



Effectiveness of proteolytic enzymes to remove gluten residues and feasibility of incorporating them into cleaning products for industrial purposes

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ABSTRACT

The development of protocols for efficient gluten elimination is one of the most critical aspects of any allergen management strategy in the industry. The suitability of different proteolytic enzymes to be included in a cleaning formulation that allows the effective elimination of gluten residues was studied. Alcalase (ALC), neutrase (NEUT) and flavourzyme (FLAV) were selected from *in silico* analysis. The presence of 1% (v/v) of linear alkylbenzene sulphonate (LAS), a common anionic detergent, improved the gluten solubility, which may favour its elimination. Chromatographic analysis showed that the three enzymes studied were able to hydrolyse gluten in the presence of LAS. The highest percentage of short peptides (< 5 kDa) was achieved with ALC, what increases the probability of reducing the gluten antigenicity. Besides, in the presence of ALC and detergent LAS have detected the lowest levels of gluten with ELISA kits. So, effective amounts of ALC and LAS were added to a cleaning formulation, where its proteolytic activity was maintained above 90% after 37 days at 4 °C and 25 °C (under dark). Preliminary validation of the effectiveness enzymatic cleaning formulation to hydrolyse gluten was performed in a ready-to-eat/frozen food company, in which previous episodes of cross-contamination with gluten have been detected. The gluten content decreased to values below 0.125 µg/100 cm² when the cleaning formulation was tested on different surfaces with different cleaning protocols, demonstrating the high suitability of the enzymatic cleaning formulation developed.

1. Introduction

The World Allergy Organization estimates in approximately 240–550 million the potential food-allergic people, with an incidence of 5–8% in toddlers (often life-threatening) and 1–2% in adults (Ficchi, Sampson, Bahna, & Lack, 2013). Food allergens exist widely in different food products (Huang, Hsu, Yang, & Wang, 2014). > 90% of food allergies are derived from cows' milk, hens' eggs, peanuts, tree nuts, soy, wheat, shellfish and fish (the 'big 8') (Verhoeckx et al., 2015). These eight food categories have been defined as the most important types that cause food allergies by the Food and Agriculture Organization of the United Nations (FAO, 1995) and adopted by the Codex Alimentarius Commission (1999).

Wheat is one of the leading food crops in the world. Wheat grain contains 8–15% of protein, of which 10–15% are albumins, and

globulins and 85–90% represent the gluten proteins or prolamins. Gluten is a complex mixture of hundreds of related but distinct storage proteins, mainly gliadins and glutenins (Biesiekierski, 2017; Verhoeckx et al., 2015). Similar proteins to the gliadin of wheat can be found as secalin in rye, as hordein in barley and as avenins in oats, or in hybrids such as triticale, malt or in other ancient wheat varieties such as spelt and kamut (Biesiekierski, 2017; Kim, 2017). In all these grains, the intact gluten has been identified as the component capable of causing the disorders immune-mediated which include the coeliac disease (CD) and wheat allergy (WA), but also the non-coeliac gluten sensitivity (NCGS) (Henggeler, Verissimo, & Ramos, 2017). The CD is a disorder caused by gluten and analogous prolamines (found in rye and barley) in individuals with a genetic susceptibility, which affects between 1 and 2% of the general population (Henggeler et al., 2017). The WA is an adverse immunological reaction to gluten and other proteins found in

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wheat that represents the highest proportion (57%) of the cases produced by food ingestion (Henggeler et al., 2017; Huang et al., 2014). The WA include symptoms such as baker's asthma and rhinitis, atopic dermatitis, urticaria or anaphylaxis (Biesiekierski, 2017; Huang et al., 2014). Also, some people suffer from NCGS disorder being a reaction to the ingestion of foods containing wheat, rye and barley. It is defined as a reaction to gluten in which allergic and autoimmune mechanisms have been excluded, but its ingestion still leads to one or more of a wide range of immunological, morphological and symptomatic manifestations (Sapone et al., 2012).

However, these diseases can only be treated with a permanent and strict gluten-free diet or wheat-free diet, in the case of the WA (García-García, Madrid, García, Martín, & González, 2018; Henggeler et al., 2017; Mena, Lombardía, Hernando, Méndez, & Albar, 2012).

To reduce the incidence of gluten-related disorders, education can raise public awareness about food allergies, but accurate information about the content of allergens in food labelling is crucial for the adequate protection of allergic people (Huang et al., 2014; Ortiz et al., 2018). Thus, there is a Codex Standard that establishes a maximum gluten level of 20 ppm for food labelled as “gluten-free”. Thus, the claim cannot be used on foods containing 20 mg Kg⁻¹ or more of gluten as a result of cross-contact with gluten-containing grains or other gluten-containing ingredients (Codex Standard 118-1979, 2015; Scherf, Wieser, & Koehler, 2018). However, many foods contain gluten even though they do not contain any gluten-containing ingredients, because of cross-contact with these ingredients (Food and Drug Administration, 2013). The labels must include not only the allergenic ingredients but also the risk of the accidental presence of allergens due to cross-contamination (Food and Drink Europe, 2013). The accidental allergenic response due to the inadvertent intake of foods containing allergens has been reported frequently (Blom et al., 2018; Huang et al., 2014; Michelsen-Huisman et al., 2018; Szychaj, Pospiech, Iwanska, & Montowska, 2018; Versluis et al., 2015). Cross-contamination in the food processing industry can occur due to a diversity of practices. These practices could include (Jackson et al., 2008): an inadequate production sequence in an equipment, which causes the transport of allergenic proteins to the next product; contamination of non-allergenic food by airborne dust and aerosols from allergenic food, due to the effect of static electricity and the use of compressed air to clean equipment; inadequate cleaning of shared processing and/or packaging equipment between products; and the existence of crossing points between the different production lines. Thus, a common objective of the food industries is the development of processing technologies that inhibit the allergenic activity or eliminate allergens in order to obtain hypoallergenic or non-allergenic foods (Huang et al., 2014). The Regulation (EU) 1169/2011 states that the food industry must include allergens in their HACCP systems to reduce the risk of unexpected allergens in the food offered to consumers (Ortiz et al., 2018). So, effective cleaning is one of the most critical aspects of any allergen management strategy (Food and Drink Europe, 2013; Wang, Young, & Karl, 2010).

The allergenic potential of wheat gluten can be reduced remarkably by enzymatic hydrolysis since its allergenic epitopes contain between 5 and 20 amino acids (Merz, Appel, et al., 2016). Some authors have studied the enzymatic hydrolysis of gluten to obtain less allergenic foods (Merz, Appel, et al., 2016; Susanna & Prabhasankar, 2011; Watanabe, Watanabe, Sonoyama, & Tanabe, 2000) as well as to improve their functional properties (Drago, González, & Añón, 2008; Kong, Zhou, & Qian, 2007; Wang, Zhao, Bao, Hong, & Rosella, 2008). On the other hand, the enzymes, and particularly proteases, are commonly used as active ingredients in detergents to improve their efficiency (dos Santos Aguilar & Sato, 2018; Hellmuth & Dreja, 2016), especially in the detergent industry for laundry and dishwasher (Saperas & Fonfría-Subirós, 2011; Vojcic et al., 2015). However, until now, and to our knowledge, no studies have described the inclusion of proteases in detergents for the elimination of gluten in equipment and work surfaces of the food industry.

The objective of the present work involved the selection of suitable proteolytic enzymes to be included in a standard cleaning product and the evaluation of their efficiency to eliminate gluten residues in a food processing industry.

2. Materials and methods

2.1. Materials

Alcalase® 2.4 L (ALC, serine *endo*-peptidase from *Bacillus licheniformis*, EC 3.4.21.62), Neutrase® 0.8 L (NEUT, metalloprotease from *Bacillus amylolique faciens*, EC 3.4.24.28) and Flavourzyme® 500 L (FLAV, complex of *exo*-peptidases and *endo*-proteases from *Aspergillus oryzae*, EC 3.4.11.1) were kindly provided by Novozymes (Copenhagen, Denmark), and are all food grade. Linear alkylbenzene sulphonate (LAS) and sodium lauryl ether sulfate (SLES) were provided by KEMEGAL QUÍMICOS S.L. (Vilagarcía de Arousa, Spain). Wheat gluten, azocasein, trichloroacetic acid (TCA), acetonitrile CHROMASOLV® Plus for HPLC (ACN, ≥ 99.9%), trifluoroacetic acid CHROMASOLV® for HPLC (TFA ≥ 99.0%) and 2-mercaptoethanol for electrophoresis (> 98%) were all purchased from Sigma-Aldrich (Munich, Germany). Tris-(hydroxymethyl)-aminomethane (Tris, extra pure) was from Scharlau. Hydrochloric acid (HCl, 37%), sodium hydroxide (NaOH), sodium dihydrogen phosphate anhydrous (NaH₂PO₄), disodium hydrogen phosphate anhydrous (Na₂HPO₄), sodium dodecyl sulphate (SDS), glycerol, Coomassie Brilliant Blue R-250 and bromophenol blue were from Panreac (Barcelona, Spain). RIDASCREEN® Gliadin (Product no. R 7001), RIDASCREEN® Gliadin Competitive (Product no. R 70021) and extraction Cocktail (Product no. R 7006) were purchased from R-Biopharm AG (Darmstadt, Germany). Sterile EUROTUBO® Collection cotton swabs were from DeltaLab (Barcelona, Spain). All solutions were prepared with distilled or ultrapure water (Milli-Q Advantage A10, Millipore, CA, USA).

2.2. Determination of protein content

The protein concentration of each sample was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) following the manufacturer's instructions and using bovine serum albumin (BSA) as standard.

2.3. Measurement of the proteolytic activity of enzymes

Proteolytic activity was determined using the method proposed by Ginther (1979), with slight modifications. Azocasein, prepared at 1.25% (w/v) in a buffer with the optimum pH for each enzyme (Table 1), was used as the substrate. 240 µL of substrate solution were mixed with 60 µL of the enzyme solution. The mixture was incubated for 15 min at 25 °C on a thermo-shaker (Biosan, mod. TS-100, Riga, Latvia), regardless of the enzymes' optimum since the final product with the enzymes will be applied at room temperature. The reaction was then stopped by adding 300 µL of 10% (w/v) TCA and rapidly cooled in an ice-water bath for 10 min. Then the solution was centrifuged (Eppendorf AG, mod. 5415 R, Hamburg, Germany) at 16,100 ×g for 15 min (4 °C) and 400 µL of the supernatant were mixed with 100 µL of 1.8 N NaOH to intensify the azo-associated colour. The absorbance of

Table 1
Reaction conditions for each enzyme.

Enzyme	Buffer	pH ^a
Alcalase	Tris-HCl 200 mM	8
Neutrase	Phosphate 200 mM	7
Flavourzyme		6.5

^a Data from Novozymes.

the solutions was measured at 420 nm in a microplate scanning spectrophotometer (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). One unit (U) was defined as the amount of enzyme needed to increase one absorbance unit per minute, under the above assay conditions. Blank samples were prepared by adding distilled water instead of the enzyme. Assays were performed in triplicate.

The proteolytic activity in the presence of ionic detergents was determined in the same way but with buffers containing 1% (v/v) of LAS or SLES. Results were expressed as the relative proteolytic activity of each enzyme considering the activity measured in the buffered solution without detergent as the 100%.

2.4. Enzymatic hydrolysis of gluten

The hydrolysis was carried out in triplicate with 5 mg of commercial wheat gluten suspended per mL of distilled water, under constant magnetic stirring at room temperature ($\sim 25^\circ\text{C}$, not controlled). The pH of the hydrolysis was the optimum for each enzyme (Table 1). Control samples, containing buffer instead of gluten or proteases, were used to confirm that the hydrolysis was only caused by the proteases action. Taking into account the proteolytic activity obtained and the previous experience of our group, the selected enzyme/substrate (E/S) ratios were 1/0.6, 1/1.6 and 1/10.7 U/mg for ALC, NEUT and FLAV, respectively.

In the case of the hydrolysis optimisation in the presence of 1% (v/v) detergent LAS, samples and controls were prepared in the same way described above, but here all the amount of commercial wheat gluten were solubilised in the distilled water (5 mg mL^{-1}). Four E/S ratios (1/0.3, 1/0.6, 1/3 and 1/6 U/mg) were tested with the three enzymes.

Samples withdrawn at 0 h (just after adding the enzyme) and after 2 h were immediately cooled on ice and frozen to stop the reaction and kept until their analysis. For the immunoassay analyses (see Section 2.8), the samples taken were acidified by diluting them $2\times$ with 2% TFA to inactivate the enzymes.

2.5. Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, according to Laemmli (1970), using 10% acrylamide separating gels and 4% stacking gels in a vertical slab mini-gel apparatus (Model SE 250; Amersham Biosciences, Umeå, Sweden) at 40 mA for 1 h. Samples were $2\times$ diluted with sample buffer (125 mM Tris-HCl buffer pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 2% of 2-mercaptoethanol (w/v) and 0.002% bromphenol blue (w/v)). Then, the mixture was stirred in a vortex, boiled at 100°C for 5 min and loaded onto the gel. After migration, gels were stained for protein detection with Coomassie Brilliant Blue R-250 following standard procedures (Morrisey, 1981). Broad range molecular weight (MW) standards from Bio-Rad (California, USA) were used: myosin (200 kDa), β -galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). Commercial gluten standard was also analysed as a reference sample.

2.6. Determination of gluten solubility in the presence of ionic detergents

The soluble protein was determined with saturated gluten solutions in the presence of different concentrations (0–2% v/v aqueous solutions) of two detergents commonly used in cleaning products, LAS and SLES. Saturated solutions were prepared, at ambient temperature, with 20 mg of commercial wheat gluten suspended per mL of distilled water. After 24 h, the excess of gluten was decanted, and a sample was removed from the supernatant and then filtered throughout $0.2\text{ }\mu\text{m}$. Protein content was also determined in control samples of detergents without gluten, and the result was negative. Six replicates were evaluated for each sample.

Gluten solubility was calculated as follows:

$$\text{Gluten solubility (\%)} = \frac{C_{\text{SUP prot}}}{C_{\text{T prot}}} \times 100 \quad (1)$$

where $C_{\text{SUP prot}}$ is the protein concentration determined in the supernatant of each sample in mg mL^{-1} , and $C_{\text{T prot}}$ is the total protein concentration corresponding to the initial concentration of gluten in mg mL^{-1} .

2.7. High-performance gel permeation chromatography (HP-GPC)

The protein profiles of the hydrolysates were obtained following the methodology described by Power-Grant et al. (2015) with some modifications. The samples ($20\text{ }\mu\text{L}$) were loaded on a TSKgel G2000 SWXL ($5\text{ }\mu\text{m}$, $7.8\text{ mm} \times 300\text{ mm}$; Tosoh Bioscience, Grove City, OH, USA) column coupled to a TSKgel Super SW Guard column ($6\text{ }\mu\text{m}$, $4.6\text{ mm} \times 35\text{ mm}$; Tosoh Bioscience, Grove City, OH, USA) and connected to an Agilent 1200 HPLC system equipped with a Variable Wavelength Detector (Agilent Technologies, Waldbronn, Germany). Protein elution was followed at 214 nm. The isocratic elution was performed at 25°C with a flow rate of 0.25 mL min^{-1} during 60 min, employing 0.1% (v/v) TFA in 30% (v/v) ACN. All the samples were filtered through $0.2\text{ }\mu\text{m}$.

The following standards were used for MW determinations: BSA (66,000 Da), β -Lactoglobulin (36,000 Da), α -Lactalbumin (14,178 Da), aprotinin (6500 Da), and Val-Pro-Pro (347 Da). The calibration curve was prepared by plotting the logarithm of the MW against the retention time (RT) of the standards. To analyse the MW distribution, the chromatograms obtained after 2 h of hydrolysis were divided into three zones: 0–26.5 min ($> 30\text{ kDa}$), 26.5–33.2 min ($30\text{--}5\text{ kDa}$) and 33.2–49 min ($< 5\text{ kDa}$), and the percentage area within a defined MW range was calculated with respect to the total area for each chromatogram.

2.8. Determination of the antigenicity of wheat gluten hydrolysates

The gluten quantitative analysis of the hydrolysates was made with the conventional sandwich R5 ELISA (for raw products, spices as well as, processed food) and competitive R5 ELISA (for fermented and hydrolysed food) with the corresponding RIDASCREEN® Gliadin kits (R-Biopharm AG, Darmstadt, Germany). The manufacturers' instructions for proper use were followed. These enzyme immunoassays use R5 monoclonal antibody that recognises several, small repetitive potentially celiac-toxic epitopes, QQPFP, LQFPF, QLPYP, QLTP, QQSFP, QQTFF, PQPFP, QQPYP and PQPFP, which repeatedly occurs in the prolamin molecules (Kahlenberg et al., 2006; Osman et al., 2001).

Gluten determinations were performed either directly in the hydrolysates or using the extraction Cocktail patented (López Villar, Llorente Gómez, & Méndez Cormán, 2002), recommended for sample preparation by the manufacturer, to elucidate the effect of the interfering compounds from the hydrolysates, mainly buffer, enzymes (although inactivated) and detergents.

2.9. Measurement of the alcalase stability in the cleaning formulation

The stability of ALC in the final cleaning formulation was evaluated during 37 days at refrigeration (4°C) and ambient (25°C) temperature in darkness. Control samples of ALC prepared in 200 mM Tris-HCl buffer at pH 8 were evaluated under the same conditions. Assays were performed in triplicate. At selected time intervals, and after thorough mixing, aliquots were withdrawn and immediately assayed for proteolytic activity using the protocol described in Section 2.3. The initial activity (at 0 h) was taken, as 100% and the results were expressed as percentages of the residual activity.

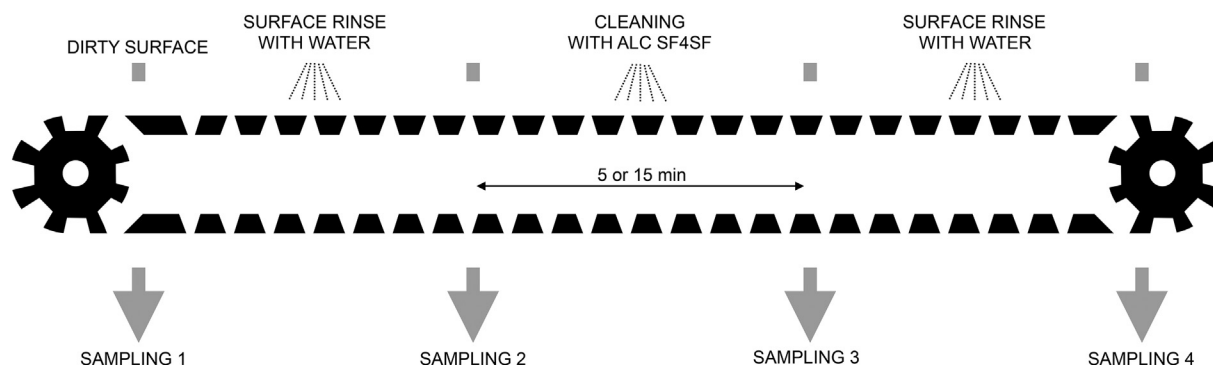


Fig. 1. Scheme of the cleaning protocol followed to test the enzymatic cleaning formulation in the production line of a food processing factory. Sampling 1: after food processing and before rinse with raw water. Sampling 2: after rinsing with raw water and before applying the enzymatic cleaning formulation. Sampling 3: after the actuation of the enzymatic cleaning formulation and before an additional rinse with raw water. Sampling 4: after the last rinse with raw water.

2.10. Validation of the enzymatic cleaning formulation in the production line of a food industry

CLAVO FOOD FACTORY (www.clavofoodfactory.com), a company which produces different types of ready to cook and breaded products (with or without gluten) in separated production lines, kindly offered their facilities in Caldas de Reis (Spain) to perform the validation. The enzymatic cleaning formulation was tested on different machines and surface materials (plastic polymer and stainless steel) from the production line (conveyor line, dusting, battering and breading machines). A scheme with the cleaning protocol followed is depicted in Fig. 1. Four samplings were made. Sampling 1 was made after processing of food products with gluten (flour, breadcrumbs), in different dirty surfaces of the production line. After that, all the production line was rinsed with raw water. Then additional reference samples were taken (sampling 2) to determine the gluten content before applying the enzymatic cleaning formulation. The cleaning process continued with the application of the enzymatic cleaning formulation, which remained in contact with the tested surfaces during two different contact times, 5 and 15 min, after which it was carried out sampling 3. After that, all the production line was rinsed again with raw water, and then a final sampling was performed (sampling 4).

The cleaning product was directly applied to the tested surfaces (10 × 10 cm) by using a low pressure-spraying device. In all the cases, sampling was carried out by the swabbing method for the analysis of allergens in the production line, following the manufacturer's instructions described for the RIDASCREEN® allergen tests. Briefly, the tested surfaces were wiped with a cotton-tipped swab, and then the cotton swab tip was transferred into a reaction vial and covered with 0.25 mL of the extraction Cocktail. Later, the sample was incubated for 40 min at 50 °C in a water bath. This extract was diluted 12.5 times. Three analytical replicates were prepared to quantify gluten with the conventional sandwich R5 ELISA, and some points were also tested with the competitive R5 ELISA.

3. Results and discussion

3.1. Selection of target enzymes

Proteolytic enzymes were first selected by *in silico* analysis, using the ExPASy proteomics server of the European Bioinformatics Institute (<https://www.expasy.org/>), which offers open access databases and analysis tools for protein sequences.

The primary sequences of several glutenins and gliadins were located and downloaded from the UniProtKB protein database (entries P10388, P10387, P10385, P04722, P18573, P02863 and P21292). Moreover, the sequence of the 33-mer peptide, which is resistant to gastrointestinal digestion and strongly allergenic (Schalk, Lang, Wieser,

Koehler, & Scherf, 2017), was also analysed. The sequences were analysed with the PeptideCutter tool (https://web.expasy.org/peptide_cutter/), which allows predicting potential protease cleaved sites in an amino acid sequence. So, the proteases that, according to the tool, will generate more peptide fragments from the gluten proteins, and in particular, from the 33-mer peptide, will be the best candidates for experimental tests.

PeptideCutter predicts that proteases such as trypsin or proline-endopeptidase do not have specific cleavage sites in the 33-mer peptide. The proline-endopeptidase hydrolyses specifically peptide bonds with Pro in the P1 position. However, it does not produce any fragment from the 33-mer peptide. This fact confirms the difficulty of hydrolysing highly immunogenic gluten peptides, which is deduced from the 33-mer sequence, composed almost exclusively by glutamine (30%) and proline (39%) (data obtained with the ProtParam tool, <https://web.expasy.org/protparam/>). It was determined that, when Pro occupies the position P1 of the peptide bond (N-terminal end), the activity of the endopeptidase is blocked if there is another Pro at the P1' position (C-terminal end). In general, at the position P2 there is a basic amino acid (Lys, His, Arg) (Cebolla, Moreno, Coto, & Sousa, 2018; Fülöp, Böcskei, & Polgár, 1998). However, these amino acids are not present in the 33-mer peptide.

On the other hand, proteases such as pepsin (pH 1–2), proteinase K and thermolysin have 6, 10 and 2 potential cut sites, respectively, in the 33-mer peptide. Since the optimum pH of pepsin is very far from that of industrial cleaning solutions (usually strongly alkaline), this protease was discarded, and the proteinase K and thermolysin were selected for the experimental tests. Thermolysin preferentially cleaves peptide bonds with bulky and aromatic residues (Ile, Leu, Val, Ala, Met or Phe) at the position P1'. Cleavage is hindered when there is an acidic residue in position P1 or a Pro residue in P2' (Keil, 1992). On the other hand, proteinase K (EC 3.4.21.64) shows an affinity for aromatic or aliphatic residues, and its hydrolytic activity is strongly promoted by long N-terminal regions of peptide substrates (Ageitos, Baker, Sugahara, & Numata, 2013).

Since these enzymes will be incorporated into industrial cleaning solutions, the use of pure enzymes was discarded because of their high cost. For this reason, equivalent enzymes of technical-grade existing in the market were searched with the BRAunschweig ENzyme DAtabase (BRENDA, <https://www.brenda-enzymes.org/>), which collects molecular and biochemical information about enzymes and metabolic pathways. Thus, under the name proteinase K there are two enzymes with EC numbers (Enzyme Commission numbers) 3.4.21.62 and 3.4.21.64, respectively. The first (EC 3.4.21.62), which appears as a broad-spectrum enzyme with a preference for hydrophobic amino acids, is marketed as technical-grade (low purity) preparation under different commercial names (Alcalase® and Savinase® of the company Novozymes). The second one (EC 3.4.21.64) that is shown as an enzyme

capable of hydrolysing keratin does not have a low-cost commercial equivalent. Thermolysin (EC 3.4.24.27) has substrate specificity very similar to the protease produced by *Bacillus amyloliquefaciens* (EC 3.4.24.28), which is marketed as a technical-grade preparation by Novozymes under the denomination Neutrase®. Therefore, the proteases selected for the hydrolysis of gluten proteins were Alcalase® (ALC) and Neutrase® (NEUT).

Flavourzyme® (FLAV, EC 3.4.11.1) derived from the *Aspergillus oryzae* strain ATCC 42149/RIB 40 and marketed by Novozymes, is an enzyme frequently used for the hydrolysis of gluten proteins (Merz, Eisele, et al., 2015), so it was also included in the subsequent hydrolysis experiments.

3.2. Enzymatic hydrolysis of gluten

The ability of the selected enzymes (ALC, NEUT and FLAV) to hydrolyse gluten proteins was initially tested using optimum pH values and the selected E/S ratios for each enzyme (see Section 2.4). Hydrolysis was performed during 2 h, at ambient temperature (25 °C), to reproduce the conditions at which the gluten elimination from the work surfaces will be foreseeably carried out.

The analyses of the hydrolysates by SDS-PAGE (Fig. 2) confirmed that all the enzymes were able to hydrolyse the gluten completely after 2 h, and also that ALC was able to hydrolyse the gluten in a few minutes (necessary time to take and process the sample), since no protein bands were observed at 0 h. This indicates that these enzymes, especially ALC and FLAV, act very quickly generating small fragments that are not detectable by this technique. These enzymes showed before succeeding by hydrolysing gluten under different conditions (Apar & Özbek, 2008; Berends et al., 2016; Choi, Lim, He, & Hwang, 2018; Kim, 2017; Kong et al., 2007). So, the proteases herein selected represent good candidates to achieve the complete hydrolysis of gluten proteins.

3.3. Solubility of gluten in the presence of anionic detergents

During the previous hydrolysis, gluten was not completely dissolved, probably due to its low solubility in aqueous solutions (Mejri, Rogé, BenSouissi, Michels, & Mathlouthi, 2005; Wieser, 2007). This can hinder the gluten extraction from the work surfaces and machines with the traditional cleaning protocols used in the food industry. So, to find the conditions that maximise the gluten protein solubility is important.

LAS and SLES are two anionic surfactants that currently represent the base formulation of a wide range of commercial detergents and

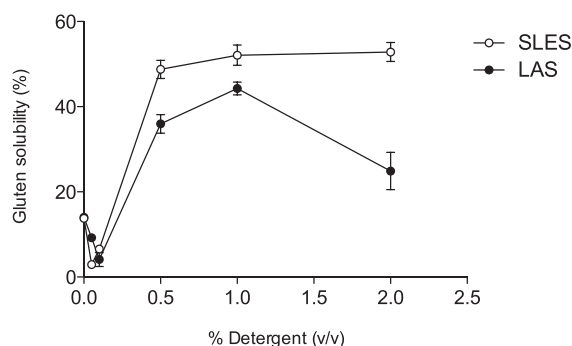


Fig. 3. Gluten solubility in the presence of the anionic detergents linear alkylbenzene sulphonate (LAS) and sodium lauryl ether sulfate (SLES).

personal care products (Liley et al., 2017). We evaluate the gluten solubility of aqueous solutions with different percentages of these surfactants. Results depicted in Fig. 3, showed that the gluten solubility was improved by increasing the concentration of detergent until reach a maximum solubility at around 1% (v/v) of both detergents. Therefore, 1% (v/v) was selected as the optimal concentration of both detergents for subsequent experiments.

3.4. Proteolytic activity of enzymes in the presence of anionic detergents

The proteolytic activity of ALC, NEUT and FLAV was analysed in the presence of the detergents LAS and SLES to know the effect of their presence on the activity of the enzymes. The activity was measured at the optimal detergent concentration determined in the previous section (at ambient temperature and the optimum pH for each enzyme). The relative proteolytic activity of each enzyme is depicted in Fig. 4 and was calculated considering the activity measured in a buffered solution without detergent as the 100%.

Anionic surfactants are more effective in removing dirt, and that is why they play a significant role in modern cleaning products (Cornwell, 2018; Kogawa, Cernic, do Couto, & Salgado, 2017; Williams, 2007). However, it is well known that ionic surfactants interact strongly with enzymes by altering their tertiary structure (denaturation) resulting in the loss of activity and, in the case of proteases, by increasing self-digestion (autolysis) (Hellmuth & Dreja, 2016; Stoner et al., 2004). Also, anionic surfactants can form complexes and remove calcium ions, destabilising the structure of many bacterial proteases (Lalonde, Witte, O'Connell, & Holliday, 1995; Russell & Britton, 2002; Stoner et al., 2004).

The activity of all enzymes decreased in the presence of the detergents, but especially in the presence of the SLES (Fig. 4). In the presence of the detergent LAS, activity decreased by 16.03%, 29.01% and 54.54% for ALC, NEUT and FLAV, respectively. However, the

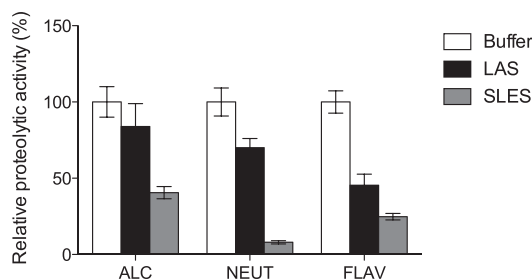


Fig. 4. Proteolytic activity in the presence of the anionic detergents linear alkylbenzene sulphonate (LAS) and sodium lauryl ether sulfate (SLES) (1%, v/v). The proteolytic activity is expressed as a percentage of the initial value obtained without detergent: 21.22 ± 1.65 U/mg for alcalase (ALC), 7.34 ± 0.48 for neutrase (NEUT), and 0.40 ± 0.02 U/mg for flavourzyme (FLAV).

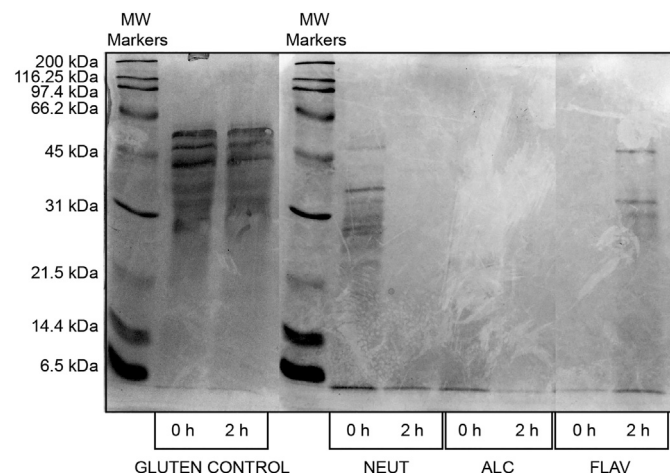


Fig. 2. SDS-PAGE profiles of the gluten control (without enzyme) as commercial standard and the gluten hydrolysates obtained with neutrase (NEUT), alcalase (ALC) and flavourzyme (FLAV) at ambient temperature for 2 h, employing the broad range molecular weight (MW) standards from Bio-Rad.

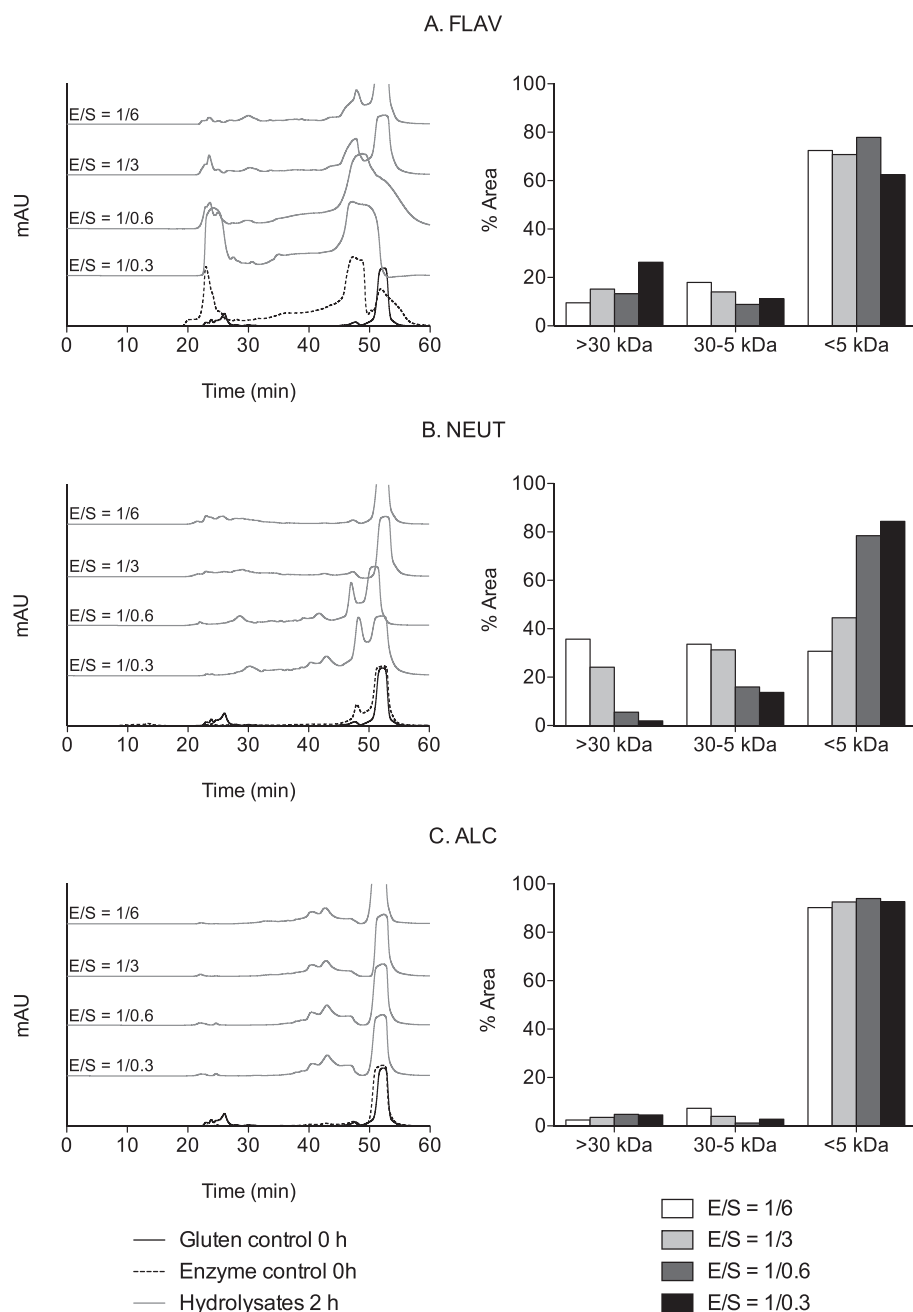


Fig. 5. HP-GPC profiles and molecular weight distribution (%Area) obtained in the hydrolysis of gluten with (A) flavourzyme (FLAV), (B) neutrase (NEUT), and (C) alcalase (ALC), at different E/S ratios and in the presence of detergent linear alkylbenzene sulphonate (LAS) 1% (v/v), after 2 h of incubation at ambient temperature.

proteases were more sensitive to the presence of SLES, which decreased the activity by 59.41%, 91.34% and 75.17%, for ALC, NEUT and FLAV, respectively. So, for the next experiments, we decided to use LAS at 1% (v/v) as detergent, which allows improving the solubility of the gluten in water maintaining acceptable levels of proteolytic activity.

3.5. Optimisation of the enzymatic hydrolysis of gluten in the presence of detergent LAS

The suitability of the three proteases (ALC, NEUT and FLAV) to be incorporated in a detergent formulation was investigated by testing their capability to hydrolyse the gluten proteins in the presence of the detergent LAS (1%, v/v). For this purpose, gluten was hydrolysed during 2 h using four E/S ratios (1/0.3, 1/0.6, 1/3 and 1/6 U/mg) with all the enzymes, and the hydrolysates obtained were analysed by HP-

GPC (Fig. 5). An analysis of the MW distribution was also performed by grouping the areas of the protein fragments as follows: > 30 kDa (0–26.5 min RT), 30–5 kDa (26.5–33.2 min RT), and < 5 kDa (33.2–49 min RT).

The HP-GPC profile (Fig. 5) of all the samples revealed that around 53 min appears a large peak that corresponded with the detergent LAS. Besides, it can be observed in the gluten control profiles that this protein fraction does not correspond to a single peak because it is constituted by a mixture of proteins (glutenins and gliadins).

The results obtained showed that, in general, the three proteases were able to hydrolyse the gluten protein in the presence of LAS (Fig. 5). At 0 h, the appearance of peptides is already observed for the three enzymes (data not shown) indicating that these proteases were able to hydrolyse gluten proteins very fast, as also showed before (see Fig. 2). Besides, after 2 h of reaction, the hydrolysis of the gluten and

the increase of the peptides produced, especially those < 5 kDa, were observed for the three enzymes (Fig. 5). However, differences in the peptide profile between the different E/S ratios and each enzyme can be observed. The results are in concordance with those described previously by other authors (Apar & Özbek, 2008; Choi et al., 2018; Kong et al., 2007).

In the case of FLAV (Fig. 5A), the results obtained should be taken with caution because the enzyme co-eluted with the gluten fraction and this hindered the quantification of the gluten hydrolysis and the production of peptides. Recent studies have shown that the commercially FLAV preparation consists of a complex mixture of eight enzymes (two aminopeptidases, two dipeptidyl peptidases, three endopeptidases, and one α -amylase) (Merz, Eisele, et al., 2015), which contribute differently in the hydrolysis of wheat gluten and, therefore influence the reproducibility of the process (Choi et al., 2018; Merz, Ewert, et al., 2015; Merz, Kettner, et al., 2016). Also, in our case, it was necessary to add too much enzyme to obtain the same activity units that with the other enzymes, since, as we discussed above, it loses much activity in the presence of LAS.

On the other hand, when the amount of NEUT was increased, the production of peptides < 5 kDa also increased, and simultaneously decreased the fragments of 30–5 kDa (Fig. 5B). However, the highest percentage of low MW peptides (< 5 kDa) was achieved with ALC (Fig. 5C), even at low E/S ratios, compared with the rest of the enzymes tested. Choi et al. (2018) also pointed alcalase as the best to hydrolyse wheat gluten into peptides among the employed enzymes (alcalase, flavourzyme, protamex and neutrase). In this work, alcalase produced hydrolysates with the highest amount of low MW peptides (< 500 Da) and the lowest amount of high MW peptides (> 10 kDa).

The fact that the main products obtained from the wheat gluten hydrolysis with ALC were fragments lower than 5 kDa increases the probability of reducing the antigenicity because, according to with AllergenOnline database (www.allergenonline.org/), the majority of the allergenic sequences contain more than eight amino acids, and therefore, will have a MW higher than 8 kDa.

In general terms, the E/S ratios 1/0.3 and 1/0.6 led to the highest percentages of short fragments with the three enzymes. However, from an industrial point of view, the E/S ratio 1/0.6 could be more suitable because less amount of enzyme is required to achieve good hydrolysis levels. Therefore, the E/S ratio 1/0.6 were selected for the subsequent experiments. At this point, based on the results described above, the ALC was postulated as a good candidate to develop a commercial formulation to eliminate gluten. Chromatographic analysis gave us a qualitative study of the effectiveness of each enzyme. Next, we carried out a quantitative analysis of the residual gluten in the hydrolysates obtained with all the enzymes to get a complete analysis that allows us to decide which enzyme would be the most suitable to include in the cleaning formulation.

3.6. Quantification of gluten in the hydrolysed samples

To evaluate the effectiveness of the enzymatic hydrolysis to eliminate gluten, samples obtained from the previous assays, with and without detergent, were analysed by R5 ELISA.

3.6.1. Optimisation of quantification

Samples from the previous hydrolysis (with different enzymes, with and without detergent, and different E/S ratios), with expected different gluten content, were selected to check the need for an extraction step before the ELISA tests. A correlation analysis between gluten content in the sample after extraction with the patented *Cocktail* and without the extraction step is shown in Fig. 6. The results did not differ significantly between the two methods used (high correlation coefficient: $r^2 > 0.97$). Therefore, the extraction step was considered not necessary for the further immunoassay analyses of the hydrolysates.

The influence of the presence of detergent LAS in the R5 ELISA was

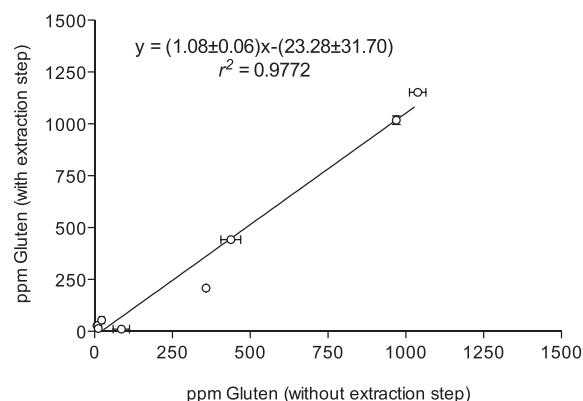


Fig. 6. Relationships between gluten content determined with the R5 ELISA after extraction with the patented Cocktail and without extraction step.

evaluated by the analysis of samples prepared by mixing the gluten control at 0 h (from the hydrolysis assay) with the detergent LAS (to obtain a final concentration of 1%, v/v). The presence of detergent LAS did not affect the quantification of gluten with the R5 ELISA as could see in Table 2. Approximately, half of the gluten detected in the absence of detergent LAS was quantified when it was diluted (1:1) with detergent LAS.

Although the enzymes in the hydrolysates were inactivated by diluting them 2 × with 2% TFA, a check was performed to make sure that they did not interfere with the R5 ELISA. Samples for the analysis were prepared by mixing the gluten control at 0 h (from the hydrolysis assay) with the different inactivated enzymes (to obtain a final E/S = 1/0.6). Results showed that both ALC and NEUT did not affect the determination, as the amount of gluten detected (half of the amount detected in the gluten control) was consistent with the dilution performed (1:1) (Table 2). In the presence of FLAV a more considerable amount of gluten was detected (higher than half of the amount detected in the gluten control) (Table 2). FLAV is sold as an industrial peptidase preparation, derived from *Aspergillus oryzae* (Merz, Eisele, et al., 2015). This preparation contains a mixture of peptidases in a non-pure form, so it includes components from the culture media that could interfere in the quantification of gluten. We had to add a more considerable amount of FLAV to achieve the same U/mg as ALC and NEUT, during the hydrolysis of gluten. So, the interferences were also increased, causing the lack of reproducibility of the assay in the presence of FLAV, as occurred in the chromatographic analysis (Fig. 5A).

3.6.2. Quantification of gluten in the enzymatic hydrolysates

Once evaluated and optimised the conditions to quantify gluten in the hydrolysates employing the R5 ELISA, the samples from the enzymatic hydrolysis in the presence of both buffer and detergent LAS were analysed. As shown in Fig. 7, the results obtained with the sandwich R5 ELISA were consistent with those obtained in the chromatographic analysis (Section 3.5). After 2 h of hydrolysis, remaining amounts of

Table 2

Gluten determination with sandwich R5 ELISA in gluten control diluted with the inactivated enzyme controls, corresponding to the samples at 0 h from the hydrolysis assay in a buffered solution.

Samples	ppm Gluten
Gluten control	1017
Gluten control diluted 1:1 with LAS	553
Gluten control diluted 1:1 with FLAV	736
Gluten control diluted 1:1 with NEUT	493
Gluten control diluted 1:1 with ALC	519

LAS: linear alkylbenzene sulphonate detergent; ALC: alcalase; NEUT: neutrase; FLAV: flavourzyme.

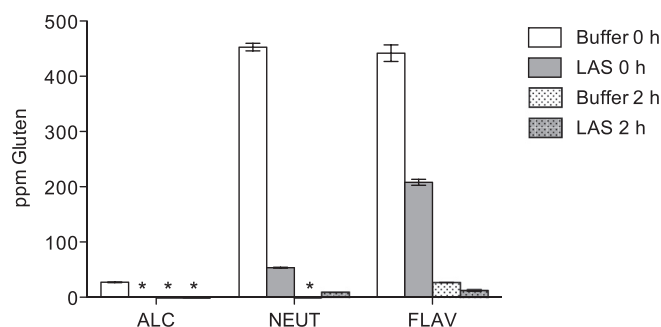


Fig. 7. Quantification of gluten with sandwich R5 ELISA in the hydrolysates obtained in the presence of buffer and detergent linear alkylbenzene sulphonate (LAS) (1%, v/v), and alcalase (ALC), neutrase (NEUT), and flavourzyme (FLAV) (E/S = 1/0.6, at ambient temperature for 2 h). *Value lower than the detection limit (< 5 ppm).

gluten were detected with NEUT and FLAV. However, the use of ALC resulted in the disappearance of gluten below the limit of detection (< 5 ppm), even at the initial time in the presence of detergent LAS (very rapid hydrolysis) (Fig. 7). In general, lower levels of gluten were detected in the presence of detergent LAS.

This analysis confirmed that ALC could be used in a matrix with detergent LAS as an effective combination for the elimination of gluten.

The sandwich R5 ELISA needs at least two specific celiac-toxic epitopes. However, prolamins presented in samples are partially hydrolysed in fragments with one or more celiac-toxic epitope. Consequently, these small fragments, containing a single celiac-toxic epitope cannot be measured by conventional sandwich R5 ELISA (the hydrolysed prolamins are always underestimated). For this reason, a competitive assay was carried out to analyse the gluten content in the hydrolysates and to compare with the results of the conventional R5 ELISA (Fig. 8). As expected, competitive R5 ELISA was more efficient than sandwich R5 ELISA in quantifying hydrolysed samples, in the presence of both buffer (Fig. 8A) and detergent LAS (Fig. 8B), as demonstrated by Dostálek, Hochel, Méndez, Hernando, and Gabrovská (2006) in the analysis of beer. The results were consistent with the previous analysis of the hydrolysates. The hydrolysis of gluten was already detected at time 0 h and after 2 h of hydrolysis with ALC gluten was not detected. Moreover, the results showed that the E/S ratio employed (1/0.6) resulted enough to eliminate the gluten in the case of ALC (Fig. 8).

3.7. Stability of proteolytic activity in the cleaning formulation

It is known that the activity of enzymes in liquid detergent formulations may be decreased as a result of the interactions with the other compounds in solution. As was mentioned in previous sections,

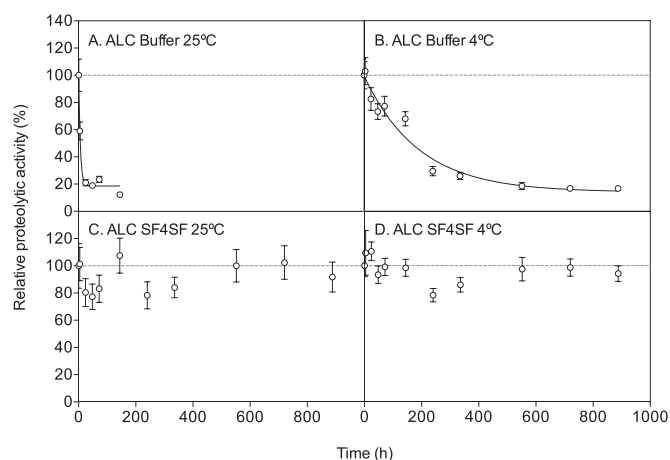


Fig. 9. Stability of proteolytic activity of alcalase in the 200 mM Tris-HCl buffer pH 8 (ALC Buffer) at (A) 25 °C and (B) 4 °C and in the cleaning formulation (ALC SF4SF) at (C) 25 °C and (D) 4 °C.

anionic surfactants such as LAS are known to reduce the stability of proteases, probably by accelerating autolysis processes, but also complexing calcium ions that stabilise the protease structure (Hellmuth & Dreja, 2016; Russell & Britton, 2002; Stoner et al., 2004). From a commercial point of view, it is necessary to incorporate additional stabilising agents to the cleaning formulation to ensure enzymatic activity after the storage time of the cleaning formulation. Among others stabilisers, monopropylene glycol and calcium chloride act blocking the active sites of the enzyme and stabilising the protease structure (Banerjee, Sani, Azmi, & Soni, 1999; Lalonde, Witte, O'Connell, & Holliday, 1995; Maurer, 2004; Stoner et al., 2004). Moreover, as recommended by the enzyme manufacturer, lowering the water content (< 60%) and ensuring a pH range around 7–8 will help to stabilise the enzyme by reducing autolysis. With these premises, a cleaning formulation was developed in collaboration with KEMEGAL QUÍMICOS S.L. (Vilagarcía de Arousa, Spain). The final cleaning formulation (ALC SF4SF) was prepared by adding the effective amounts of ALC and LAS, tested in the previous laboratory assays, into an aqueous cleaning matrix based in an equilibrated mixture of monopropylene glycol and calcium chloride as stabilising agents, together with the needed amount of an alkaline agent for adjusting the pH to the optimal activity of the ALC (pH = 8).

The stability of the proteolytic activity in the final cleaning formulation was evaluated during 37 days at refrigeration (4 °C) and ambient temperature (25 °C) in darkness. Besides, samples of ALC prepared in 200 mM Tris-HCl buffer at pH 8 were included as controls.

In the presence of the buffered solution (Fig. 9A, at 25 °C, and Fig. 9B, at 4 °C), the proteolytic activity of the ALC decreased

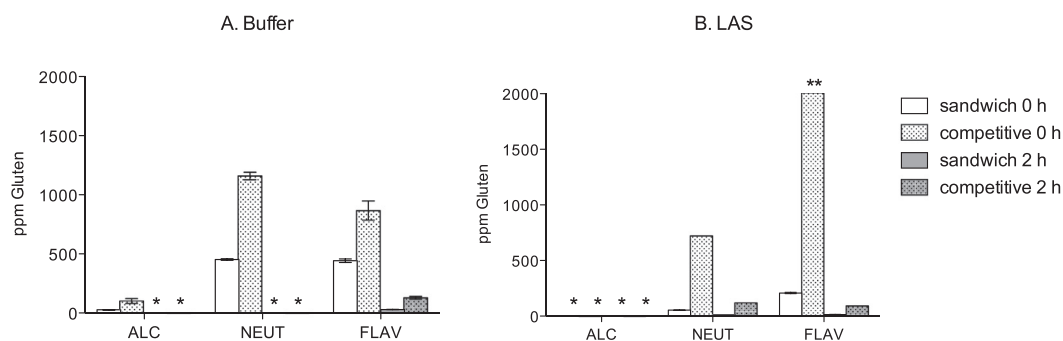


Fig. 8. Quantification of gluten with sandwich and competitive R5 ELISA in the gluten hydrolysates obtained with alcalase (ALC), neutrase (NEUT), and flavourzyme (FLAV) (E/S = 1/0.6, at ambient temperature for 2 h), in the presence of (A) buffer and (B) detergent linear alkylbenzene sulphonate (LAS) 1% (v/v). *Value lower than the detection limit (< 5 ppm, for sandwich R5 ELISA, and < 10 ppm for competitive R5 ELISA). **Value was higher than the detection limit (> 2000).

Table 3

Inactivation kinetic parameters (average $\pm$ standard deviation) of ALC incorporated in 200 mM Tris-HCl buffer pH 8, at two different temperatures, calculated with Eq. (2).

	A_0 (%)	A_{min} (%)	K (h ⁻¹)	Half-life (h)	r^2
25 °C	99.90 $\pm$ 1.55	18.55 $\pm$ 0.80	0.1728 $\pm$ 0.0110	4.01 $\pm$ 0.25	0.9579
4 °C	99.64 $\pm$ 1.22	14.17 $\pm$ 1.49	0.0052 $\pm$ 0.0003	132.23 $\pm$ 7.88	0.9333

A_0 : initial relative proteolytic activity of ALC; A_{min} : minimal relative proteolytic activity; K : inactivation rate.

Table 4

Gluten concentration ($\mu\text{g}/100\text{ cm}^2$) determined by sandwich R5 ELISA in different points in the production line of food industry, before and after cleaning with the enzymatic cleaning formulation (ALC SF4SF).

Machine	Type of surface	Sampling 1	Sampling 2	Contact time with ALC SF4SF (min)	Sampling 3	Sampling 4
Conveyor belt	Plastic roller	2.47	0.80	15	–	< 0.125 ^a
	Stainless steel grid	0.93	1.50	15	–	< 0.125 ^a
	–	–	1.85	5	< 0.125	< 0.125
	–	–	1.47	5	< 0.125	< 0.125
	–	–	1.58	5	< 0.125	< 0.125
Dusting machine	Plastic roller	–	1.45	15	–	< 0.125 ^a
	–	–	0.45	15	–	< 0.125
Breading machine	Stainless steel grid	–	1.53	5	< 0.125	< 0.125
Battering machine	Stainless steel grid	–	9.80	5	< 0.125	< 0.125

Sampling 1: after food processing and before rinse with raw water. Sampling 2: after rinsing with raw water and before applying the enzymatic cleaning formulation. Sampling 3: after the actuation of the enzymatic cleaning formulation and before an additional rinse with raw water. Sampling 4: after the last rinse with raw water.

^a These points were also analysed by competitive R5 ELISA with the same results.

significantly, so, it was possible to fit the inactivation data obtained to a one-phase exponential decay model in order to estimate the half-life of the protease under the different storage conditions:

$$A_t = (A_0 - A_{min}) \cdot e^{-k \cdot t} + A_{min} \quad (2)$$

where A_0 is the initial relative proteolytic activity of ALC, A_t is the remaining relative proteolytic activity at time t , A_{min} is the minimal relative proteolytic activity achievable in the different matrices, K is the inactivation rate (h⁻¹), and t is the sampling time (h). The proteolytic activity half-life (h) can be calculated as $\frac{\ln 2}{K}$.

As shown in Table 3, the half-life of the ALC incorporated in Tris-HCl buffer was barely 4 h at 25 °C and almost 6 days (~132 h) at 4 °C.

When the ALC was incorporated in the cleaning formulation, the stability increased substantially at both 25 °C and 4 °C (Fig. 9C and D, respectively), compared with that obtained when the protease was incorporated in the buffered solution (Fig. 9A and B). Thus, the activity remained above 90% even after 37 days of incubation, whether the cleaning formulation was kept at 25 °C or 4 °C (Fig. 9C and D, respectively), under dark. Stoner et al. (2004) also showed how the subtilisin degradation rate, in a detergent formulation, was reduced up to 21 times respect to that obtained in a buffered solution, when stabilising agents were incorporated into the formulation.

These results indicated that the formulation of the cleaning formulation was optimum to maintain ALC activity for an extended period.

3.8. Validation of the enzymatic cleaning formulation (ALC SF4SF) in a food industry

The efficacy of the ALC SF4SF was tested on a food-processing factory. For this purpose, different surface materials (plastic polymer and stainless steel) containing gluten residues (wheat flour, bread) as a result of routinely food processing activity were selected from the production line (conveyor belt, dusting, battering and breading machines). The cleaning protocols tested in the different surfaces are depicted in Fig. 1. All the samples were analysed with the sandwich R5 ELISA, and some points were also tested with the competitive R5 ELISA.

Two points were analysed with the surface completely dirty – remains of flour and breadcrumbs – (sampling 1), one in a plastic roller and another in the stainless steel grid, both in the conveyor belt

(Table 4). The values of gluten concentration were positive ($> 0.125\text{ }\mu\text{g}/100\text{ cm}^2$) in this sampling. This values decreased after rinsing only with water (sampling 2) but remained positive in all the points analysed (Table 4). After application of the ALC SF4SF, and rinse with water after 15 min, the gluten content decreased to values lower than $0.125\text{ }\mu\text{g}/100\text{ cm}^2$ (detection limit of the assay) (Table 4).

When the ALC SF4SF was applied during a contact time of only 5 min, the gluten content also decreased to values below $0.125\text{ }\mu\text{g}/100\text{ cm}^2$ (Table 4). Besides, those points analysed with the competitive R5 ELISA also gave negative results (Table 4), which confirm that in the hydrolysates did not remain potentially celiac-toxic epitopes.

Therefore, the results demonstrated the high suitability of the ALC SF4SF to eliminate the gluten from different surfaces of a food factory.

4. Conclusions

The hydrolysis conditions to develop a useful product that allows eliminate gluten from the work surfaces and avoid cross-contamination in a food processing factory were optimised. The presence of LAS helped to improve gluten solubility and enzymatic hydrolysis, despite its negative influence on the proteolytic activity. Alcalase (ALC) was demonstrated to be an efficient enzyme to incorporate in a cleaning formulation together with the detergent LAS and hydrolyse gluten to obtain short peptides and low levels of gluten detected with R5 ELISA, even at very short times and ambient temperature, which increases the probability of reducing its antigenicity. The proteolytic activity of alcalase into the cleaning formulation was stable after 37 days under dark. Preliminary validation of the effectiveness of the enzymatic cleaning formulation (ALC SF4SF) has been carried out in a real food processing industry. The results showed that gluten content was always reduced under the detection limit ($< 0.125\text{ }\mu\text{g}/100\text{ cm}^2$), in different surface materials (plastic polymer and stainless steel) when the ALC SF4SF was applied following different cleaning protocols, what verifies the high efficiency of the formulation developed.

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