases I and II were optimally active at pH 4.0 and 5.5 and (Figure 1) and 30 and 40°C (Figure 2), respectively. Their apparent K_m values, determined by the usual Lineweaver-Burk plot, were 5.0 and 3.33 mg protein/ml, respectively.

Most proteases are optimally active at alkaline pH values but there are exceptions. For example, the protease of *Staphylococcus epidermidis* (Teufel & Götz 1993) had a pH optimum between 5 and 7 with casein as substrate and the serine protease from *Sta. aureus* (protease I) was optimal at pH 4.0 (Drapeau 1978).

Thermostability of the proteases was tested by exposure of the cell-free culture supernatants at different temperatures for 15 min. Protease I was thermostable up to 60°C; 10% inactivation occurred at 70°C and 25% activity still remained after 15 min at 80°C. Protease II was only stable below 50°C; 14% inactivation occurred above 50°C and only 15% of the activity remained after incubation at 70°C. The two proteases are thus different in their thermotolerance. Other proteases also show thermotolerance: that from *Sta. epidermidis* (Teufel & Götz 1993) was stable up to 50°C and 20% of its activity remained after incubation at 80°C for 25 min. These results are comparable with those of protease I, whereas the protease of *Sta. hyicus* subsp. *hyicus* (Ayora *et al.* 1994), which was only stable below 55°C with 40% activity remaining after 10 min at 65°C, is comparable with protease II.

The proteolytic activities of the cell-free culture supernatants were not affected when stored without any protector for ten days at 4, −20 or −70°C. After 30 days, however, both proteases were inactivated by 40% at all storage temperatures. Adding 20% glycerol did not alter these results. Different results were reported by Cowman *et al.* (1967) for the proteinase of *Streptococcus lactis*, which, on storage for 24 h at 3°C, lost 75% activity.

The action of some protease inhibitors is listed in Table 2. Metalloproteinases inhibitors, EDTA and *o*-phenanthroline did not affect the enzyme activities, indicating that a metal ion was not required. The proteases were not inhibited by iodoacetamide or *p*-hydroxymercuribenzoate, indicating that sulphydryl groups were not involved in their activities. The enzymes were also resistant to PMSF, a serine protease inhibitor. They were, however, strongly inhibited by 10 mM cysteine and β-mercaptoethanol and partially inhibited by dithiothreitol, indicating that disul-

Table 2. Effect of inhibitors on the extracellular proteases I and II of *Leuconostoc oenos* X_2L.*

Inhibitor	Concentration (mM)	Activity (% of control value) of:†	
		Protease I	Protease II
Control	–	100	100
Cysteine	1	83	98
	10	0	0
o-Phenanthroline	10	89	95
PMSF	10	109	110
β-Mercaptoethanol	1	129	144
	10	0	0
p-Hydroxymercuri-benzoate	1	85	106
	10	77	87
1,4-Dithiothreitol	1	78	80
	10	43	45
Iodoacetamide	10	112	113
EDTA	10	93	103

*Proteases incubated with inhibitor for 30 min at 30°C before addition of substrate (autoclaved grape juice) and incubation then continued for 60 min.

†Values are the average of duplicate experiments; 100% values were 7.6 and 12.0 U/mg protein for proteases I and II, respectively.

phide bonds are probably present at the active sites. From these results alone it is impossible to designate the enzymes as serine, cysteine, metal or aspartic proteinases.

To our knowledge this is the first characterization of proteases from *Leuconostoc oenos* isolated from wine.

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