

EFFECT OF ENZYME TYPE AND HYDROLYSIS TIME ON ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF WHEY PROTEIN HYDROLYSATES

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ABSTRACT

There is an elevated need for novel antimicrobial preservatives in the food industry, and hydrolysis of waste products from the same sector has for decades been viewed as a potential source of these. In the current study we have purified bovine whey protein using size-exclusion chromatography (SEC), and hydrolyzed it by trypsin, pepsin, alcalase, savinase, and neutrase at different times 30, 60, 120, 240, and 300 min. The highest active time hydrolysate was subsequently fractionated by SEC, monitored for antibacterial and antioxidant activities, and characterized using UHPLC-MS/MS. Alcalase and savinase displayed higher degree of hydrolysis, higher antibacterial activity in their hydrolysates at 60 and 30 min, respectively compared to the other enzymes. The alcalase hydrolysates exhibited significantly the highest antioxidant activity rescuing 89% of the yeast cell from Hydrogen peroxide induced oxidative stress at 120 minutes. Proteomic analysis of the highly active fractions identified peptides from α -lactalbumin with structural similarity to known antioxidant peptides. Thus, our results support the using food grade enzymes like alcalase and savinase in the food industry.

Key words: *Size-exclusion chromatography; UHPLC-MS/MS; Bioactive milk peptides.*

نصار وآخرون

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تأثير نوع الإنزيم ووقت التحلل على النشاط المضاد للبكتيريا ومضادات الأكسدة لمتحللات بروتين الشرش

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قسم الرقابة الصحية على الاغذية. كلية الطب البيطري. جامعه بنها. مصر

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المستخلص

هناك حاجة متزايدة إلى مواد حافظة جديدة مضادة للميكروبات في صناعة الأغذية ، وكان يُنظر إلى التحلل الإنزيمي لمنتجات النفايات من نفس القطاع على مدى عقود كمصدر محتمل لهذه المواد. في الدراسة الحالية ، تم تنقية بروتين شرش اللبن البقري باستخدام كروماتوجرافيا الترشيح الهلامي (SEC) ، وقمنا بتحليله عن طريق الترسين والبيسين والكالاز والسافيناز والنيوتراز في أوقات مختلفة 30 و 60 و 120 و 240 و 300 دقيقة. تم تجزئة التحلل الإنزيمي لأعلى وقت نشط لاحقاً بواسطة SEC ، ومراقبة الأنشطة المضادة للبكتيريا ومضادات الأكسدة ، وتميزت باستخدام UHPLC-MS / MS. أظهر الكالاز و السافيناز درجة أعلى من التحلل ونشاط مضاد للجراثيم أعلى في التحلل الخاص بهم في 60 و 30 دقيقة ، على التوالي ، مقارنة بالإنزيمات الأخرى. أظهرت التحلل المائي للكالاز بشكل ملحوظ أعلى نشاط مضاد للأكسدة حيث أنقذ 89% من خلايا الخميرة من بيروكسيد الهيدروجين الناجم عن الإجهاد التأكسدي في 120 دقيقة. حدد التحليل البروتيني للأجزاء عالية النشاط الببتيدات من α -lactalbumin مع التشابه الهيكلي مع الببتيدات المضادة للأكسدة المعروفة. وبالتالي ، فإن نتائج هذه الدراسة تدعم استخدام إنزيمات الغذاء مثل الكالاز و السافيناز في صناعة المواد الغذائية.

الكلمات المفتاحية: جهاز الكروماتوجرافى - UHPLC-MS/MS - ببتيدات الحليب النشطة بيولوجيا

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INTRODUCTION

Over the last decade, attention has been focused on identifying novel antioxidant and antimicrobial agent as a results of safety concerns associated with the use of synthetic antioxidants, a significant increase in bacterial drug resistance to conventional antibiotics, as well as an increase in the incidence of oxidative stress-related mitochondrial diseases such as rheumatoid arthritis, atherosclerosis, and cancer. Therefore great efforts have been paid toward the discovery of various bioactive peptides from natural origin like milk protein (35, 42). Whey proteins mainly including β -lactoglobulin, α -lactalbumin, bovine serum albumin, immunoglobulins, and other minor proteins with high biological and functional activities (39). Furthermore, through enzymatic hydrolysis of whey protein, smaller biologically active peptides are released. These peptides possess a wide range of bioactivities like antibacterial, antioxidant, immunomodulatory, and anticancer effects (3, 6). The released bioactive peptides (length and sequences) are primarily dependent on type of enzyme and time of hydrolysis, therefore, introduction of various proteases of microbial origin like alcalase and neutrase offers numerous advantages i.e. they are inexpensive, commercially available and release a variety of peptides with enhanced antibacterial and/or antioxidant activities (46). Time of hydrolysis greatly affects the activity of the resulted hydrolysates as in case of peptic hydrolysis of camel whey protein, which showed higher antioxidant activity after 2 h (15), while chymotryptic hydrolysates of bovine whey protein exerted higher antioxidant activity after 5 h (41). On other hand, DPPH-free radical inhibitory activity of cumin protein hydrolysates was highest after 160 min when using pancreatin while reaching maximum activity after 240 min when using alcalase (43). Antibacterial activity of goat whey protein by alcalase treatment is best achieved after 240 min (31), meanwhile, peptic hydrolysates of bovine whey achieved best activity against *Listeria ivanovii* after 90 min of hydrolysis (52). The main cause behind the variation in the activity at different times may be due to the differentially released peptides and amino acids (12). Therefore the current

work mainly depends on the impact of enzyme type and time of hydrolysis on antibacterial and antioxidant activity of whey protein hydrolysates and their fractions. In the current study we hydrolysed bovine whey protein using different enzymes and digestion times, followed by size-exclusion chromatography fractionation and testing for antimicrobial and/or antioxidant properties. The composition of the most active fractions were further characterized using UHPLC-MS/MS. Using a food grade broad spectrum endoprotease enzyme, savinase (12) we generated whey protein hydrolysates with both antibacterial and antioxidant activity.

MATERIALS AND METHODS

2.1. Materials

Bovine milk (Holstein-Friesian) was obtained from the farm at Faculty of Agriculture (Faculty of Agriculture, Benha University, Egypt). Alcalase enzyme 2.4 L (2.4 AU-A g^{-1}) 112 FG (EC. 3.4.21.62), trypsin enzyme (Pancreatic Trypsin PTN 6.05 salt free) 800 USP mg^{-1} (EC.3.4.21.4), neutrase enzyme 0.8 L (0.8 AU-N g^{-1}) (EC.3.4.22) and savinase enzyme 16 L (16 KN-PU-S/G) (EC.3.4.21.62) were kindly provided from Novozymes A/S Denmark. Pepsin enzyme 1:2500 (≥ 2500 units mg^{-1} protein) (EC.3.4.23.1) was purchased from Sigma (St. Louis, USA). All chemicals were of analytical grade.

2.2. Extraction of whey

Raw bovine milk (3.3% protein) was obtained under complete aseptic conditions from healthy cows, then kept at 4°C in sterile containers until it arrived at the laboratory. Milk was heated to 37 °C, then centrifuged at 2500 $\times g$, 30 min at 10 °C for removal of fat. Casein was separated from whey proteins by addition of 10% acetic acid to reach pH 4.6 (isoelectric point of casein), followed by centrifugation at 5000 $\times g$, 30 min at 4 °C). Finally, the whey was collected, lyophilized, and stored at -20 °C until use

2.3. Purification of whey protein

Purification of whey protein was done according to Rojas et al. (40), using Sephadex G-25 medium grade resin on an Äkta™ pure (Uppsala, Sweden). Whey was injected at a concentration of 40 mg/mL and fractionated with distilled water (mobile phase) at a flow rate of 2 mL/min. All fractions were collected

at regular interval using fractions collector and lyophilized, to determine protein concentration by Bradford analysis and protein composition by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reductive conditions.

2.4. Hydrolysis of whey protein

Hydrolysis was carried out using different enzymes (trypsin, pepsin, alcalase, neutrase and savinase) and within their optimal temperature and pH range (Table 1). Whey protein was dissolved in deionized water at concentration of 2.5% (w/v). The pH was maintained constant during hydrolysis either by 0.5 M NaOH or 0.5 M HCl. The degree of hydrolysis was determined at different time points (30, 60, 120, 240, and 300 min). The hydrolysis was terminated by heat inactivation of the enzyme at 95 °C for 10 min. Hydrolysates were subjected to centrifugation at 3000 ×g for 10 min, then the supernatant was collected and lyophilized and stored at -20 °C. The hydrolysis process was repeated 3 times as previously described (1).

Table 1. Conditions for hydrolysis of whey protein by different enzymes

Enzyme	pH	Temperature	Enzyme/substrate ratio
Trypsin	8	37 °C	2%(w/w)
Pepsin	3	37 °C	2%(w/w)
Alcalase	7.8	50 °C	2%(v/w)
Neutrase	7	50 °C	2%(v/w)
Savinase	9	50 °C	2%(v/w)

2.5. Degree of hydrolysis

Degree of hydrolysis was determined according to (14). Briefly the hydrolyzed mixture was mixed with 20% trichloroacetic acid (1:1), incubated at 4 °C for 30 minutes, followed by centrifugation at 3000 ×g for 10 min at 4 °C. The protein content in the supernatant was measured by Bradford analysis and expressed as mg of protein. The degree of hydrolysis was calculated as the ratio between Soluble protein content in 10% g(w/v) trichloroacetic acid (TCA) (mg) to total protein content (mg) according to the following formula

$$DH\% = \frac{\text{Soluble protein content in 10\% g}\left(\frac{w}{v}\right)\text{ TCA (mg)}}{\text{Total protein content (mg)}} \times 100$$

2.6. SDS-polyacrylamide gel electrophoresis: The protein profile of the hydrolysates in each enzyme group was determined by using SDS-polyacrylamide gel

electrophoresis under reducing conditions on 4-15% acrylamide gel. All bands appeared by using commassie brilliant blue R250 according to standard protocol (25).

2.7. Fractionation of whey protein hydrolysates:

Highly active whey protein hydrolysates were fractionated by SEC using Sephadex G-100 superfine grade resin on an Äkta™ pure (Cytiva, Uppsala, Sweden). Whey protein hydrolysates (20 mg/mL) were injected and eluted with distilled water at 0.8 mL/min. All fractions were collected and lyophilized to determine their antibacterial and antioxidant activities. Protein concentration in all fractions were measured by using NanoDrop (Thermo Scientific, San Jose, US).

2.8. Antibacterial activity

The antimicrobial activity of bovine whey, bovine whey hydrolysates and fractions eluted from SEC was determined against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 as described earlier (36) with slight modification. Test samples were dissolved in sterile distilled water and filtered with a 0.45 µm membrane filter. Single colonies of bacteria were transferred to sterile Muller Hinton broth (MHB) and grown overnight at 37 °C under constant agitation (200 rpm). The overnight cultures were diluted 1:50 in fresh growth medium and incubated with agitation at 37 °C to mid-logarithmic phase (optical density at 600nm reaching 0.4). The bacterial cultures were further diluted in fresh MHB (1:500) to obtain final inoculum concentration ranging from 2-8 ×10⁵ CFU mL⁻¹. Finally, 50 µL of inoculum was mixed with 50 µL of the test samples to give 10 mg/mL sample concentration and subsequently mixed with 100 µL of MHB. The mixtures were incubated at 37 °C for 2 h and ten-fold serially diluted in PBS before plating on Muller Hinton agar. Colony forming units were counted after incubation for 24-48 hours at 37 °C. All assays were performed in triplicates. Antibacterial activity is represented as log N/N1 where N refers to the control number of colonies without antibacterial material, and N1 refers to the number of colonies containing whey protein hydrolysates after an incubation period of 2 hour

2.9. Minimum inhibitory concentration

Minimum inhibitory concentration was measured by the microtiter broth dilution method for highly active hydrolysates as described earlier (44). Briefly, 90 μL of bacterial inoculum ($2\text{--}8 \times 10^5 \text{ CFU mL}^{-1}$) in mid-exponential phase were mixed with 10 μL of the tested sample (10 -0.625mg). Then made tenfold serial dilutions. The microtitre plates were incubated at 37 °C for 24 hours. The minimum inhibitory concentration (MIC) was determined as the lowest sample concentration resulting in no visible growth.

2.10. Yeast model for oxidative stress

Saccharomyces cerevisiae has been shown to parade a moderate sensitivity to H_2O_2 induced oxidative stress, so it acts as a model system for studying mitochondrial diseases. As described earlier (15), in brief, *S. cerevisiae*, suspended in yeast extract peptone dextrose broth at concentration 10^4 CFU mL^{-1} , were incubated with protein samples (bovine whey, whey hydrolysates and SEC fractions) at concentrations ranging from (1 to 0.1 mg mL^{-1}) for 1 h at 30 °C, then the oxidizing agent H_2O_2 was added (4 mmole L^{-1}) and incubation extended for 48 h. After that 10 μL portion was extracted, ten-fold serially diluted in yeast extract peptone dextrose broth containing 4 $\text{mmole L}^{-1} \text{H}_2\text{O}_2$ and spotted on yeast extract peptone dextrose agar plates containing 4 $\text{mmole L}^{-1} \text{H}_2\text{O}_2$. Plates were incubated at 30 °C for 48 h. Data were collected from three independent experiments and presented as survival percentage to non-treated cells.

2.11. Peptide profile by ultra-high performance liquid chromatography/tandem mass spectrometry : Peptides were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using an ultra-high performance liquid chromatograph (UHPLC). Five μL of the SEC fraction of each enzyme group was injected. Peptides were trapped on a C18 column (5 μm , 5mm, 0.3mm) and separated on a 15 cm fused silica column (75 μm inner diameter) pulled and packed in-house with 1.9 μm C18 beads (Reprosil-AQ Pur, Dr. Maisch) on an Ultimate 3000 system connected to a LTQ Velos Orbitrap (Thermo Scientific, San Jose, US). The peptides were separated with a 110 min gradient with increasing buffer B (90% ACN and 0.1% formic acid), going from 5 to 30% in 70 min,

30 to 50% in 15 min, 50 to 95% in 20 min followed by a 5 min wash and re-equilibrating step. All steps were performed at a flow rate of 250 nL/min. The LTQ Velos Orbitrap was operated in data-dependent top 15 mode. Full scan mass spectra were recorded in the orbitrap at a resolution of 60,000 at m/z 200 over the m/z range 375–1600 with a target value of 1×10^6 and a maximum injection time of 500 ms. CID-generated product ions were recorded in the iontrap with a maximum ion injection time set to 100 ms and a target value set to 1×10^4 . Spray voltage was set to 2.2 kV, S-lens RF level at 50, and heated capillary at 300 °C. Normalized collision energy was set at 35 and the isolation window was 2 m/z .

2.12. Data and bioinformatic analysis

LC-MS/MS data were processed using MaxQuant version 1.5.0.38 (7) with default settings. The data was searched against bovine (taxonomy ID 9913) protein sequence database from UniProt (<http://www.uniprot.org>). The Milk Bioactive Peptide Database (<http://mbpdb.nws.oregonstate.edu>) was used to identify bioactive peptides (28). The database was searched to compare resulted peptide sequences with the identity to the database sequences. A peptide coverage map was made using Geneious version 2021.1 created by Biomatters (<http://www.geneious.com>).

2.13. Statistical analysis

Statistical comparisons were made by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons, using GraphPad Prism 8. $P < 0.05$ was considered to indicate a statistically significant difference

RESULTS & DISCUSSION

Purification of whey protein: Whey protein concentrate is defined as protein that contains at least 25% protein after separation and lyophilization (22). Bovine whey protein was efficiently separated and purified from milk salts and sugar using SEC (sephadex G-25). It was fractionated into 2 fractions which were lyophilized (Fig. 1A), and the dried whey fractions allowed for determination of the whey protein concentration at 98% (w/w) in F1, which corresponds to previous findings (40). For visualization the two peaks eluting from the SEC was run on a SDS-PAGE gel

(Fig. 1B), illustrating a general desalting with all main proteins eluting in fraction 1 (F1) so

we complete the work on F1 (purified whey protein).

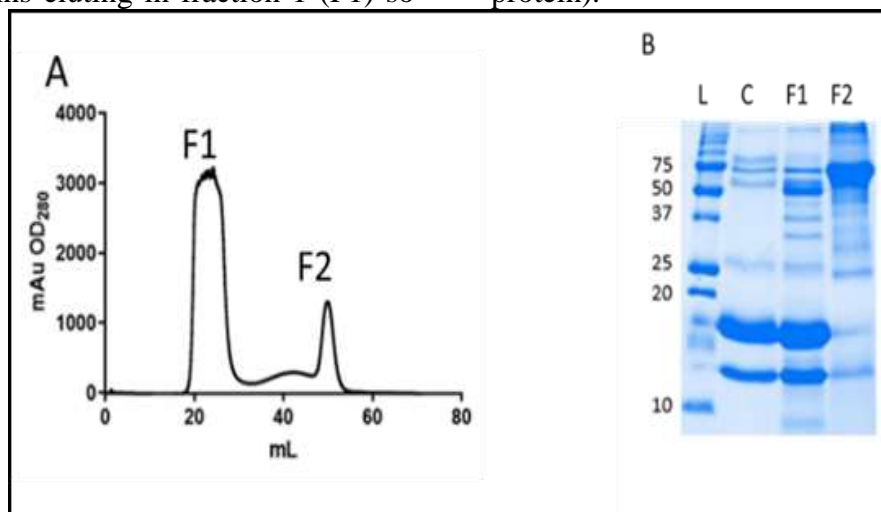


Fig. 1. A: Electrophoretic patterns of bovine whey proteins on size exclusion chromatography G-25. B: SDS-PAGE of crude whey protein before purification (C), peak 1 (F1) and peak 2 (F2) and the molecular ladder (L).

Fraction 2 (F2) was blank when loaded at the same concentration as F1, but when up-concentrated about 100 times, it demonstrates protein in this fraction too (Fig. 1B).

Production of whey protein hydrolysates

Hydrolysis of concentrated whey protein by enzymes released peptides and amino acid collectively known as whey protein hydrolysates (29). In the current work, purified whey protein (F1) was hydrolysed by five enzymes of different origin, i.e. trypsin, pepsin (animal origin), savinase, alcalase and neutrase (bacterial origin), to obtain various whey protein hydrolysates. The magnitude of hydrolysis was determined by degree of hydrolysis and visualized on SDS-PAGE (53). The degree of hydrolysis is a critical variable in predicting the extent of whey protein fragmentation in response to time exposure to the enzyme (55). In the current work the degree of hydrolysis of purified whey protein ranged from 0% to 28.8% (Fig. 2). There is a variation in the degree of hydrolysis between the different enzymes, which largely depends on the protease type, enzyme to substrate ratio and the incubation time (24) from zero to 300 min. For trypsin and pepsin there is a gradual increase of the degree of hydrolysis after 30 min until it reaches a plateau phase after 240 min which was 14.5% and 25.1%, respectively as in case of Kadhim&Shakir (21). The significantly lower degree of hydrolysis by trypsin has also been reported earlier, eluting to the importance of enzyme to substrate ratio,

where a ratio of 1% resulted in a significantly higher degree of hydrolysis (23) while a ratio of 0.4% gave a degree of hydrolysis of bovine whey protein of 11% (52) also 4000 U/g E/S ratio gave 14.62% after 5 h (16). In comparison, pepsin hydrolysis of bovine whey protein using only 0.005% enzyme to substrate ratio, has earlier been reported to yield 25% degree of hydrolysis after 240 min at 37 °C (32). Respectively, neutrase gave a slow and steady degree of hydrolysis at 50°C, peaking with 13.9%, while contrary to the other enzymes, time seem to be less relevant for hydrolysis with this enzyme. The degree of hydrolysis by neutrase is also corroborated with previous protocols using similar conditions (34). The highest degree of hydrolysis was achieved by alcalase and savinase, with 27% and 28 %, respectively after only 30 min at 50 °C. It was noticed that alcalase and savinase achieved the significantly ($p < 0.05$) highest hydrolytic activity. The superior efficacy of alcalase has been reported earlier (31, 34), while savinase hydrolysis of bovine whey protein has never before been investigated. The reasoning for their high degree of hydrolysis is their broad specificity, as they recognize more binding sites compared to the other enzymes (48). Conversely, the decrease in degree of hydrolysis after 30 min, coincides with findings by Fenoglio et al. (11), who explained this by reduction in the number of peptide bond and inhibition of enzyme activity after

intense hydrolysis. It has also been suggested that a lower degree of hydrolysis in trypsin and neutrase hydrolysates could be explained by aggregation and masking of the peptide bonds, as the hydrolysis was carried out at a pH close to the isoelectric point of the bovine whey protein (1). These variations can also be visualized using SDS-PAGE. The lower degree of hydrolysis in the case of trypsin and pepsin (Fig. 3 A,B) are clearly a result of the higher resistance of β -lactoglobulin (18 kDa) to be degraded by these two enzymes, which previously have been explained by the stabilizing effect of the intermolecular disulfide bridges (27). On the contrary, alcalase and savinase characterized by complete hydrolysis and removal of all major protein bands (Fig. 3 C,D), which was explained by the low substrate specificity of these enzymes (38). Neutrase has by far the lowest degree of hydrolysis, where most of the whey protein bands resist hydrolysis (Fig. 3 E). The activity of the neutrase appears rather good after 60 min, and larger molecular bands seem to reappear particularly around 240 min. This reproducible phenomenon are likely due to partial oxidation of cysteines, resulting in inter-polypeptide aggregation and formation of novel larger polypeptide chains (45).

Antibacterial activities of whey protein hydrolysates: The bioactivities of numerous protein hydrolysates are governed by the degree of hydrolysis. In the current work, five different enzymes were used, each with its own specificity and cleavage site to produce various bovine whey protein hydrolysates. Antibacterial activity of the hydrolysates was measured from zero to 240 minutes of hydrolysis. The best group was confirmed by using microtiter broth dilution method to measure MIC. At concentration 10 mg mL^{-1} , unhydrolysed bovine whey protein exerts no antibacterial activity against either *E. coli* or *S. aureus* (data not shown). Similarly, tryptic hydrolysates have no antibacterial activity

during the hydrolysis process (Fig. 4 A). This result was consistent with previous findings concluding that tryptic and chymotryptic hydrolysates of whey proteins had no antibacterial activity(52), despite previous studies elucidating antibacterial peptides from tryptic hydrolysis of α -lactalbumin, β -lactoglobulin, and bovine whey proteins (36,37,53). Absence of activity in case of trypsin may be traced back to its specific cleavage site between Arginine and Lysine, thus arguing for degradation of peptide fragments with high structural resemblance to classical cationic antimicrobial peptides (18). Pepsin hydrolysates exerted antibacterial activity after 60 min of hydrolysis and no significant drop in the antibacterial activity was observed as hydrolysis was allowed to continue to 240 min (Fig. 4 B). When monitoring the antibacterial potential of the hydrolysate obtained after 120 min, it was observed a log reduction of 2.54 and 2.06, for the growth of *E. coli* and *S. aureus*, respectively. These results are similar to the antibacterial activity reported by Théolier et al. (52), however it contradicts findings (52) which may return to the growth media, which might indicate that tryptic soy broth can inhibit the antibacterial potential of the pepsin hydrolysates. alcalase with one of the highest degree of hydrolysis, resulted in hydrolysates with overall very modest antibacterial properties, with the of the 60 min hydrolysate, which reduced *S. aureus* growth by a log of 2.06, while *E. coli* respond non-significantly (Fig. 4 C). Similarly, activity appear by hydrolysis of goat whey protein by alcalase for 4 h (31). Contrary to alcalase, savinase which also demonstrated very potent digestion of the whey protein, resulted in production of a hydrolysate after 30 min which possessed a very potent and significantly ($p < 0.05$) higher antibacterial activity against both *E. coli* and *S. aureus*, with reduction exceeding 2 log (Fig. 4 D).

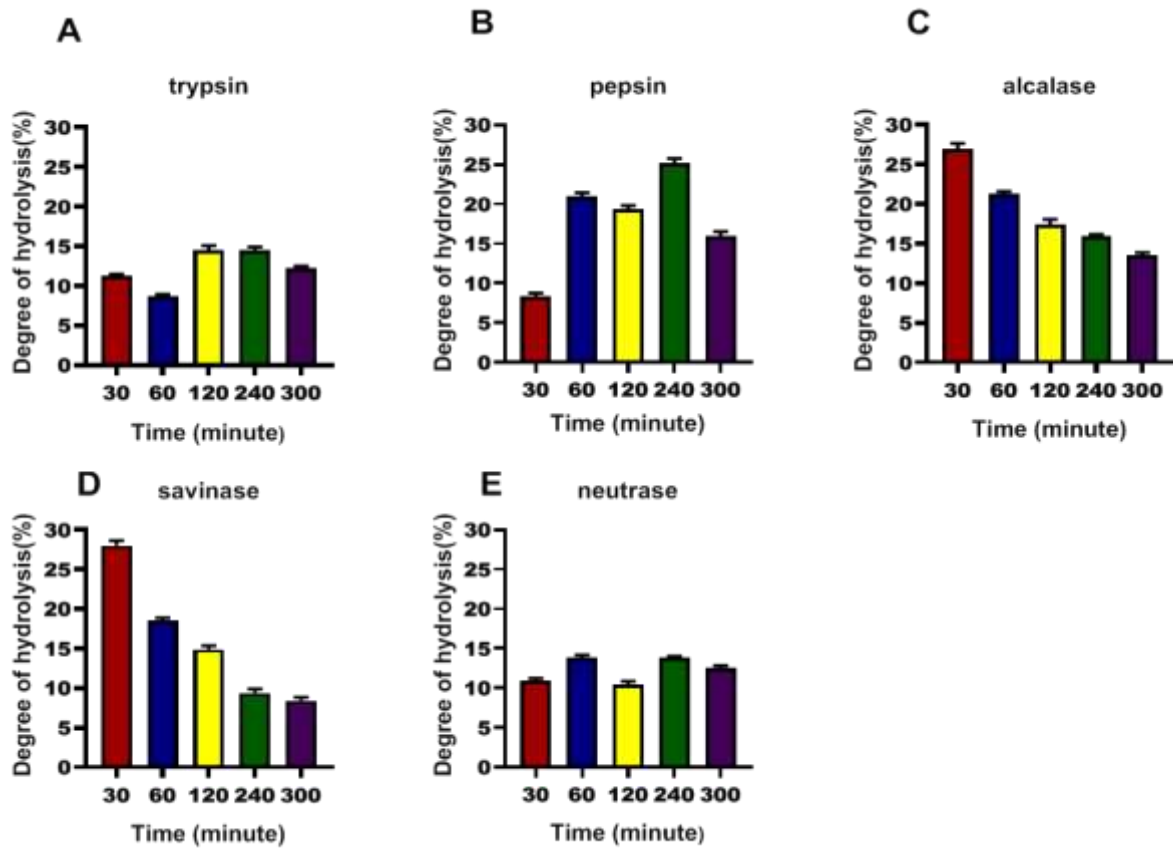


Fig. 2. Degree of hydrolysis (DH) of whey protein by using different enzymes: trypsin (A); pepsin (B); alcalase (C); savinase (D); neutrase (F). The experiment of WPC hydrolysis was carried out in triplicate (± SEM)

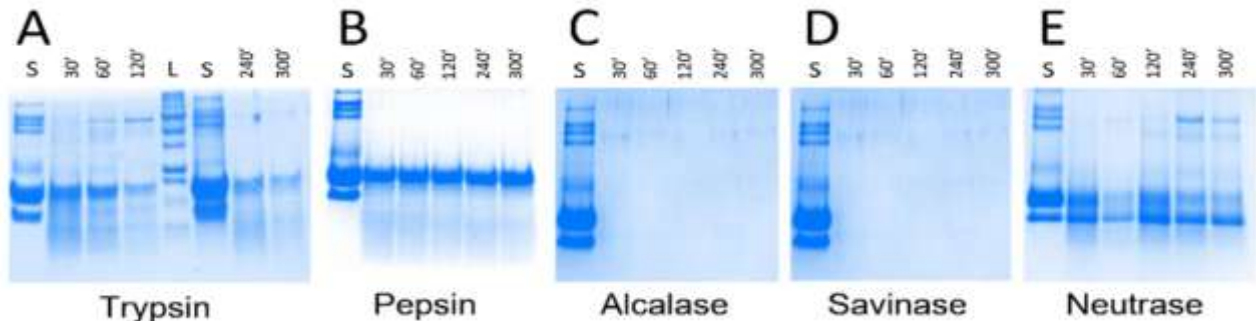


Fig. 3. Bovine whey protein before and after hydrolysis, visualized on SDS-PAGE. Whey protein before hydrolysis (S), molecular weight ladder (L), and whey protein samples digested for 30, 60, 120, 240, and 300 minutes

As the enzyme is allowed to work, the antibacterial activity is slowly decreasing in the consecutive hydrolysates. Neutrase which gave a very poor degree of hydrolysis, exerted non-significantly antibacterial activity in any of the hydrolysates against either *E. coli* or *S. aureus* (Fig. 4E). The lack of production of antimicrobial peptides by neutrase hydrolysis from bovine whey has not been reported before, but the findings are supported by similar lack of antimicrobial activity when digesting porcine blood proteins with the same enzyme (20). The results of highly active

groups were confirmed by measuring MIC, MIC of pepsin, alcalase, savinase and neutrase hydrolysates against *E. coli* was 1.25, 2.50, 2.50, 5 mg/mL respectively, while against *S. aureus* was 2.50, 2.50, 2.50 and 5 mg/mL, respectively.

The effect of various whey protein hydrolysates on yeast cell oxidative stress tolerance: Oxidative stress is a condition linked to a variety of diseases, including rheumatoid arthritis and cancer, due to damage to vital molecules such as DNA, lipids, and proteins, which is primarily caused by an

increase in free radicals such as reactive oxygen species (ROS) and H_2O_2 . As a result, natural antioxidants are crucial in preventing oxidative stress (16). In this study, we used bovine whey protein hydrolysates from different time points of hydrolysis, and monitored how they could affect survival of yeast cell. *S. cerevisiae* was incubated with the hydrolysates for 1 h, then exposed to H_2O_2 (4 mmole L^{-1}). The survival of the treated cells was determined by spotting on agar plates containing 4 mmole/L of H_2O_2 , then viability was represented as survival percentage to non-treated cells (Fig. 5). All whey protein hydrolysates groups exhibited a significant

improvement in the survival rate of yeast cells, and increased hydrolysis time generally seemed to produce hydrolysates with higher antioxidant properties. Furthermore, the different enzymes resulted in significantly different antioxidant effects and could be ranked as follows alcalase > pepsin > neutrase > trypsin > savinase with maximal survival rate of 81% > 59% > 51% > 46% > 41%, respectively as compared to stressed yeast cells. From the result it is apparent that the antioxidant activity of hydrolysates isn't correlated with the degree of hydrolysis, a result which also has been documented previously (48).

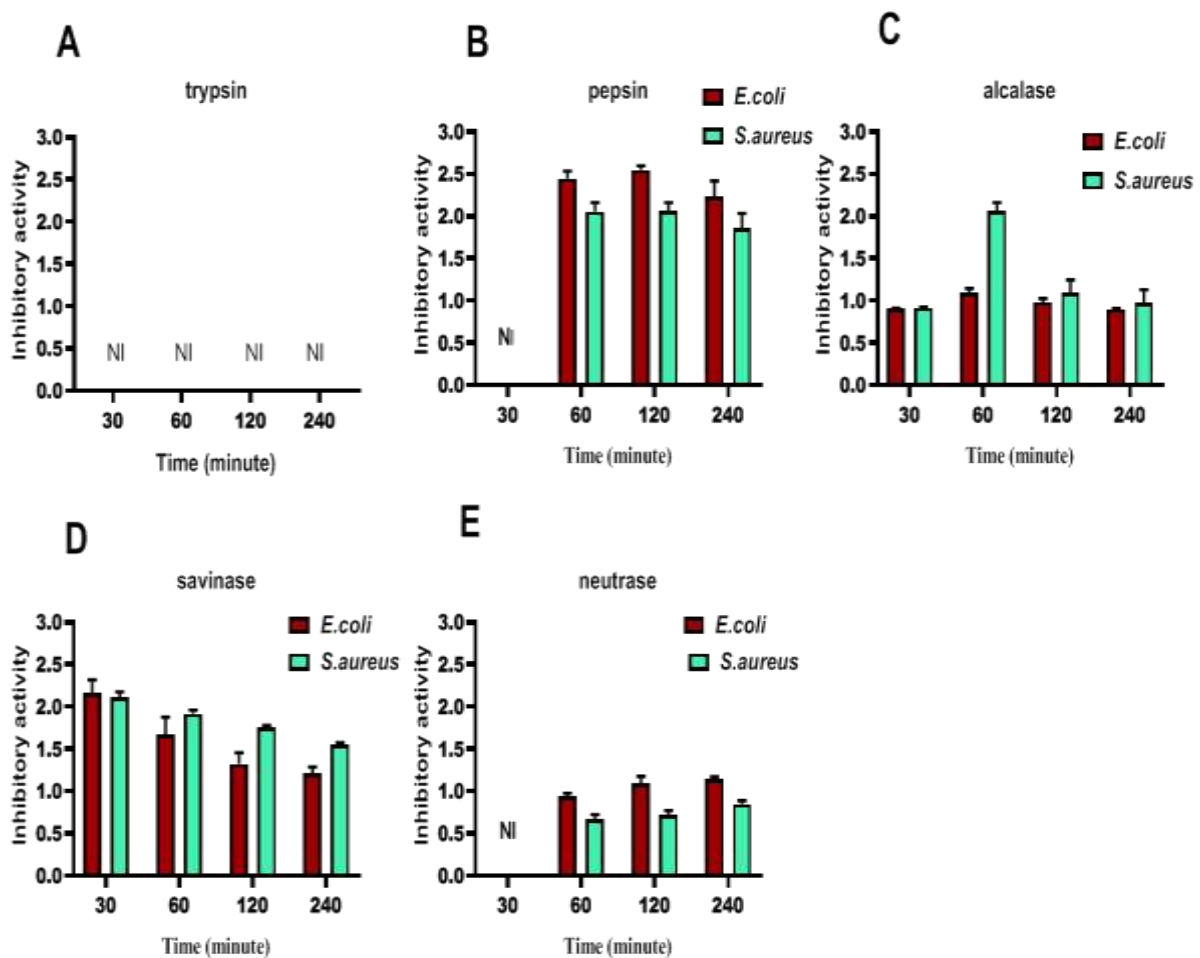


Fig. 4. Antibacterial activity of whey protein hydrolysates of different enzymes against *E. coli* and *S. aureus*. Antibacterial activity is represented as log N/N1 where N refers to the control number of colonies without antibacterial material, and N1 refers to the number of colonies containing whey protein hydrolysates after an incubation period of 2 hours. The assays were performed in triplicate. >1 indicate bactericidal activity

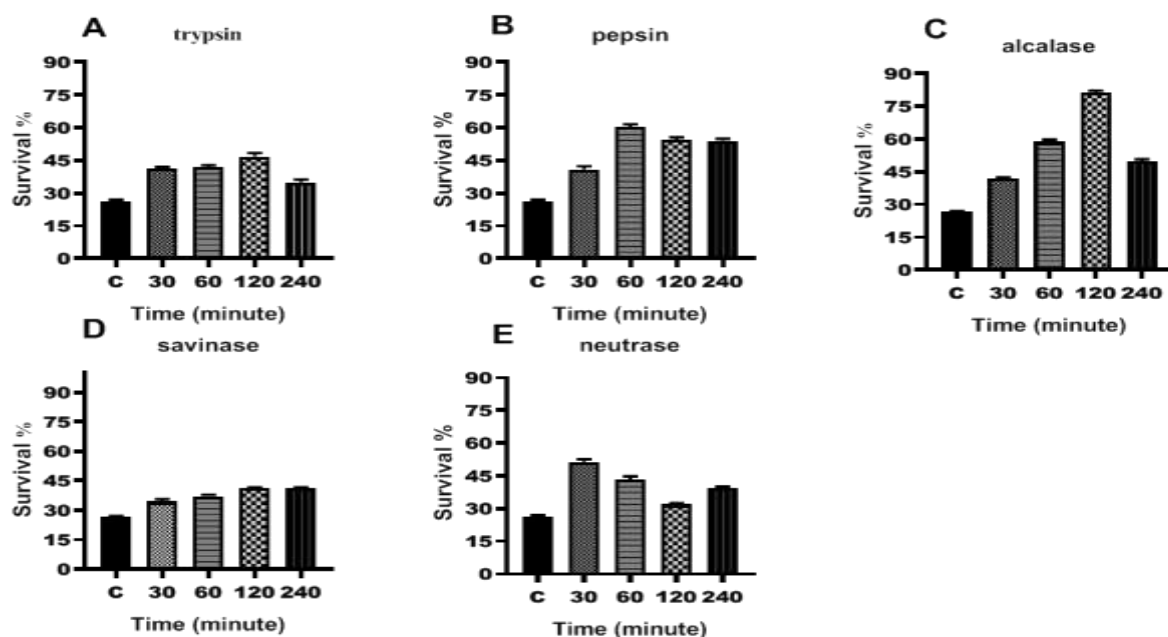


Fig. 5. Viability % of yeast cell oxidized by H_2O_2 by using different enzymes whey protein hydrolysates. Viability of H_2O_2 -treated yeast cells was measured by colony counting on yeast extract peptone dextrose broth agar plates. Viability% was represented as survival percentage to the non-induced cells

Fractionation of hydrolysates by size exclusion chromatography:

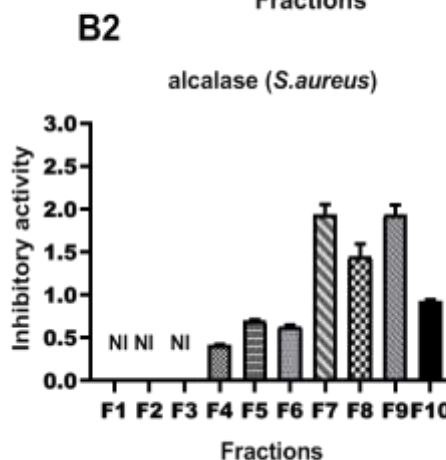
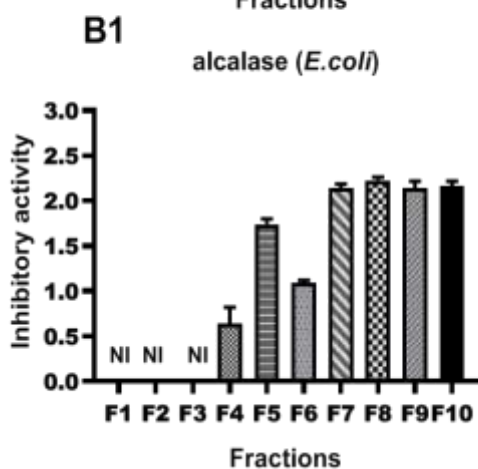
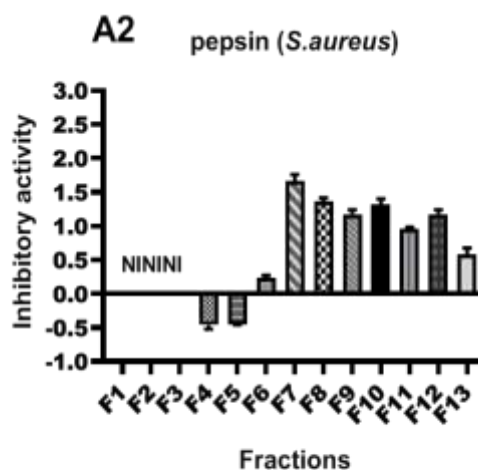
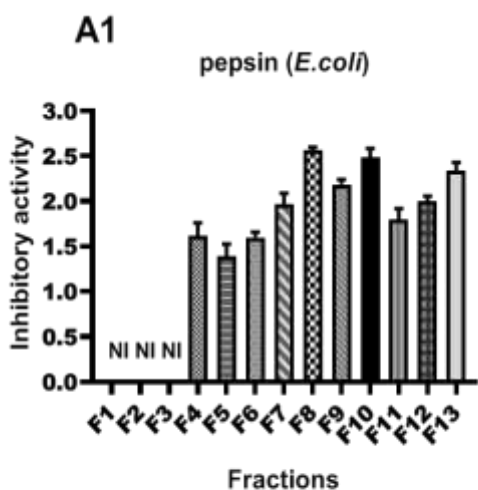
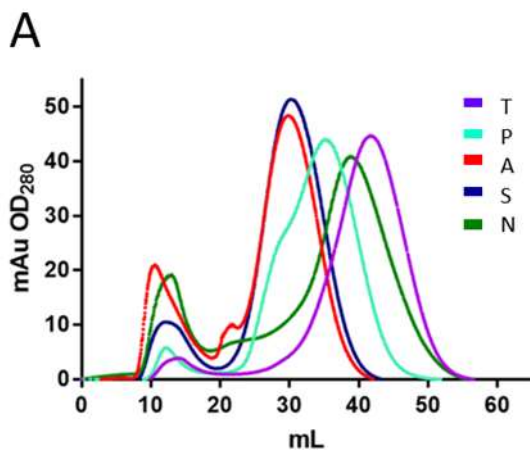
The most active time dependent hydrolysates of each enzyme was fractionated by SEC (Fig. 6A), and result has high similarity to previous published work (20). The elution profile mainly consists of two peaks and the second peak was fractionated in multiple smaller segments. The antibacterial activity of all hydrolysate fractions were assayed against *E. coli* and *S. aureus*. The first peak from all enzyme treatments exerts no antibacterial activity, which might be explained by the same size of the proteins in these fractions (Fig. 6A1-D2). Furthermore, none of the fractions of the trypsin hydrolysates did not have detectable antibacterial activity (Data not shown). In the case of pepsin the highest antibacterial activity against *E. coli* and *S. aureus* was observed in the center of the second peak (Fig. 6 A1-2), the activity against *E. coli* was also quite high towards the very end of peak, while the *S. aureus* activity then was lost. Similarly, the activity of the fractions from the alcalase hydrolysate was more active against *E. coli* and *S. aureus*, and the activity was generally found in the last smallest fragments eluting at the end of the second peak (Fig. 6 B1-2). Fractionation of the hydrolysates from savinase and neutrase resulted in an inhibitory

activity below 1.0, thus stimulating bacterial growth, through production of peptide fragments and free amino acids which can be utilized by both *E. coli* and *S. aureus* as e.g. a source of energy (Fig. 6 C1-D2). Overall, it allows us to conclude that in the current work, the antibacterial activity has higher selectivity for *E. coli* than *S. aureus*. A similar conclusion has been drawn earlier in a study on goat whey protein hydrolysates, and the authors used transmission electron microscopy to visualize destruction of *E. coli* bacterial cell wall, while their hydrolysates only had bacteriostatic activity against *S. aureus* (31). The size-exclusion chromatography fractions were also assessed for their effect on oxidative stress tolerance of yeast cell. Fractionated hydrolysates from all enzymes at concentration (0.2 mg/mL) showed a remarkable increase in *S. cerevisiae* survival compared with control group (yeast cell + 4 mmole L^{-1} H_2O_2) (Fig. 7A-E). Corresponding fractions from the center region of the second peak had all highest antioxidant properties, ranging from 80-89% yeast cell viability. From these results it is apparent that all highly active fractions exhibit comparable antioxidant activity, but when ranking them according to potency [savinase (S5) > alcalase (A4) > trypsin (T7) and neutrase (N7) > pepsin (P7)],

it is also apparent that they differ slightly in their elution time. In conclusion, hydrolysed bovine whey protein is a good source for peptides which can be used to protect live eukaryotic cell against oxidative stress and prevent cell damage.

Role of proteomics in enzyme specificity determination: From the SDS-PAGE (Fig.3) it is obvious that the five enzymes have different specificity resulting in different

degradation product, which is further corroborated by the peptide coverage maps derived from the proteomics analysis of the fractionated hydrolysates (Fig.8 and Table 2). Particularly trypsin is very narrow in its cleavage, cutting on the C-terminal side of Arginine and Lysine. In comparison, alcalase and savinase are found in the other end of the spectrum producing a more diverse mixture of peptide fragments.



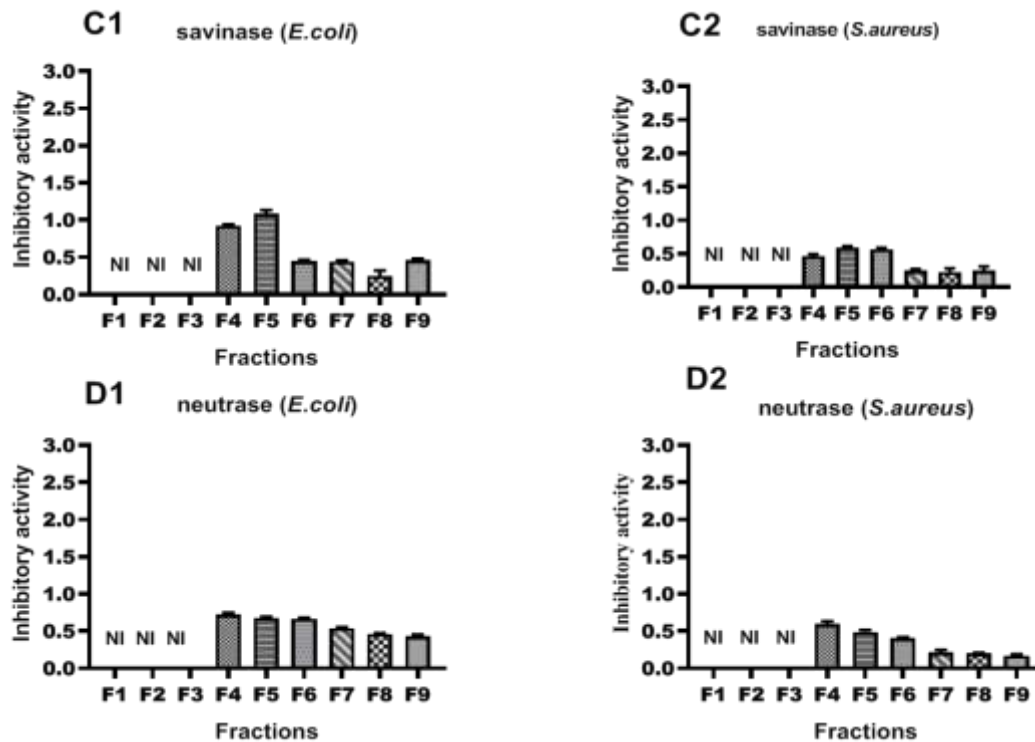


Fig. 6. A. Size exclusion chromatography separation of enzyme hydrolysates. A1-D2: Antibacterial activity of whey protein peptides from different enzymes groups against *E. coli* and *S. aureus*. antibacterial activity is represented as log N/N1 where N refers to the control number of colonies without antibacterial material, and N1 refers to the number of colonies containing antibacterial material (whey protein hydrolysates) after an incubation period of 2 hours. The assays were performed in triplicate. >1 indicate bactericidal activity

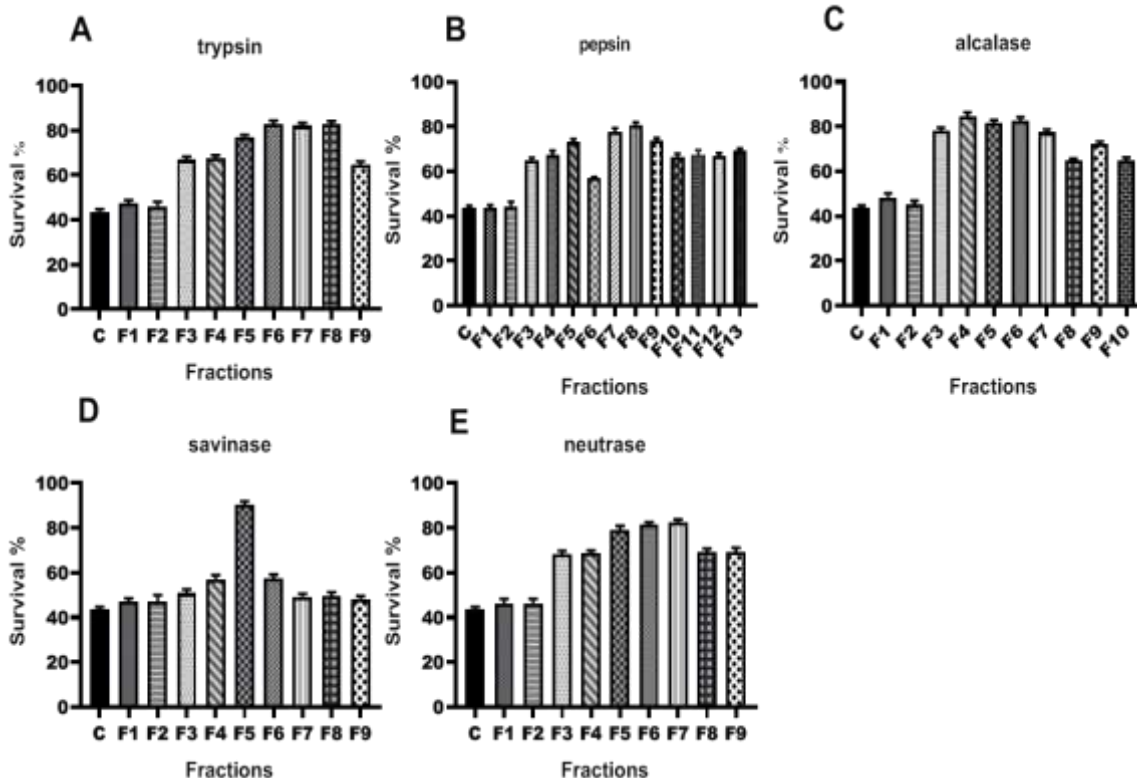
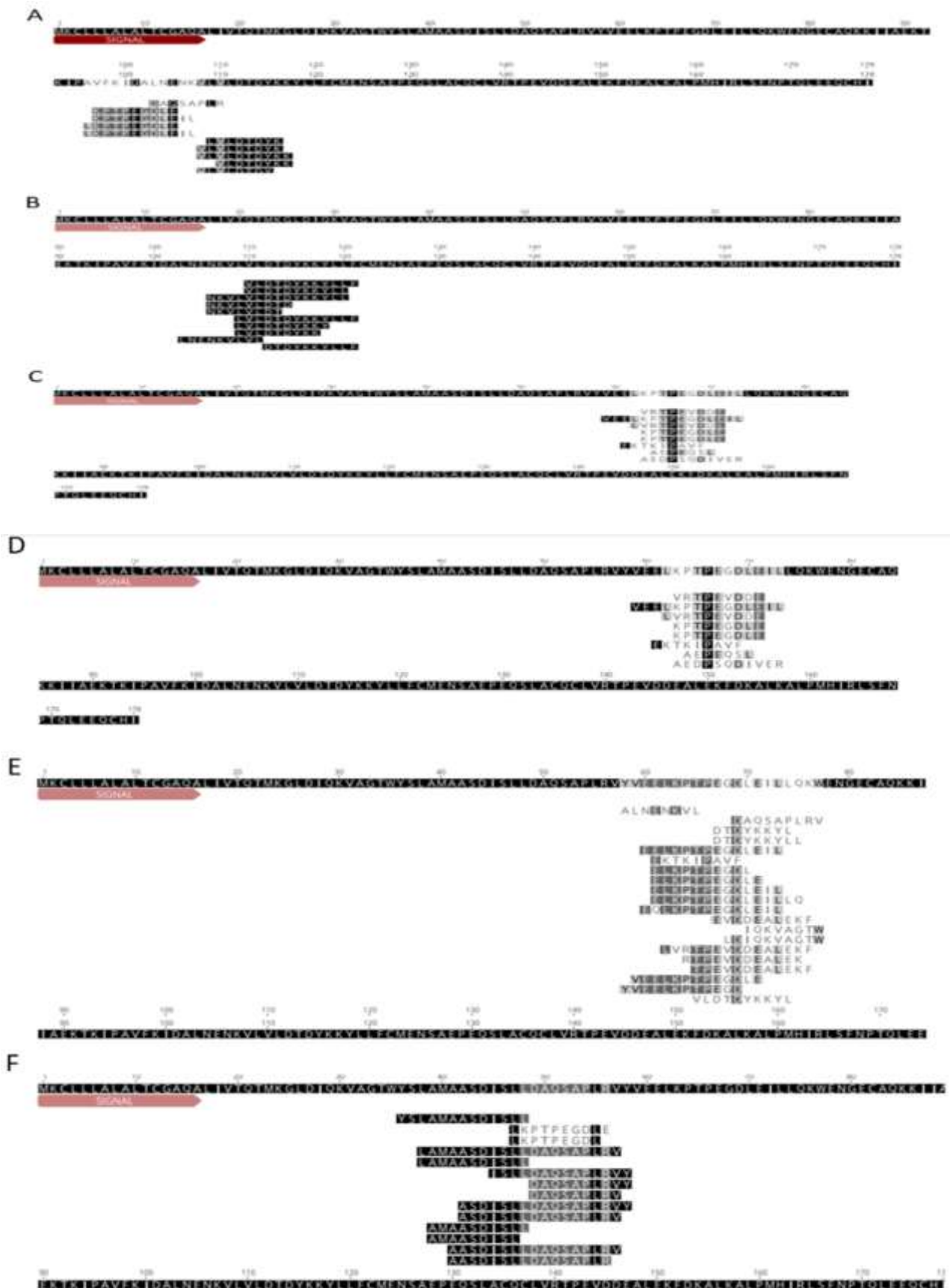


Fig. 7. Viability % of yeast cell oxidized by H₂O₂ by using different enzymes whey protein peptides. Viability of H₂O₂-treated yeast cells was measured by colony counting on yeast extract peptone dextrose broth agar plates. Viability was represented as survival percentage to the non-induced cells



demonstrated to possess inhibitory activity of the enzyme dipeptidyl peptidase IV (DPP-IV), which is an attractive target for treatment of type 2 diabetes (26, 30). These sequences are also contained in fragments derived in this study using trypsin, pepsin, alcalase, savinase, and neutrase. In fact, the peptides ILDKVGINYWLAHK and VGINYWLAHK produced by trypsin were also previously been

identified as DPP-IV inhibitors (19). Similarly, using all enzymes except trypsin, we did also produce matching or containing elements of the previously identified ACE-inhibitor peptide LKGYGGVSLPEW (33). This findings indicate for the presence of multifunctional activities within bovine whey protein hydrolysates.

Table 2. Sequences of bioactive peptides from Lactoferrin, α -lactalbumin and β -lactoglobulin found in active fractions of the different enzyme groups

Peptide source	Peptide Sequences	Molecular mass	Biological properties	
Trypsin hydrolysate fraction 7				
Lactoferrin	EKYGYGTGAFR	1354.5		
	SVDGKEDLIWK	1289.5	Antioxidant	This paper; (54)
α -lactoalbumin	ILDKVGINYWLAHK	1670.0	ACE-inhibitor; DPP-IV inhibitor	(19,26,51)
	VGINYWLAHK	1200.4	DPP-IV inhibitor	(19)
β -lactoglobulin	DAQSAPLR	856.9		
	KPTPEGDLEIL	1211.4		
	LVLDTDYK	966.1	Antibacterial; DPP-IV inhibitor	This paper; (50)
	VLVLDTDYK	1065.2	Antibacterial; DPP-IV inhibitor	This paper; (50)
Pepsin hydrolysate fraction 7				
Lactoferrin	LSKAQEKFGKNKRSRF	1855.1	Antibacterial	This paper; (5,9)
	SVDGKEDL	861.9	Antioxidant	This paper; (54)
	LFKSETKNLL	1192.47	Proliferation differentiation	(47)
α -lactoalbumin	AIVQNNDSTEYGL	1423.5		
	DKVGINYW	994.1	ACE-inhibitor	(51)
	GGVSLPEW	843.9	Potential ACE-inhibitor	(33)
	ILDKVGINY	1034.2	Potential ACE-inhibitor, DPP-IV inhibitor	(26,33,51)
β -lactoglobulin	LVLDTDYK	966.1	Antibacterial; DPP-IV inhibitor	This paper; (50)
	LVLDTDYKKYLLF	1630.9	Antibacterial; DPP-IV inhibitor	This paper; (50)
Alcalase hydrolysate fraction 4				
Lactoferrin	GRDPYKLRPV	1200.4		
α -lactoalbumin	GGVSLPEWV	943.1	Potential ACE-inhibitor	(33)
	KILDKVGINY	1162.4	Potential ACE-inhibitor; DPP-IV inhibitor	(26,33,56)
β -lactoglobulin	EKTKIPAVF	1032.2		
	VLDTDYK	852.9		
Alcalase hydrolysate fraction 7				
Lactoferrin	EDLIWKL	916.1	Antibacterial	This study;(49).
	KARDRQYE	1024.1	Antibacterial	This study; (12).
α -lactoalbumin	DKVGINY	807.9	ACE-inhibitor	(51)
β -lactoglobulin	ELKPTPEGDLEIL	1453.7	Antibacterial; antioxidant	(2,4)
	LVRTPEVDDE	1172.3		
	QLKPTPEGDLE	1226.3		
	VRTPEVDDE	1059.1		
Savinase hydrolysate fraction 5				

Lactoferrin	DLIWKLL	1029.2	Antibacterial	This study; (44).
	SVDGKEDLIW	1161.3	Antioxidant	This study;(54)
α -lactoalbumin	GGVSLPEW	843.9	Potential ACE-inhibitor	(33)
	KILDKVGIN	999.2		
β -lactoglobulin	DAQSAPLRV	956.1		
	ELKPTPEGDLEIL	1453.6		
	IQKVAGTW	902.1		
	RTPEVDDEALEK	1401.5		
	VLDTDYKKYL	1257.4		
	YVEELKPTPEGDL	1489.6		
Neutrased hydrolysate fraction 4				
Lactoferrin	FKSETKNL	966.1	Enhance proliferation and differentiation	(47)
α -lactoalbumin	IVQNDSTYGLF	1499.6		
	LKDLKGYGGVSLPEW	1661.9	Potential ACE-inhibitor	(33)
β -lactoglobulin	AMAASDISLL	991.2		
	DAQSAPLRVY	1119.2		
	LKPTPEGDLE	1098.2		
	SDISLLDAQSAPLRV	1584.8		
	AASDISLLDAQSAP	1358.5		
	YSLAMAASDISLL	1354.6		
Neutrased hydrolysate fraction 7				
Lactoferrin	VAAEIYGTKESPQTHYYA	2028.2		
	VDGKEDLIWK	1202.4	Antioxidant	This study; (54)
α -lactoalbumin	DKVGINYW	994.1	ACE-inhibitor; potential DPP-IV inhibitor	(30,51)
	FHTSGYDTQA	1126.1		
	ILDKVGINY	1034.2	Potential ACE-inhibitor, DPP-IV inhibitor	(30,51)
	LKGYGGVSLPEW	1305.5	ACE-inhibitor	(33)
β -lactoglobulin	AASDISLLDAQSAP	1358.5		
	ENKVLVLDTDYKKY	1728.0		
	VLVLDTDYKKYL	1469.7		

Note: References are for sequences either identical or significantly overlapping with characterized peptides

Conclusions and future work In the current work, the purified whey protein was hydrolysed by five enzymes (pepsin, trypsin, alcalase, savinase and neutrased) demonstrating different degree of hydrolysis. The impact of hydrolysis times was also clearly affecting the activity of the hydrolysates. Alcalase and savinase were superior to the other three enzymes, characterized by the highest degree of hydrolysis and producing a large number of peptides with both antioxidant and antibacterial properties, while trypsin and neutrased are the poorest in regards to degree of hydrolysis and antibacterial activity. Proteomic analysis of the hydrolysates allowed to identify peptides with multifunctional properties, as antibacterial, antioxidant, ACE-inhibitory, and DPP-IV inhibitory activities. These findings may argue the future application of bovine whey protein either in dairy- and/or nutraceutical industry.

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