Characterization of the ozone effect over an α-amylase from *Bacillus licheniformis*

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Abstract

Starch usually soils industrial process equipment, hence demanding specific washing procedures to ensure optimal products and reliable process performance. α-Amylases have been included in detergent formulations to hydrolyse starch making easier its elimination. Ozone is frequently used as disinfectant but could also help to remove the starchy soils improving the cleaning process. To study the effect of ozone on the enzyme, the ozonation of an α-amylase from *Bacillus licheniformis* at pH 7.5 and 25 ºC was carried out. Enzyme activity assays showed that the relative α-amylase activity after ozonation decreased with increasing ozone/enzyme molar ratio exponentially. On the other hand, the ozone concentration after the reaction was negligible as some enzymatic activity remained, being the ozone consumption fast due to the high reaction kinetics of aromatic and sulfur-containing amino acids residues of the enzyme. The UV and MALDI-TOF mass spectra confirmed the oxidation of these amino acids, while the peptide bonds were unaffected. Therefore the loss of the α-amylase activity observed would be caused by the oxidation of amino acids residues directly involved in the hydrolysis mechanism such as tyrosine and histidine and/or by denaturation of the enzyme upon amino acid residues oxidation.

Keywords: α-amylase; *Bacillus licheniformis*; ozonation; starch; enzyme activity; amino acids oxidation.

1. Introduction

Amylases constitute one of the most outstanding enzyme families for biotechnological processes, being used, among others, in food, fermentation, textile, papermaking, pharmaceutical, and detergent industries [1,2]. Starch in turn, is a widespread raw material, which strongly adheres to hard surfaces [3] and therefore forms deposits on process equipment and pipes. As a result, frequent washing cycles are essential in order to achieve high quality and hygienic products, and optimal process performance. The amylases hydrolyse starch producing lower molecular weight dextrins, oligosaccharides and sugars, which are more soluble than the original starch, thus making easier to remove starchy soils; for this reason, amylases have been included in detergent formulations [4,5]. Another advantage of enzyme application in detergents is the much milder operating conditions, such as more moderated pH and lower washing temperatures, compared with enzyme free detergents [1,6,7]. In addition, amylases can provide an environmentally friendly solution, substituting other chemicals with higher impact on wastewaters [6,7]. The amylase family comprise several enzymes subclasses with different hydrolysing starch mechanisms; the α-amylase (1,4-α-D-glucan-glucanohydrolase, EC 3.2.1.1) is likely the most appropriate for starch solubilisation and it is usually employed for its liquefaction as it randomly cleaves the internal α-1,4 glucosidic bonds of the amylose and amylopectin chains, the polysaccharides present in starch [1,8]. So, it is the amylase subclass mostly used in detergents [4,5,9]. α-Amylases can be obtained from different sources, being the most important the microbial ones [1]. *Bacillus licheniformis* α-amylase, which shows a higher thermostability in comparison with other bacterial or fungal α-amylases [2,10], would be more suitable for cleaning processes than other microbial α-amylases as relatively high temperatures may be required.

Ozone, a strong oxidant, has been commonly used as disinfectant [11], but in addition, it could be also employed to remove starchy soils as it has been found to modify starch properties [12] and to depolymerize polysaccharides [13]. Hence, ozone would not only prevent microorganism growth on the starchy soiling, but it could also be used, in addition to the enzyme, to enhance the adhered starch removal. From an environmental point of view, ozone could as well replace other oxidising disinfecting agents such as chlorine that may form

secondary pollutants [14]. Furthermore, once the cleaning process had finished, ozone would allow an initial biodegradation of the surfactants present in the washing liquor [15], thus helping to the subsequent sewage treatment. Taking in account all these facts, ozone provides a new chance for the improvement of enzymatic-based cleaning processes for the starch consuming industries. Nevertheless, ozone has also been found to react with proteins and so affecting enzymes activity [16-19]. Therefore the effect of ozone on the α-amylase deserves a specific study that will provide essential data for a later optimization of the ozone dose and cleaning procedure to facilitate the enzyme performance and improve the washing efficiency due to the action of the ozone on the starchy soil. Thus in the present work we have experimentally, quantitatively and qualitatively, studied the action of ozone on a commercial α-amylase from *Bacillus licheniformis* which is used in detergent formulations.

2. Materials and Methods

2.1. Materials

α-Amylase from *Bacillus licheniformis* was purchased from Sigma-Aldrich Co. (USA). This enzyme is provided to Sigma-Aldrich by Novo Nordisk A/S (Denmark) with the commercial name of Termamyl® 120 and it is recommended for detergents formulations. Soluble potato starch was purchased from Panreac Química S.A. (Spain). Starch moisture content, 17.0±0.2% (w/w), was determined drying triplicate starch samples on an infrared balance [7]. Pierce® BCA Protein Assay Kit was purchased from Thermo Scientific Pierce Protein Biology Products (USA). All other chemicals used were analytical grade.

2.2. Enzyme protein determination

α-Amylase protein content, 24.09±0.17mg/mL, was obtained by the Pierce® BCA Protein Assay Kit that is based on the bicinchoninic acid method for the colorimetric detection and quantitation of total protein [20]. Bovine serum albumin (BSA) was used as standard and triplicate assays were done.

2.3. Enzyme activity assay

The α-amylase activity was measured at 25 ºC and pH 7.5 by the iodometric method [21] following the procedure described in previous works [6,7]. Enzyme activity assays were done by triplicate. The activity unit, UA, was defined as the amount of enzyme which degrades 1g of starch (dry matter) per minute under the established experimental conditions. The relative enzymatic activity, UAr, was defined as the ratio of enzyme activity after and before enzyme ozonation.

2.4 Ozone production and concentration measurements

Ozone was produced from an O_2 gas stream in a COM-AD-02 high voltage gas discharge ozone generator. The ozone gas stream concentration was measured by non-dispersive UV absorption in an Ozomat GM-6000-PRO ozone analyser. Both equipment were from ANSEROS Klaus Nonnenmacher GmbH (Germany).

The concentration of aqueous ozone solutions was determined by the iodometric method [22]. According to this method 2N sulphuric acid was added to 50 mL of aqueous ozone sample to adjust pH within 3-4 followed by 1g of potassium iodide dissolved in 15mL of deionised water. The iodine liberated by ozone was then titrated with standardised 0.01N sodium thiosulphate solution in an automatic titrator 736GP Titrino from Metrohm AG (Switzerland).

The residual aqueous ozone after enzyme ozonation, Or, was expressed as the fraction of ozone remaining after reaction with respect to its initial concentration in the reaction beaker.

2.5. UV-Vis and mass spectrometry measurements

UV-Vis spectra, 190-490 nm, of α-amylase samples were measured against a 0.04g/L sodium sulphite solution in 0.1M phosphate buffer, pH 7.5, as blank, using a Cary 100 Bio spectrophotometer from Varian Inc. (USA). Difference absorption spectra were constructed by subtraction of the unozonised enzyme spectrum from the ozonised enzyme spectra.

Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectra of α-amylase samples were done in an AUTOFLEX mass spectrometer from Bruker Daltonik GmbH (Germany). The mass spectrometer was run in linear mode and positive ion detection with 20kV accelerating voltage. Each well on the MALDI target was irradiated by a pulsed nitrogen laser (337nm) and operated at a 5Hz repetition rate to acquire mass spectra. Mass spectra samples were prepared as follows: 40μL of α-amylase sample were dissolved in an equal volume of a 33% v/v TFA (0.1% v/v trifluoracetic acid aqueous solution) in acetonitrile solution. 2μL of this solution were mixed with an equal volume of SA matrix (saturated sinapinic acid solution in TFA). 2μL of this mixture were put over a MALDI-TOF plate well and left to dry. Afterwards, 2μL of TFA were added and left to stay over the well for 30s, subsequently been discarded; this procedure was repeated twice to remove sample salts. Finally 2μL of SA matrix were added to each well, left to dry, and afterwards the mass spectrum was recorded within 800-80000 mass/charge range, m/z.

2.6. Enzyme ozonation experiments

2.6.1. Study of ozone influence on the enzymatic activity

Enzyme-ozone one minute reaction tests, at 25 ºC and pH 7.5, were made at different ozone/enzyme molar ratios, R ($mole_{ozone}/mole_{enzyme}$), within 10 to $4.5 \cdot 10^7$. The procedure was as follows: 250mL aqueous ozone buffered solution was prepared at 25 ºC by bubbling a 40NL/h 73.0gO_3/Nm^3 oxygen/ozone gas stream in a 0.1M phosphate buffer solution, pH 7.5, until ozone saturation (it was achieved after 10 min of ozonation, being checked in previous tests by measuring ozone concentration vs. ozonation time); 25mL ozone buffered solution was added over 25mL of α-amylase in 0.1M phosphate buffer solution, pH 7.5, and left to react in a thermostated beaker, 25 ºC, with magnetic stirring, 100rpm, for 1 minute; the enzyme buffered solution protein content was previously adjusted to achieve the desire R ratio; 2mL were withdrawn from the reaction beaker and added over 0.5mL 2g/L sodium sulphite solution in 0.1M phosphate buffer, pH 7.5, to stop the ozone-enzyme reaction, and later used for enzymatic activity assay; at the same time, the remaining 48mL of the reaction beaker were employed to determine the unexpended ozone after reaction. Enzymatic activity and

ozone concentration were also measured before the reaction and so the relative enzymatic activity and residual ozone were calculated.

2.6.2. Qualitative characterization of ozone attack

UV-Vis and mass spectrometry analysis of the α-amylase, before and after ozonation, were performed. A 150mL 0.481mg/mL α-amylase solution in 0.1M phosphate buffer, pH 7.5, was ozonised at 25 ºC for five minutes by bubbling a 5NL/h 42.5gO_3/Nm^3 oxygen/ozone gas stream. 2mL samples were withdrawn from the ozonised enzyme solution at 1 minute intervals (including zero-time sample) and added over 0.5mL 2g/L sodium sulphite solution in 0.1M phosphate buffer, pH 7.5, to stop the ozone-enzyme reaction. 1.75mL of the final samples were ten-fold diluted with a 0.04g/L sodium sulphite solution in 0.1 M phosphate buffer, pH 7.5, and their UV-Vis spectra were measured. The remaining 0.75mL volume of the final samples was frozen and later analysed by MALDI-TOF mass spectrometry.

3. Results and Discussion

3.1. Ozone effect on the enzymatic activity

The commercial α-amylase exhibited an activity of 7.02±0.30UA/mL before ozonation. For the ozonised enzyme, ozone-enzyme reaction tests showed that the relative enzymatic activity diminished with increasing ozone/enzyme molar ratio (Figure 1). The enzyme activity was completely lost for values of R approximately higher than 400-500 and so it was not experimentally measured for R values beyond 900 as it was unnecessary. Regarding ozone concentration, its initial value before the reaction was 8.85±0.36mg/L, and the residual ozone measured after reaction was found to be negligible as some enzyme activity remained, afterwards ascending and finally reaching values almost equal to 1 for very high R values (Figure 1). Additionally, the rate of ozone decomposition in 0.1M phosphate buffer solutions with pH ≤ 8 has been found to be very low and can be neglected [23]. These facts points towards a fast ozone reaction with the amino acids residues of the enzyme within the one-minute reaction time. The reaction kinetics of ozone and amino acids

have been studied in several works [23,24]. Amino acids reacts with ozone mainly through their α-amino groups, in the form of unprotonated bases, or through highly reactive side-chain groups such as aromatic and thio moieties present in some amino acids [23,24]. Furthermore, the reaction rate raises with increasing dissociation for protonated reactive species [23,24]. On the contrary, amides have been found to show very low reaction kinetics with ozone, and so, as in proteins and polypeptides the α-amino groups are linked in amide bonds, i.e. the peptide bonds, they are unlikely to react [23]. Therefore the attack of ozone should be focused on the aromatic and thio groups containing amino acids residues of the enzyme, i.e., cysteine, cystine, histidine, methionine, phenylalanine, tryptophan and tyrosine. Tables 1 and 2 summarize reaction rate constants found in bibliography for these amino acids and other chemical species with similar functional groups when reacting with ozone in aqueous solutions. All these amino acids have shown high reaction rate constants even for protonated reactive moieties (Table 1). Similarly, functional groups of these amino acids such as imidazole, phenol, sulfide and thiol have also shown high reaction rates in other chemical species, while amides hardly react (Table 2). According to these data, and bearing in mind the pK values of the protonated reactive moieties (Table 1) and our experimental pH assayed, 7.5, the amino acids can be sorted on decreasing order of reaction rate with ozone for our test conditions: cysteine > tryptophan > methionine > tyrosine > histidine > phenylalanine > cystine. Thus, these high reaction rates support the fast ozone consumption observed for its reaction with the α-amylase, the ozone being completely exhausted within the one-minute reaction time as long as there were amino acids residues susceptible to ozone attack and as result deactivating the enzyme.

As the kinetics of deactivation of the α-amylase by ozone seems to be very fast, it would be more interesting from a practical point of view to find a mathematical relationship between the enzymatic activity and the ozone dosage rather than active enzyme with time. Therefore equation (1) is proposed in order to establish the dependence of the relative enzymatic activity with the ozone/enzyme molar ratio:

$$UAr = exp(-k \cdot R) \qquad (1)$$

The experimental data from the ozone-enzyme one minute reaction tests fitted this equation (Figure 2) with a coefficient of determination of 0.99, obtaining a k value of $9.34 \cdot 10^{-3}$ mol_{enzyme}/mol_{ozone}.

3.2. Qualitative study of the ozone reaction with the enzyme

To achieve a better understanding of the ozone attack on the α-amylase UV-Vis and MALDI-TOF spectra of ozonised and unozonised enzyme were done.

The UV spectra of proteins is usually divided into near, 250-300 nm, and far, < 250 nm, UV regions. The absorption in the near-UV region is mainly due to aromatic amino acid residues: phenylalanine, tyrosine and tryptophan. The cystine residues also contribute to the total absorption in this region through electronic transitions of their disulfide bonds. Meanwhile, the absorption observed within 180-240 nm in the far-UV region is mainly due to transitions of the peptide bond; aromatic amino acid residues and histidine also absorb in this region [27] but their contribution is much less intense compared with the amide bonds due to its low concentration in comparison with the peptide bonds; in addition, the absorption of aromatic amino acids in the far-UV region is caused by combined absorptions of the side chain chromophore and of the carboxylate group; since in proteins the latter is consumed in the formation of peptide bonds, an aromatic amino acid residue absorbs at less intensity than its free form in this region [28]. Table 3 summarizes wave lengths for maximum absorbance peaks and their corresponding extinction coefficients for amino acids and peptide bond electronic transitions in neutral aqueous solutions. The pH affects the wavelength and extinction coefficients of ionisable chromophores such as tyrosine and histidine depending on their protonation state [27], but since our experimental pH, 7.5, is very close to the neutral one only a small red shift of the absorption band and a light increase in the extinction coefficient could be expected for these amino acids with respect to data in Table 3.

The UV spectra and difference absorption spectra of the α-amylase at different ozonisation times are shown on Figures 3 and 4 respectively. The 390-490nm wavelengths have been omitted in these figures since no absorption was detected within this range. For the unozonised enzyme, t = 0 min, the 200-220 nm peak would correspond mainly to the transitions of the peptide bonds, the 220-230 nm shoulder would be attributed

to histidine absorption, and the 240-280 nm peak would be due to the absorption of the aromatic amino acid residues, tryptophan, tyrosine and phenylalanine in minor contribution, together with a less intense absorption contribution of the disulfide bonds from cystine. When ozonation took place, the 200-220 nm band almost did not change with increasing ozonation time, the small absorption decrease observed being attributable to the aromatic amino acid residues oxidation, as they also slightly contribute to absorption in these region, thus suggesting negligible peptide bond breaking by ozone; this resistance of the peptide bond to ozone attack has also been suggested for other proteins and enzymes [16,23]. The 220-230 nm shoulder was observed to reduce with rising ozonation time being most likely due to the oxidation of the histidine residues. The same behaviour was observed for de 240-280 nm absorption band which should be attributed to the oxidation of tryptophan, tyrosine, phenylalanine and cystine residues. Furthermore, proteins have been found to be resistant against a dry ozone gas stream being only reactive with ozone in aqueous solution [16]. Therefore the amino acids attacked during ozonation should be located on the exterior surface of the protein where the hydrophilic environment allow for the aqueous ozone to react, while those amino acids buried in an internal hydrophobic region of the protein should be protected from ozone reaction. Finally a new increasing absorption band with ozonation time at 290-370 nm was detected and should correspond to oxidation products of the amino acids, such as N-formylkynurenine and kynurenine from tryptophan which absorbs at 320 and 360 nm respectively [17,19].

The MALDI-TOF mass spectra mass/charge peaks detected for enzyme ozonation at one minute intervals during five minutes are shown on Table 4, together with the resulting molecular weight of the enzyme. As an example, in the Supplementary Material section, Figures 1S and 2S show the mass spectra within 3000-80000 m/z obtained for unozonised and 4 minutes ozonised α-amylase respectively; below 3000 m/z no peaks where detected. Before ozonation, only two peaks could be observed on the spectrum, 55578.1 and 27843.3 m/z, corresponding to mono-charged and dual-charged ions of the α-amylase, thus obtaining a molecular weight of 55578.1 g/mol, i.e. approximately 55.6 kDa for the commercial enzyme. These mono and dual charged ions of the enzyme are also detected for all the ozonation time intervals analysed, being the estimated

molecular weight of the enzyme almost unchanged for the first three ozonation minutes and detecting a very small decrease at minutes four and five (Table 4). When ozonation took place some new m/z peaks were also detected within $8.7 \cdot 10^3$ - $3.3 \cdot 10^3$ m/z; some of these peaks, $6.82 \cdot 10^3$ m/z and $5.06 \cdot 10^3$ m/z, could correspond to multiples charges of the enzyme, 8 and 11 respectively, but the other should correspond to polypeptides other than the enzyme and likely originated from its ozonation. In any case, it can be seen that the change in the molecular weight of the enzyme with ozonation time is negligible as commented before, and the miniscule decrease from 55.6 kDa to 55.4kDa does not correspond with a generalized rupture of the α-amylase peptide bonds within the molecular mass range of the other polypeptides detected, 8.7-3.3 kDa assuming they are mono-charged, and so it should be admitted that the amide backbone of the enzyme is resistant to the ozone attack.

Therefore based on these UV and mass spectra the attack of ozone seems to be mostly aimed to the aromatic amino acids residues and to the disulfide bonds, the primary structure of the α-amylase been basically unaffected, in agreement with the reaction rate constants of the amino acids commented in section 3.1.

The generally accepted catalytic mechanism of the α-amylases involves two aspartates and one glutamate as important residues for catalysis [5,8,10]. In addition, histidine, arginine, and tyrosine play a role in positioning the substrate into the correct orientation into the active site, proper orientation of the nucleophile, transition state stabilization, and polarization of the electronic structure of the substrate [8]. The aspartic acid, glutamic acid and arginine have side-chain groups, carboxylic acids and guanidinium, known to react very slowly with ozone (Table 2). Guanidine derivatives have a similar structure to amides and have been used to produce rubbers and inks resistant to ozone degradation [30,31]. Therefore the ozone would not oxidise the active catalytic amino acid residues, aspartic and glutamic acid, but would react with histidine and tyrosine, as suggested before (Figures 3 and 4), which would lose their function in the catalytic mechanism of starch hydrolysis. Furthermore, the oxidation of these and other susceptible amino acid residues of the enzyme, i.e., cysteine, tryptophan, methionine, phenylalanine and cystine, would also be able to induce changes in the native folded structure of the protein [16], thus denaturing the α-amylase. All these facts would justify the loss of the

α-amylase biocatalytic activity observed.

4. Conclusions

Ozone has been found to deactivate the α-amylase through fast reactions with aromatic and sulfur-containing amino acids residues of the enzyme, and the enzymatic relative activity decrease of the enzyme after ozonation has fitted an exponential relationship with ozone/enzyme molar ratio. Hence, for a combined use of α-amylases and ozone that could improve the cleaning efficiency of washing processes intended to remove starchy soiling from industrial equipment, the following options are proposed to be explored:

- Using the α-amylase and the ozone in separated steps of the cleaning process, optimizing their quantities and action times in order to enhance the washing cycle performance.
- Trying to find and test chemical species able to increase the enzyme resistance against ozone attack.
- Protein engineering of the assayed α-amylase by substituting the amino acid residues susceptible to ozone attack by other more resistant, or trying to bury them in hydrophobic inner regions of the enzyme.
- Looking for other commercial α-amylases with improve resistance to oxidising agents and checking their suitability.

5. Acknowledgements

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6. References

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Figure Captions

Figure 1. Relative enzymatic activity (○) and residual ozone (△) after α-amylase ozonation at pH 7.5 and 25 ºC versus ozone/enzyme molar ratio.

Figure 2. Fitting of the relative enzymatic activity after ozonation at pH 7.5 and 25 ºC with the ozone/enzyme molar ratio.

Figure 3. UV spectra of the α-amylase at different ozonation times at pH 7.5 and 25 ºC.

Figure 4. UV difference absorption spectra of the α-amylase at different ozonation times at pH 7.5 and 25 ºC.

Figure 1S. MALDI-TOFF mass spectrum for the unozonised α-amylase.

Figure 2S. MALDI-TOFF mass spectrum for the α-amylase after four minutes of ozonation at pH 7.5 and 25 ºC.

Table 1

Table 1. Reaction rate constants, reactive moieties and pK for proton dissociation of the protonated reactive moieties for different amino acids in reactions with ozone.

Amino acid	k, $M^{-1}s^{-1}$	Reactive moiety	pK
Cysteine	$3.0 \cdot 10^{4} \pm 1.0 \cdot 10^{4}$ [b]	Thiol [*]	8.14 [a,c]
	$4.2 \cdot 10^{4} \pm 1.4 \cdot 10^{4}$ [a]	Thiol [*]	8.14 [a,c]
	$2.4 \cdot 10^{10} \pm 0.1 \cdot 10^{10}$ [a]	Thiol [**]	8.14 [a,c]
Cystine	$5.5 \cdot 10^{2} \pm 10$ [b]	Disulfide	-
Histidine	$2.7 \cdot 10^{3} \pm 6.0 \cdot 10^{3}$ [a]	Imidazole [*]	6.00 [a], 6.04 [c]
	$2.12 \cdot 10^{5} \pm 0.1 \cdot 10^{4}$ [a]	Imidazole [**]	6.00 [a], 6.04 [c]
Methionine	$>5.0 \cdot 10^{5}$ [b]	Sulfide	-
	$4.0 \cdot 10^{6}$ [a]	Sulfide	-
Phenylalanine	$5.37 \cdot 10^{2} \pm 7.74 \cdot 10^{2}$ [a]	Benzene	-
Tryptophan	$7.0 \cdot 10^{6}$ [a]	Indole	-
Tyrosine	$0.4 \cdot k_{Tryptophan}$ [a†]	Phenol	10.1 [a,c]

[a] Pryor et al. 1984 [23]

[b] Hoigne et al. 1983 [24]

[c] David R. Lide, ed., CRC Handbook of Chemistry and Physics, Internet Version 2005.

[*] Protonated

[**] Unprotonated

[†] Total reaction rate constant for protonated and unprotonated phenol moieties at pH=7.2

Table 2

Table 2. Reaction rate constants, reactive moieties and pK for proton dissociation of the protonated reactive moieties for different chemicals species in reactions with ozone.

Chemical specie	k, $M^{-1}s^{-1}$	Reactive moiety	pK
N,N-dimethylacetamide	0.7 ± 0.1 [ao]	Amide	-
N-methylacetamide	0.6 ± 0.1 [ao]	Amide	-
Benzene	2 ± 0.4 [c]	Benzene	-
Acetic Acid	$\leq 3.0\cdot10^{-5}$ [b]	Carboxylic acid [*]	4.75 [b,e]
Acetic Acid	$\leq 3.0\cdot10^{-5}$ [b]	Carboxylic acid [**]	4.75 [b,e]
Propionic Acid	$<4.0\cdot10^{-4}$ [b]	Carboxylic acid [*]	4.9 [b], 4.87 [e]
Propionic Acid	$1\pm0.5\cdot10^{-3}$ [b]	Carboxylic acid [**]	4.9 [b], 4.87 [e]
Imidazole	22 ± 5 [b]	Imidazole [*]	7.1 [b], 6.99 [e]
	$2.14\cdot10^{2}\pm7.0\cdot10^{2}$ [a]	Imidazole [*]	6.95 [a], 6.99 [e]
	$2.37\cdot10^{5}\pm0.4\cdot10^{4}$ [a]	Imidazole [**]	6.95 [a], 6.99 [e]
	$4.0\cdot10^{5}$ [b]	Imidazole [**]	7.1 [b], 6.99 [e]
Phenol	$1.3\cdot10^{3}\pm0.2\cdot10^{3}$ [b]	Phenol [*]	9.9 [b], 9.99 [e]
	$1.4\cdot10^{9}\pm0.4\cdot10^{9}$ [b]	Phenol [**]	9.9 [b], 9.99 [e]
Dipropyl sulfide	$>2.0\cdot10^{5}$ [c]	Sulfide	-
Hydrogen sulfide	$32.0\cdot10^{4}$ [d]	Hydrogen sulfide	7 [d†], 7.05 [e†]
Hydrogen sulfide ion	$1.1\cdot10^{6}\pm0.4\cdot10^{6}$ [d]	Hydrogen sulfide ion	7 [d†], 7.05 [e†]
Sulfide ion	$3.0\cdot10^{9}\pm1.0\cdot10^{9}$ [d]	Sulfide ion	19 [e‡]
Ethyl mercaptan	$>2.0\cdot10^{5}$ [c]	Thiol	-

[a] Pryor et al. 1984 [23]

[b] Hoigne et al. 1983 [24]

[c] Hoigne et al. 1983 [25]

[d] Hoigne et al. 1985 [26]

[e] David R. Lide, ed., CRC Handbook of Chemistry and Physics, Internet Version 2005.

[*] protonated

[**] unprotonated

[†] H_2S/HS^-

[‡] HS^-/S^{2-}

[o] At pH=7

Table 3

Table 3. Amino acids and peptide bond UV absorption wave lengths and their corresponding extinction coefficients for maximum absorbance peaks in neutral aqueous solutions.

Chemical specie	λ_{max}, nm	ε, $M^{-1}cm^{-1}$
Tryptophan	280[a], 279[b]	5600 [a], 5000 [b]
Tyrosine	274[a,b]	1400 [a,b]
Phenylalanine	257[a,b]	200 [a], 190 [b]
Histidine	211[a]	5900 [a]
Cystine (disulfide bond)	250[a], 250-270[b]	300 [a,b]
Peptide bond	220 [b]	100 [b]
	190 [b]	7000 [b]

[a] Freifelder. 1982 [27]

[b] Rodger et al. 2010 [29]

Table 4

Table 4. MALDI-TOF mass spectra mass/charge peaks and molecular weight of the α-amylase at different ozonation times.

Ozonation time (min)	Peaks (m/z)	MW (g/mol)
0	55578.1, 27843.3	55578.1
1	55557.1, 27823.1, 6824.4	55557.1
2	55576.1, 27843.3, 8604.9, 6824.8	55576.1
3	55570.4, 27869, 6823.3, 5791.7, 5060.4, 4370.6, 4123.8, 3969.4	55570.4
4	55415.4, 27940.5, 7922.5, 7032.2, 6824.9, 5791.2, 5085.8, 4743.7, 4503.4, 4367.3, 3581.2, 3306.7	55415.4
5	55388.5, 27797.6, 5785.7, 5056,	55388.5

Figure 1 (revised)

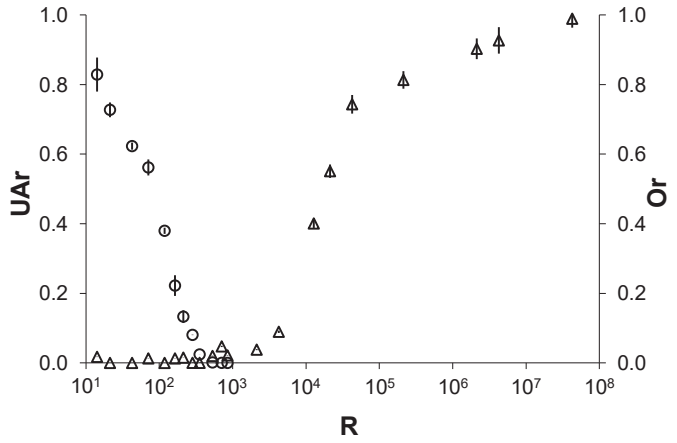


Figure 2

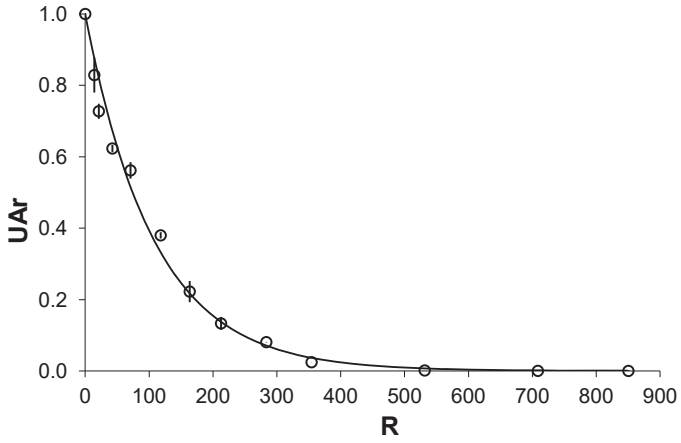


Figure 3

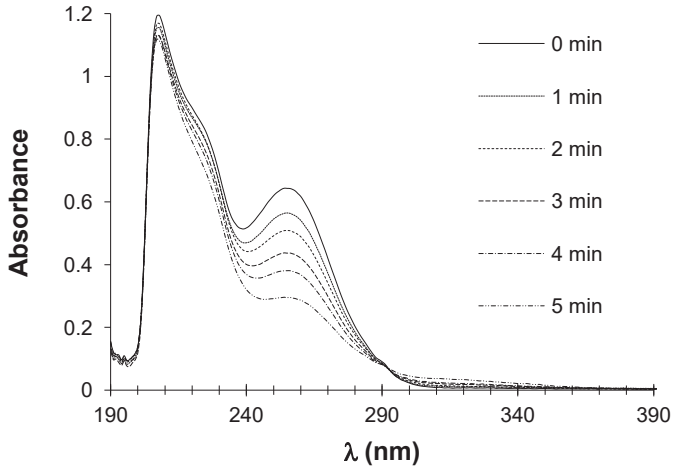

1.2
1
0.8
0.6
0.4
0.2
0
Absorbance
190
240
290
340
390
λ (nm)
0 min
1 min
2 min
3 min
4 min
5 min

Figure 4

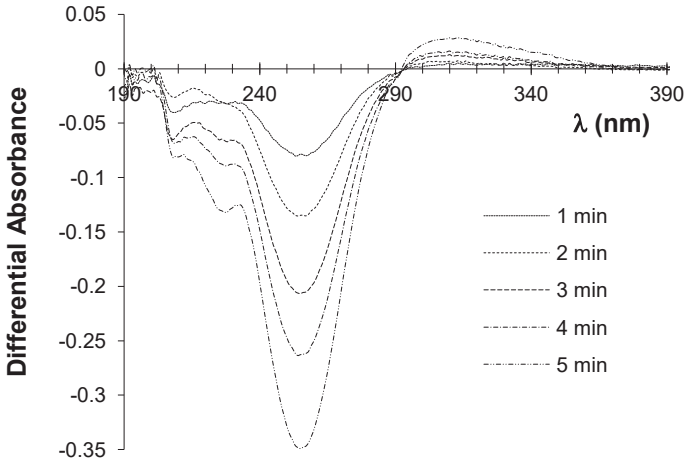
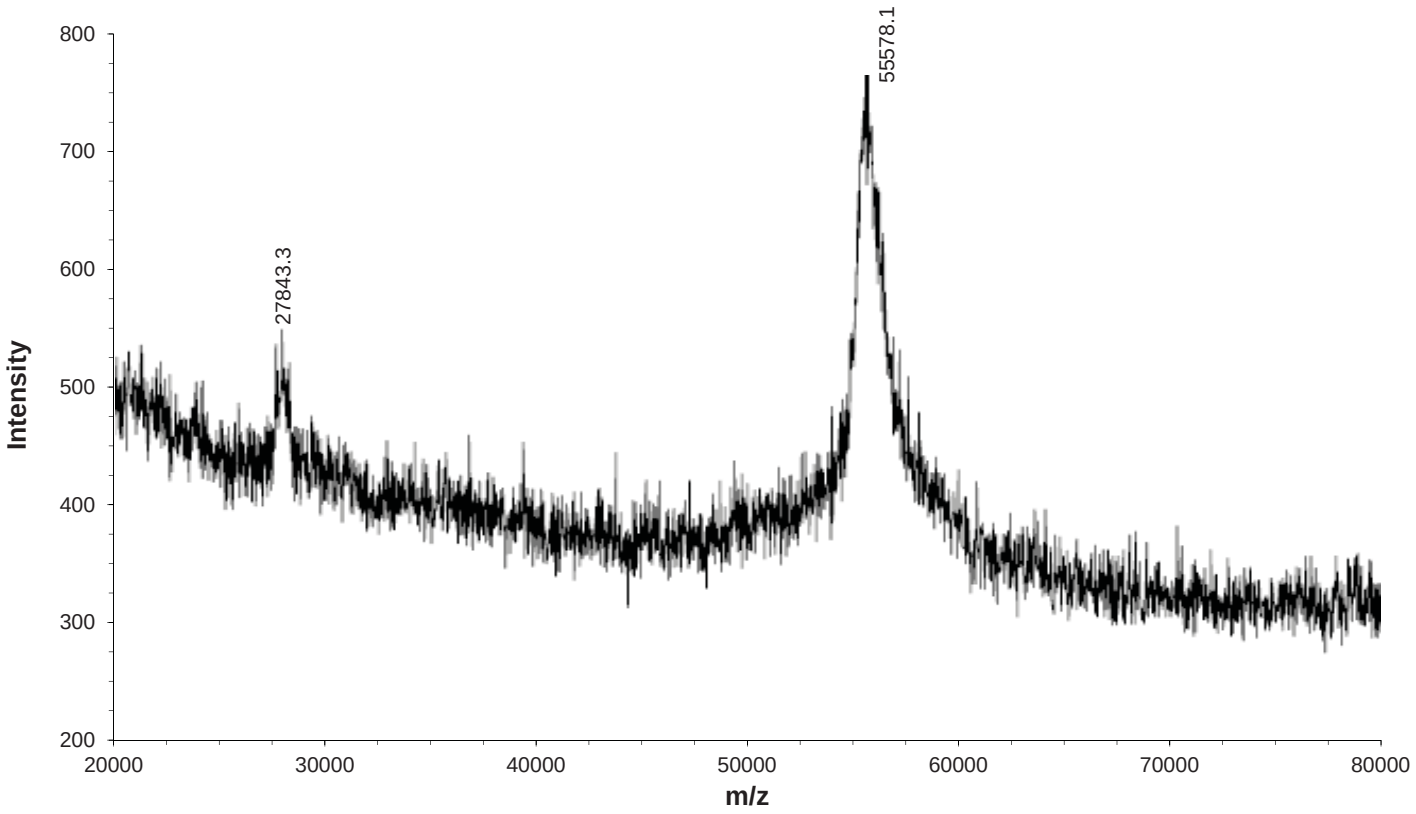

0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.3
-0.35
190
240
290
340
390
λ (nm)
Differential Absorbance
1 min
2 min
3 min
4 min
5 min

Figure 1S (Supplementary Materia)l



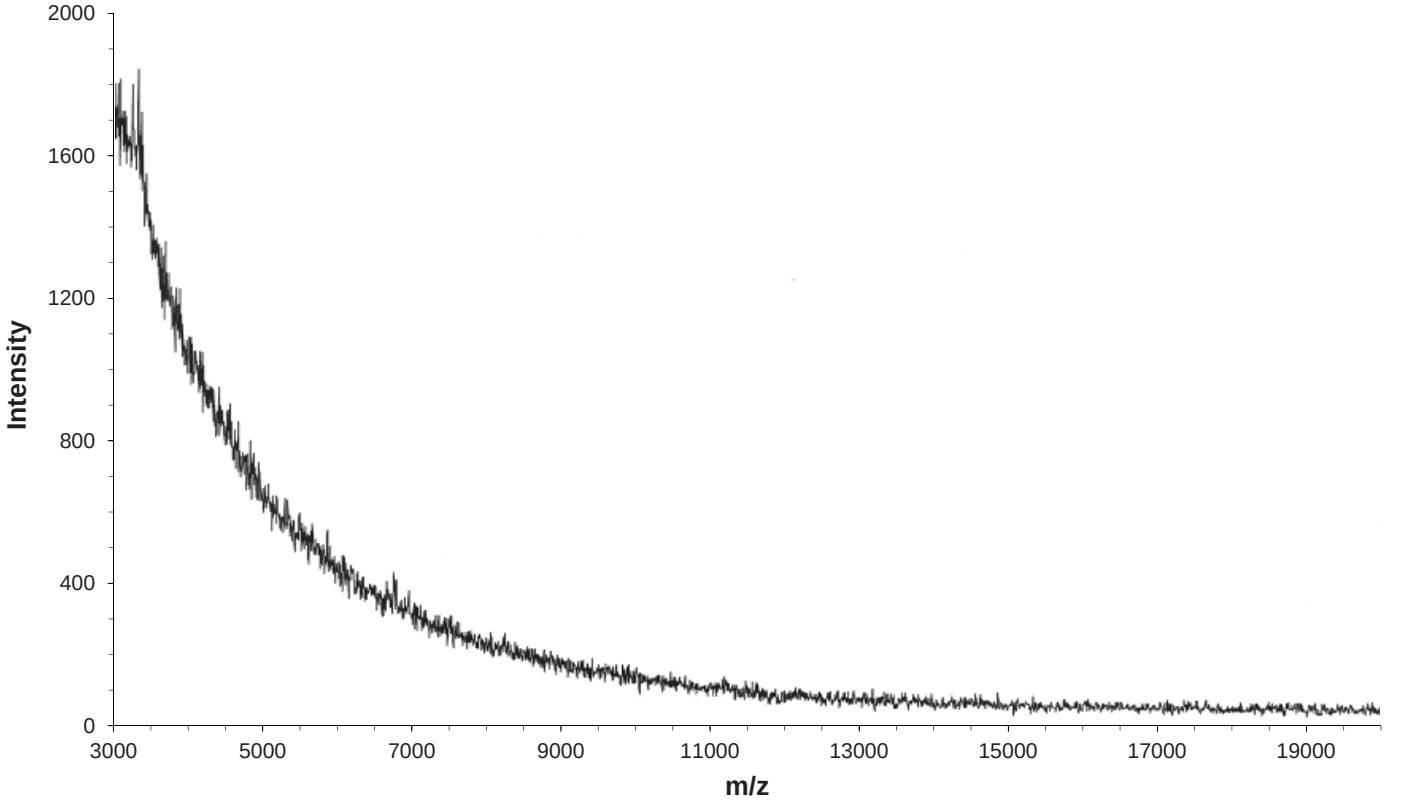


Figure 2S (Supplementary Materia)l