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Original research

Functional properties and emulsion stability of wheat gluten hydrolysates produced by endopeptidases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*

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A B S T R A C T

Gluten hydrolysates (GHs) were prepared using endopeptidases, alcalase, and neutrase. Characteristics of both enzymes including UV spectrophotometry, protein content, enzyme activity, and productivity, temperature, pH, hydrolysis time, and the combination ratio of the enzymes were determined. The degree of hydrolysis (DH) and alcalase to neutrase ratio was determined 2:1 ratio with 97% product yield. A study on emulsification properties of gluten hydrolysates revealed that oil in water emulsions obtained by a combination of two enzymes and 8 hours' hydrolysis time were more stable compared with the resulting hydrolysates with less or more than 8 hours. The molecular weight of the resulting peptides compared with natural gluten by sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) indicated 20-245 KDa for gluten and 45-55 KDa for gluten hydrolysates. Comparison of the effect of the combination of sodium caseinate (SC), sodium polyphosphate (SP), and xanthan gum (XG) with gluten hydrolysates on oil in water emulsion stability indicated that a combination of hydrolysates with sodium caseinate has been the best effect on emulsion stability.

Keywords: Gluten hydrolysates; Endopeptidases; Alcalase; Neutrase; Oil in water emulsions

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1. Introduction

The protein content of wheat grain varies from 8 to 17%, and it depends on genetic differences and environmental factors. An outstanding feature of the wheat flour is that the gluten proteins in the flour have the ability to polymerize and form the largest polymers in nature. Gluten is a very large complex primarily comprised of polymeric glutenins (with aggregated proteins caused by the formation of interchain disulfide bonds) and monomeric gliadin proteins (can be solubilized in 70% aqueous ethanol). Gliadin is monomeric in its native form but forms polymers through intermolecular disulfide bonds in dough and during processing (Ceresino et al., 2020; Johansson et al., 2013; Kuktaite et al., 2004).Gluten protein contains glutamine (35%), hydrophobic amino acids (19%), and proline (15%). Due to its amino acid composition, gluten has certain limitations in mechanical and emulsifying properties and solubility (Ceresino et al., 2019). Enzymatic modification of protein is becoming increasingly important in food and non-food industries to enhance certain properties of gluten (Wouters et al., 2017).

Enzymatic hydrolysis of proteins has been the most common way of producing bioactive peptides or hydrolysates (Nwachukwu & Aluko, 2019). Control of the parameters of the enzymatic hydrolysis allows for acquiring polypeptides of various sizes and improved biological properties. The biological activity of protein hydrolysates depends on many factors including enzyme to substrate ratio, type of protease enzyme, incubation duration, substrate concentration, and pH (Halim et al., 2016). Protein hydrolysates are one such compound, being a source of hydrolysates or bioactive peptides (Alirezalu et al., 2019). To reduce the cost of the final product and to achieve sustainable management of food resources, protein hydrolysates are made from the protein of the food industry by-products and waste(Lafarga & Hayes, 2014). Numerous studies have shown that the hydrolysates

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or bioactive peptides produced from rice bran protein (Cheetangdee & Benjakul, 2015), milk casein (Hernandez-Ledesma et al., 2007), soy protein (de Oliveira et al., 2015; Oliveira et al., 2014), sorghum kafirin (Xu et al., 2019a; Xu et al., 2019b) and corn protein (Li et al., 2019a,b; Liu et al., 2015; Zhu et al., 2019). Cereal grain protein hydrolysates are versatile food commodities and play the main role in food additives. Depending on the degree of hydrolysis, these hydrolysates can be used to gelatinization, emulsification, or as a seasoning.

To acquire a hydrolysate which could be employed as a food additive, it has to be created using food approved, commercially available enzyme preparation or enzyme, the cost of which would be acceptable for the manufacturer (Tavano et al., 2018). pH, temperature, and duration have been identified as critical parameters of the hydrolysis process, and by using various combinations of those parameters, the produced product may have different biological activity (Nongonierma et al., 2017).

In the present study, the enzymatic hydrolysis of wheat gluten is broadly utilized as a part of food commercial enterprises to enhance its functional properties, for example, solubility, emulsification, and film-framing property, or to get ready widely hydrolyzed proteins for hypoallergenic newborn child diets and nutritional treatment. Diverse proteases have been attempted, for example, alcalase 2.4L, neutrase 0.8L, and blend alcalase/neutrase. The protein hydrolysates are broadly utilized as nutritional supplements, functional fixings, and flavor enhancers in foods, individual consideration items, cosmetics, espresso whiteners, confectionery, and in the fortress of juices and sodas. Protein hydrolysates are additionally utilized as a part of sauces, soups, meat items, snacks, flavors, and other exquisite applications. The chemicals utilized as a part of protein change incorporate business proteins, for example, alcalase, neutrase, and blend alcalase/neutrase fungal protease.

2. Material and Methods

2.1. Materials

Wheat gluten powder was supplied by Arian Glucose Company (Zanjan, Iran). All chemicals and reagents used in this work were food-grade or reagent-grade. Alcalase 2.4L (EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g) and neutrase 0.8 (EC 3.4.24.28, from *Bacillus amyloliquefaciens*, 0.8 AU/g) were donated by Novo Nordisk (Bagsvaerd, Denmark). The prestained protein ladder 10-250 KDa for SDS-PAGE was purchased from the SinaClon Company of Iran. All other chemicals were prepared from Merck Company of Germany.

Table 1. Conditions for the hydrolysis of wheat gluten with different endopeptidases.

Proteases	pH	Temperature (°C)
Alcalase 2.4 L	6.5±0.01	600±0.01
Neutrase 0.8 L	6±0.02	60±0.01
Alcalase/Neutrase	6.5±0.03	60±0.01

2.2. Proteolysis with different enzymes

Hydrolysates were prepared according to the method of Zhang et al. (2020) with minor modifications. Briefly, wheat gluten was hydrolyzed with different enzymes, under the conditions given in Table 1, based on optimum hydrolysis conditions. Wheat gluten was dissolved as a 5.5% (w/v) solution, the suspension was adjusted to appropriate temperature and pH, depending on the used enzyme, and incubated for 240 min. After all, samples to determine the degree of hydrolysis were tested with Ninhydrin assay.

Determined BSA standard curve by the Bradford method Set up several Falcone containing standard protein (BSA, 0.1 to 1 mg/ml). After 5ml of coomassie reagent to each Falcone. Mix well and incubate at room temperature and measure absorbance for each Falcone at 595 nm between 5 minutes and 10 after mixing (He, 2011).

2.3. Determination of moisture content

The moisture content was measured (Nielsen, 2010). Briefly Weigh accurately dried pan with the lid then Place 2–3 g of sample in the pan and weigh accurately next Place in a forced draft oven at 130°C for 3 h. then Remove from oven, realign covers to close, cool, and store in a desiccator until samples are weighed next Calculate percentage moisture (w/w) as described below.

$$\text{Moisture (\%)} = \frac{\text{weight of } H_2O \text{ in sample}}{\text{weight of wet sample}} \times 100 \quad (1)$$

2.4. Determination of ash content

Ash content of the wheat gluten was measured according to the AOAC method. Briefly weigh about 5 g sample into the crucible. Heat over low Bunsen flame with the lid half covered. When fumes are no longer produced, place crucible and lid in the furnace. Heat at 550°C overnight, during heating, do not cover the lid after complete heating to prevent loss of fluffy ash, cool down in the desiccator. Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for further ashing.

$$\text{Ash (\%)} = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100 \quad (2)$$

2.5. Determination of protein content

The total protein content of wheat gluten was calculated according to the kjeldahl method (Latimer, 2016). Approximately 1 g of raw material was hydrolyzed with 15 mL concentrated sulfuric acid (H_2SO_4) containing two copper catalyst tablets in a heat block at 420°C for 2 h. After cooling, H_2O was added to the hydrolysates before neutralization and titration. The amount of total nitrogen in the raw materials was multiplied with both the traditional conversion factor of 5.7.

2.6. Enzyme protein assay

The Bradford protein assay is a spectroscopic analytical procedure used to measure the concentration of protein in a solution. It is subjective, i.e., dependent on the amino acid composition of the measured protein. The Bradford protein assay was developed by Marion M. Bradford. The concentration of 500 and 700 µl enzyme was added to 5 mL of Coomassie Blue to each Falcone and mixed by a vortex. After 5 to 10 minutes, the

absorbance of each sample was measured at 595 nm wavelength (Zhang et al., 2020).

2.7. Optimum conditions for enzymatic hydrolysis

2.7.1. Thermal treatment and enzymatic hydrolysis of wheat gluten

Wheat gluten was dissolved as a 1% (w/v) solution in 100 mM phosphate Buffer. It was incubated in a water bath at a specific temperature (40, 50, 60, 70, and 80°C) for 30, 60, 90, 120, 150, 180, 210, and 240 min respective.

2.7.2. The effect of pH on enzyme activity

Enzymes are affected by changes in pH. The most favorable pH value - the point where the enzyme is most active is known as the optimum pH. Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes. As with activity, for each enzyme, there is also a region of pH optimal stability. Wheat gluten was dissolved as a 1% (w/v) solution in 100 mM phosphate Buffer. Iit was incubated in a water bath at specific pH (5-10) for 30, 60, 90, 120, 150, 180, 210 and 240 min.

2.8. Effect of enzyme and substrate concentration on enzyme activity

Changing the enzyme and substrate concentrations affect the rate of reaction of an enzyme-catalyzed reaction. Controlling these factors in a cell is one way that an organism regulates its enzyme activity and so it's Metabolism.

2.8.1. Effect of substrate concentration on enzyme activity

Increasing substrate concentration increases the rate of reaction. This is because more substrate molecules will be colliding with enzyme molecules, so more products will be formed. However, after a certain concentration, any increase will not affect the rate of reaction, since substrate concentration will no longer be the limiting factor. The enzymes will effectively become saturated and will be working at their maximum possible rate. Wheat gluten was dissolved as a 1% (w/v) solution in 100 mM phosphate Buffer. It was incubated in a water bath for 30, 60, 90, 120, 150, 180, 210 and 240 min.

2.8.2. Effect of enzyme concentration on enzyme activity

Increasing enzyme concentration will increase the rate of reaction, as more enzymes will be colliding with substrate molecules. However, this too will only affect up to a certain concentration, where the enzyme concentration is no longer the limiting factor.

Wheat gluten was dissolved as a 1% (w/v) solution in 100 mM phosphate Buffer. It was incubated in a water bath at specific Concentration (5-80 µl) for 30, 60, 90, 120, 150, 180, 210 and 240 min.

2.9. Solubility of gluten hydrolysates

To determine the solubility, 0.5g protein hydrolysate sample was dispersed in 50 ml of 0.1 M sodium chloride (pH = 7). The mixture was stirred at room temperature for 1 hour and centrifuged at 6200 rpm for 30 min. the supernatant was filtered through Whatman paper No.1 and the nitrogen content in the total fraction was analyzed by the Bradford method. The solubility index (NSI) was calculated as follows:

$$\text{NSI (\%)} = \frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}} \times 100 \quad (3)$$

2.10. Determination of enzyme activity

Wheat gluten was dissolved as a 1% (w/v) solution in 100 mM phosphate Buffer. After it was incubated in a water bath for 30, 60, 90, 120, 150, 180, 210 and 240 min. Enzyme activity was calculated according to the Ninhydrin assay. Concentration 70µl of all samples (30, 60, 90, 120, 150, 180, 210, and 240 min) was added to 700µl Ninhydrin reagent and the absorbance of each sample was measured at 575 nm.

2.11. Determination of degree of hydrolysis

Trichloroacetic acid (TCA) assay for peptic hydrolysis, DH was determined by the ratio of the percentage of 10% TCA-soluble nitrogen to total nitrogen in the sample (Drago & Gonzalez, 2000). One-gram wheat gluten was dissolved in trichloroacetic acid; After 30 min, the mixture was centrifuged at 3000 g and TCA-soluble peptide yield (Ysp) was also calculated using the following formula (Ketnawa & Ogawa, 2019):

$$\text{Ysp (\%)} = \frac{\text{A}}{\text{B}} \times 100 \quad (4)$$

where A is the peptide content of the supernatant, and B is the total protein content of the sample before hydrolysis.

2.12. Determination of protein recovery of GHs

Protein recovery was calculated as the ratio of the amount of protein (% N × 5.7) present in the hydrolysates to the initial amount of protein present in the reaction mixture.

2.13. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDA-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on all samples by 2- ME according to the discontinuous electrophoresis method of using a 4% stacking gel and 13% acrylamide gel (Haider et al., 2012). The reduction of disulfide bonds was performed at 100°C for 5 min. All samples were centrifuged at 4000 g for 10 min, and the supernatants were used to load the gels. The separating gel was run at a constant current of 20 to 60 mA for about 3.5 hours. The gels were stained in Coomassie brilliant blue. Molecular weights of protein hydrolysates were estimated by using the protein ladder with 10-250 KDa respectively.

2.14. The emulsion activity and stability index

The emulsifying activity of wheat gluten hydrolysates and wheat gluten was determined by the turbid metric method. To prepare the emulsions, 9 ml of soybean oil and 21 ml of 0.2% protein solution (pH 7.0) were shaken together and homogenized in a blender at 20,000 rpm for 2 min. Emulsion (50 µl) was taken from the bottom of the container after different time intervals (0 and 30 min) and diluted with 5 ml of 0.1% SDS solution. The absorbance of diluted emulsions was then determined at 500 nm and indicated as turbidity.

The emulsifying activity was determined from the absorbance measured immediately after emulsion formation. The emulsion activity index (EAI) was calculated as follows:

$$\text{EAI } (m^2/g) = 2\,(2.303 \times A500)N \times 10 - 4/\varphi LC \quad (5)$$

where A500, absorbance; φ, the volume fraction of dispersed phase; L, the light path in meters; C, the concentration of protein before the formation of the emulsion and N, diluted folds.

The emulsion stability (ES) was estimated by:

$$\text{ES } (\%) = \left(\frac{A30}{A0}\right) \times 100 \quad (6)$$

where *A*0, initial absorbance; *A*30, the absorbance determined at 30 min after emulsion formation.

2.15. Statistical analysis

All the values expressed were mean ± standard deviation of three replicate experiments. All data were analyzed using SPSS v17.0 statistical computer software.

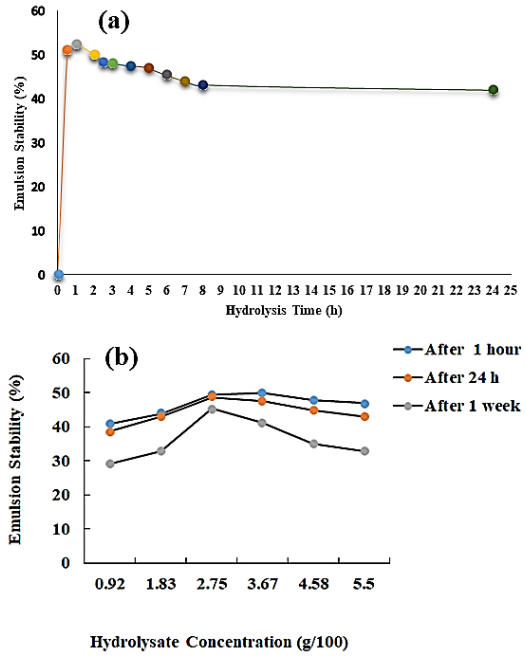

Fig.1. Stability percentage of gluten hydrolysate emulsion relative to hydrolysis time (a) and the percentage of emulsion stability relative to the concentration of gluten hydrolysates (b).

3. Results and Discussion

3.1. Determining the emulsifier properties of GHs

An emulsion is a heterogeneous system consisting of two immiscible liquids. Such systems have a minimum of stability due to the presence of interfacial tensile force between the components of the two liquids. Emulsifiers are the most important factor that can provide stability and non-rupture in emulsions (Tadros, 2009).

The results in Fig. 1a showed that the highest rate of oil in water emulsion stability was related to the emulsions containing hydrolyzate with 60 minutes. But then, with increasing the duration of hydrolysis up to 24 hours the emulsifier power of hydrolyzate was provided that showed a decreasing trend, so that the stability of the emulsion for 60 min hydrolysate reduced from 52.25 to 42%. This means that the emulsifier power of 24-hour hydrolysate reduced from 19.6% than an hour hydrolysate (Fig. 1a). The emulsifying capacity was the highest in 60 min, which could be due to the limited opening of protein chains, which, compared to their original state, the chains are not cumulative and complex, and the hydrophilic and hydrophobic groups are in a more balanced position and facilitate The placement of these groups on the surface between water and fat leads to an increase in emulsifying capacity, and this property results in the partial opening of the space structure of the protein chain and the loss of some of the bonds. However, if the amount of hydrolysis is higher than this amount, the amount of short-chain peptides resulting from enzymatic hydrolysis will increase and because these peptides are short, they can form less stable protein layers around the oil droplets, resulting in less emulsifying properties (Kong et al., 2008; Olanca & Özay, 2010).

The Fig. 1b shows the percent of emulsion stability to the concentration of gluten hydrolysate and suggests that it increased with 0-1.83 (gram %) with increasing the concentration of emulsifiers but with the increase of concentration from 2.75 to 3.67 the maximum stability of the emulsion was observed up to 50%. We can say that the most percentage of emulsion stability was seen at concentrations close to 3% (Fig. 1b). This is due to the production of hydrolysates with hydrophobic and hydrophilic groups are more available and they have an effect on emulsification. Smaller peptides have weaker emulsifying properties. Smaller peptides migrate rapidly and are absorbed in the interface between the two phases. However, they show less efficiency in reducing interfacial tension because compared to larger peptides, it can open and orientation at an interface, which makes the emulsions stable. These results were consistent with the results of (Wang et al., 2006).

3.2. Determining the emulsifier activity and emulsion stability index

The emulsifier activity index and emulsion stability index were determined and these results were consistent with the results of (Agyare et al., 2009; Agyare et al., 2008; Kong et al., 2007; Olanca & Özay, 2010). Emulsion activity index in the concentration of 0.1 with the amount of 170 allocated the highest amount to it and showed a decreasing trend by increasing the hydrolysis time (Fig. 2A).

The highest Emulsion Stability Index value was 120 for the concentration of 0.35 and by increasing the hydrolysis time, the figure of emulsion stability index showed a downward trend (Fig. 2B).

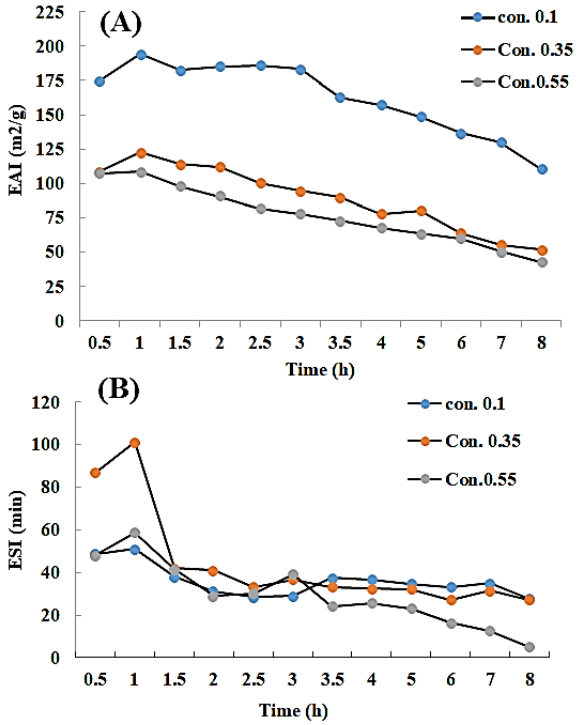

Fig. 2. Emulsifier activity index to hydrolysis time (A) and emulsion stability index of the hydrolysis time (B).

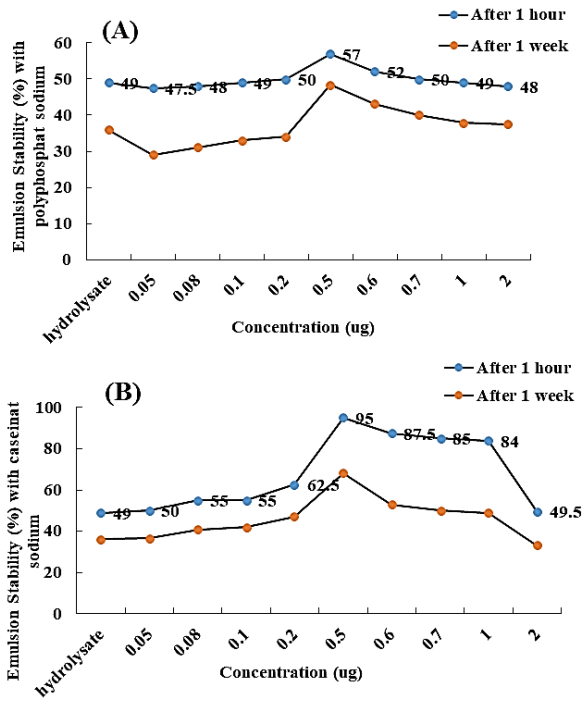

Fig. 3. stability percentage of emulsion relative to the combination of gluten hydrolysates (2.75g/100) with different concentrations of sodium polyphosphate (A) and stability percentage of emulsion relative to the combination of gluten hydrolysates (2.75g/100) with different concentrations of sodium caseinate (B).

The percentage of emulsion stability at constant hydrolysate concentrations (2.75 g /100) was measured with different concentrations of sodium polyphosphate (Fig. 3A) and sodium caseinate (Fig. 3B). The results showed that the addition of casein stabilizer increased the stability of the emulsion and had the most stability of the emulsion at a concentration of 0.5% with a value of 95%.

The emulsification mechanism of peptide samples is defined based on the adsorption of peptides on the surface of oil droplets, coating of oil droplets and inhibition of re-binding of these particles after homogenization. Emulsifying power depends on the solubility, degree of hydrophobicity and molecular weight of the peptides. Peptides containing hydrophobic amino acids, longer chain and higher solubility have higher emulsifying power (Klompong et al., 2009). When hydrolysis occurs, there will be an increase in the solubility of the peptides and their migration to the space between the water and the oil (Li et al., 2007). In addition, protein hydrolysates can be considered as surfactants because they contain hydrophilic and hydrophobic amino acids that are essential for surface properties (Klompong et al., 2009). Balancing the hydrophobic and hydrophilic parts of the peptides will ultimately lead to an increase in their emulsifying power (Li et al., 2007).

3.3. Determination of protein recovery of GHs

The yields of protein hydrolysates with different proteolytic enzymes were given in Table 2. This is an important measurement because a maximum recovery is desired in the production of hydrolyzed food proteins. Although many factors affected the yield of hydrolysis, the type of enzyme used had a marked effect on the yield and features. Recovery of Protein hydrolysate made from wheat gluten was measured according to the method stated in Section 2- 12 for 120 min. Calculations showed that most proteins retrieved by a mixture of two enzymes (alcalase/neutrase) were achieved with the amount of 97.4% and neutrase had the lowest protein recovery (39.88%).

Table 2. The amount of protein hydrolysate produced by proteolytic enzymes.

Enzyme	Protein content (%)	Protein recovery (%)
Alcalase	54.73 ± 0.01	74.8±0.03
Neutrase	29.193 ± 0.02	39.88±0.04
Alcalase/Neutrase	71.3 ± 0.01	97.4±0.07

3.4. Determination of degree of hydrolysis

The degree of hydrolysis (DH) affects the size and amino acid composition of the peptides. In high DH, the production of bitter-tasting hydrophobic peptides affects the taste of protein hydrolysis (Silva & Silveira, 2013). As the degree of hydrolysis increases, more peptide bonds are broken and the peptide strands become shorter, their molecular weight decreases, and finally the total amount of free amino acids in the substrate increases (Živanović et al., 2011). Gluten hydrolysate was provided using several commercially available proteases such as alcalase 2.4 L and the combination of alcalase and neutrase. The degree of hydrolysis was 14.7, 8.1, and 23.13 and the combination of alcalase and neutrase after 7 hours for the hydrolysis of wheat gluten had the highest degree of hydrolysis (23.13). The control of the enzymatic reaction

was dependent on the mechanism of proteolytic reactions between the solution enzyme and the insoluble substrate (wheat gluten). Wheat gluten hydrolysis was very fast in the first 60 min and then was slowed down over time and indicated that the highest hydrolysis of proteins occurs in the first 60 min (Fig. 4A).

The protein hydrolysis of wheat gluten by alcalase and Nutrase enzyme increased with a high slope, but then the rate of increase of the degree of hydrolysis decreased. The duration of hydrolysis is very important and its reduction can be due to excessive decomposition of the substrate and also the inhibitory effect of the final product, which as a competitive inhibitor by binding to the active site of the enzyme has a negative effect on enzyme activity on the substrate. The degree of hydrolysis parameter is specifically affected by the direct process time (Sienkiewicz-Szłapka et al., 2009). Also, this decrease in the rate of hydrolysis may be due to a decrease in the concentration of peptide bonds available for hydrolysis, enzyme inhibition and enzyme inactivation (Silva & Silveira, 2013). In another study on whey protein concentrate, a degree of hydrolysis by pancreatin was observed at intervals of up to 5 hours, which increased to about 30.52% over time (Silva & Silveira, 2013).

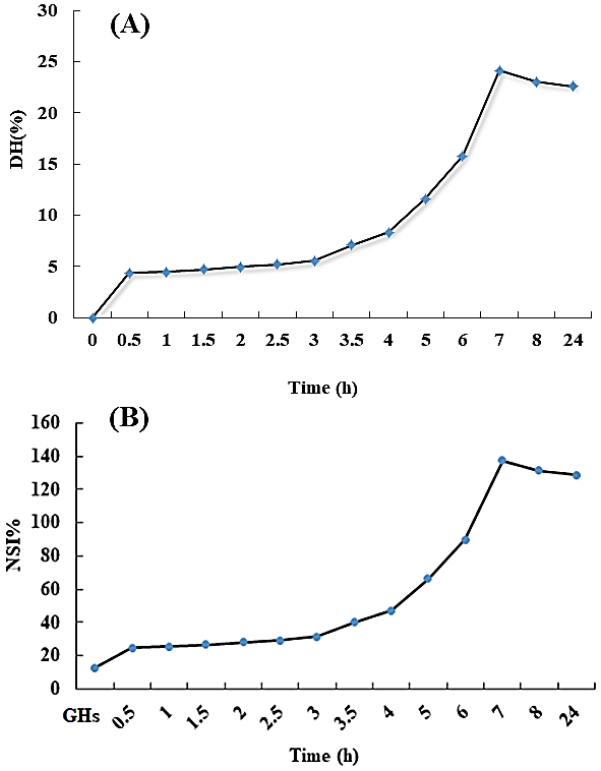

Fig. 4. The degree of hydrolysis ratio the hydrolysis time (A) and effect of enzymatic hydrolysis (combination of two enzymes alcalase and neutrase) on solubility of gluten hydrolysate (B).

3.5. *Determining the solubility of GHs*

A significant part of the functional properties of food is related to proteins (Assadpour et al., 2011). Any change in the structural arrangement of proteins also causes changes in the functional properties of proteins (Aluko & Yada, 1995).

Solubility is one of the most important and generally the first functional properties that are examined during the production of new protein components because it has a significant effect on other functional properties (Kinsella & Melachouris, 1976). Low solubility causes unsuitable appearance and sandy mouth feeling in the final product (Mahmoud et al., 1992; Molina Ortiz & Cristina An, 2000).

So far, several studies have been conducted on the effect of enzymatic hydrolysis process on the functional and antioxidant properties of hydrolyzed proteins obtained from agricultural products. Hydrolysis of barley (Yalçın & Çelik, 2007), tea seed (Li et al., 2014), peanut (Jamdar et al., 2010) and pea (del Mar Yust et al., 2010) proteins increased protein solubility, especially at acidic pHs.

All WGHs presented a similar pattern of NSI values, and the solubilities of those hydrolysates were pH-independent over the studied range.The results obtained for the solubility of gluten hydrolysate showed an increasing trend than the hydrolysis time. Hydrolysis of gluten proteins increased by increasing the hydrolysis time and reached from the solubility of 19% for the control group (wheat gluten) for all hydrolysis samples approximately to 66 % because the size of the proteins is reduced and the molecular weight of the hydrolysis is less and hydrophilic and hydrophobic groups have increased (Fig. 4B).

In a study, hydrolysis of pea protein with alcalase enzyme increased the solubility at all pHs and with increasing the degree of hydrolysis, the solubility at the isoelectric point showed a significant increase (del Mar Yust et al., 2010).During enzymatic hydrolysis of proteins, molecular weight decreases, a group of free amino acids, di, tri, and oligopeptides are formed, and polar and ionizing groups increase in surface area. Therefore, the solubility of hydrolyzed compounds increases, the functional properties of proteins change, and their functional quality and availability increase (Ghribi et al., 2015; Mullally et al., 1995).

Combined hydrolysis of bean protein isolates using pepsin and pancreatin enzymes increased the solubility and saturation of hydrolyzed proteins compared to protein isolates at the isoelectric point (Polanco-Lugo et al., 2014).This is due to changes in protein conformation due to the effect of endopeptidase activity of enzymes. Minor modifications in the molecular weight of protein chains increase their solubility in aqueous media (Liu et al., 2011).

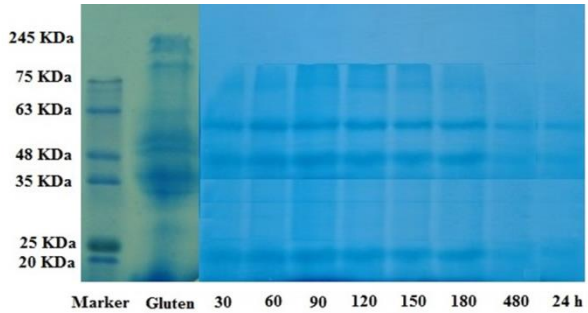

Fig. 5. SDS-PAGE is related to gluten and gluten hydrolysates. Line 1. Molecular weight index, line 2. Gluten before hydrolysis, line 3. Gluten hydrolysate with both endopeptidase enzymes (Alcalase, Neutrase) for 30 minutes, line 4. 60-minutehydrolysate, line 5. 90-minute hydrolysate, line 6. 120-minute hydrolysate, line 7. 150-minute hydrolysate, line 8, 180-minute hydrolysate, line 9, 8-hour hydrolysate, line 10, 24-hour hydrolysate.

3.6. SDS- PAGE of gluten and GHs

To study the effect of different enzymes on protein breakdown in wheat gluten, SDS-PAGE of WGHs was performed to obtain information on their molecular (Fig. 5). In SDS-PAGE technique, the resolution of proteins is very good due to the use of sodium dodecyl sulfate (SDS) and also the excellent properties of polyacrylamide gel. SDS is an anionic detergent that denatures proteins by binding to hydrophobic regions. In fact, the SDS molecule binds to proteins to cover their natural charge, creating a uniform distribution of negative charges on it. As a result, proteins are isolated solely on the basis of their molecular weight. Small proteins or peptides in all samples indicate hydrolysis. The results of SDS-PAGE related to gluten and gluten hydrolysate showed that gluten had numerous bands in terms of molecular weight in the range of 20-245 KDa but 30-60 KDa bands are seen with more concentration. After the hydrolysis of gluten at 58-60°C and a pH of 6.5 by the set of endopeptidase enzymes (alcalase and neutrase), bands with a molecular weight greater than 63 KDa disappeared and then appeared due to the enzymatic hydrolysis in the molecular weight range of 45-55 and with less concentration in the molecular weight range of 20 KDa and with much less concentration in the range of 70 KDa. This results matched with Kong et al. (2007).

3.7. Foaming properties of GHs

Foams are colloidal systems with a continuous liquid or aqueous phase and a dispersed phase of gas or air. Food foams are generally caused by severe shaking or agitation, and the method used is effective on the properties of the resulting foam. Flooring capacity and floor stability are two common characteristics of floors. The stability of the foam depends on the strength of the protein film and its impermeability to air. Dietary proteins have the ability to create good foam. Proteins in dispersions reduce surface tension at the surface between climates, resulting in foaming (Surowka & Fik, 1992). Proteins are good foaming agents because they can rapidly spread to the water-air interface and there create a strong cohesive and elastic film by partial unfolding. Foaming properties are associated with the number of hydrophobic amino acids exposed at the surface of the protein molecule. Dispersed proteins lower the surface tension at the air-water interface, thus forming foaming capacity. The result in (Fig. 6A) showed that the most foam stability in hydrolyzed samples was in the amount of 18.52% for 60 minutes and also the foam stability reduced by increasing the hydrolysis time and reached 7% after 24 hours of hydrolysis. Increased foaming of wheat grain protein (gluten) under the influence of enzymatic hydrolysis can be attributed to the production of biophilic peptides (amphiphilic) after hydrolysis. Molecular weight reduction also increases flexibility and the formation of a more stable layer in the interfacial region. As a result, foaming increases with increasing propagation speed to the interface (Liceaga-Gesualdo & Li-Chan, 1999). Studies have shown that partial hydrolysis of pea protein isolates with alkalase enzyme increased foaming; if the pea protein isolate had no foaming power (del Mar Yust et al., 2010). Hydrolyzed soy protein by pepsin and papain enzymes at pH 4, 5.5, 7 showed more foaming than soy protein isolate (Tsumura et al., 2005). Other studies have shown that canola protein hydrolyzed by alkalase has more foaming than the original protein (Chabanon et al., 2007). According to reports in hydrolyzed wheat germ protein, at low degrees of hydrolysis, a 74% increase in foaming capacity and maximum foaming stability was observed (Claver & Zhou, 2005).

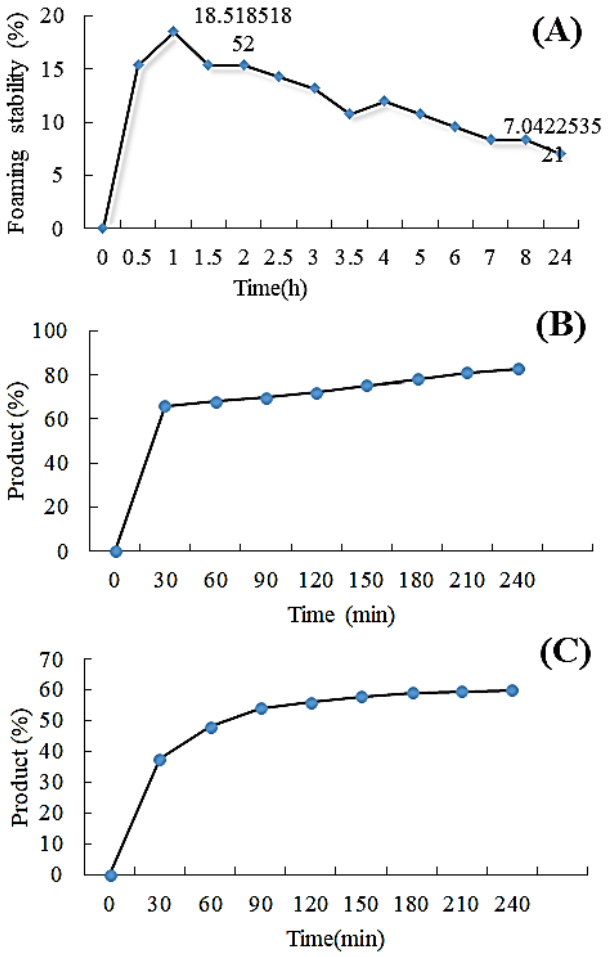

Fig. 6. Gluten hydrolysates foaming stability to the hydrolysis time (A) and alcalase enzyme activity according to the amount of produced hydrolysates to the hydrolysis time (B) and alcalase enzyme activity according to the amount of produced hydrolysates to the hydrolysis time (C).

Table 3. Protein content of neutrase and alcalase.

Enzyme	Protein concentration (mg/ml)
Neutrase	1.987±0.03
Alcalase	2.295±0.01

Table 4. Wheat gluten properties.

Ash (%)	Moisture (%)	Protein (%)	pH
1.4 ± 0.02	1.46 ± 0.06	73.2 ± 0.01	6.05 ± 0.07

3.8. The amounts of enzyme protein

The amount of protein in alcalase and neutrase enzymes was measured (Table 3). The results showed that the amount of alcalase enzyme as 2.97 was more than neutrase.

The amount of ash, moisture percent, protein percent pH and the amount of gluten were tested by the procedures described and the results were obtained as shown in Table 4.

3.9. The activity of alcalase and neutrase

Free enzyme activity was drawn based on the amount of produced peptide. The activity diagram of the alcalase enzyme (Fig. 6B) and nutrase (Fig. 6C) increases logarithmically in the first 30 minutes but then reduced gradually over time.

3.10. Optimum temperature for enzyme activity

To determine the optimum temperature for free enzyme activity, the level of enzyme activity has been determined and the highest activity of alcalase and neutrase enzymes was achieved at 60°C. At temperatures above 60°C, the level of enzyme activity decreased. Since the optimum temperature for the activity of both enzymes was 60°C, thus the optimum temperature for the combination of two enzymes was considered as 60°C.

3.11. Determining the optimum pH for enzyme activity

The results of determining the optimum were set for the free enzyme activity. Alcalase free enzyme at pH = 6.5, neutrase enzyme at pH = 6, and the combination of two enzymes (alcalase/neutrase) at pH = 6.5 showed the highest activity and these amounts of pH were chosen as the optimum pH.

3.12. Determining the optimum concentration of enzymes

The optimum concentration of the enzyme was determined for the combination of two enzymes. And the ratio of 2 to 1 of alcalase to neutrase showed the highest activity.

4. Conclusion

Gluten hydrolysates (GHs) were prepared by endopeptidases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*. It was observed that alcalase to neutrase with a 2:1 ratio has an efficient hydrolysis yield. The results of the emulsification properties of gluten hydrolysates revealed that oil in water emulsions obtained by a combination of two enzymes and 8 hours' hydrolysis time were more stable compared with the resulting hydrolysates with less or more than 8 hours. Determining the molecular mass of gluten hydrolysates by SDS-PAGE implied that, the main peptide bands were formed around 45-55 KDa for gluten hydrolysates. Comparative analysis of sodium caseinate, sodium polyphosphate, and xanthan gum with gluten hydrolysates on oil in water emulsion stability indicated that a combination of hydrolysates with sodium caseinate has been the best effect on emulsion stability. The results showed that the use of WGHs as an alternative to other gums is possible in mayonnaise production.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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