Characterization of the Nitrogen Compounds Released during Yeast Autolysis in a Model Wine System

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The nitrogen composition of wines aged with yeast for a long period of time, as in the case of sparkling wines, depends on the composition of the base wine and on the compounds released by the yeast. In this paper, the release of the different classes of nitrogen compounds during autolysis of one of the strains of yeast used in the manufacture of sparkling wines has been studied. The yeast, *Saccharomyces bayanus*, was suspended in a model wine buffer, pH 3.0 and 10% ethanol, and incubated at 30 °C. Samples of the autolysate were taken after 4, 24, 48, 72, 168, and 360 h of autolysis. An electrophoretic and chromatographic study was conducted of the proteins, peptides with molecular weights higher and lower than 700 Da, and amino acids released during the autolysis. Using SDS−PAGE, it was observed that it was predominantly polypeptides with molecular weights lower than 10 000 that were released. Through HPLC of the fraction lower than 10 000 Da, it was observed that it is polypeptides with molecular weights of between 10 000 and 700 Da that are released first and that these later break up to give rise to peptides with molecular weights lower than 700 Da, which in turn break down into amino acids. This indicates that the nature of the nitrogen compounds present in wines aged with yeast depends on the aging time, being less polymerized as the aging time increases.

Keywords: *Wine; yeast autolysis; proteins; peptides; amino acids*

INTRODUCTION

The release of yeast compounds into wine during autolysis is of great importance in the manufacture of wines that remain in contact with yeast for several months or even years, as in the case of "sur lie" Burgundy wines and sparkling wines manufactured by the champenoise, or traditional method, in different countries.

Autolysis is a very slow process of breaking down that may be induced by increase of temperature, addition of plasmolyzers, mechanical intervention, and other factors facilitating activation of lytic cell enzymes (Babayan et al., 1981). Because of their ethanol content (10−12%), low pH (3.0−3.5), and low aging temperature (usually below 15 °C), sparkling wines are not a suitable medium for the process of autolysis, which takes a long time to begin, usually lasts a long time, and seems to depend on the strains of yeast involved (Hernawan and Fleet, 1995). Most of the studies on yeast autolysis are carried out under conditions that accelerate the process in order to obtain findings in a reasonable period of time. The assays are almost always conducted at relatively high temperatures, usually above 40 °C (Vosti and Joslyn, 1954a,b; Babayan et al., 1981; Feuillat and Charpentier, 1982; Lurton et al., 1989; Leroy et al., 1990; Hernawan and Fleet, 1995). Because the temperature affects the process of yeast autolysis, in that intracellular enzymatic activities depend on temperature, not all findings appearing in the literature can be extrapolated to what occurs in the aging of wines, and at times the findings obtained by different authors are even contradictory. For example, while Babayan et al. (1981), in induced autolysis assays conducted between 40 and 70 °C, do not detect enzymes in the extracellular medium, Hough and Maddox (1970), in autolysis assays with beer yeast at 45 °C and pH 6.5, say that it is evident that fairly extensive proteolysis is occurring outside the cell.

Recent studies of the aging of sparkling wines with yeast (Moreno-Arribas et al., 1996) indicate that although cell wall integrity is not lost during autolysis and that no break in the membrane is detected by microscopic techniques (Charpentier et al., 1986; Charpentier and Feuillat, 1993; Hernawan and Fleet, 1995), changes do occur in the composition of wine that suggest that not only are low and medium molecular weight nitrogen compounds released but also hydrolytic enzymes break them down. Thus, this study was conceived with the objective of characterizing the nitrogen compounds released by yeast during the process of autolysis under conditions close to those of wine manufacture. For this purpose the autolysis of one of the strains of yeast used in the manufacture of sparkling wines was induced, in a model wine buffer, at 30 °C, a temperature at which the enzymes involved in autolysis are not inhibited. Free amino acids and different size peptides and proteins were studied in the model wines after different times of autolysis.

MATERIALS AND METHODS

Yeast Strains and Autolysis Conditions. A commercial active dry yeast, *Saccharomyces bayanus* EC1118, supplied by Lallemand (Madrid, Spain) was used. The yeast was rehydrated according to the manufacturer's instructions. After rehydration, yeast cells were harvested by centrifugation at 7000*g* for 10 min at room temperature and washed three times with 0.9% NaCl. The washed yeast was suspended in a model wine buffer (Feuillat, 1987). The wine buffer contained ethanol

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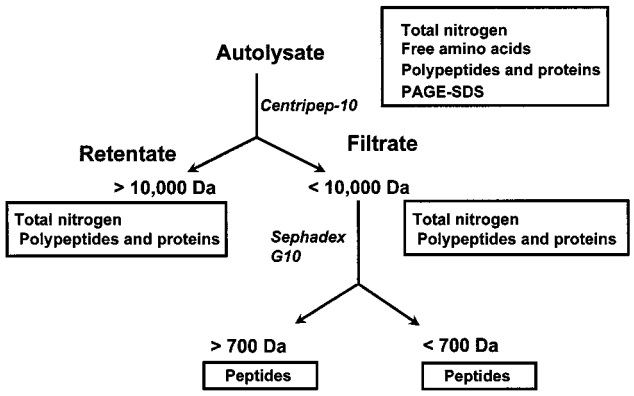

Figure 1. Diagram of the fractionation steps of autolysates and analytical determinations.

(10%, v/v), tartaric acid (4 g/L), malic acid (3 g/L), acetic acid (0.1 g/L), potassium sulfate (0.1 g/L), and magnesium sulfate (0.025 g/L). The pH was adjusted to 3.0 with sodium hydroxide. Autolysis was conducted by incubating the cell suspensions at 30 °C for up to 15 days with shaking at 100 rpm. During autolysis, samples of the suspensions were taken for analysis. The whole rehydration, washing, and yeast autolysis process was conducted in individual flasks (1 g of yeast dry weight/100 mL of model wine buffer), and a flask was taken at each sample taking point.

Fractionation of the Autolysates. Figure 1 indicates schematically the method used for the fractionation of the autolysates and the determinations carried out in each of the fractions obtained. An aliquot of 15 mL of the autolysates was ultrafiltered through a Centripep-10 device (Amicon, Inc, Beverly, MA) with a 10 000 Da cutoff membrane. Two fractions were obtained: the filtrate and the retentate. The filtrate was refractioned by low-pressure liquid chromatography in a Sephadex G10 column (Pharmacia Fine Chemical, Uppsala, Sweden) 92 cm long × 2.5 cm i.d. under the conditions described by Moreno-Arribas et al. (1996). Eluent was 3% acetic acid, the flow rate was 2.5 mL/min, the absorbance at 280 nm was measured with a model 2138 Uvicord S, LKB detector and a model 2210 LKB recorder (Pharmacia Fine Chemical). Two peptide fractions containing the compounds with molecular weights above and below ~700 Da, respectively, were obtained.

Total Nitrogen. This was determined by the Kjeldahl method with a Tecator digestion system and a Kjeltec 1030 autoanalyzer (Tecator AB, Höganäs, Sweden).

Polypeptides and Soluble Proteins. These were determined by the Bradford dye-binding assay (Bradford, 1976).

Amino Acid Analysis. Free amino acids were determined in the autolysates. Analyses, in duplicate, were carried out by HPLC using a Waters (Milford, MA) liquid chromatograph controlled by a Waters Maxima 820 chromatography workstation. Samples were submitted to an automatic precolumn derivatization with *o*-phthaldialdehyde (González de Llano et al., 1991) to determine primary amino acids. The separation of amino acids was performed on a Waters Novapak C-18, 60 Å, 4 μm column (3.9 cm × 150 mm). Detection was by fluorescence using wavelengths of excitation and emission at 340 and 425 nm, respectively. All reagents used were HPLC grade. Variation coefficients of the amino acids determination were less than 6%.

Preparation of the Sample for Electrophoretic Study. The samples of unfractionated autolysate were dialyzed against running water on the Spectra POR 3 membranes (Spectrum Medical Industries, Los Angeles, CA). The retentates were lyophilized, and the resulting residues were dissolved in 1 mL of pH 8.3 buffer (0.6 g of tris(hydroxymethyl)aminomethane + 2.9 g of glycerine per liter of water).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) for Proteins with Molecular Weights of over 10 000. Laemmli's method (Laemmli, 1970) for discontinuous electrophoresis was performed [*T* (acrylamide plus bisacrylamide, w/v) = 15; *C* (bisacrylamide:acrylamide plus bisacrylamide, w/v) = 3, resolving gel (*T* = 4%, *C* = 2.7%, stacking gel)]. Electrophoresis was carried out on plates of 140 mm × 130 mm × 0.75 mm at room temperature at a constant current setting of 15 mA per gel for approximately 4 h. A Pharmacia Fine Chemicals (Pharmacia LKB, Uppsala, Sweden) low molecular weight electrophoresis calibration kit (14400−94000 Da) was used as a marker. The gel was silver-stained (Blum et al., 1987).

Table 1. Nitrogen Compounds Content of the Autolysates at Different Autolysis Times (h)

	autolysis time (h)					
	4	24	48	72	168	360
total nitrogen (mg/L)	<10	233.8	279.4	282.8	331.8	328.8
polypeptides and soluble proteins (mg BSA/L)	6.1	90.0	64.4	61.1	51.7	35.9
amino acids (mg/L)						
Asp	3.21	11.25	14.63	12.16	14.44	12.81
Glu	4.64	15.26	19.05	15.82	19.44	18.51
Asn	1.95	6.86	8.68	9.18	10.18	9.40
Ser	5.12	7.49	10.05	10.29	11.51	10.21
Gln	2.73	12.01	12.27	13.88	15.08	14.64
Hys	2.14	2.03	2.98	6.48	7.10	7.04
Gly	2.89	4.97	7.36	5.67	5.53	5.13
Thr	2.73	6.39	9.04	9.61	9.32	9.30
Arg	3.51	13.57	16.06	15.59	14.02	13.56
β-Ala	2.26	2.18	1.42	2.39	4.44	4.16
α-Ala	5.08	35.82	39.66	33.52	37.43	36.77
GABA[a]	3.48	40.20	52.42	52.42	62.73	60.04
Tyr	3.01	8.21	10.21	9.05	10.50	10.05
α-Aba	1.46	1.72	1.58	1.59	1.65	1.73
Met	1.73	4.75	6.01	6.87	8.66	7.51
Val	2.57	10.48	12.62	10.86	10.75	9.88
Trp	3.15	3.48	3.45	4.45	6.59	6.32
Phe	3.04	15.12	20.54	21.54	28.16	26.23
Ile	2.55	7.64	8.81	8.10	8.06	7.45
Leu	3.60	18.11	22.84	23.27	24.72	23.92
Orn	4.61	6.51	8.84	7.22	7.18	7.32
Lys	2.53	2.78	2.39	3.74	4.5	3.60
sum of free amino acids	67.98	236.82	288.71	293.67	332.02	315.56

[a] GABA = γ-aminobutyric acid.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) for Peptides with Molecular Weight from 5000 to 20 000. Schäger and von Jagow's method (Schägger and von Jagow, 1987) for discontinuous electrophoresis was performed using the samples prepared as indicated previously. *T* = 16.6%, *C* = 6.06% resolving gel and *T* = 16.5%, *C* = 3.03% stacking gel were used. Electrophoresis was performed on plates of 140 mm × 130 mm × 0.75 mm at 30 V until the sample had completely left the sample pocket and at a constant voltage setting of 105 V until the tracking reached the lower part of the plate. The gel was silver-stained (Blum et al., 1987). A Pharmacia Fine Chemicals (Pharmacia LKB, Uppsala, Sweden) peptide marker kit (2512−16949 Da) was used as marker.

Separation of Peptides by HPLC. Peptides from the fractions higher and lower than ~700 Da were separated by reversed-phase HPLC under the conditions described by Moreno-Arribas et al. (1996) on a Waters Nova Pak C-18 column (150 mm × 3.9 mm i.d., 60 Å, 4 μm) (Milford, MA). A Beckman (Beckman Instruments, Fullerton, CA) M168 diode array detector was used. Eluent A was 0.1% trifluoroacetic acid in water, and eluent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient of B was increased from 0 to 40% over 70 min. The flow rate was 1 mL/min.

RESULTS AND DISCUSSION

Autolysate Analysis. Table 1 shows the concentrations of total nitrogen, polypeptides, and soluble proteins determined by the Bradford dye-binding method, and the proportion of free amino acids, in the autolysates

after different autolysis times. In the first 4 h a slight increase can be observed in total nitrogen, polypeptides, proteins, and free amino acids. The greatest increase in total nitrogen occurs between 4 and 24 h of autolysis, when it reaches a value of 233.8 mg/L. Total nitrogen content continues to rise between 24 and 168 h but at a slower rate. At 360 h of autolysis, total nitrogen concentration is approximately the same as at 168 h. Table 1 also indicates that the greatest release of polypeptides and proteins occurs, as in the case of total nitrogen, between 4 and 24 h. Between 24 h and the end of the assay polypeptide and protein content decreases. Table 1 also shows the amino acid content (mg/L) of the autolysates after different times of autolysis. In the first 4 h, 67.98 mg/L of free amino acids are released. The greatest increase in free amino acid content in the autolysates occurs between 4 and 24 h, as was the case with total nitrogen, peptides, and soluble proteins. α-Alanine, γ-aminobutyric acid, phenilalanine, leucine, glutamic acid, and arginine are the most prevalent amino acids and accounted for some 50% of total amino acids. Similar findings were obtained by Hernawan and Fleet (1995). Most of the amino acids continued to increase up to 168 h. Between 168 and 360 h there were no important changes in the amino acid concentration. Moreno-Arribas et al. (1998) observed a reduction in free amino acids in sparkling wines aged with yeast after 18−24 months of aging. The difference between the total nitrogen content and the sum of the nitrogen of the free amino acids and of the polypeptides and proteins indicates that over 80% of the nitrogen released corresponds to small-size peptides not considered by the Bradford method.

The release of amino acids in the first 4 h, approximately 30% of the total released, may be due to a process of exsorption of the internal content of the yeast before the self-proteolysis process begins. These findings coincide with those of Lurton et al. (1989) in studies of autolysis in an acid medium. This rise in amino acids is accompanied by an increase in other compounds that could be wall peptides, not dependent on the action of the proteases (Charpentier et al., 1986). The large increase in amino acids and in proteins and polypeptides between 4 and 24 h indicates that the process of autolysis has begun and that there is a breakdown of the wall, enabling relatively large molecules to escape (Charpentier and Feuillat, 1993). The reduction in proteins observed after 24 h indicates that yeast proteases have been released that act on the outside on the yeast proteins.

In the electrophoretic analysis of the unfractionated autolysate, conducted using SDS electrophoresis for proteins with a molecular weight over 10 000, some poorly resolved bands appeared on the edge of the gel, and no band was observed in the area of higher molecular weights. Owing to the fact that with the Bradford dye-binding method, which is the method used conventionally to quantify proteins, peptides with a molecular weight of over 3500 Da are also quantified (Sedmak, 1977), we estimate that the compounds determined here correspond mainly to polypeptides. The molecular masses of polypeptides were estimated by using the silver-stained polypeptides SDS−PAGE shown in Figure 2. The results are presented in Table 2. In the autolysates of under 168 h, bands were detected with relative molecular masses (M_r) ranging from 16 900 to 4400. In the 4 h autolysate, the major band had a M_r of 16 900. Lower intensity bands were also detected with M_r 11 400, 9800, 8000, and 6000. Between 4 and 24 h, the highest M_r band, 16 900, diminished and the intensity of the lower M_r bands increased while a new band with M_r 4400, appeared. Later, the intensity of all the bands decreased, and no electrophoretic band was detected after 168 and 360 h. The greatest concentration of polypeptides was detected in the 24 h autolysate, which coincides with the data obtained in the Bradford dye-binding analysis (Table 1).

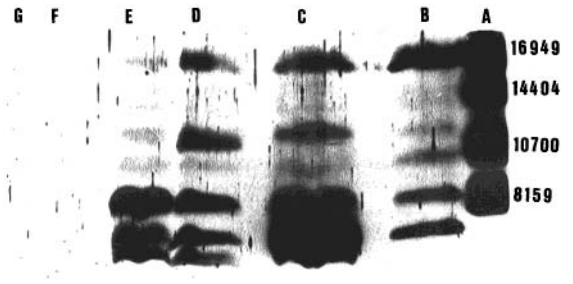

Figure 2. Results of a 16.6% T, 6.06%C silver-stained polypeptides SDS−PAGE. The lanes contain samples of different autolysis times: (A) molecular mass marker; (B) 4 h; (C) 24 h; (D) 48 h; (E) 72 h; (F) 168 h; (G) 360 h. Molecular weights of standards are given on the right side of the gel.

Table 2. Electrophoretic Bands Detected by SDS−PAGE in the Autolysate at Different Autolysis Times

relative molecular mass	autolysis time (h)					
	4	24	48	72	168	360
16900	+++	++	+	−	−	−
11400	+	++	+++	+	−	−
9900	+	+	+	+	−	−
6800	+	+++	++	++	−	−
4800	+	+++	++	++	−	−
4400	−	+++	++	++	−	−

Table 3. Total Nitrogen (mg/L) and Polypeptides and Soluble Proteins (mg BSA/L) Content in the Centripep-10 Retentate and Filtrate at Different Autolysis Times

	Centripep-10 retentate		Centripep-10 filtrate	
autolysis time (h)	total nitrogen	polypeptides and soluble proteins	total nitrogen	polypeptides and soluble proteins
4	<5	0	<5	6.0
24	7.4	34.6	211.3	55.9
48	<5	12.9	269.2	52.1
72	<5	15.9	271.0	46.2
168	8.4	12.6	311.5	38.0
360	6.4	2.2	309.0	31.1

Centripep-10 Retentate and Filtrate Analysis. Table 3 shows the total nitrogen, and polypeptide and protein content of the fractions retained and eluted from the Centripep-10. The total nitrogen, and the protein and polypeptide content of the fractions retained by Centripep-10 is very low and changes very little during autolysis. The concentration varies between 8.4 mg/L at 168 h and unmeasurable quantities at 4, 48, and 72 h. Greater variations are observed in the protein and soluble polypeptide concentrations in these fractions during autolysis. No proteins were detected in this fraction in the first 4 h. Between 4 and 24 h of autolysis, 34.6 mg/L were released. Later, the concentration decreased to 2.2 mg/L after 360 h of autolysis. The fact that there was no coincidence between nitrogen concentration and polypeptide and protein concentration leads to the assumption that this fraction is made up

of a mixture of polypeptides with a different response to the Coomassie Blue reagent used in the Bradford method.

The Centripep-10 filtrate fraction contains the majority of the total nitrogen in the autolysates, and its behavior follows the same tendencies as those observed in the unfractionated autolysates. That is, the greatest increase in total nitrogen occurs in the first 24 h, it continues to increase up to 168 h, and remains practically constant between 168 and 360 h of autolysis.

The concentration of proteins and polypeptides in this fraction is also higher than that detected in the fraction with a molecular weight of over 10 000 (Table 3). As in the findings obtained for the unfractionated autolysates, the greatest increase is observed after 24 h of autolysis, after which these compounds decrease. These findings indicate that most of the nitrogen compounds released during autolysis have molecular weights of less than 10 000 and coincide with the findings obtained in the electrophoretic analysis (Table 2 and Figure 2).

Owing to the fact that the findings described above indicate that the nitrogen fraction of the autolysates is made up mainly of free amino acids and peptides and owing to the absence of data in the bibliography on the peptide fraction, the fraction with molecular weights of under 10 000 was refractionated, using molecular exclusion chromatography, and each of the subfractions obtained was subjected to a chromatographic study. The fractions obtained by molecular exclusion chromatography on Sephadex G-10, under the prevailing conditions, had approximate molecular weights above and below 700 (Moreno-Arribas et al., 1996).

Figure 3 shows the chromatograms obtained by HPLC analysis of the peptides with approximate molecular weights higher (Figure 3a) and lower (Figure 3b) than 700. An extra sample was taken at 18 h for the peptides analysis. It can be observed that between 4 and 18 h of autolysis the concentration of peptides with molecular weights higher than 700 increases and continues to rise up to 24 h, after which time it continually decreases during the subsequent aging period. Peptides with a molecular weight lower than 700 also increase between 4 and 18 h, but the increase is even faster between 18 and 24 h and especially between 24 and 48 h, after which they decrease, as in the case of the larger peptides. From a joint observation of all the chromatograms, it can be deduced that larger peptides are released first and that these are later broken down, giving rise to other smaller ones. These results confirm that changes in the peptide fraction of wines during aging with yeast, observed by Moreno-Arribas et al. (1996), are due to the release of yeast enzymes during autolysis.

To summarize the findings obtained, it can be said that during autolysis of yeast in wine manufacturing conditions, not only amino acids, the nitrogen compounds conventionally studied in autolysis assays, but also polypeptides with M_r 16 900 and 11 000 and, above all, peptides with M_r lower than 10 000 are released. These compounds are transformed over time by the enzymes released by the yeast into smaller peptides. These peptides may modify the quality of wine during aging with yeast, since the presence of peptides in foodstuffs has been related, among other properties, to the appearance of sweet and bitter tastes (Polo et al., 1992; Turgeon et al., 1992; Bumberger and Berlitz, 1993) and to the modification of surfactant properties (Razafindralambo et al., 1998).

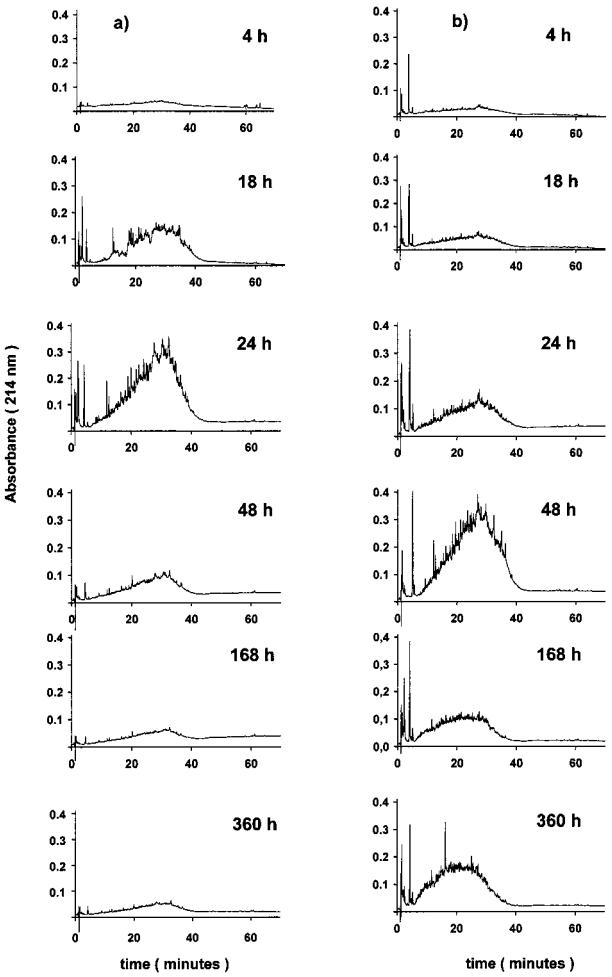

Figure 3. HPLC profiles of the autolysate peptide fractions with approximate molecular weight higher (a) and lower (b) than 700 at different autolysis times.

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