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Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions

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Determination of protease A activity during alcoholic fermentation of a synthetic must (pH 3.5 at 25°C) and during autolysis showed that a sixfold induction of protease A activity occurred after sugar exhaustion, well before 100% cell death occurred. A decrease in protease A activity was observed when yeast cell autolysis started. Extracellular protease A activity was detected late in the autolysis process, which suggests that protease A is not easily released. Evolution of amino acids and peptides was determined during alcoholic fermentation and during autolysis. Amino acids were released in early stationary phase. These amino acids were subsequently assimilated during the fermentation. The same pattern was observed for peptides; this has never been reported previously. During autolysis, the concentration of amino acids and peptides increased to reach a maximum of 20 and 40 mg N l^{-1}, respectively. This study supports the idea that although protease A activity seemed to be responsible for peptides release, there is no clear correlation among protease A activity, cell death, and autolysis. The amino acid composition of the peptides showed some variations between peptides released during alcoholic fermentation and during autolysis. Depending on aging time on yeast lees, the nature of the peptides present in the medium changed, which could lead to different organoleptic properties. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 235–240.

Keywords: autolysis; protease A; amino acids; peptides; *Saccharomyces cerevisiae*

Introduction

Yeast autolysis represents an enzymatic self-degradation of cellular constituents, which occurs after cell death or causes cell death. The main events during autolysis are the breakdown of cell membranes, which allows release of hydrolytic enzymes, and, subsequently, accumulation of degradation constituents in the autolytic medium [2]. Therefore, hydrolytic enzymes are of major concern during autolysis. Among all the enzymes involved in the process (phospholipases, glucanases, nucleases), proteases have been studied extensively [1,2,4]. According to Behalova and Beran [4], the proteolytic activity could serve as the indicator of the rate of the autolysis process. In order to accelerate the process of autolysis, most studies are conducted at high temperature [18], with different pH values (pH 3.5 in the fermentation medium or pH 6.0 in the growth medium). Inducers have also been used to increase the autolysis kinetics [20]. Autolysis studies have been conducted using either fresh yeast or active dry yeast. All these different yeast autolysis conditions lead to contradictory results as reported in the literature. Kollar *et al.* [20] observed a decrease in protease activity during a 12-h autolysis, while Sato *et al.* [32] observed an increase of proteolytic activity during a 24-h autolysis. Slaughter and Nomura [34] reported that during autolysis, protease A activity was not detectable outside the cells, while in beer, extracellular proteolytic activity has been observed [29]. The medium also affects the autolysis process. Under acid conditions, e.g., using protease inhibitors, Lurton *et al.* [23] showed that protease A activity might be responsible for 80% of the nitrogen released during autolysis under optimum conditions. The pH of wine is unfavourable for most yeast proteases, so that autolysis in wine is a specific case. Protease A is the only acid protease found in yeast, which explains its role in nitrogen release. Many studies on autolysis in wine have focused on the evolution of nitrogen compounds, especially amino acids, during aging on yeast lees [8,11,21,26,33]. However, protease A is an endoprotease; consequently, its action should result in peptide release.

This has not been well documented [26,27]. Furthermore, peptides have been studied in sparkling wines [26] and during yeast autolysis using active dry yeast in a model wine system which has not undergone alcoholic fermentation [27].

In wines aged on yeast lees, autolysis is an important event since it results in enrichment of the medium in nitrogen compounds [23], glucans, and mannoproteins [21]. These molecules could lead to the formation of new aroma compounds [10,30]. Therefore, autolysis could modify the wine quality.

Autolysis conditions reported in the literature are far from those encountered during wine aging (i.e., low temperature, low pH, presence of ethanol) [11,23,27]. For example, the work of Lurton *et al.* [23] was conducted at 40°C, pH 3.5, using active dry yeast in a non-ethanolic medium. In order to better characterize autolysis during wine aging on yeast lees, we have followed evolution of the protease A activity and nitrogen fractions, especially amino acids and peptides, during alcoholic fermentation of a synthetic must and autolysis under conditions closer to those encountered in wine processes.

Materials and methods

Alcoholic fermentation

Synthetic must used for fermentation has the following composition for 900 ml: glucose (75 g), fructose (85 g), tartaric acid (2 g), malic acid (10 g), ammonium chloride (1.5 g); the pH was adjusted to 3.5 with NaOH (8 N). After sterilisation (110°C, 5

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min), 100 ml containing 11.7 g of yeast carbon base (Difco) was added. The synthetic must was then inoculated from a 24-h-old culture (synthetic must containing 2% (p/v) glucose) with an enological *Saccharomyces cerevisiae* strain (3079 isolated by the Bureau Interprofessionnel des Vins de Bourgogne) to a final concentration of 10^6 cells ml^{-1}. The alcoholic fermentations were conducted in duplicate in 3 l of medium in 5-l conical flasks at 25°C. At suitable times, flasks were shaken, samples were withdrawn, centrifuged, and analyzed for sugars [6]. After alcoholic fermentation, the wine was left on yeast lees for autolysis for 80 days at 25°C. Viable and total cells were determined in a Malassez counting cell. Viability of populations was measured by staining cells with methylene blue [13].

Enzyme activities

Intracellular protease A activity: At each time point, 200 ml of medium was withdrawn and centrifuged at 5000×*g* for 10 min at 4°C. The supernatant was kept for measuring extracellular protease activity and for nitrogen fractions analysis. The pellet was washed in pH 4.8, 0.1 M citrate buffer. Protease A was extracted by breaking the cells with glass beads (0.45 mm) in a Braun homogeniser apparatus cooled with CO_2. The cell debris was separated by centrifugation at 12,000×*g* for 10 min at 4°C. The protease A activity of the extract was determined by the haemoglobin test according to the method of Jones [19]. Briefly, 100 μl of cell-free extract were added to 2 ml solution (1 ml haemoglobin solution and 1 ml 0.2 M glycine, pH 3.2) and the mixture was incubated at 37°C. At 0, 5, 10, 15, and 30 min, 0.4 ml was removed and added to 0.2 ml of 1 N perchloric acid and maintained on ice before centrifugation for 5 min at 1650×*g*. One hundred microliters of supernatant was added to 100 μl of 0.5 M NaOH. Tyrosine-containing peptides in the neutralized 0.2 ml samples were determined by the Lowry method [22].

Extracellular protease A activity: The supernatant obtained as described above was ultrafiltered on a 5000-Da cutoff membrane (Amicon, Beverly, MA). The filtrate was kept for amino acid and peptide analyses. The extracellular protease activity was determined on the retentate by the haemoglobin test [19]. Incubation time was 6 h. Protein content was determined by the Lowry method [22].

Amino acids and peptides analysis

The amino acids present in the filtrate fraction were determined by high-performance liquid chromatography (HPLC) using a Waters™ liquid chromatograph. Briefly, derivatization was carried out on 200 μl of filtrate; 40 μl of internal standard consisting of α-aminobutyric acid (2.5 mmol l^{-1}) was added together with 760 μl of MilliQ water. To 10 μl of the above solution, 70 μl of borate buffer and 20 μl of AQC reagent (6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate) were mixed and then placed at 55°C for 10 min [35]. For liquid chromatography, two pumps (Waters™ 626), a system controller (Waters™ 600S), and an autosampler (Waters™ 717 plus) were combined with a scanning fluorescence detector (Waters™ 474). Separation was carried out using a Nova-Pak™ C18 Column (3.9 mm×150 mm) filled with silica spheres (pore size 6.0 nm; particle size 4 μm). The precolumn had the same characteristics and was 2 cm long. Eluant A was sodium acetate buffer (pH 5.8), eluant B was acetonitrile, and eluant C was water. The elution started with 100% of eluant A. The ratio of eluant B was increased from 0% to 14% over 35 min and was maintained at this concentration for 16 min. The elution ended isocratically with 60% of eluant B and 40% of eluant C. The concentration of each amino acid was determined from a standard curve.

Acidic hydrolysis of a 250-μl sample containing peptides was conducted in the presence of 0.65 ml of HCl, 6 M and 0.1 ml of internal standard (AabasI, 2.5 mM) at 110°C during 24 h. Amino acids were then analysed by HPLC. The concentration of peptides was deduced from the difference in amino acids concentration before and after hydrolysis.

Results and discussion

Intracellular protease A activity

We report here for the first time the production of protease A activity in yeast cells during alcoholic fermentation and autolysis in synthetic must under enological conditions. The method used [19] allowed us to specifically measure the protease A activity. Addition of pepstatin, a specific protease A inhibitor, to the incubation medium led to 95% inhibition of the enzyme activity (data not shown). As shown in Figure 1a, protease A was present at all time points sampled during alcoholic fermentation. It is noteworthy that protease A activity increased after sugar exhaustion (Figure 1b); protease A activity at 25 and 32 days showed a six- and sevenfold increase compared with the activity measured during alcoholic fermentation. Another striking observation was that very high activity was observed at day 25 while the viability was still 80%. On the other hand, the activity started to decrease before 100% mortality was reached (Figure 1a). Thus, it can be concluded that there is no correlation between protease A activity and cell death. Furthermore, our experience pointed out that protease A activity is not a good criterion to follow autolysis in wine, conversely to what has been reported for autolysis conducted under optimum conditions [4].

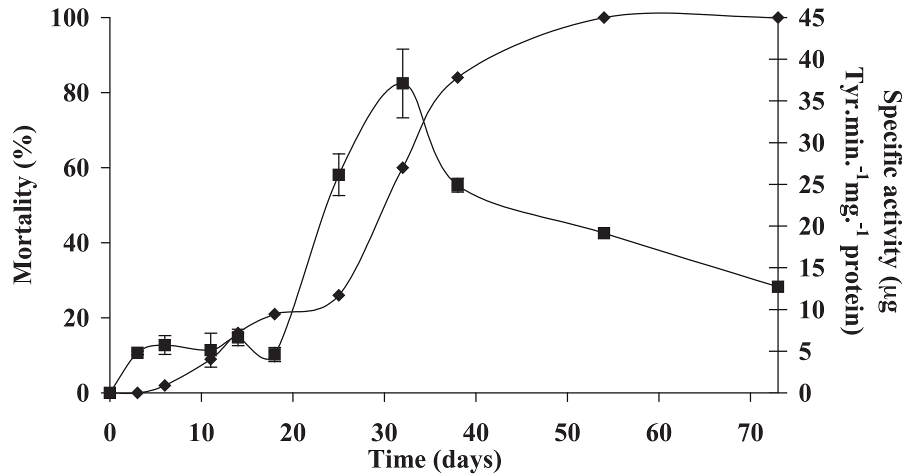
Protease A activity or its expression is induced during stress conditions, especially nutritional stress such as nitrogen starvation [28]. It has been shown that the expression of protease A encoded by the PEP4 gene is sensitive to nitrogen catabolite repression [28]. However, at the end of the fermentation, our medium still contained 500 mg l^{-1} nitrogen. Thus, induced protease A activity cannot be attributed to nitrogen starvation. It is more likely that under our conditions, the increase in protease A activity is linked to glucose exhaustion. This is supported by the study of Hansen *et al.* [16] who showed that synthesis of proteases appeared to be repressed by glucose.

During 75 days of autolysis, the protease A activity continuously decreased, which is in agreement with previous reports [33]. Since protease A activity decreased while autolysis proceeded, protease A activity cannot be used as an autolysis marker.

Extracellular protease A activity

Secretion of proteases is not an ability commonly attributed to *S. cerevisiae*. The available data are controversial; some of them indicate the presence of proteolytic activity released in the medium during either alcoholic fermentation or autolysis [12,29], and others the complete absence of proteolytic activity [4,24].

To measure an extracellular proteolytic activity, we have concentrated the fermentation and the autolysis medium up to



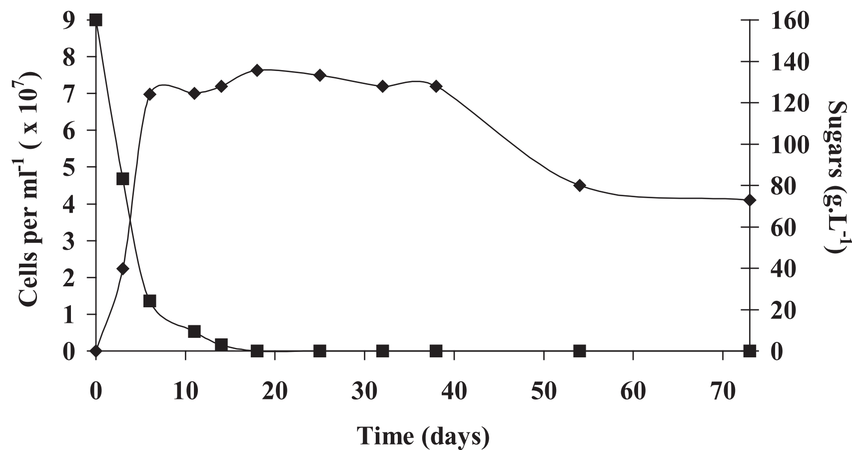


Figure 1 (a) Protease A activity (■) during alcoholic fermentation and autolysis in a synthetic must (pH 3.5 at 25°C). Proteolytic activity was determined by the haemoglobin test. Percent mortality (◇). (b) Sugar consumption (■) and cell biomass (◇) throughout the alcoholic fermentation of a synthetic must (pH 3.5 at 25°C) and during autolysis. Data are the means ± SD of two independent experiments.

200-fold by ultrafiltration. No protease activity was detected during the alcoholic fermentation. On the other hand, a small but reproducible activity was measured in the autolysate from day 54 (Table 1). It should be noted that extracellular protease activity is 3- to 30-fold lower than intracellular activity measured during alcoholic fermentation and autolysis, respectively. Results obtained during autolysis in enological conditions support previously reported data [21,33]. The very low extracellular activity raised the question of whether or not the protease activity can diffuse outside the cell, and whether autolysis awaits breakdown of vacuolar membrane so that the protease is released. Once in the cytoplasm, the protease must cross the cell wall to be released in the medium. It is likely that an extensive degradation of the cell wall should occur before the proteases are effectively released. Actually, during autolysis, the cell wall of dead cells, although thinner than living cells, remains unbroken [8], which means that it could still act as an efficient barrier. Moreover, the cell wall porosity of cells, which affects release of macromolecules, is very low in the stationary growth phase [6,9].

Table 1 Extracellular protease A activity during alcoholic fermentation of a synthetic must (pH 3.5, at 25°C) and during autolysis

Time (days)	Activity
0	ND*
10	ND
15	ND
28	ND
54	1.5
73	3.0

Activity is expressed in μg Tyr min^{-1} mg^{-1} protein.
*ND, non-detectable.

Evolution of amino acids and peptides during alcoholic fermentation and autolysis

An enrichment of the medium in amino acids was observed on day 6 and reached a maximum on day 11. Subsequently, the

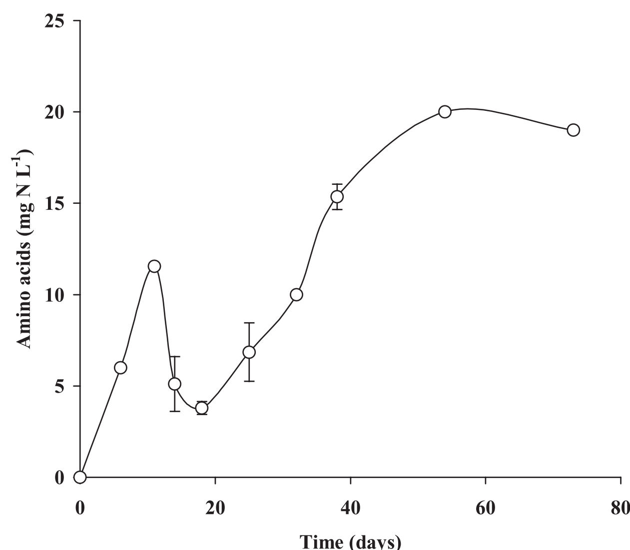


Figure 2 Profile of the amino acids present in the medium during alcoholic fermentation of a synthetic must (pH 3.5 at 25°C) and during autolysis. Amino acids were analysed by HPLC (○) method. Data are the mean ± SD of two independent experiments.

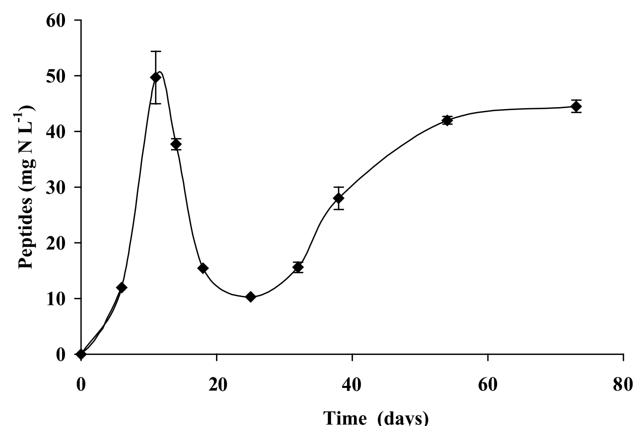


Figure 3 Evolution of peptides concentration during alcoholic fermentation of a synthetic must (pH 3.5 at 25°C) and during autolysis. Peptides were quantified from the difference between amino acid analysis of the molecular weight fraction < 5000 Da before and after acid hydrolysis. Data are the mean ± SD of two independent experiments.

concentration decreased until the end of the alcoholic fermentation (Figure 2). Amino acid excretion appeared at the end of the exponential phase and during early stages of the stationary phase of growth (Figures 1b and 2). During this phase, proteolysis occurred [14], which could lead to amino acid excretion. This excretion might also reflect the composition of our medium, which contained ammonium chloride as the sole nitrogen source; this has been reported to favour amino acid excretion [5]. It is likely that the released amino acids, which constitute a nitrogen source, are assimilated during the course of the fermentation between days 11 and 20.

After alcoholic fermentation, the amino acid content increased during autolysis (Figure 2), which agrees with previous reports [2,8,11,33]. The kinetics of liberation has been well described [23,25]. In the first stage, amino acid liberation results from a passive diffusion from the intracellular pool. In a second stage, amino acid enrichment is linked to the action of protease activity [23], especially exoprotease like carboxypeptidase [33].

Table 2 describes the content of amino acids during alcoholic fermentation and autolysis. Lys, Val, Ala, Pro, Gln, Gly, and Gluc represent about 75% of the total amino acids present in the medium during alcoholic fermentation. During autolysis, other amino acids such as Asp, Arg, α-aminobutyric acid (GABA), His, Leu, Thr,

Table 2 Free amino acid composition of the medium during alcoholic fermentation of a synthetic must (pH 3.5 at 25°C) and during autolysis

Amino acid	Percent at (day)									
	0	6	11	14	18	25	32	38	54	72
Asp	0	1.8	2	0	0	2.9	3.9	3.9	3.1	4.8
Glu	0	10.6	8.5	12	13.2	10.3	6.9	5.2	5.1	6.3
Ser	0	5.2	1	4	0	0	1.9	1.9	1.5	2.1
Asn	0	3.5	2.8	4	5.2	4.4	3.9	3.3	3.1	4.2
Gly	0	15.8	12.1	16	15.8	10.3	9.7	7.9	6.6	9
Gln	0	15.8	14	18	18.5	10.3	7.8	5.2	2.6	1.6
His	0	0	0	0	0	1.5	3.9	3.1	2.6	0
Thr	0	0	4	0	0	0	0	1.9	3.6	3.7
Arg	0	0	7.4	0	0	20.6	25.2	25	27.6	14.3
Ala	0	8.8	1.9	8	10.5	11.8	10.7	11.2	8.2	8.5
GABA	0	0	9.3	0	2.6	4.4	4.8	7.8	11.7	7.4
Pro	0	14	26.1	14	18.4	10.3	8.7	7.8	6.6	5.8
Tyr	0	1.7	1	2	0	0	1	1.3	1	5.3
Cys	0	0	0	0	0	0	0	0	0	0
Val	0	12.2	4	4	2.6	2.9	1.9	1.9	2	9
Ile	0	1.7	1	2	0	1.5	1.9	2.6	2	4.2
Leu	0	0	0	10	13.2	8.8	6.8	6.5	7.1	9
Lys	0	8.7	2.8	6	0	0	1	1.3	5.6	2.2
Phe	0	0	2	0	0	0	0	1.9	0	2.6

Table 3 Amino acid composition of the peptides present in the medium during alcoholic fermentation of a synthetic must (pH 3.5 at 25°C) and during autolysis

Amino acid	Percent at (day)									
	0	6	11	14	18	25	32	38	54	72
Asx*	0	16.6	9.9	3.1	7.7	4.4	18.7	18.7	20	11
Glx*	0	0	9.9	2.4	9.6	26.1	28.1	27.2	21.2	9.2
Ser	0	11.5	7.4	5	7.7	0	9.4	11.4	2.5	0
Gly	0	19.5	7.4	6.2	0	17.4	0	0	3.8	6.2
His	0	7.7	4.5	3.1	3.8	4.4	0	4.2	7.5	19
Thr	0	3.8	54	3.1	7.7	0	15.6	13.5	6.2	4.9
Arg	0	10.2	9.9	15	21.8	17.4	0	0	0	18.7
Ala	0	6.4	9.5	5	5.8	13	0	0	0	0
GABA	0	0	0	0	0	0	0	0	0	0
Pro	0	0	0	4.3	0	13	0	0	3.8	0
Tyr	0	2.6	1.2	3.7	0	0	0	0	0	0
Cys	0	1.3	0	2.5	0	0	0	0	0	0
Val	0	3.8	13.2	37.2	7.7	0	0	8.3	10	0.6
Ile	0	0	4.1	1.9	5.8	0	0	0	3.8	3.7
Leu	0	6.4	7.4	5	7.7	4.3	0	0	0	4.2
Lys	0	8.9	7.8	1.9	11.6	0	21.9	14.6	13.7	22.7
Phe	0	0	2	0	3.8	0	6.3	0	7.5	0

*Asx and Glx correspond to the sum of aspartic acid and asparagines and glutamic acid and glutamine, respectively.

and Ser appeared. The appearance of serine and threonine during late autolysis could reflect the degradation of mannoprotein the cell wall. The C-terminal region of the cell wall is rich in serine and threonine [38].

Although protease A activity has been well studied, curiously the products of its action (i.e., peptides) are not well documented. The evolution of peptides during alcoholic fermentation and autolysis is depicted in Figure 3. It is noteworthy that, like amino acids, an increase in peptide concentration in the medium was observed when cells entered the stationary phase of growth (Figure 1b). As far as we know, this phenomenon has not been reported previously. We hypothesize that the peptides came from active proteolysis that occurred during entrance into the stationary phase of growth [14].

Disappearance of these peptides during the alcoholic fermentation could be due to assimilation. A di- and tripeptides transporter exists in *Saccharomyces* [3]. This is supported by the fact that analysis of peptides present in wines indicated mainly small peptides (less than 700 Da) [7]. Furthermore, the periplasmic aminopeptidase yscII, which hydrolyses intra- or extracellular peptides [31], might be involved in the disappearance of these compounds. During autolysis, a constant increase in peptide concentration was observed (Figure 3). These peptides are released by the protease A activity since it is the only endoprotease active at this pH. Although recently peptide fractions have been studied during autolysis of active dry yeast resuspended in a model wine system [25], we report for the first time peptide evolution during alcoholic fermentation and autolysis in enological conditions.

The percentage of each amino acid, which made up these peptides, is shown Table 3. The data for asparagine and/or aspartic acid and glutamine and/or glutamic acid are reported as Asx and Glx, respectively, because asparagine and glutamine are partially converted into aspartic and glutamic acid during acid hydrolysis. Apart from GABA, Pro, and Cys, all the other amino acids are constituents of peptides released during alcoholic fermentation. On the other hand, peptides present in the medium during autolysis consist mainly of Asx, Glx, Ser, His, Val, and Lys. A similar amino acid composition of peptides was observed in sparkling wines [27]. During autolysis, the proportion of Asx and Glx did not change in peptides until day 54; then an approximately 50% decrease was observed. On the other hand, Ser and Val decreased during autolysis while an enrichment in Lys was noted (Table 3). These results showed that, depending on aging time on yeast lees, peptide composition could vary, leading to peptides of different organoleptic properties. It should be noted that Phe-, Tyr-, and Leu-containing peptides seemed to represent a minor fraction of peptides released during autolysis, which is important since it has been shown that such peptides were responsible for bitter taste [17,18]. Nevertheless, a more in-depth study of isolated peptides must be done to confirm our observation.

Not only the amino acids currently studied, but also peptides are important components released during autolysis. The concentration of this nitrogen fraction could reach as much as 40 mg N l^{-1}, which is more significant than the amino acid concentration. Peptides as nitrogen compounds favour malolactic fermentation in wines [15]. Peptides could also interact with phenolic compounds, which have been shown to improve fining in wines [39]. Furthermore, they also contribute to organoleptic properties of food [37]. Because of the potential of peptides affecting wine quality, we are currently studying the composition of these peptides.

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