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FUNCTIONAL AND BIOLOGICAL PROPERTIES OF ENZYMATICALLY MODIFIED WHEAT GLUTEN

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FUNKCIONALNA I BIOLOŠKA SVOJSTVA PŠENIČNOG GLUTENA MODIFIKOVANOG ENZIMSKIM POSTUPCIMA

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Greetings

To the spirit of my pure father, to my beloved dear mother (the most precious thing I have in existence), to my beloved wife who stood with me and helped me on this scientific journey …

To my dear sons, daughters, brothers and sisters …

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I present this modest work …

Functional and biological properties of enzymatically modified wheat gluten

ABSTRACT

In order to confirm the gluten potential for inclusion into functional foods, the synergistic effect of the heat treatment and controlled enzymatic hydrolysis on the functional and the antioxidant properties of alcalase-assisted wheat gluten hydrolysates (AWGHs) have been first discussed. For this purpose, wheat gluten was heat-treated during 30 min at 75 °C and intensively hydrolyzed with alcalase at degree of hydrolysis (DH) 16.1%, 22.9%, and 30.2%. All the hydrolysates had excellent solubility over a pH range of 2–12. Emulsifying activity and stability were also improved, while proteolysis was deleterious to foam capacity and stability, water-holding capacity, fat-binding capacity and did not show improvement at higher DH (22.9% and 30.2%). As well, controlled hydrolysis of heat-treated gluten resulted in a remarkable improvement in antioxidant activities. The results show that the heat-treated AWGHs were superior to the untreated hydrolysate in the functional and antioxidant properties tested.

The further aim of the thesis was to find the optimal operational andprocess parameters for the enzymatic hydrolysis of wheat gluten in abatch stirred bioreactor regarding degree of hydrolysis, functional properties andantioxidant capacity of the obtained hydrolysates. It appeared thatimpeller geometry and agitation speed influenced the mass transferresulting in enhanced gluten hydrolysis. The highest initial reaction rate (0.83 ± 0.02 min^{-1}) and degree of hydrolysis (30.47%) were achievedwith the pitched four-bladed impeller and agitation speed of 350-450rpm, conditions which provided proper balance between requirements foradequate mass/heat transfer and low shear stress. The highest DH was achieved with the lowest gluten concentration and higher pH value. These results may be connected to an increased product and/or substrate inhibition, insufficient mixing, steric hindrance and lack of cleavage sites at higher gluten concentration. The low solubility of gluten at neutral pH might have contributed to low DH obtained and this was pronounced athigher gluten concentration.

The impact of selected process conditions including gluten concentration, temperature, pH andenzyme-gluten (*E*/*S*) ratio on the enzymatic reaction was also

investigated by applying a Box-Behnken experimental design from the viewpoint of degree of hydrolysis, functional properties and antioxidant activity determined by two methods. Eight mathematical models obtained allowed calculation of the hydrolysis degree, solubility, foam capacity (*FC*), foam stability (*FS*), emulsion activity index (*EAI*) and emulsion stability index (*ESI*) as well as both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulpfonic acid) radical scavenging activity from a given set ofreaction conditions with good predictability. The statistical analysis showed that each variable had a significant effect on degree ofhydrolysis and the antioxidant capacity of both tested systems. Hydrolysis up to around DH 15% improved DPPH radical scavenging activity, while excessive hydrolysis worsened it. The ABTS activity of the hydrolysates was not associated with the DPPH activity nor with the DH, revealing that it was not possible to fulfill all desirable qualityrequirements (maximum degree of hydrolysis and protein yield, maximum DPPH and ABTS scavenging activities) by using the same reactionconditions.

The analysis revealed that all tested variables had a significant effect on the solubility, within the experimentally tested ranges. The most relevant variable seemed to be *E*/*S* ratio, but the effects of gluten concentration, pH, temperature and gluten concentration and *E*/*S* interactions were also significant. The quadratic term of gluten concentration was also significant, indicating that a response was a quadratic function with a local maximum. It can be concluded that the high solubility of the wheat gluten hydrolysates was achieved at a low temperature of the reaction of enzymatic hydrolysis (40 °C) and a pH of 9, using minimal amounts of substrate and a rather low amount of enzymes, since the optimal [*E*]/[*S*] ratio seemed to be 0.5 AU/g of gluten.

The analysis revealed that all tested variables had a significant effect on the *EAI*, within the experimentally tested ranges. The most relevant variable seemed to be interaction of the gluten concentration and temperature ($p<0.0001$). The quadratic terms of gluten concentration and temperature were also significant, indicating that a response was a quadratic function with a local maximum. It appeared that the highest *EAI* of 2782.9 m^2/g was achieved at pH 8.0 and at a temperature of 50 °C, [*E*]/[*S*] of 0.75 AU /g gluten and a substrate concentration of 1%.

The obtained results revealed that the foam capacity range of the wheat gluten hydrolysates were in the range of 24.2-80.3%, depending on the independent variables that were tested. Overall, it was apparent that the gluten hydrolysates had a relatively good foam capacity, since in a large number of experiments this ability was higher than 50%.

It seemed that the most relevant variables for the foam stabilitywere gluten concentration and pH with estimated effects of 4.64 and 4.58, respectively. The effects of temperature and *E*/*S*ratio were also significant ($p<0.05$). It appeared that while substrate concentration and all other parameters had a positive effect, the gluten concentration and *E*/*S* ratio interaction had a significant negative influence on foam stability. According to the statistical analysis, the maximum foam stability can be obtained at high level of gluten concentration and pH value.

The hydrolysate which showed the highest DPPH and ABTS radical quenching ability, was further separated by sequential ultrafiltration intothree major peptide fractions, GF I (10–30 kDa), GF II (3–10 kDa) and GF III (<3 kDa) and fractions were compared based on their protein content and their radical scavenging activity against DPPH, ABTS and superoxide radical. It appeared that the hydrolysate showing the highest DPPHand ABTS activity (DH 13.6%) had the highest percentage of peptides with medium molecular masses (3–10 kDa). This peptide fraction seemed to be primarily responsible for all three scavenging activities.

Overall, the study might contribute to approve wheat gluten,a by-product of wheat starch industry, as an accessible and cheap source of bioactive compounds for the development of novel nutraceuticals, cosmetics and drugs.

Keywords: wheat, proteins, hydolisates, antioxidant, optimization

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Funkcionalna i biološka svojstva pšeničnog glutena modifikovanog enzimskim postupcima

ABSTRAKT

U početku je ispitan uticaj kombinovanog postupka koji se sastoji u toplotnom pretretmanu i kontrolisanoj enzimskoj hidrolizi pšeničnog glutena pomoću alkalaze (proteaze iz *Bacillus licheniformis*) na funkcionalna i antioksidativna svojstva dobijenih hidrolizata. U tom cilju, pšenični gluten je inkubiran u toku 30 min na 75 ^{o}C, a potom je vršena enzimska hidroliza do dostizanja stepena hidrolize 6,1%, 22,9% i 30,2%. Svi dobijeni hidrolizati su pokazali veliku rastvorljivost u intervalu pH od 2-12, značajno veću u poređenju sa netretiranim glutenom. Takođe su bile značajno poboljšane emulgujuća aktivnost i emulgujuća stabilnost, dok je hidroliza dovela do smanjenja kapaciteta penjenja kao i kapaciteta vezivanja vode i ulja pri različitim stepenima hidrolize (DH 22,9% i 30,2%). Dobijeni hidrolizati su imali značajno veći antioksidativni potencijal i unapređena funkcionalna svojstva u poređenju sa netretiranim glutenom.

Sledeći cilj ove disertacije bio je da se utvrde optimalni operativni i procesni parametri za izvođenje enzimske hidrolize pšeničnog glutena u šaržnom reaktoru sa mehaničkim mešanjem u pogledu stepena hidrolize, funkcionalnih svojstava i bioloških svojstava dobijenih hidrolizata. Pokazano je da geometrija mešalice i brzina mešanja značajno utiču na prenos mase i brzinu reakcije. Najveće početne brzine reakcije (0,83±0,02 min^{-1}) i stepeni hidrolize (30,47%) postignuti su sa mešalicom sa četiri zakošene lopatice (DH 30,47%, 105 min) i pri brzini mešanja 350-450 rpm, uslovi koji su omogućili kompromis između zahteva za efikasnim prenosom mase i toplote i smanjenom dezaktivacijom enzima usled smicajnih sila. Kod ovih mešalica mešanje se manifestuje aksijalnim strujanjem na dole, sa pojačanim smicanjem, koje ne prouzrokuje dezaktivaciju enzima u toku 2 h reakcije uz intenzivno mešanje. Najviši stepeni hidrolize su postignuti pri najmanjim početnim koncentracijama glutena i višim pH vrednostima, što se može pripisati inhibicijom enzima supstratom u višku, maloj rastvorljivosti glutena, difuzionim limitacijama ili istovremenom utcaju više navedenih faktora.

U daljem radu ispitan je uticaj četiri procesna parametra i to koncentracije supstrata, odnosa enzim-supstrat (odnos [*E*]/[*S*]), pH i temperature na stepen hidrolize i odabrana

funkcionalna i antioksidativna svojstva hidrolizata primenom Box-Behnken-ovog eksperimantalnog plana. Konkretno, ispitani su uticaji navedenih parametara na stepen hidrolize, rastvorljivost, emulgujuću aktivnost i stabilnost, kapacitet i stabilnost pene, kao i na antioksidativnu aktivnost određenu pomoću dve metode. Metoda odzivne površine je korišćena da se proceni značaj svakog pojedinačnog faktora uzimajući u obzir njihove interakcije, kao i da se utvrde optimalni uslovi hidrolize glutena katalizovane alkalazom iz *B. licheniformis.*Dobijene su matematičke funkcije sa definisanim maksimumima (osam funkcija) koje adekvatno opisuju uticaj temperature, pH, koncentracije glutena i odnosa enzim/gluten na svako od navedenih svojstava i ukazuju na međusobni uticaj i interakcije između procesnih parametara. Statistička analiza rezultata pokazala je da sva četiri procesna parametra imaju značajan uticaj na stepen hidrolize i antioksidativnu aktivnost hidrolizata određenu u dva eksperimentalna sistema. Pokušano je da se uspostavi korelacija između stepena hidrolize i sposobnosti inhibicije DPPH radikala i utvrđeno je da sa porastom stepena hidrolize do oko 15% povećava se i DPPH antioksidativna aktivnost, da bi pri većim stepenima hidrolize naglo opala. Sposobnost inhibicije katjonskog ABTS radikala nije bila u korelaciji ni sa DPPH inhibitornom aktivnosti, kao ni sa stepenom hidrolize. Ovi rezultati su pokazali da nije moguće primenom istih reakcionih uslova istovremeno postići i visok stepen hidrolize (veliki prinos peptida), maksimalnu DPPH i ABTS inhibitornu aktivnost kao i da je zadržavanje određenih delova sekundarne strukture, koja se nalazi u dužim oligopeptidima, neophodno za antioksidativnu aktivnost.

Nakon statističke analize dobijenih rezultata pokazano je da sva četiri ispitivana parametra imaju značajan uticaj na rastvorljivost hidrolizata u intervalu ispitivanih vrednosti. Odnos enzim/supstrat pokazao je statistički najveći uticaj na rastvorljivost, ali uticaj koncentracije glutena, pH, tempertaure kao i interakcije koncentracije glutena i odnosa *E*/*S* bio je takođe statistički značajan. Uticaj kvadratnog člana koncentracije glutena bio je takođe statistički značajan, što ukazuje na to da odzivne površine imaju lokalne maksimume. Najveća rastvorljivost hidrolizata (~ 97% i ~ 92%) postiže se pri niskim temperaturama (40 ^{o}C) i pH 9, i pri niskim koncentracijama supstrata (1%) i [*E*]/[*S*] odnosu (0,5 i 0,25 AU/g glutena, redom).

Regresiona analiza je pokazala da sva četiri procesna parametra pokazuju statistički značajan uticaj na sposobnost emulgovanja hidrolizata, izražen kao indeks emulgacione aktivnosti. Interakcija između koncentracije glutena i temperature imala je statistički najveći uticaj (p<0,0001). Sposobnost emulgovanja hidolizata je povezana sa stepenom hidrolize, pri čemu niži stepen hidrolize dovodi do veće emulgujuće aktivnosti. Uopšteno je prihvaćeno da limitirana hidroliza poboljšava emulgujuća svojstva proteina i njihovih hidrolizata. Najveća emulgujuća aktivnost hidrolizata proteina glutena od 2782,9 m²/g postiže se pri slabo baznoj sredini od 8,0 i temperaturi reakcije od 47,5 °C, pri koncentraciji glutena 1% i *E*/*S* odnosu 0,75 AU/g glutena. Što se tiče emulgujuće stabilnosti hidrolizata proteina, uzimajući u obzir sve značajne faktore prilikom određivanja maksimalne emulgujuće stabilnosti, dolazi se do zaključka da se ista postiže pri [*E*]/[*S*] odnosu od 0,75 AU/g glutena i koncentraciji supstrata od 9,0%.

Kapacitet penjenja bio je u intervalu od 24-80,3% u zavisnosti od procesnih parametara. Generalno je pokazano da hidrolizati glutena imaju odgovarajući kapacitet penjenja, koji je bio u većini slučajeva veći od 50%.Parametri koji su pokazali najveći značaj na stabilnost pene su koncentracija glutena i pH sa procenjenim efektima od 4,64 i 4,58, redom. Uticaj temperature i *E*/*S* odnosa je takođe bio statistički značajan (p<0,05).Iako su koncentracija glutena i ostali parametri imali pozitivan uticaj na stabilnost pene, interakcija između koncentracije glutena i *E*/*S* odnosa pokazali su negativan uticaj. Maksimalne vrednosti stabilnosti pene postignute su pri velikim koncentracijama glutena i pH vrednostima.

Odabrani hidrolizati, koji su pokazali najveću antioksidativnu aktivnost, koncentrovani su i razdvajani po veličini na četiri frakcije pomoću ultrafiltracije sa tri membrane različitih veličina pora (30 kDa, 10 kDa, 3kDa), a potom na više frakcija primenom gel permeacione hromatografije. Antioksidativna aktivnost ultrafiltracionih frakcija hidrolizata glutena određena je na osnovu sposobnosti neutralizacije 2,2ʾ-difenil-1-pikrilhidrazil (DPPH) i 2,2ʾ-azinobis (3-etilbenzotiazolin-6-sulfonska kiselina)-diamonijum so (ABTS) radikala, superoksid radikala kao i sposobnosti heliranja jona metala. Ispitivanjem antioksidativnih svojstava svih uzoraka pomoću četiri različite metode, došlo se do zaključka da frakcije proteina glutena manje molekulske mase pokazuju bolju

sposobnost u borbi protiv slobodnih radikala, ali i da njihova učinkovitost zavisi od uslova odvijanja samog procesa hidrolize. Naime, hidrolizat koji je imao najveću sposobnost neutralizacije DPPH i ABTS radikala imao je najveći sadržaj peptida molekulsih masa u intervalu od 3-10 kDa. Generalno je pokazano da frakcije glutenina i glijadina poseduju specifične sekvence aminokiselina koje kada se oslobode iz kompleksne strukture nativnog proteina postaju veoma vredan izvor antioksidanasa.

U ovoj tezi pokazano je da se parcijalnom enzimskom hidrolizom glutena u kontrolisanim uslovima mogu značajno unaprediti funkcionalna i biološka svojstva ovog proteina i valorizovati njegova primena u razvoju novih nutraceutika, funkcionalne hrane i formulacija za farmaceutsku i kozmetičku industriju.

Ključnereči:pšenični gluten, proteini, hidrolizati, antioksidanti, optimizacija

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UDC number:

CONTENT

1. INTRODUCTION

The increasing human population over the last decades has greatly influenced the demand for food products and the use of these proteins have attracted particular interests. Research has demonstrated that nutrition plays a crucial role in the prevention of chronic diseases. Some food can be regarded as a functional if it has beneficial effects on target functions in the body as a source of mental and physical well-being, contributing to the prevention and reduction of risk factors for several diseases or enhancing certain physiological functions, beyond adequate nutritional effects. On the other side, functional properties of food components should not be confused with functional foods or biological activity. Techno-functional properties represent complex interactions between the conformation, structure, composition and physicochemicalproperties of proteins under the influence of other food components and environment. They are important from the point ofview of food technology and include protein ability to form interfacial films createing stable systems, solubility, emulsifying ability, foaming, gelation, viscosity and many others. Most functional properties affect the sensory characteristics of food and can play a major role in the physical behavior of foods or food ingredients during their preparation, processing and storage.

In recent years, cereals and their ingredients are accepted as functional foods because of providing dietary fibre, proteins, energy, minerals, vitamins and antioxidants required for human health and among them, wheat products are notified the most common cereal based functional foods. Gluten, the major wheat protein, has attracted the attention of food processors for new product development or for use in existing food products. It is an economically important co-product in the recovery of wheat starch in wet processing of wheat flour, and presents an abundant plant protein source. It is also well known for their high nutritional value and versatile functional properties in food products.

Wheat gluten is unique in its functional properties that are the direct consequence of its amino acid composition and protein structure. Modern understanding of the complementary nature of proteins from different sources in a mixed diet positions wheat

protein as a significant contributor to human nutrition. Recent studies of health and well-being particularly in relation to food consumption and obesity have indicated the value of food proteins as they have the effect of increasing feelings of satiety and reducing the desire to consume as much food. Gluten is sought after as a protein additive because of its desirable viscoelastic properties.

Although wheat gluten is in abundant supply as a byproduct of the wheat starch industry, its utilization is limited by its insolubility. Researchers have been focusing on chemical and enzymatic modifications of wheat gluten proteins to enhance their functional and biological properties. Such modifications are critical for the commercial use of wheat gluten proteins in food formulations. Wheat proteins` unique functional and nutritional properties may offer enormous possibilities for use not only in existing food applications but also in new food product formulations.

A commercial production of wheat protein hydrolysates may offer an inexpensive protein source for use in various food products and for nutritional supplementation. It has been shown that functional and nutritional properties of food proteins can be improved by enzymatic hydrolysis. Protein hydrolysates possess properties that make them attractive as a protein source in human nutrition. Hydrolysates are used in products for special nutrition, such as diets for elderly and patients with impaired gastrointestinal absorption, hypoallergenic infant formulas, sports nutrition, and weight-control diets, as well as in consumer products for general use. Thus, the protein hydrolysates could also be used as flavor enhancers in foods, coffee whiteners, cosmetics, personal care products, and confectionery, and in the fortification of soft drinks and juices. In addition, they are used in soups, sauces, gravies, snacks, meat products, and other savory applications. In any case, it has been shown that protein hydrolysates should be rich in low molecular weight peptides which offer advantages for dietary purposes. Depending on the type of product in which peptides will be incorporated, the degree of hydrolysis required is different. Moreover, certain oligopeptides released during protein hydrolysis have been shown to possess distinctive physiological activities, such as anti-hypertensive activity, antioxidant activity, immunostimulating activity, and ACE (angiotensin converting enzyme) inhibiting activity and as such may contribute to enhanced biological activities and health benefits of the

hydrolysate. However, the enzymatic hydrolysis process is still ill-defined and difficult to control at the industrial scale raising a number of technological challenges as well as regulatory and marketing issue.

Based on literature data, studies of protease action on wheat gluten proteins have had three major objectives. The first was to relate the physicochemical properties of gluten proteins with their functional properties. In particular, a number of authors have tried to modify the solubility of the gluten fraction enzymatically. The second objective was to identify peptides with biological activity, especially with respect to celiac disease (gluten intolerance). The third objective was to obtain structural or genetic information by comparing the composition of peptide fractions from different proteins. Because of the insoluble nature of wheat gluten and the complexity of their composition, enzymatic hydrolysis of wheat gluten must usually be studied in heterogeneous dispersed systems and the products of hydrolysis are difficult to isolate and to characterize. Different proteases have been tried, such as pepsin, trypsin, papain, bromelain, subtilisin and others. Wheat gluten hydrolysis resulted in peptide mixtures with high solubilities and altered foaming and emulsifying characteristics, depending on the degree of hydrolysis.

In spite of the large number of excellent studies dedicated to characterizing structure and functionality of gluten proteins, important gaps of knowledge has to be filled by future research. One of the priorities should be a better understanding of the relations between structure and functionality of gluten proteins. Furthermore, protein hydrolysates should be investigated for their potential bioactive and techno-functional properties.

2. THE BASIC PROTEIN PROPERTIES

The word “protein” is derived from the Greek word “proteios”, which means “of primary importance”. In fact, proteins play an important role in all biochemical and physiological body processes; they act as enzymes, hormones, receptors, antibodies and are required for the structural integrity of cells (Wallis,1971).

Proteinsare one of macromolecules and they are also the most abundant. They are also very important constituent of our body and a great source of nutrient and energy. Proteinsare constructed from their monomers which are amino acids. These amino acids are linked covalently by peptide bonds.

The functions of proteins are for growth, maintenance, and repair of cells by proteins also action as:

1. enzymes that catalyze metabolic reactions;
2. structural proteins that maintain the shape of cells;
3. hormones that regulates cell activities;
4. antibodies that provide defense mechanism;
5. contractile proteins, transport intracellular structures;
6. proteins, toxins, and components of energy source (Dey et al., 2014).

In addition to their contribution to human nutrition, proteins are functional ingredientsthat can affect organoleptic, kinesthetic, hydration, surface and rheological/texturalproperties of foods, pharmaceuticals, and health-care products (Phillips et al., 1994; McClements et al., 1993). Protein functionality is a direct manifestationof protein structure and molecular interactions. Protein structure-functionalityrelationship can be modified enzymatically, chemically, genetically or physically (Dickison and McClements, 1996; Damodaran, 1996). However, in the area of food chemistry andtechnology, the protein functionality has always been among the most inconsistently andambiguously defined topics (Pour-El, 1981). Thus the establishment of methods and/orgathering of data to monitor possible changes in structure of proteins and in structure-functionrelationships due to chemical, enzymatic or physical modification have been akey goal of food scientists.

2.1. Protein denaturation

Generally speaking, proteins can be either in a native or a denatured state. The native state is the conformation adopted by a protein under standard environmental conditions and the denatured state is a confirmation that protein molecules adopt when their chains are completely unfolded to form a highly flexible random coil. Changes in environmental conditions can break hydrogen bonds or hydrophilic interactionsbetween protein molecules, leading to the denaturation of proteins.

Denaturation of proteins involves the disruption and possible destruction of both the secondary and tertiary structures. In tertiary structure there are several types of bonding interactions between "side chains" including hydrogen bonding, salt bridges, disulfide bonds, and non-polar hydrophobic interactions which may be disrupted.Since denaturation reactions are not strong enough to break the peptide bonds, the primary structure (sequence of amino acids) remains the same after a denaturation process. Denaturation disrupts the normal alpha-helix and beta sheets in a protein and uncoils it into a random shape.

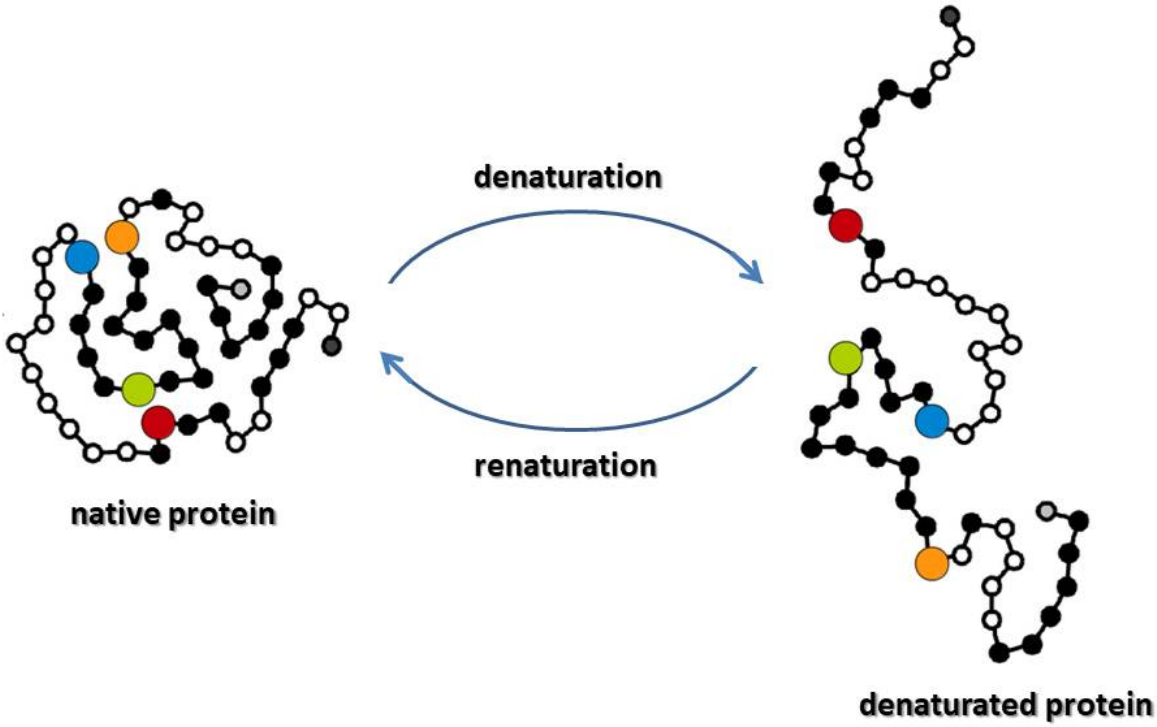


Figure 1. Schematic illustration of the denaturation of proteins

Denaturation of proteins can occur under a variety of physical conditions, including high temperature, very low or high pH, and very high pressure (Tanford, 1968; Klibanov, 1983). In many cases, the denaturation is only partially reversible. Thermal denaturation in particular often results in irreversible inactivation.During the thermal denaturation, intact proteins are gradually unfolded into a denatured conformation and sufficient energy is required to prevent a fold back to its native conformation. The probability for interactions with other denatured proteins is increased, thus allowing hydrophobic interactions between exposed hydrophobic parts of the proteins. Protein aggregation, which is one of the outcomes of thermal denaturation, is easily observed after thermal denaturation. The protein aggregation may be the reason for the thermal denaturation facilitates digestion in most cases. Protein aggregates are believed to be the oligomerization products of the denatured form of a protein. In general, hydrophobic parts of the protein are located inside and relatively less hydrophobic parts of the protein are exposed to the aqueous environment (Klibanov, 1983).

The major factor stabilizing the native state of globular proteins is believed to be the hydrophobic effect. The molecule tends to adopt a compact arrangement that minimizes thermodynamically unfavorable hydrophobic interactions by having the non-polar amino acids located in the interior and polar amino acids located at the exterior. The major factor favoring the denatured state of proteins is configurational entropy which increases with increasing temperature. Globular proteins therefore tend to unfold when they are heated above a certain temperature because the forces favoring the denatured state (i.e., configurational entropy) increase compared to those favoring the native state (i.e., hydrophobic interactions) (Betz, 1993).

Denaturation may be reversible or irreversible. Frequently, small, denatured proteins will spontaneously revert to their native structure after denaturant has been removed or it has cooled down below its melting temperature. Some small domains fold and unfold on the millisecond time scale and are constantly sampling their denatured states *in vivo*. At enough high concentrations, nearly all proteins denature irreversibly because their denatured state aggregates and precipitates. Some proteins will not spontaneously renature because they have been subjected to modification during the denaturation, such as

the removal of stretches of amino acids or just simple proteolytic cleavage. Proteins that have multiple disulfide bridges are particularly intractable to renaturation if those bridges are cleaved by reduction as part of the denaturation process. Incorrect bridges may be formed on reoxidation.This problem is overcome during protein biosynthesis by disulfide-shuffling enzymes, which function as molecular chaperones. Some proteins do not renature because a partly denatured state is kinetically more stable and the structure becomes trapped. Denaturation can also be caused by chemical changes in a protein, such as oxidation of cysteine or methionine residues, or by deamidation of glutamine or asparagines (Johnson, 2013).

2.2. Functional properties of proteins

Functional properties of proteins are those physicochemical properties that govern their performance and behavior in foodsystems during their preparation, processing, storage andconsumption. A number ofstrategieshave been suggested to improve the functional properties ofproteins, including chemical and enzymatic modifications. Enzymatic hydrolysis may be preferable to chemical treatments becauseofmilder process conditions, higher specificity and minimalformation of by products. To improve functional properties is generally admitted that a limited hydrolysis, between 1 and 10%, is needed (Asunción et al, 2000; Yust et al., 2010).

Proteins represent a most important class of functional ingredients because they possess a range of dynamic functional properties (Table 1). They show versatility during processing, they can form networks and structures and they provide essential amino acids. In addition, they interact with other components and improve quality attributes of foods. The functional properties of proteins are a primary factor determining their utility in food products. For the proteins to be used in foods they must possess or contribute characteristics that are appropriate in interaction with other food components (e.g. water and lipids) or be suitable for processing. The functional properties that are required from a protein vary with different food applications and food systems. Solubility, foaming and emulsification are desired properties in many industrial food processes and the

improvementof proteins` functionality is still a challenge for industries and needs to be considered for their practical application in food products (Kinsella, 1982).

Table 1. Functional properties of proteins of importance in food applications (Kinsella, 1982)

General property	Functional properties
Organoleptic	Color, flavor, mouthfeel
Kinesthetic	Smoothness
Surface properties	Emulsification, foaming, film forming
Rheological	Viscosity, texture, gelation, gel strength,elasticity
Hydration	Solubility, water absorption
Structural	Surface hydrophobicity, conformational structure, ability for aggregation

The type of functional requirements that are needed of a protein in different food systems is shown in Table 2. However, no single protein can exhibit all the functional properties (Vaclavik and Christian 2003).

Table 2. Functional properties performed by functional proteins in food systems (Vaclavik, 2003)

Functional property	Mode of action	Food system
Solubility	Protein solvation	Beverages
Water binding capacity	Hydrogen bonding of water Entrapment of water molecules (no drip)	Meat, sausages, breads, cakes
Viscosity	Thickening	Soups, gravies
Gelation	Protein network formation and setting	Meats, cheese
Cohesion-adhesion	Protein act as adhesive material	Meats, sausages, baked goods, pasta
Elasticity	Hydrophobic binding in gluten, disulfide links in gels	Meats, bakery
Emulsification	Formation and stabilization of fat emulsions	Sausages, bologna, soups, cakes
Fat binding capacity	Binding of free fat	Meats, sausages, doughnuts
Foaming ability	Form stable film to entrap gas	Whipped toppings, chiffon desserts, angel cakes

One reason why proteins possess such different functional properties is the fact that all proteins are built up by different amino acids (Nakai, 1983). The amino acid composition affects the functional properties of a protein according to how they are disposed in the polypeptide chain, as well as what type and how many of those amino acids that are present (Kinsella, 1981).

2.2.1. Solubility

As the name implies, solubility is the amount of a solute that can be dissolved in a solvent at certain temperature. Each protein has a distinct and characteristic solubility in a defined environment and any changes to those conditions (buffer or solvent type, pH, ionic strength, temperature, etc.) can cause proteins to lose the property of solubility and precipitate out of solution. For example, the precipitation of protein in a solution of high ionic strengthcaused by shielding by ions in solution is presented in Fig. 2.

a) b)

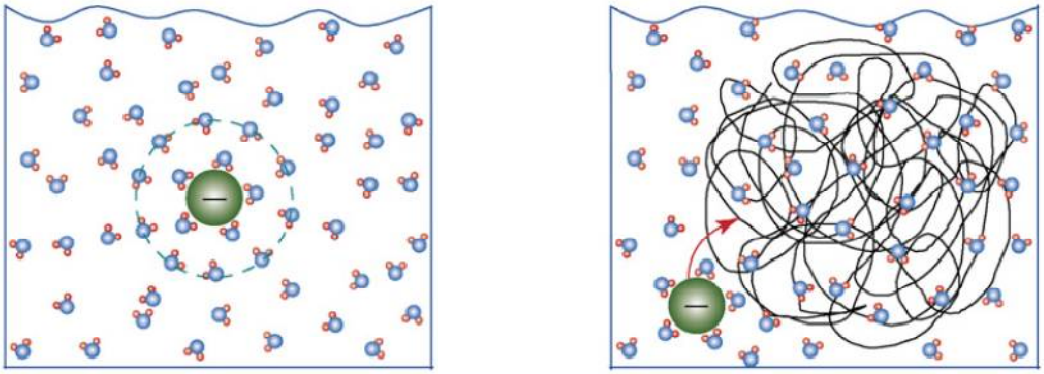


Figure 2. The effect of ion concentration on protein folding. *Source: Zhang and Cremer, 2006*.

The solubility of proteins seems to be a key functional property for their food application since the protein needs to be soluble to be used for some purposes. Other important functional properties such asemulsification, foaming, and gelation are dependent on the solubility of proteins (Vaclavik and Christian 2003). It has been proposed that the reduction of the secondary structure of a protein and the enzymatic release of smaller polypeptide units are responsible for the increased solubility of hydrolyzates compared to the original intact protein. The solubility of a protein is related to the pH, andit is apparently minimal at the isoelectric point. This makes the environmental pH the crucial factor when it comes to the degree of protein solubility. The solubility is also influenced by temperature

and ionic strength (Bolontrade et al., 2013). Food processing including freezing, heating, drying and shearing are also factors that have an influence of protein solubility in food systems (Vaclavik and Christian 2003). Insoluble proteins are not good for food applications and thus it is important that denaturation caused by e.g. heat is controlled so that the protein solubility not will be affected in a negative way (Raikos et al., 2007).

2.2.2. Emulsifying properties

Food emulsions are defined on a molecular level as complex colloidal systems comprising two immiscible phases, one dispersed in the other. The study of emulsions is complicated by the interactions that can occur when multiple components are present; the fact is that the systems are easier to study in dilute solutions, under conditions that may not apply to those likely to be found in foods. It is possible to explain how proteins work in emulsions from the knowledge of the forces that operate during emulsion formation and from information about protein structure (Halting, 1981; Kato and Nakai 1980).The correlation between the changes in the protein structure and changes occurring in emulsification properties of the proteinis of a crucial importance for their further improvement and application.

A more common defect in food emulsions results from the phenomenon known as creaming. If enough time is given or a centrifuge force is applied, a depletion of the lipid from the bulk aqueous phase occurs with the formation of a compact cream layer containing the majority of the lipid (Fig. 3). The extent of the emulsion shelf life depends on the fact that the density of the fat globules must be made identical to that of the continuous phase or the viscosity must be high enough so that the yield value is greater than the acceleration due to buoyant differences.

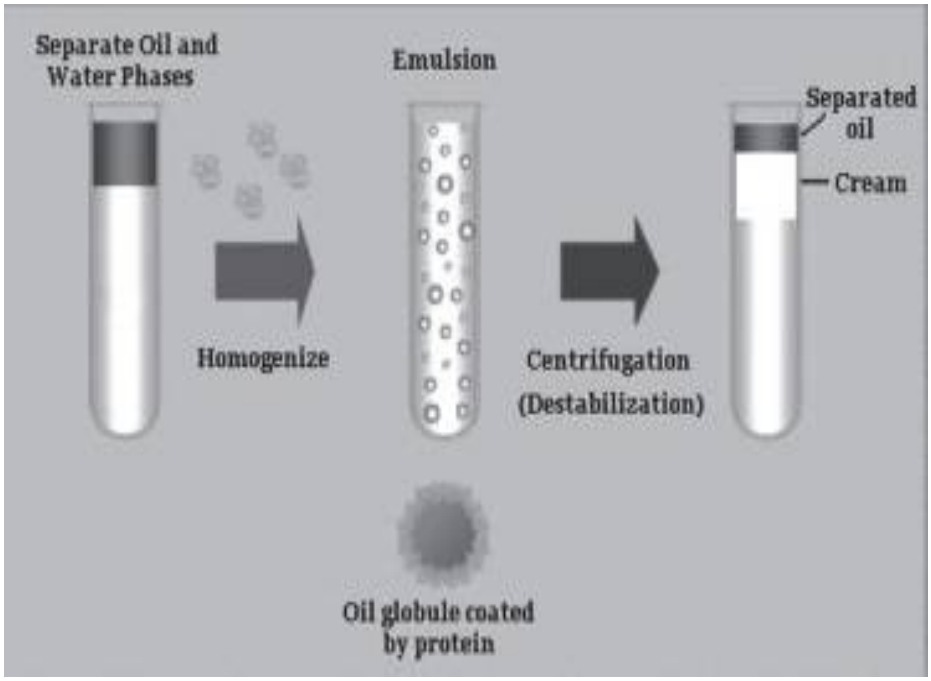


Figure 3. Emulsion destabilization to measure the emulsion stability by appliance of centrifuge force. *Source: Cabra et al., 2003.*

The ability of proteins to interact with lipids and form stable emulsions is essential to yield a stable food product. Enzymatic hydrolysis of protein often results in improved emulsification properties. For example, rapeseed protein hydrolysates have higher emulsifying activity (at least 20% greater) and stability than rapeseed protein isolates (Vioque et al., 2000). Enzymatic hydrolysis of soy protein also results in an increased emulsification activity of the obtained hydrolysates (Wang et al., 2014). Studies examining protein hydrolysates from different crop sources suggest that the emulsifying capacity of the hydrolysates is related to the degree of hydrolysis. It seems that a low degree of hydrolysis (DH) (3–5%) is often associated with increasing emulsifying capacity and a high degree of hydrolysis (~8%) with decreasing (Vioque et al., 2000; Achouri et al., 1999; Kong et al., 2007). Ultrafiltered rapeseed protein hydrolysate has been demonstrated to have greater emulsification stability compared to that of whole egg (Yoshie-Stark et al., 2008) and wheat germ protein hydrolysates hasshown higher emulsification capacity, activity and stability than bovine casein (Chobert et al., 1988). In another study with gluten,

the authors have also stated that the DH should be lower, <5%, in order to obtain desirable emulsifying and foaming properties (Kong et al., 2007). It is generally accepted that limited hydrolysis improves the emulsification properties of proteins by exposing hydrophobic amino acid residues (which may interact with oil), while the hydrophilic residues interact with water (Vioque et al., 2000). Similarly, an increase in hydrophilicity as a result of acid modification has been shown to increase the emulsion activity index (EAI) of okara protein isolates (Chan, 1999).

2.2.3. Foaming properties

Foams represent colloidal systems in which air bubbles (dispersed phase) are dispersed within a liquid or solid continuous phase (Fig. 4). The best foaming agent found in the food industry are food-derived proteins which act as the main surface-active agents that help in the formation and stabilization of the dispersed air bubbles (gas phase).

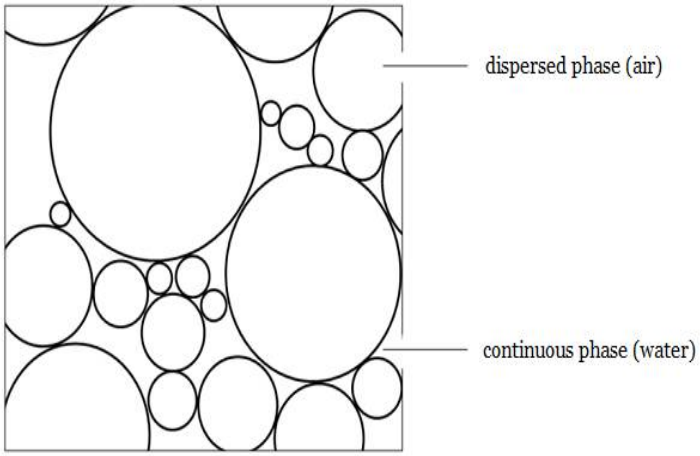


Figure 4. Colloidal systems of foam formation.

The two most important features defining the quality of foam are its volume and its stability. Foam volume depends on the ability of the foaming agent to adsorb at the interface and rapidly reduce interfacial tension, as well as during the energy input such as

whipping, whereas foam stability depends on the ability of the foaming agent to form a stable interfacial film by forming a viscous continuous phase. Foam stability is assured by a multitude of forces, including the viscosity of the liquid phase as well as the electrostatic and steric forces between the proteins. On the other hand, destabilizing forces such as electrostatic attractions or repulsions (in highly charged proteins) and hydrophobic forces between the molecules, will tend to minimize foam formation and break down the foam.

Factors that affecting a foam formation include protein concentration, film thickness, ionic strength, pH, temperature and presence of other components in the food systems. In general, an increase of the protein concentration causes the formation of a thick lamellar film which yields more stable foam. Foaming properties have been reported to be primarily affected by (a) surface hydrophobicity of the proteins (increased surface hydrophobicity usually results in better foam ability), (b) protein charge density and charge distribution (excessive charge leads to excessive repulsion and poor foam stability), (c) proteins flexibility (increased flexibilityleads to more rapid foam formation) (Kinsella, 1981; Yang and Baldwin 1995). The main characteristics which define a good foaming agent can besummarised as follows: 1) to be able to adsorb rapidly to the air bubble surface (air-water interface), 2) to have flexible molecules undergoing rapid conformationalchange at the interface, and 3) to form a cohesive viscoelasticfilm via intermolecular interactions. There are some reports showing that mildheat-treatments of proteins or other mild tretamant can improve their foaming properties. Namely, the treatment changes the protein structure from a “rigid” conformational state to an intermediate stable partiallyunfolded state, so-called the “molten globule” state, increasing the foaming power.

Wheat germ has been shown to have poor foaming properties (German et al., 1985). However, the enzymatic treatment of wheat germ appears to increase foam volume/height but decrease foam stability. The trend of increased foam volume coupled with decreased foam stability has been reported in the previous studies on rice bran protein hydrolysate and acid modification of okara protein hydrolysate (Chan and Ma, 1999; Zhang et al., 2010). The absence of large protein components, which function to stabilize the foam, may contribute to the observed lack of foam stability (Sarmadi and Ismail 2010). In a similar

study of wheat germ hydrolysates, it was found that foaming capacity was increased at a degree of hydrolysis (DH) of 5%, resulting in a 74% increase in foam volume compared to the control. The foam was also most stable at DH of 5%, with 40% of foam volume sustained after 60 minutes. There was an inverse relationship between DH and foam stability, with stability in the order of DH 5% > DH 10% > DH 15%. Similar to suggestions by Claver et al., the stability of the foams was attributed to the presence of larger component proteins and a partial hydrolysis, whereas a higher DH increased the number of polypeptide chains which do not have the ability to stabilize foams (Claver et al., 2005; Kong et al., 2007). The study of both soy (Tsumura et al., 2005) and rapeseed (Vioque et al., 2005) protein hydrolysates produced comparable results. Regarding the effect of pH on foaming, barley hydrolysates had greater foam stability at basic pH values and very low stability at acidic pH (Yalcın et al, 2008).

2.2.4. Wheat proteins

Wheat is a major global commodity, with annual production ~600 million tonnes. Around 65% of wheat is estimated to be milled for flour production, potentially generating ~90 million tonnes of wheat bran.

Wheat contains approximately 7-14% storage protein. Wheat flour is a complex natural system containing carbohydrates, amino acids, proteins, dietary fiber, fat, water, minerals and vitamins. It is a basic ingredient in a variety of baked goods and flour dishes. Its properties, important for the production of bread, pasta and noodles, are related mainly to the amount of gluten which, according to some definitions, is also found in rye, barley, oats or their crossbred varieties and derivatives. Some people are intolerant to gluten. It is insoluble in water and 0.5 M sodium chloride solution.

Wheat gluten is an abundant and relatively inexpensive industrial by-product from wheat starch processing and in Europe also from manufacturing of bioethanol fuel. During the process wheat flour dough is washed with water to remove starch. A matrix of gluten is obtained which is palletized, dried, and ground into powder. Wheat gluten possesses unusually good cohesive and unique viscoelastic properties making it very suitable for

baking and food products. It is mainly used for baking although its worldwide use varies, including applications such as flour fortification, imitation of meat and fish, and processed foods. Its non-food uses are also diverse, including usage in pet food and cosmetics. It has also successfully been used in making biodegradable films and coatings for food and non-food applications. Moreover, foams based on wheat gluten are a new application area.

Approximately 80% of wheat gluten consists of wheat storage protein. The rest is composed of traces of starch and non-starch polysaccharides (10-14 %), lipids (6-8 %), and minerals (0.8-1.4 %). From the chemical standpoint, gluten is the composite of two storage proteins, glutenins and gliadins. Glutenins, the major proteins of flour, are poorly soluble in alcohols because they are capable to form large polymers that are stabilized by intermolecular disulfide bonds and hydrophobic interactions. In contrast to glutenins, gliadins are soluble in aqueous alcohol (for example 60–70% ethanol) and are mainly present in gluten as monomers interacting by non-covalent forces. They arearesingle-chained polypeptides with a molecular mass (MM) between30,000 and 80,000, whereas glutenins are multi-chained polypeptideswith MM ranging from 80,000 to several millions. Both gliadins and glutenins are rich in proline and glutamine. The amount of amino acid residues with charged side groups is a rather low giving a hydrophobic character. The pH value at the isoelectric point of wheat gluten is around 7.3, but it should be kept in mindthatthe wheat gluten, including gliadin and glutenin, is composed of polymorphic polypeptides, showing more than 60 different molecular weights ranging from 30,000 to 90,000 Da (Wang et al. 2007a, b).

Gliadins and glutenins mainly contribute to the unique baking quality of wheat. Hydrated gliadins contribute to the extensibility and the viscosity of the dough system, while hydrated glutenins contribute with elasticity and strength. Gliadins interact with the glutenin polymers via non-covalent hydrophobic interactions and hydrogen bonding. The structure of both proteins iscrucial in the breadmaking process, but is also connected with gluten allergenicity. Of these two proteins, gliadins are considered strong food allergens. They cause IgE-mediated allergies such asasthma, atopic dermatitis or celiac disease (Payne, 1987).

The gluten proteins (gliadins and glutenins) are often being denominated monomeric and polymeric, respectively, even though they are macromolecules. Nevertheless, these terms refer in this case to the quaternary structure of the proteins. The monomeric gliadins consist of a heterogeneous mixture of distinct polypeptide chains with molar masses ranging from 30,000 to 60,000 g/mol. In contrast, the polymeric glutenins are comprised of several polypeptide chains (subunits) bonded together by interchain disulfide bonds and the molar masses extend from approximately 500,000 g/mol to above 107 g/mol. The gliadins can be further divided into α-, β-, γ-, or ω-gliadins due to differences in their amino acid sequence, while the subunits of the glutenins are separated into high molecular weight (HMW) and low molecular weight (LMW) groups (Fig. 5) (Bietz and Wall 1983; Bietz and Wall 1980; Kanazawa and Yonezawa1973).

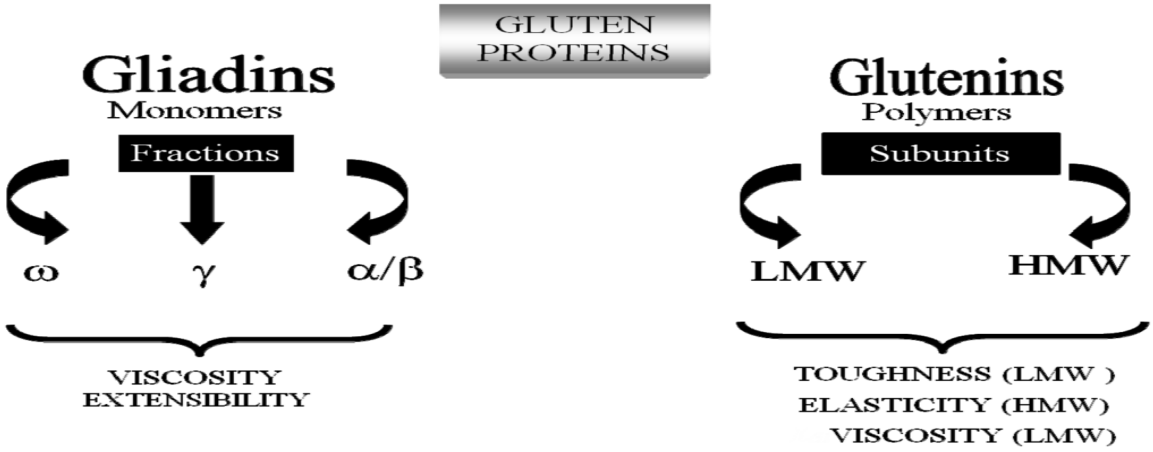


Figure 5. Gluten proteins: fractions and technological properties *(Source: Lamacchia et al.,2013).*

The new system of classification, based on the availability of complete amino acid sequences, divided the gluten proteins in three broad groups: sulphur-rich (S-rich), sulphur-poor (S-poor), and high molecular weight (HMW) prolamins (Fig. 6). Traditionally, gluten proteins have been divided according to their solubility in alcohol-water solutions (e.g., 60% ethanol), as the soluble gliadins and the insoluble glutenins. These properties are largely determined by the inter-chain disulphide bonds, with the glutenins consisting of disulphide-stabilized polymers. Reduction of these interchain bonds allows the separation of the glutenin subunits into low molecular weight (LMW) and high molecular weight

(HMW) groups (Fig. 6). In contrast, most of the α/β- and γ-gliadins contain six or eight cysteine residues, respectively, forming intrachain disulfide bonds, while most ω-gliadins lack cysteine. The subunits of the glutenins contain also several cysteine residues, forming intra- and interchain disulfide bonds, or being present as free thiol groups (Shewry and Halford 2002; Shewry and Tatham 1990; Osborne, 1924).

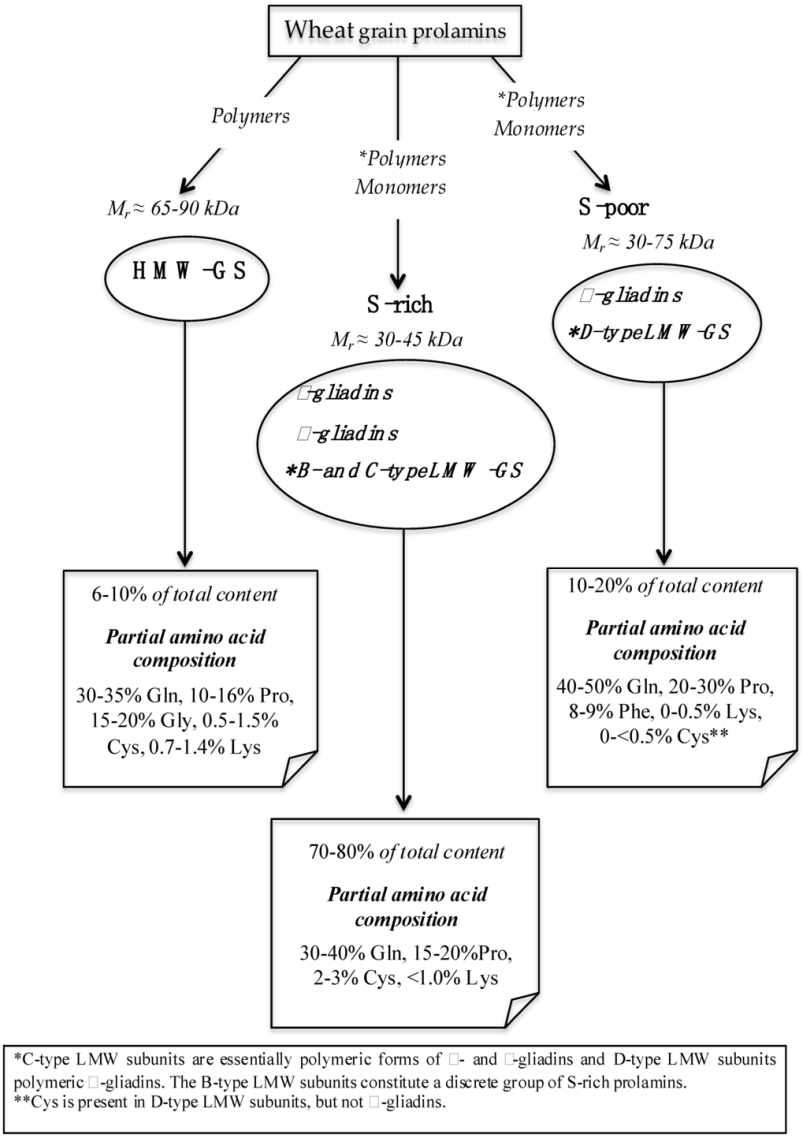


Figure 6. Schematic presentation of types of wheat gluten proteins. *Source:(Shewry and Halford 2002)*

3. ENZYMATIC HYDROLYSIS OF PROTEINS

3.1. Enzymatic hydrolysis of proteins as a mean to improve protein functionality

The increasing human population, over the last few decades, has greatly influenced a progressive increase in demand for value-added food products. Substantial effort has expressly focused on various relatively inexpensive sources of plant proteins that are considered as an economic and adaptable substitute for animal proteins in functional food stuff formulations. With the expansion of wheat starch production, wheat gluten, the major protein of wheat and a by-product from the wet processing of wheat flour, is a renewable and an abundant plant protein source available in large amounts with relatively moderate prices. Native wheat gluten is a deluxe bread improver and may be utilized as a functional protein additive in multifarious nonbakery food stuffs due to its desirable structure-enhancing properties (Gontardand Ring, 1996). The utilization of wheat gluten protein (WGP) resources would be economically interesting for wheat starch factories, but lack of some desirable functional properties, such as solubility, emulsifying, and foaming ability limited their expanding utilization in foodstuff formulations (Popineau et al., 2002).

Controlled enzymatic hydrolysis of plant proteins is an effective way to modify and upgrade the functional and biological properties of native proteins, including wheat gluten proteins (WGPs), without affecting their nutritive value (Kristinsson and Rasco, 2000). Moreover, enzymatic hydrolysis disturbs the molecular weight and hydrophobicity of protein hydrolysates consequently affecting the functional properties and their utilization in food systems. Mainly, the high solubility of proteinhydrolysate over a wide range of pH improves an essentially appropriate feature for a lot of food applications and influences the emulsifying and foaming properties (Klompong et al., 2007). Depending on the degree of hydrolysis (DH), limited proteolysis may improve the solubility and enhance the foam properties and emulsifying performances of WGPs.

Protein hydrolysates possess properties that make them attractive as a protein source in human nutrition. Hydrolysates are used in products for special nutrition, such as diets for

elderly and patients with impaired gastrointestinal absorption, hypoallergenic infant formulas, sports nutrition, and weight-control diets, as well as in consumer products for general use. Peptide-based formulas have been useful because of their high solubility especially under acidic conditions; even during heat treatment, the peptides remain in solution (Frøkjaer, 1994). In addition to their solubility in a wide range of pH and other functional properties, such as improvement of texture and water binding capacity (Lin et al., 2013), protein hydrolysates are physiologically better than intact proteins because their intestinal absorption appears to be more effective (Ziegler et al., 1998). On the other hand, proteases used in the preparation of protein hydrolysates have the ability to catalyze peptide synthesis under appropriate conditions. Enzymatic synthesis has potential application in the food industry to remove bitterness of protein hydrolysates, to improve amino acids composition, or to modify some functional property (Lozano and Combes 1992).

The DH and the enzyme type are the substantial factors which influence on hydrolysates' performances, such as amino acid and molecular weight distributions and the amount of residual intact protein, thus influencing their functionality (Stefanović et al., 2014). The opposite, the extensive proteolysis can have enormous negative impact on the functionality (Kristinsson and Rasco, 2000). Several authors have suggested that there is an optimum molecular size or chain length for peptides to provide good functional properties (Klompong et al., 2007; Liu et al., 2010; Zhu et al., 2011). Meanwhile, slow and incomplete hydrolysis produces the hydrolysateswith bitter taste, low yield of the desired peptides and leads to difficulty of separation for hydrolysates but the peptide profile highly depends on protease used.

Papain, pepsin, and trypsin are common enzymes utilized in wheat protein or gluten modification (Yang and McCalla 1968). Many previous studies have focused on the processing properties and nutritional and medical functions of modified proteins (Babiker et al., 1996; Wang et al., 2009). However, there only a little information is available of the influence of the enzymatic hydrolysis of protein or gluten on the pasting properties of wheat flour, and the behind mechanisms of these processes also remain unknown. It's known that the insolubility of gluten in aqueous solutions is one of the major limitations for its more extensive use in food processing. Because of that, researchers were enzymatically

hydrolysed wheat gluten by several commercially available proteases (alcalase 2.4L, PTN 6.0S, pepsin, pancreatin, neutrase and protamex) and protein recovery was 81.3%, 42.5%, 53.3%, 61.6%, 46.3% and 43.8%, respectively. The hydrolytic efficiency of these proteases on wheat gluten was also compared. Alcalase showed the best performance for the preparation of wheat gluten hydrolysates with the maximum degree of hydrolysisof 15.8%. Subsequently, the solubility of wheat gluten hydrolysates obtained with those enzymes was comparably evaluated. The products had excellent solubility (>60%) over a pH range of 2–12 obtaind with all tested enzymes. Also, their results showed that with the increasing of DH values, a large amount of smaller polypeptideswas obtained (Kong et al., 2007).

It is very important to facilitate the proteolysis of food proteins by means of applying the treatment before proteolysis. Before preparation of protein hydrolysates, it is substantial to perform treatments to increase the accessibility of the enzyme to cleavage sites and consequently to raise the DH. Furthermore, because of the presence of specific serine protease inhibitors in some plant and animal proteins, the hydrolysis of demands a heating process to inactivate the inhibitors before protease action and facilitate the enzymatic hydrolysis.

Heat treatment is beneficial for ameliorating proteolysis and considered to be an eco-friendly processing technology since no chemicals are added and decreasing toxic compound formations (Prieto et al., 2008). In food industry, the heat treatment of proteins before the proteolysis can lead to rearrangements of the inter- and intra-molecular binding with accompanying changes in protein conformation, making proteins more susceptible to enzyme, due to the exposure of previously hidden cleavage sites, thereby enhancing hydrolysis (Saha et al., 2013). Likewise, such method leads to destruction of some functional properties which are often desirable for food manufacture (van der Plancken et al., 2003). In this thesis WGPswill besubjected to heat treatment and the DH of WGHs will beanalyzed. The objective isto evaluate whether the DH affects the functional and antioxidant properties of the resulting alcalase wheat gluten hydrolysates (AWGHs) and the main aim isto obtain gluten hydrolysates having an improved solubility and free- radical scavenging profiles with good foaming and emulsifying ability using a commercial food-grade bacterial protease like alcalase.

3.2. Enzymatic hydrolysis of proteins as a mean to improve protein biological activity

Enzymatic hydrolysis of proteins is an important bioprocess to improve the physical, chemical, functional and nutritional properties of original proteins. It is also an effective method to prepare active peptides, which possess many physiological properties likeantioxidant activity, antihypertensive activity, mineral binding, opioid activity, antimicrobial activity, growth enhancer for *bifidobacteria*, anticancer activity, regulation of the blood pressure or the immune system and others (Schlimme and Meise 1995). In recent years, many types of peptides have been applied in food, drug, cosmetic, and some other fields because of their biological activity and exceptional properties.

However, enzyme hydrolysis of natural proteins is a complex process because of the specificity pattern of proteases and the diversity of the substrates involved, i.e., peptide fragments are products as well as reactants of next reaction. In addition, a number of inherent simultaneous inhibition and enzyme inactivation reactions occur during hydrolysis.

Compared to acid or alkali hydrolysis, the enzymatic hydrolysis of protein, using selective proteases, provides more moderate conditions of the process and few or no undesirable side reactions or products. In addition, the final hydrolysate after neutralization contains less salts and the functionality of the final product can be controlled by selection of specific enzymes and reaction factors (Chiang et al., 1999; Madsen et al., 1997; Darwicz et al., 2007). Enzymatic modification of proteins using selected proteases to split specific peptide bonds is widely used. The peptides produced have a smaller molecular size than proteins. Thus, their functional properties are differentas previously stated: increased solubility over pH range, decreased viscosity, and significant changes in foaming, gelling and emulsifying properties. The functional properties of hydrolysed proteins are governed to a large extent by their molecular size and their hydrophobicity (Turgeon et al., 1992).

Besides the improvement of functional properties of protein hydrolysates, enzymatic hydrolysis is an efficient tool for the production of low molecular peptides with antioxidant properties, including the capacity to inhibit the linoleic acid peroxidation or to

quench the DPPH, ABTS or other radicals (Kong et al., 2008; Wang et al., 2006). In this case, processing conditions, specificity of the proteolytic enzymes used, and the degree of hydrolysis also greatly affect theantioxidant properties of the obtained hydrolysate. Thus, the degree of hydrolysis should be controlled because the efficiencies of antioxidant capacity increase with increasing the degree of hydrolysis (decreasing peptide chain length) up to a critical value after which the efficiency can decrease.

3.3. Proteolytic enzymes

Proteolytic enzymes, also known as protease, proteinase or peptidase, constitute a large group of enzymes that catalyze the hydrolysis of the peptide bond in protein or peptide molecules producing a lower molecular weight peptides and amino acids. The hydrolysis reaction is reversible, and the protease can, under certain conditions, catalyzed the return reaction of the synthesis of the peptide with the exclusion of water. According to the International Nomenclature, proteases belong to the third group of the enzyme-hydrolase subgroup 3.4.-peptide hydrolase. Since proteins are widely distributed in nature, these enzymes have the greatest industrial importance. Their annual production accounts for around 60% of total production of industrial enzymes. Traditionally, proteases are used in the food industry and industrial detergents but their application in the leather industry and in therapeutic is of a recent date. Industrial proteolytic enzymes are produced from a variety of commercial strains depending on the purpose, whereas a modern approach based on the production of recombinant enzymes precisely defines properties for the needs of industry and other detergents. The largest amounts of proteases produce by microorganisms as extracellular enzymes, but some proteases of plant and animal source are still of a commercial importance. The world's largest producers of proteases are Denmark (Novozymes), Netherlands (Gist - Brocades) and the USA (Genencor International) (Knežević-Jugović, 2008).

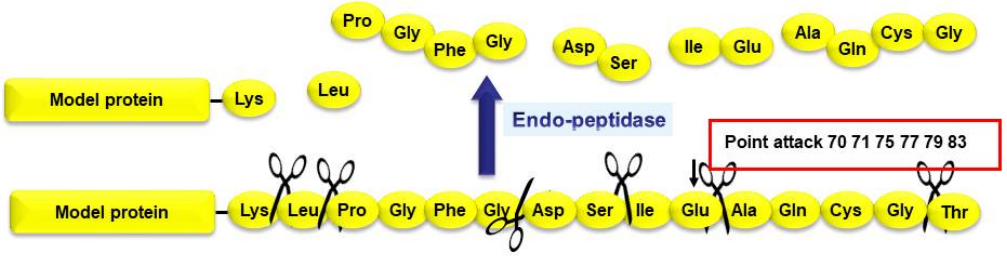


Figure 7. Illustration of hydrolysis of peptide bonds in proteins and polypeptides. Points attack depends on protease specificity.

Although the chemical reactionswhich they catalize are essentially the same, the different proteases have various preferences for diverse peptide bonds as modified by other groups in their neighborhood. Namely, some proteases have a preference to the C-terminus or the N-terminus of the peptide chain. These exopeptidases are specific for the free α-carboxyl group or the free α-amino groups. Other proteases act inside the polypeptide chain. The specific of these endopeptidases is mainly dependent on the amino acid side chains encountered in the vicinity of the peptide bond (Polgár, 1930).

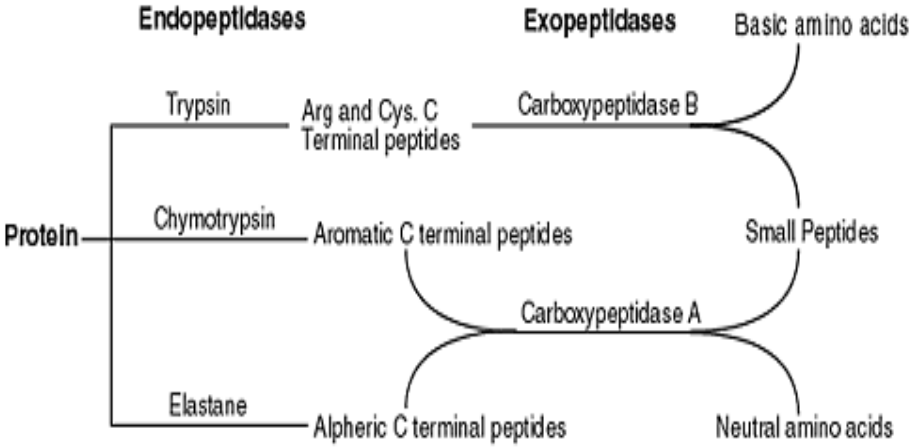


Figure 8. Schematic overview of reaction mechanism of some endopeptidase and exopeptidase

Proteases are hydrolase which can be divided by their mechanisam of actionon proteins into fourgroups: 1) serine protease: 2) cystein proteases; 3) aspartate proteases and 4) metalloproteases.The specificity criterion which serves as a basis of dividing the enzymes into exo- and endopeptidases has a priority over the mechanism criterion in the classification proposed by the International Union of Biochemists (Polgár, 1930). In the active sites of serine and cysteine proteases, the residue is usually paired with a proton-withdrawing group to promote nucleophilic attack on the peptide bond. Aspartate proteases and metalloproteases activate a water molecule to serve as the nucleophile, rather than using a functional group of the enzyme itself. However, the overall process of peptide bond scission is essentially the same for all protease classes.

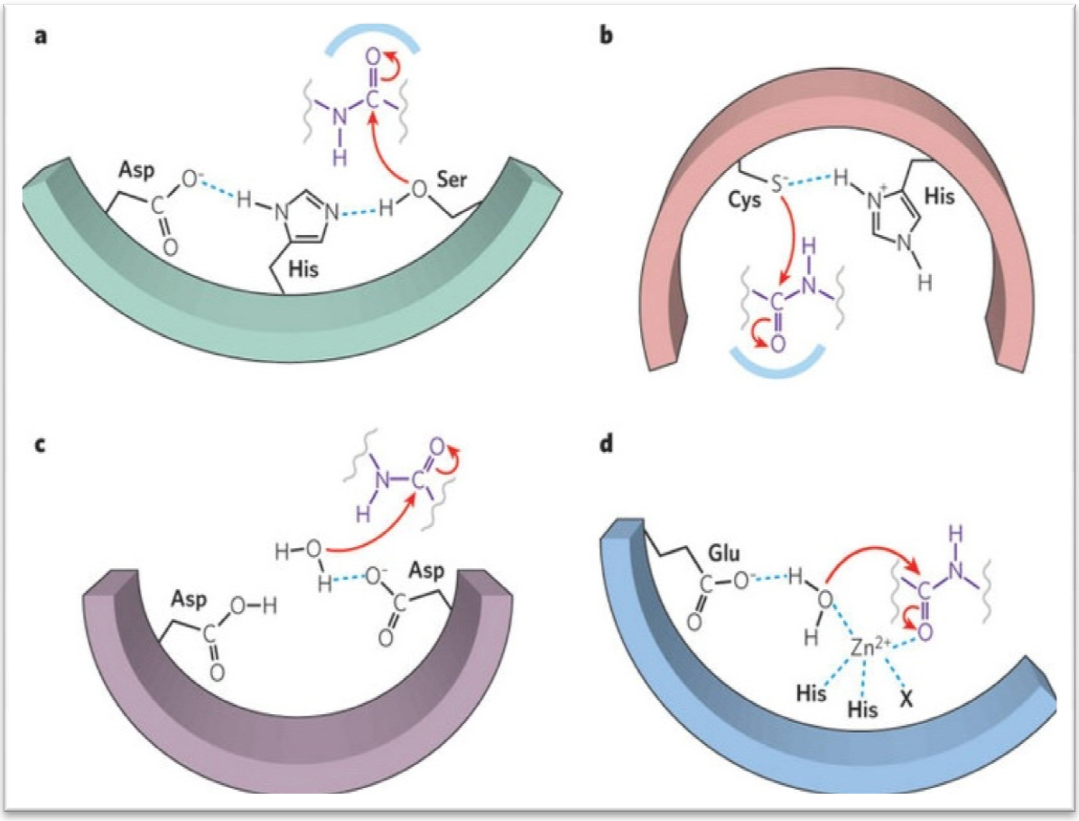


Figure 9. Mechanisms of action of selected proteases (a) serine proteases, (b) cystein proteases, (c) aspartyl proteases, (d) metalloproteases*(Source: Neitzel, 2010)*.

Serine proteases are characterized by the fact that in the composition of their active center serine is substituted by an amino acid residue which has an important function in the catalytic activity. They are very widespread in nature and are found in secretions of animal digestion and juices and tissues of plants. A large number of microorganisms produce them mainly extracellular. Except for a few exceptions, they are relatively small molecules in a range of 18 to 35 kDa and show the maximum activity at a pH from 7 to 11. In the important representatives of these groups of enzymes can be classified chymotrypsin, trypsin, thrombin, elastase, subtilisin, and others. These enzymes show great similarity in the structure and mechanism of action.

Serine protease mainly synthesized in the cells as an inactive precursor proenzyme or zymogen which must be converted to the active form by removal of part of the peptide chain which masks the active center of enzyme. Thus, for example, cells in the pancreas chymotrypsin are synthesized as an inactive precursor, chymotrypsinogen, which is eliminated in the duodenum and then converted to the active form by using trypsin or any other proteases. The same is the case with other enzymes: trypsin synthesized by cells of the pancreas in an inactive precursor trypsinogen; elastase is also obtained from its precursor, proelastase (Knežević-Jugović, 2008).

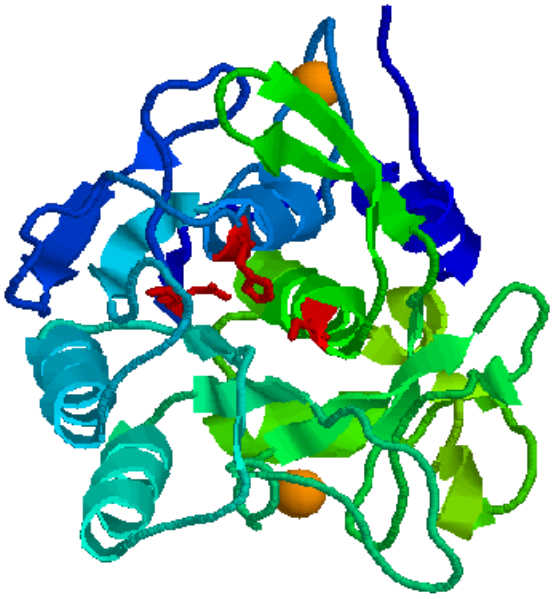


Figure 10. Serine protease – subtilisin

Cystein proteases are widespread in nature and composition of their active center included two amino acid residues: cysteine and histidine. They become active under the influence of certain regulatory agents such as ascorbic acid, hydrogen sulfide, glutathione, etc. The highest activities are in a neutral environment. They are known as plant proteases because they spread in the juices and tissues of plants. The most important representatives are papain, ficain and bromelain (Knežević-Jugović, 2008). Papain is a well-known sulfhydryl protease frompapaya latex with various industrial applications. It consists of 212 amino acid 20 residues including 7 Cys residues and is stabilized by three disulfide bridges which contribute to the stability of the enzyme.

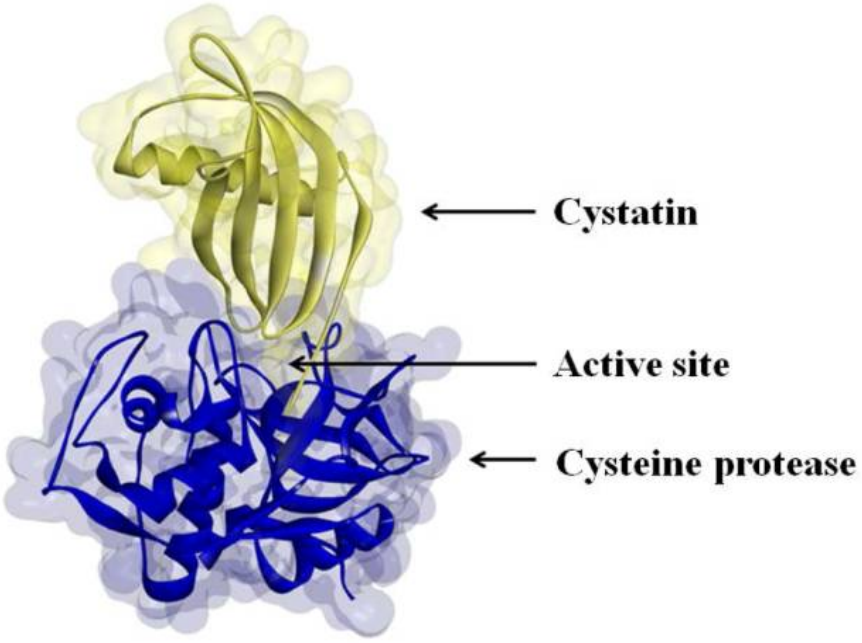


Figure 11. Cystein protease *(Source: Vorster et al., 2013)*

Aspartate proteases represent the group of acidic proteases, whose active center contains residues of aspartic acid. They show a maximum activity in the acidic medium at a pH 3 to 4.5. The isoelectric point also was located in an acidic environment. Two of the residues aspartic acid, sometimes on different subunits, can be participate in the reaction mechanism. The most famous representatives of this group are pepsin and renin and the animal's stomach enzymes. Microbial aspartic proteasesare mainly produced by *Aspergillus*, *Rhizopus*, *Mucor* and *Penicillinum* strains (Knežević-Jugović, 2008).

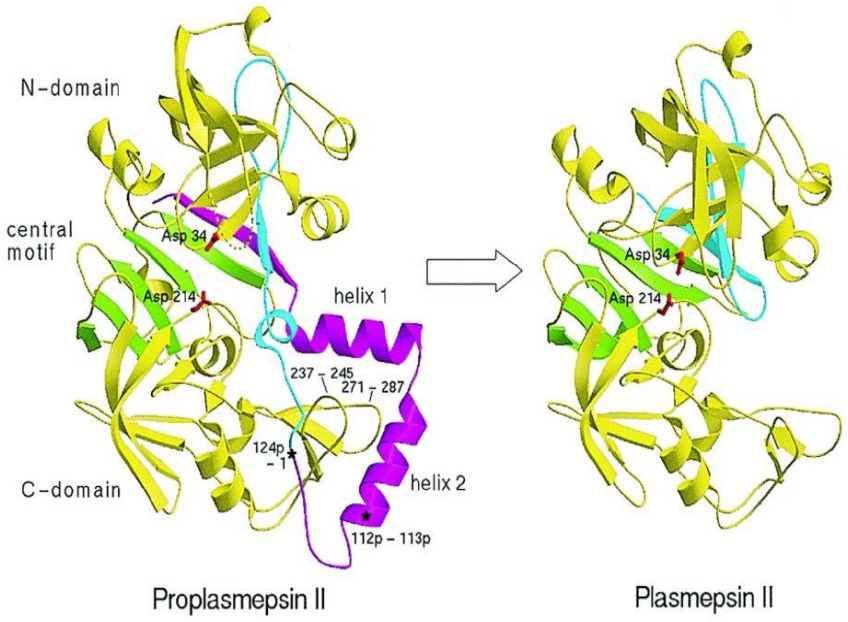


Figure 12. The activation pathways of aspartic protease

Metalloproteases constitute a group of proteases whose catalytic activity has been associated with the action of the divalent metal ions Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} etc. Metal can be loosely (complex) attached to a protein part of the enzyme, and canbe easy separated from it. This is the case forleucine aminopeptidase. Another subgroup of this protease in the molecule contains a metal that is tight (covalent, ionic) linked with a protein part of the enzyme, such as, for example, in carboxypeptidase. A large number of metalloproteases has a maximum activity in the neutral and acidic media. The well-knownrepresentative of this group is thermolysin, one thermostable neutral protease (Knežević-Jugović, 2008).

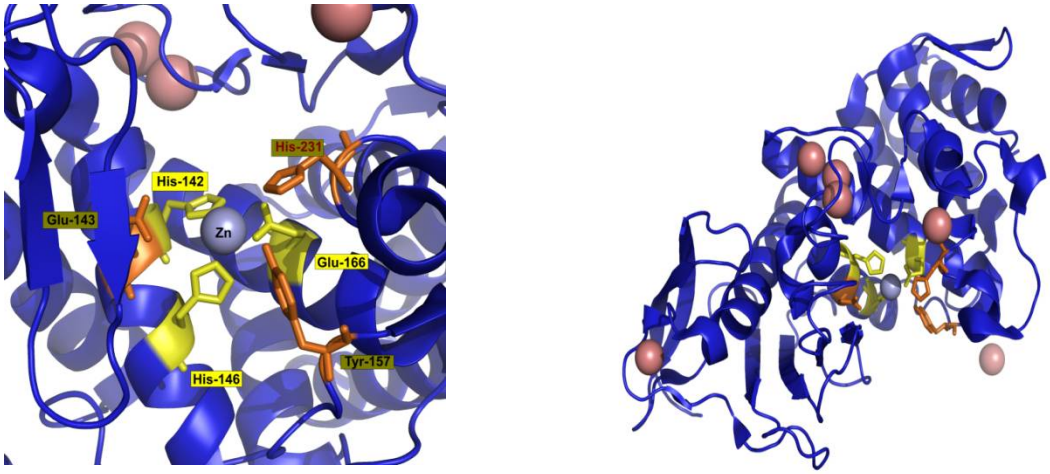


Figure 13. Thermolysin – metalloprotease (Protein structures from PDB).

3.4. Optimization of process condition for enzymatic hydrolysis of wheat gluten

Over the past years, many natural sources including some dietary protein compounds have beenused to establish the antioxidant potential of proteins due to thepotential health hazards of some synthetic antioxidants. The researchers, have so far been identified the numerous peptides isolated fromfood protein hydrolysates with remarkable antioxidant activities. Also, the protein hydrolysates rich in bioactive peptides, released during proteolysis of the native proteins, are very interesting and beneficial touse as dietary supplements (Kitts & Weiler, 2003).

The use of low grade and renewable starting material including agricultural crops for the new bioactive ingredient production is still a significant challenge. Gluten, a by-product of wheat starch industry, is a promising source due to its natural origin, availability, low cost and health-related benefits associated with their hydrolysates (Matsui et al., 2000; Kong et al., 2008). The rapid growth of wheat starch production leads to large amounts of this by-product that may provide a novel application in food, cosmetic and pharmaceutical industries as a source of valuable bioactive and functional ingredients in the form of soluble protein hydrolysates. Recent research has also shown that the wheat gluten and its

hydrolysates, as the most complex protein and peptides, have higher concentration of disulfide bonds (45.37 nmol/mg) and total cysteine (93.88 nmol/mg) compared to other tested proteins (maize proteins, pea protein isolates), showing the highest ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulpfonic acid) radical scavenging activity (74.39 mmol Trolox/kg). The antioxidant capacity is positively correlated with total cysteine and –S–S bond concentration indicating a high effect of the protein structure on its antioxidant properties (Žilić et al., 2012).

Enzymatic hydrolysis offers significant advantages over chemical methods, especially with respect to the mild conditions employed and the possibility to control the degree of hydrolysis (DH) and molecular weight of produced peptides based on enzyme specificity (Kong et al., 2007; Koo et al., 2014). Previous studies have shown that the controlled and mild enzymatic hydrolysis could be successfully applied to improve and upgrade the functional properties of gluten. Zhu et al. (2006) were prepared wheat hydrolysates by hydrolysis of defatted wheat germ proteins using alcalase and were verifiedtheir antioxidant and free-radical scavenging activities. Later, the same group of authors was hydrolysed wheat gluten under ultrasound and was found that the wheat gluten hydrolysate (WGH) prepared by a low frequency ultrasound evinced the potential antioxidant activities. They have also found that the low molecular weight peptide fraction had the highest activity (Zhu et al., 2011).

However, an excessive hydrolysis affects the functional properties of the hydrolysates negatively and may reduce their industrial application. For example, it has been reported that the solubility of the hydrolysates obtained with a fungal protease from *Aspergillus oryzae* increases as the DH increases, but foaming properties seem to be impaired beyond a certain DH (14%) (Drago and González, 2001). The authors have concluded that a minimum protein structure has to be retained, in order to keep good foaming properties. In another study, the authors have stated that the DH should be even lower, 5% in order to obtain desirable emulsifying and foaming properties (Kong et al., 2007).

Processing conditions, specificity of the proteolytic enzymes used, and the degree of hydrolysis also greatly affect the antioxidant properties of the obtained hydrolysate and

should be controlled. The conventional batch process is industrially advantageous as it can be easily performed with mild reaction conditions in terms of process control. However, the batch process has limited productivity and yield, due to the unfavorable reaction equilibrium, product inhibition, and enzyme inactivation to the enzyme autolysis and/or hydrodynamic stresses. Especially, the system with gluten is very complicated, since gluten dispersions are highly viscous and exhibit non-Newtonian flow properties (Song and Zheng, 2009). This creates problems with both mass and heat transfer within the batch reactor as well as mechanical problems related to pumping and efficient mixing of the reaction mixture. The selection of impeller types and configurations and the understanding of the performance of mixing are, thus, important parts of the process design. Regarding generally protein hydrolysis, mixing in the batch reactor is achieved by orbital shaking or by means of hotplate stirrer and only little information on the effect of mechanical agitation on reaction rate and productivity is available (Jakovetić et al., 2015; Nouri et al., 1997).

The design and optimization of the gluten hydrolysis is a rather difficult task requiring a fundamental understanding of all the variables potentially affecting the reaction rate and performance of obtained hydrolysates. When many different factors need to be considered, the response surface method (RSM) is an effective tool for optimization of conditions (Valdez-Flores et al., 2016). Rational design of process parameters could lead to improved protein yield and adjusted properties, such as solubility, and antioxidant capacity (Deng et al., 2016; Ng et al., 2013). In spite of the industrial importance of the gluten as a multifunctional ingredient of food/cosmetic interest, very little is found in the literature about the production of peptides with antioxidant activities from wheat gluten and the relationship between operating conditions, degree of hydrolysis and antioxidant capacity. Functional and biological properties of corn gluten hydrolysates has been intensively studied recently with particular emphasis on *in vivo* and *in vitro* antioxidant activity (Jin et al., 2016; Wang et al., 2016), but little is known about the biological activity of enzymatic hydrolysates originating from wheat gluten (Cian et al., 2015). Generally, systematic investigations of the effects of process operating conditions and reactor design on the proteins hydrolysis regarding antioxidant capacity of the obtained hydrolysates have been reported only for a few cases (Jakovetić and others 2015).

4. BIOLOGICAL ACTIVITY OF PEPTIDES FROM WHEAT

Basically, biologically active peptides can be generated from precursor proteins in multiple ways, including: enzymatic hydrolysis (either by digestive enzymes or enzymes derived from microorganisms and plants) and microbial fermentation (Korhonen and Pihlanto, 2006). In South East Asian countries such as China, Japan and Korea, the fermentation is used widely as the oldest way to preserve food. It is believed that fermentation can increase the nutraceutical value of foods, besides the long storability, possibly due to fragmentation of proteins to bioactive peptide by microbial proteases (Rajapakse et al., 2007).

4.1. Antioxidant activity of peptides from food proteins

The importance of oxidation processes in the body and groceries has been widely recognized. The formation of free radicals and other reactive oxygen species (ROS), which can induce oxidative damage, is a consequence of the oxidation metabolism which is necessery for the subsistence of cells. From all ROS, superoxide anion radical $(O_2^{\bullet})^-$, hydroxyl radicals ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2) are supposed to be the most frequent in biological systems (Cui, 2008). Since they are the products of normal body reactions, the body possesses its own defense system against ROS and oxidative stress happens when they overload the body's antioxidant defense mechanism which may be a significant causer of some lifestyle mediated diseases, including cancer, atherosclerosis, arthritis, diabetes, coronary heart disease and stroke, myocardial infarction, inflammation and others.

The control of oxidative stress appears to be one of the essential steps in retarding the advancement of these diseases or preventing their complications. In groceries, oxidative reactions cause deteriorations in food quality, unacceptable taste or texture, color, loss of nutritive value and shortening of shelf life. To prevent groceries from subject spoilages and to provide protection against lifestyle mediated diseases, it is necessary to inhibit the

formation of free radicals occurring (Halliwell, 1995). Its known thatantioxidants could terminate chain reactions of lipid peroxidation by donating an electron or a hydrogen atom to free radicals produced from lipids in food. Using the naturally derived antioxidants as alternative to toxic synthetic ones is essential for inhibition of the oxidation stress (Zarei et al., 2014).

Antioxidant compounds in groceries play an important role as a health-protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may cause oxidation of nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxyl or peroxyl-fatty acid radical and, thus, inhibit the oxidative mechanisms that lead to degenerative diseases. There are a number of clinical studies suggesting that the antioxidants in fruits, vegetables, tea and red wine are the main factors for the observed efficacy of these foods in reducing the incidence of chronic diseases including heart disease and some cancers(Molyneux, 2004).Antioxidant capacity is a generic term gathering various chemical mechanisms and may be delineated by two main. Antioxidant biomolecules act as free radical scavengers (R) andeither involve a single electron transfer (SET-based reactions) ora hydrogen atom transfer (HAT-based reactions) (Huang et al., 2005).

Several peptides from protein ingredients have been found topossess antioxidant capacity, and their biological activity has beenwidely studied since the effect was first reported by Marcuse (Marcuse, 1960). Since then, multitudinous peptides with various bioactivities have been identified. More than 1500 different bioactive peptides have been reported in a database named ‘Biopep’, as well as database BioPD (bioactive polypeptide database) has also reported more than 1300 peptides with different biological activity.Antioxidant peptides contain of 5–16 amino acid residues, but some peptides

(lunasin from soy contains 43 amino acids) have long chain of amino acids. Their activity is dependent on their composition, structure and hydrophobicity (Chen et al., 1996). The antioxidant peptides from foods are considered to be safeand healthy compounds with low molecular weight, low cost, high activity and easy absorption. They have some advantages in comparison to enzymatic antioxidants and the most important is thatpeptides with simpler structures have more stability in different situation and no hazardous immunoreactions. Despite the aforementioned, it is important to point out that these peptides possess nutritional and functional properties (Xie et al., 2008).

The researches which were examined the health effects of bioactive peptides apply themin two different forms: either as hydrolysates of precursor proteinsor in pure form of selected bioactive peptides. Hydrolysate is a mixture that ismainly composed of peptides and amino acids which are producedthrough protein hydrolysis by enzyme, acid or alkali treatment orfermentation. Enzymatic hydrolysis of proteins can be performed with endogenous or exogenousproteases. In the first case, the term „autolysate“isusually used rather than “hydrolysates”. Bioactive peptides, on the other hand, are severalpeptides purified from hydrolysates. It seems that in the area of nutritional sciences application ofnon-purified protein hydrolysate can have certain benefits overthose of purified peptides since the absorption of oligopeptides canbe increased in the presence of sugar and amino acids. Further, it has been shown that hydrolysate exerts higher antioxidantactivity than purified peptides which is explained by the synergistic effect between the peptides (Chen et al., 1995).

Numerous researches have been carried out to examine antioxidantproperties of hydrolysates or bioactive peptides from plant or animalsources like peanut kernels (Hwang et al., 2011), rice bran (Revilla et al., 2009), sun flower protein (Megias et al., 2008), alfalfa protein (Xie et al., 2008), corn gluten meal (Li et al., 2008), frog skin (Qian et al., 2008), yam (Nagai et al., 2007), egg-yolk protein (Sakanaka and Tachibana 2006), egg white proteins (Knežević-Jugović et al., 2012; Stefanović et al., 2014; 2017; Jovanović et al., 2016), (milk-kefir and soymilkkefir (Liu et al., 2005), medicinal mushroom (Wachtel-Galor et al., 2004), mackerel (Wu et al., 2003), curry leaves (Ningappa and Srinivas 2008), cotton leafworm (Vercruysse et al., 2009), casein (Suetsuna et al., 2000), algae protein waste

(Sheih et al., 2009) and buckwheat protein (Tang et al., 2009).A list of some studies on theantioxidant activities as well as structural properties of peptidesand hydrolysatesare presented in Table 3.

Antioxidant properties of the isolated peptides are more related to their composition, structure and hydrophobicity. The researchers found that amino acid Tyr, Trp, Met,Lys, Cys, and His caused antioxidant activity (Wang and De Mejia 2005), such as that amino acids with aromatic residues can donate protons to electron deficient radicals (Rajapakse et al., 2005). It is proposed that the antioxidant activity of His-containing peptides is in relation with the hydrogen-donating, lipid peroxyl radical trapping and/or the metal ion-chelating ability of the imidazole group (Rajapakse et al., 2005.). On the other hand, SH group in cysteine has an independently crucial antioxidant action due to its direct interaction with radicals (Qian et al., 2008). Further, Saito et al.(2003) was reported that any change in the organization of amino acid sequence in tripeptideled to different antioxidant activities. Table 4 offers additional information in respect of the impact of amino acid compositions and their correct positioning in peptide sequences. Moreover, it has been stated that the configuration of peptides can also affect antioxidant activity. It appeared that the substitution of L-His by D-His in an antioxidant peptide led to reduction of the activity and was concluded that the exact positioning of imidazole group wasthe chief factor affecting the antioxidant activity (Chen et al., 1995).

Table 3. Antioxidant peptides derived from different sources

Source of peptides	Sequence	Preparation	Reference
Walnut proteins	Ala-Asp-Ala-Phe (423.23 Da)	Pepsin hydrolysate obtained by 3 h	Chen et al. (2012)
Sardinelle (*Sardinella aurita*) protein by-products	Seven antioxidant peptides were isolated (Leu-His-Tyr, Leu-Ala-Arg-Leu, Gly-Gly-Glu, Gly-Ala-His,	Different proteases	Bougatef et al., 2010

	Gly-Ala-Trp-Ala, Pro-His-Tyr-Leu Gly-Ala-Leu-Ala-Ala-His)		
Porcine collagen hydrolysate	Antioxidative peptides (Gln-Gly-Ala-Arg)	Cocktail mixture of protease bovine pancreas, protease *Streptomyces* and protease *Bacillus* spp.	Li et al., 2007
Grass carp muscle hydrolysates	Antioxidant peptides (Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val)	Alcalase	Ren et al., 2008
Algae protein waste	VECYGPNRPQF	Pepsin	Sheih et al., 2009
Rice endosperm protein	FRDEHKK and KHDRGDEF	Five different proteases, Neutrase was the most effective	Zhang et al., 2010
Corn gluten meal	Peptides fraction of 500–1500 Da	Alcalase	Li et al., 2007
Buffalo and bovine casein hydrolysates	Peptides with a molecular weight less than 1 KDa that consisted of hydrophobic and specific hydrophilic amino acids	Alcalase	Shazly et al., 2017

Apart from the mentioned above facts, the antioxidant activity can be affected by the other factors: the operational conditions applied to isolate proteins, degree of hydrolysis, type of protease, peptide structure and peptide concentration (Saito et al., 2003). The effect of protein concentration on antioxidant activity was reported for peanut protein hydrolysates (Chen et al., 2007). In addition, molecular weight (MW) of peptides can influence the antioxidant activity and it was discovered that the antioxidant activity of corn gluten meal hydrolysates was related to the concentration and molecular weight of hydrolysates. Antioxidant activity of peptides of MW 500–1500 Da was stronger than that of peptides above 1500 Da and peptides below 500 Da (Li et al., 2008). However, it has

been assumed that the total antioxidant activity must be attributed to the integrative effects of these actions rather than to the individual actions of peptides (Chen et al., 1996).

Table 4. Amino acid compositions and their positioning in relation with peptide antioxidant activity

Amino acids	Mechanisam of action	References
Acidic acid residues (Glu, Asp)	Carboxyl and amino groups in the side chains as chelator of metal ions as hydrogen donor	Saiga et al., 2003; Sarmadi et al., 2010
Aromatic amino acid like Phe	direct electron transfer to reactive oxygen species (ROS)	Quiam et al., 2008
Hydrophobic amino acids, for example sequence Ala-AspAla-Phe	High antioxidant activity in the linoleic acid model system	Chen et al., 2012
Cysteine	SH group is a rather efficient radical scavenger, protecting tissue from oxidative stress, improving the glutathione activity	Sarmadi et al., 2010

4.2 Antioxidant hydrolysates and peptidesobtained from wheat

The antioxidant activities of wheat gluten hydrolysate and its fractions obtained by papain hydrolysis and membrane ultrafiltration were investigated in comparison with that of commercial antioxidant in two different oxidation systemsby scavenging effect of DPPH radical and TBA method (Wang et al., 2007). The gluten hydrolysate and its ultrafiltrationfractions showedstrong antioxidant activities in the linoleic acid oxidationsystem and DPPH radical scavenging activity and were evident that the scavenging effect of these fractionsincreased with increasing concentrations of the protein in the samples (Fig. 14). The authors were emphasized that ultrafiltration fractions were superior to the hydrolysate in terms of the antioxidant activities. These results revealed that

the wheat gluten hydrolysate and its fractions possibly contained some substrates, which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction (Wang et al., 2007). Similarly, enzymatic hydrolysates of buckwheat showed excellent antioxidant potential, scavenging (DPPH) radical, inhibiting linoleic acid peroxidation and possessing reducing power (Tang et al., 2009).

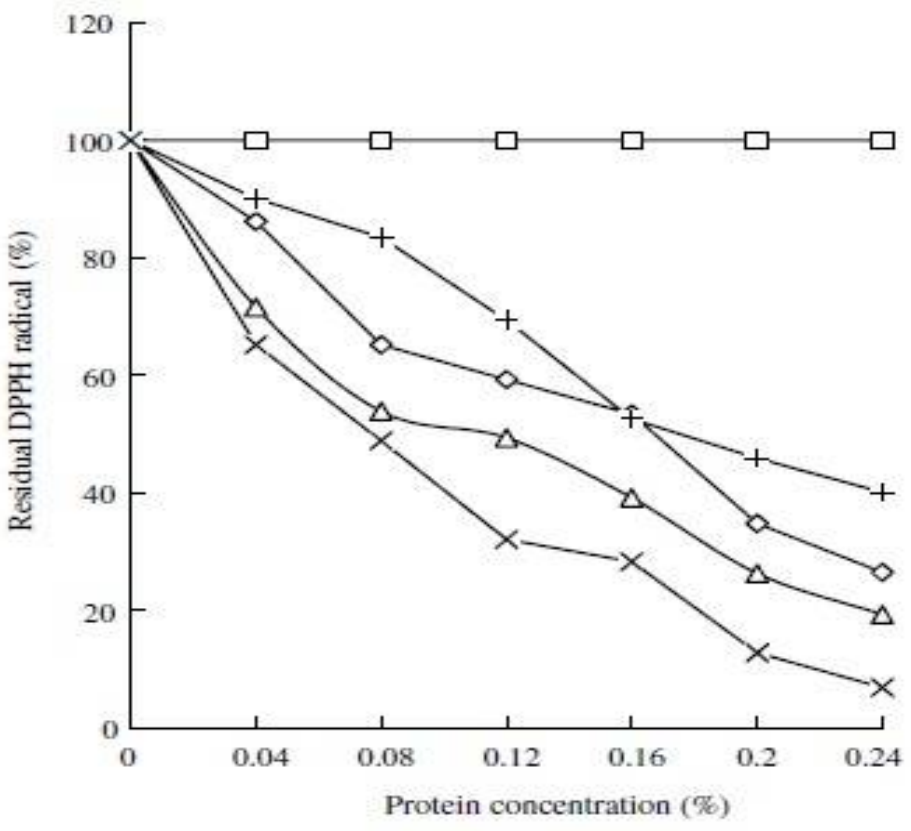


Figure 14. DPPH radical scavenging activity of the hydrolysate and its ultrafiltration fraction. Control (square), the hydrolysate (rhomboid), P fraction (triangle), 5-K fraction (plus), α-tocopherol (×) *(Source: Wang et al., 2007)*.

The antioxidant activity was evaluated from wheat gluten hydrolysates fractionated by different pH (4.0, 6.0 and 9.0) (Cian et al., 2017). Antioxidant properties were analysed using assays with the followingmechanisms: scavenging of $ABTS^{+}$ radical (TEAC), reducing poweractivity assay, copper-chelating activity by assay of β-carotene oxidation

and β-carotene-linoleic acid assay. The obtained results were presented in Figure 15 and the authors demonstrated that gluten hydrolysate possesseda rather high antioxidant activity.

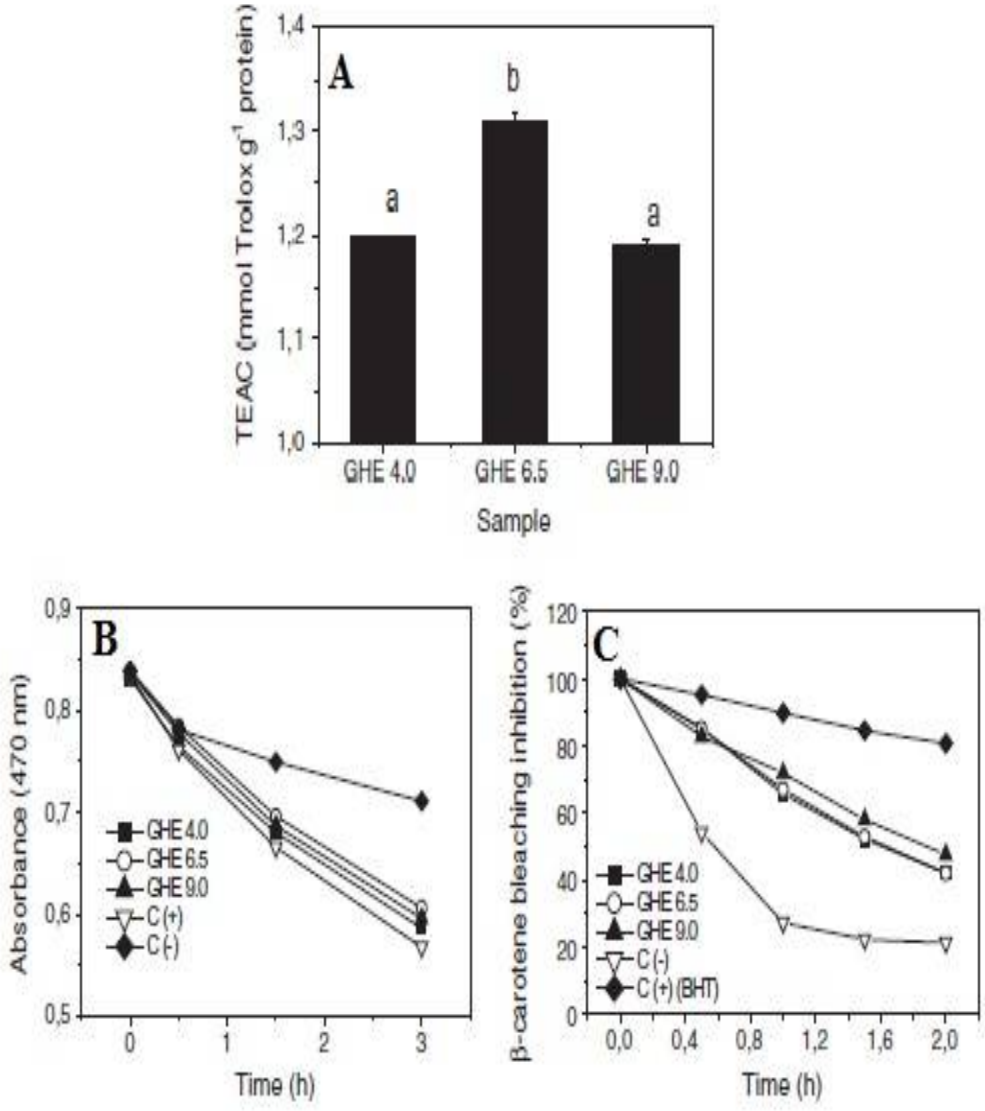


Figure 15. Trolox equivalent antioxidant capacity (TEAC) (A); copper-chelating activity by assay of β-carotene oxidation vs. time (3 h) (B); and carotene bleaching inhibition (%) vs. time (2 h) of GHE (C) *(Source: Cian et al., 2015)*.

Enzymatic hydrolysis of various wheat proteins with acid and neutral proteases (neutrase) was studied. The antioxidant properties of the produced peptides depended on the properties of both proteins and enzymes used. It was shown that when using acid

protease, peptides derived from wheat albumin possess 10-12 times higher reducing power than peptides derived with neutral protease (Boboev et al., 2012). Recent studies showed that some wheat peptides may show the noteworthy antioxidant potency against the peroxidation of lipids or fatty acid. Tang et al. (2012) was demonstrate that heat and melandialdehyde (MDA)-oxidized wheat peptides lost their surface hydrophobicity and reducing power and showed the relative lower free radical-scavenging activity *in vitro*. The authors were emphasized that the peptide modifications led to gradual formation of aggregates in wheat peptides and induced more reactive oxygen species (ROS) production *in vivo.*Wheat gluten has been hydrolyzed with protease through two treatments – single enzyme (alcalase) and double (alcalase-flavourzyme) enzyme and antioxidant activity of the hydrolysates were measured (Wang et al., 2014). The reducing power, DPPH, superoxide anion and hydroxyl radical scavenging activity of single enzymatic hydrolysates with mass concentration of 3.0 mg/mL reached 0.81, 71.82%, 56.83% and 70.82%, respectively. The corresponding indictors of double enzymatic hydrolysate reached 1.01, 85.33%, 74.84% and 84.33% respectively. The authors have made a conclusion that there is a positive correlation between antioxidant activity of wheat hydrolysates and the content of small molecular weight peptides orcontent of hydrophobic amino acids.

Researchers hydrolyzed wheat gluten by alcalase 2.4L under ultrasound and various antioxidant assays *in vitro*, suchas ferrous ion-chelating activity, reducing power, inhibition of linoleic acid emulsion peroxidation and ABTS radical scavenging activity,were employed to evaluate the antioxidant activities of the wheat gluten hydrolysate (Zhu et al., 2011). It wasfound that hydrolysate obtained by low frequency ultrasound exhibited the strongestantioxidant activities, with an EC_{50}value of 0.513 mg/mL for ferrous iron-chelating activity, as well as ahigh and dose-dependent reducing power. In the linoleic acid system, a longer induction time indicated asignificant decrease of lipid peroxidation. Also, this hydrolysate exhibited notable ABTS radical scavengingactivity.

Based on a review of available literature data, in addition to the above results, significant research in the field of determining antioxidant activity and improve existing one has been performed. Thus, Žilić et al. (2012) studied the distribution of phenolic compounds and yellow pigments in wheat grains and their relation to the total antioxidant

capacity and their results showed that the bran fraction contained significantly higher concentrations of phenolic acids, flavonoids and yellow pigments and that can potentially provide natural antioxidants. On the other hand, Liu et al. (2010) used the DPPH assay to investigate antioxidant activities and emphasized that Charcoal purple wheat had the highest antioxidant activity (up to 6899 µmol/100 g) followed by red Fife wheat and yellow Luteus wheat, as well as that the antioxidant activity was positively correlated to phenolic contents in these grains. The major phenolic composition identified in wheat grains consisted of phenolic acids, flavonoids, and anthocyanins. Ferulic acid was reported as the most dominant phenolic acid (74-87 mg/100 g). Vanillic, *p*-coumaric, and sinapic acids were found in moderate levels (about 2 mg/100 g), whereas caffeic acid was present in the least amount (2.58 mg/100 g) and ferulic acid (>81.38 mg/100 g). It's worth emphasizing a research conducted by Anson and associates whose main goal was to investigate the antioxidant and antiinflammatory capacity of bioaccessible compounds from wheat fractions after gastrointestinal digestion (Anson et al., 2010). In their study, the bioaccessible compounds from aleurone, bran and flour were obtained from a dynamic *in vitro* model of the upper gastrointestinal tract. They found that bioaccessible compounds from aleurone had the highest antioxidant capacity and provided a prolonged antiinflammatory effect as compared to bran and flour.

Bhanja et al., 2009 investigated the antioxidant potential of ethanolic extract of non-fermented and fermented wheat grains with two filamentous fungi (*Aspergillus oryzae* and *Aspergillus awamori nakazawa*). They found that the total phenolic content and antioxidant property of wheat (54% ethanol extraction) were drastically increased when fermented with *A. oryzae* and *A. awamori nakazawa*. To enhance the extractable antioxidant properties of wheat, Moore and associates were evaluated the potential of solid-state yeast fermentation (Moore et al., 2007). Their illustrated results proved that the solid state yeast treatment significantly increased releasable antioxidant properties ranging from 28 to 65%, 0 to 20%, 23 to 19%, 0 to 25%, 50 to 100% and 3 to 333% for scavenging capacity against ORAC, ABTS, DPPH, TPC, and phenolic acids, respectively.

4.3. Angiotensin I-converting enzyme inhibition activity of wheat proteins

Angiotensin I-converting enzyme (ACE, EC 3.4.15.1.), a metallo-carboxypeptidase, plays a double part in regulating blood pressure by convertingangiotensin I into angiotensin II, a vasoconstrictor, and degradingbradykinin, a vasodilator (Murray et al., 2007). Antihypertensivepeptides can decrease blood pressure by inhibiting ACEand are present in both animal and plant protein hydrolysates (Murray et al., 2007; Jiang et al., 2010; Qu et al., 2010). The molecular properties ofthe active peptides are rather diverse (Murrayet al., 2007; Hartmannan Meisel, 2007). Most of them contain 2 to 9 amino acid residues (Kitts and Weiler, 2003). Structure-activity relationships of synthetically produced analoguesof ACE inhibitory peptides have indicated that competitivebinding to ACE is strongly influenced by the C-terminal tripeptidesequence. At these positions, the presence of hydrophobic aromatic amino acid, such as Tyr, Phe, Trp, or amino acid with hydrophobic branched sidechains, such as Val, Leu and Ile, generally guides to enhance ACEinhibitory activity. Many ACE inhibitory peptides also contain aPro residue at their C-terminal position (Murray et al., 2007; Cheung et al., 2009). Furthermore, several ACE inhibitory peptides have an Arg or Lys residue at their C-terminal end, suggesting that the positive charge on the ε-aminogroup contributes to their inhibitory activity (Murray et al., 2007). The inhibitory activity towards enzymes can be expressedas an IC_{50} value, referring to the concentration of inhibitor neededto inactivate 50% of the enzyme activity under the experimentalconditions.

On the basis of the amino acid profiles, researchers were anticipated that enzymatic hydrolysis of gliadin will release significantamounts of ACE inhibitory peptides. Gobbetti et al. (1997) found ACE inhibitory activity in tryptic glutenhydrolysates. Furthermore, Saigaet al. (2002) hydrolyzed commercial glutenwith trypsin, chymotrypsin, papain and actinase and obtainedhydrolysates with IC_{50} values of 0.31, 0.42, 0.04 and 0.03 mg/ml, respectively. By subsequent ultrafiltration of gluten hydrolysed with actinaseyielded permeates with enhanced ACE inhibitory activity. Another researchers from Japan, Motoi and Kodama (2003), hydrolyzed gluten, gliadinand glutenin sequentially with pepsin and protease M (Figure 16) and received IC_{50} valuesfor ACE inhibitory activity of gliadin

peptides were inferior (about0.4 mg/ml) than those of glutenin hydrolysates. Peptide Ile–Ala–Pro, purifiedfrom the gliadin hydrolysate, hadan IC_{50} value of 2.7 µM.Enzymatic hydrolysis of gluten heat-treated at 120 and 150 ºC with pronase E was obtained the mixture of peptides with IC_{50}values 1.39 and 1.56 mg/ml, respectively.

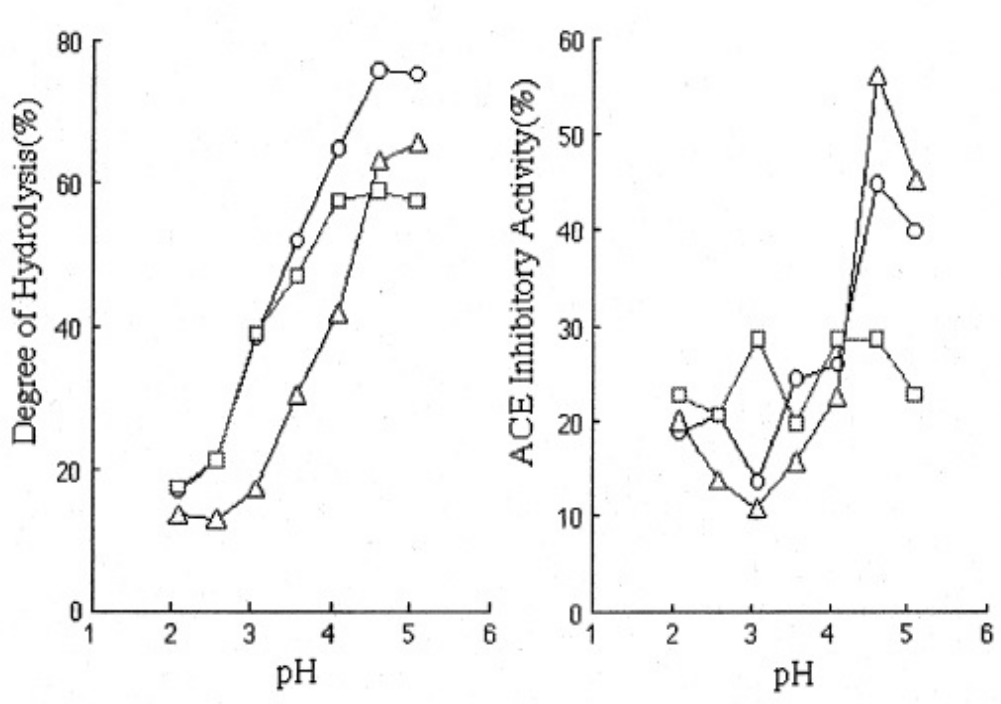


Figure 16. ACE inhibitory activity and degree of hydrolysis of gluten, gliadin and glutenin hydrolysates, which were hydrolyzed with protease M at different pH. The protein concentration for ACE inhibitory assay was 500 µg/mL. Gluten (circle); gliadin (triangle); glutenin (square) *(Source: Motoiand Kodama, 2003)*.

Motoi and Kodama (2003), were used the gliadin hydrolysates for isolation of the potent active inhibitory peptides. Figure 17, presents the ion-exchange chromatography profiles of gliadin hydrolysate on the SP-Toyopearl 550C. The fraction with the highest ACE inhibitory activity (SP-1) was further fractionated with size-exclusive chromatography on the Bio-gel P-2 column (Figure 17). Then, the active fractions were pooled and separated with PR-HPLC on the ODS column. Isolated peptideswere analysed by amino acid analyzer and was located that it was composed of isoleucine, alanine and proline per mol of molecule.

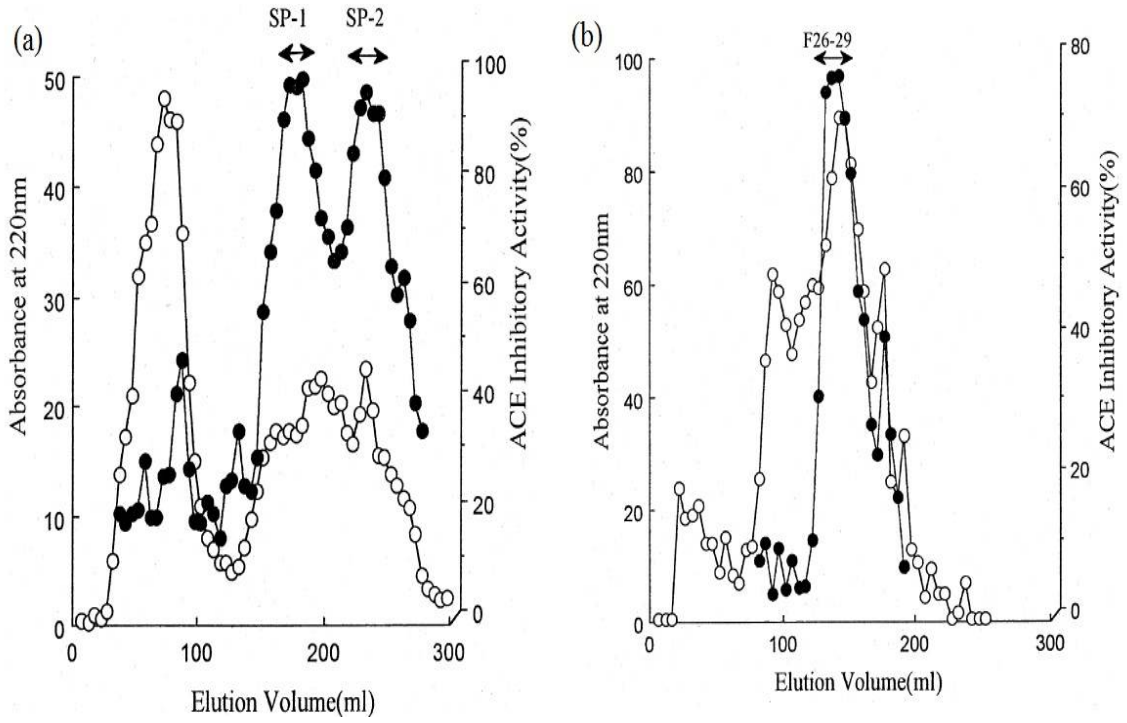


Figure 17. Isolation and purification of gliadin hydrolysates (a) Ion-exchange chromatography of wheat gliadin hydrolysate on SP-Toyopearl 550C; (b) Size-exclusive chromatography of SP-1 fraction on Biogel P-2 (*Source: Motoi et al, 2003*).

The ACE inhibitory activities of tryptic gliadin hydrolysate fractions related to the central and terminal domains of gliadins were studied before and after additional hydrolysis. Researchers were difficult to conclude whether the terminal domain is a better precursor for ACE inhibitory peptides than the central domain or *vice versa*. Thermolysin was very useful for yielding high ACE inhibitory activity (Thewissen et al., 2011). Purification of the related fraction resulted in a peptide fraction with a decreased IC_{50} value (0.02 mg/mL), lower than values for protein hydrolysates mentioned in literature (Saiga et al., 2002; Motoi et al., 2003; Del Castillo et al., 2007). Also, they were emphasized that further hydrolysis of the tryptic hydrolysate fractions with thermolysin, clarex, alcalase and esperase increased ACE inhibitory activities (Thewissen et al., 2011).

4.4. Some other biological activity of wheat proteins

Based on the accessible literature date, it can express a conclusion that wheat proteins evince biological activity in a wind diapason and that containe many potential bioactive peptides. Wheat proteins have been shown to be a good stimulator of cholecystokinin and glucagonlikepeptide 1 (GLP-1) release when exposed to human duodenaltissue, which could encourage wheat protein use as a dietary ingredientin weight management (Geraedts et al., 2010). The wheat storage proteins contained a variety of bioactive peptideswith many different activities within the body and it is presented in Table 5. In general, low molecular wheat glutenin was high in ACE inhibitors, general inhibitors such as DPP-IV, celiac toxic peptides and contained a small amount of opioids (A=0.003), which could act as analgesics or as antioxidant peptides (A=0.0206).High molecular wheat glutenin was also high inACE-inhibitor peptides, as well as opioid, antithrombotic, DPPIV inhibitor, it had the highest occurrence frequency of antioxidant peptides out of all the wheat proteins studied and contained some anticancer peptide sequences. *Alpha*-gliadin contained high amounts of ACE inhibitors, as well as DPP-IV and PEP inhibitors. It was also one of the few proteins evaluated in this investigation that had some neuropeptideactivity. Both *gamma*- and *omega*-gliadin had similaroccurrence frequencies of ACE-inhibitor, DPP-IV-inhibitor, andantioxidant peptides, though *omega*-gliadin was higher in celiactoxic peptides, while *gamma*-gliadin contained some hypotensive (rennin-inhibitor) and DPP-IV-inhibitor peptides (Cavazos et al., 2013).

Table 5. Biologically activesequences in low and high molecular wheat glutenin (Cavazos and Meija, 2013)

Biological activity	Active sequences	Description of biological activity	References
Antimicrobial	IQY	Active against Gram- positive bacteria	Lopez-Exposito et al., 2006
Antihypertensive	Short peptides like IR, LY, IY, VF, VY, PR, LAA, LQP,G, GI, GV, GQ, GT, PP, PQ, HP, PH, IQY	ACE inhibitor	Nogata et al., 2009
Antioxidant	LY, IY, AH, PHQ, RHE, IQY, IR, VY	Oxygen radical-scavenging activity	Huang et al., 2010
Opioid	YYP, RYYP, SYYP	Analgesic activity	Fukudome and Yoshikawa 1992
Anticancer	VVV	Dvl-protein binding	Li et al., 2009

The occurrence frequency (A) of bioactive fragments with a particular activity was calculated by the equation: $A = a/N$, where a is the number of amino acid residues forming fragments with given activity in protein and N is the number of amino acid residues of the protein (Cavazos and Mejia, 2013). The occurrence frequency of each wheat storageprotein was illustrated in Figure 18.

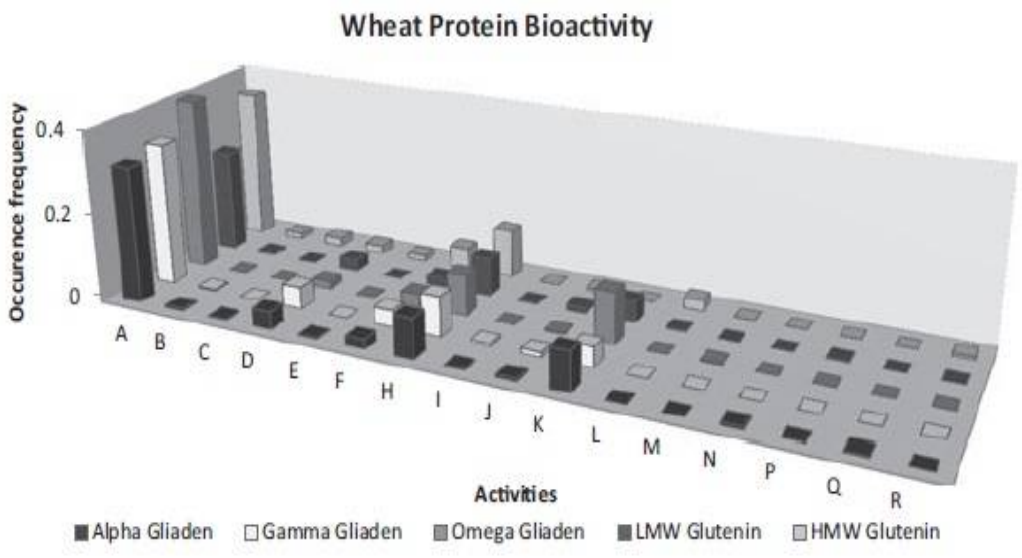


Figure 18. Wheat protein bioactivity: A, ACE inhibitor; B, antiamnestic; C, antithrombotic; D, stimulating; E, regulating; F, antioxidant; G, bacterial permease ligand; H, DPP-IV inhibitor; I, hypotensive; J, activated ubiquitin-mediated proteolysis; K, celiac toxic; L, opioid; M, immunomodulating; N, neuropeptide; O, contracting; P, antibacterial; Q, stimulated different activities; R, anticancer; S, embryotoxic *(Source: Cavazos and Mejia, 2013)*.

5. OPTIMIZATION STUDY

5.1. Response surface methodology and design

The response surface method, theso-called "*response surface methodology*" is a set of mathematical and statistical techniques used to construct empirical models based on experimental data using linear or polonomy functions.By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). The method provides us with information on:

- all interactions that exist between the variables / conditions analyzed;
- variables/conditions that have the greatest impact on the result;
- the conditions of the experiment that provide the best result.

An experiment is a series of tests (runs), in which changes are made in the input variables in order to identify the reasons for changes in the output response. Originally, RSM was developed to model experimental responses (Box and Draper, 1987), and then migrated into the modelling of numerical experiments. In RSM, the errors are assumed to be random.

The application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods and their associated numerical noise. In classical experiments, process parameters are optimized by the method of variation of one factor at constant values of other factors. This method requires a very extensive experimental work in the case when the measured parameter is influenced by several factors. Therefore, the system of planned experiments is a very effective method, since a small number of tests is sufficient to test a large number of variables. RSM can be used for any type of optimization in the technological process.

The surface represented as function of two variables, $f(x_1, x_2)$ is called a response surface. The response can be represented graphically, either in the three-dimensional formor as contour plots. Contours are curves of constant response drawn in the x_i, x_j plane keeping all other variables fixed. Each contour corresponds to a particular height of the response surface, as shown in Figure 19.

A) B)

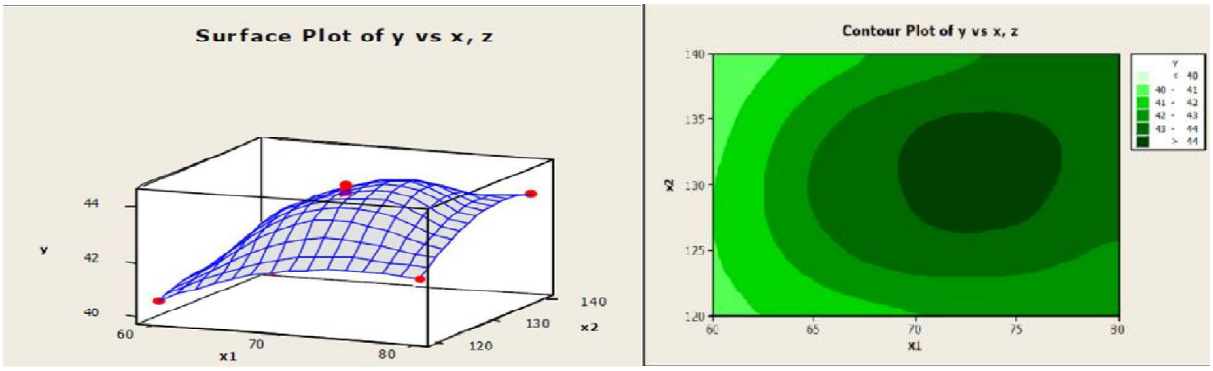


Figure 19. Examples of A) three-dimensional response surface and B) the corresponding contour plot *(Source: Nuran, 2007)*.

The aim for response surface analysis is to find the optimum response or, in the case when there is more then one response, to find the compromise optimum for several responses. The underlying mechanism is usually not fully understood, and the experimenter must approximate the unknown function g with appropriate empirical model $y = f(x_1 , x_2 ,..., x_k) + \varepsilon$, where the term ε represents the error in the system. Usually the function f is a first-order or second-order polynomial. In the practical application of RSM it is important to develop an approximating model for the true response surface.The first-order multiple linear regression modelwith two independent variables is often applied first and it is:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \varepsilon \qquad (1)$$

Models those are more complex in appearance than Equation (1) may often still be analyzed by multiple linear regression techniques. For example, considering adding an interaction term to the first-order model in two variables like follows:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \varepsilon \quad (2)$$

Lack of fit of the first order model happens when the response surface is not a plane. Than, a more highly structured model like second order model may be applied to find the optimum:

$$y= \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_{11}^2 + \beta_{22} x_{22}^2 + \beta_{12} x_1 x_2 + \varepsilon \qquad (3)$$

In order to obtain the most effective results in the approximation of polynomials, the corresponding experimental design is applied in data collection, and an assessment is made of the importance of the influence of parameters on the output size through the polynomial equation of the model. The results of the validation that are determined using the ANOVA analysis are as important as the predictive model, since they compare the experimental results obtained with the predictive one according to the appropriate model, defining their deviation. The method of least squares is typically used to estimate the regression coefficients in a multiple linear regression model.

There are several designs available for fitting a second order model. The design to be used depends on several factors like the number of variables, the number of levels selected, the number of tests etc. Some of the standard designs are full factorial design (with 2 variables), full factorial design (with 3 variables), Box-Behnken (with 3 variables) and the central composite design is also very popular. A researcher can strat with 2q factorial point, and then add central and axial points to get central composite design. Adding the axial points allows quadratic terms to be included in the model. Second order model describes quadratic surfaces and these surfaces can take many shapes.

5.2. The Box-Behnken design

In this work, the Box-Behnken design has been used. The appropriate method is chosen based on the required sensitivity, time required, cost-effectiveness, laboratory capabilities. This designis very common in experimental research, modeling and adaptive management of multifactorial processes.

Box-Behnken is a type of experimental design of the response surface method. In 1960, this method was invented by George E.P. Box and Donald Behnken which helped them to achieve the following objectives:

- to examine the influence of 3 factors / 3 independent variables that are aligned equally to 3 coded levels -1, 0, 1;

- it is necessary that the design be a square model, that it contains a square member and produces 2 factors;

- the ratio between the number of experimental points and the number of coefficients in the quadratic model should be reasonable (to maintain the ratio in the ratio 1.5: 2.6);

• the variation of the variables is estimated based on the distance from the center and should not deviate much from the smallest cubes that contain the experimental points (the ability to rotate).

In the Bex-Behnken design the tested combinations are at the midpoints of edges of the process space and at the centre. The design is rotatable and requires three levels of

each variable. The design has a limited capability for orthogonal blocking compared to the central composite design. This design requires fewer tested combinations then a centra composite design. Its “missing corneres” could be useful when the researcher want to avoid combined factor extremes. In this way, a potential loss of data can be avoided. The both Bex-Behnken and Central composite design are graphically presented in Fig. 20.

A) **B)**

Figure 20. A) Central composite design and B) Bex-Behnken design

The significance of the application of both centrally composite design and Bex-Behnken design in the RSM methodology of optimizing the bioprocesses is all the more important because the specific design of the matrix of experiments allows different approaches in the data analysis.

In addition to statistical and mathematical techniques that through the equation of the model represent the influence and significance of independent variables on the output size, the graphical representation of the response surfaces is also of importance in determining the optimum and analyzing the influence of the parameters on its determination. The response surface method is a convenient approach in developing, improving and optimizing components, products and processes.

6. EXPERIMENTAL PART

6. 1. Materials

Gluten from wheat was purchased from MP Biomedicals(Santa Ana, California, USA) and gliadin from wheat was obtained from abcr GmbH & Co. (Karlsruhe, Germany). Alcalase 2.4L (protease from *Bacillus licheniformis*)was obtained from Sigma Aldrich (St Louis, MO,USA). The enzyme activity was ≥2.4 U/g Anson Units, where one Anson unit is defined as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute, giving the same color with Folin-Ciocalteu Phenol reagent as one milliequivalent of tyrosine at 25 °C and at pH 7.50. Other chemicals used in this research were:

- methanol - HPLC grade (Prochem, Weles, Germany),
- sodium hydroxide (Lach-Ner, Neratovice, Czech Republic),
- bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, SAD)
- hydrochloric acid (Lach-Ner, Neratovice, Czech Republic),
- 2,2′-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich, St. Louis, SAD),
- 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (Sigma Aldrich, St. Louis, SAD),
- Folin-Ciocalteu phenol reagent (Sigma Aldrich, St. Louis, SAD),
- potassium sodium tartrate (zur analyse Merch Dermstadth, Germany).
- copper (II) sulphate (Zorka Pharma, Šabac, Republic of Serbia),
- sodium carbonate (Lach-Ner, Neratovice, Czech Republic).
- 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS) (Sigma Aldrich, St. Louis, SAD).
- trichloroacetic acid (TCA) (Zorka Pharma, Šabac, Republic of Serbia).

6. 2. Methods

6.2.1. Preparation of protein hydrolysates

The enzymolysis apparatus consisted of a stirred tank reactorequipped withan impeller-agitator (Heidolph RZR 2020, Germany), heating unit (C-MAG HS 7, IKA, Germany), a pH meter (Eutech instrument, Netherlands), thermometer (ETS-D6, IKA, Germany) and burette. The stirred tank reactor consisted of a 600 mL glassvessel with an inner diameter of 8 cm, flat bottom and at a working volume of 330 mL. The distance from the bottom wall was kept constant at 3.2 cm throughout the experiments. For selection of the most appropriate impeller type andconfiguration, four impeller geometries were considered and compared with each other; a single helical ribbon (diameter 75 mm, width 7.5 mm), anchor impeller (diameter 72 mm anda blade width 7.1 mm), four blades pitched impeller (with a diameter 68.2 mm and a bladewidth of 10 mm), and beater paddle (diameter 42.7 mm).

The substrate for enzymatic hydrolysis was aqueous dispersionof untreated or heat-pretreated wheat gluten (typically 5% $w_{protein}/v$) which was adjusted to optimum pH for enzyme activity with 0.8 M HCl or 0.8 M NaOH, then stirred and allowed to equilibrate to the working temperature for 20 min. The reactions were started by adding the appropriate amount of alcalase. Enzymatic hydrolysis was carried out at constant pH, temperature and agitation (typically 200 rpm) while the progress of the reaction was followed using a pH-stat method. When the reaction achieved an equilibrium state, the enzyme was inactivated by heat treatment at 90 ^{o}C for 15 min. The hydrolysates were then rapidly cooled to 25 ^{o}C, and then centrifuged (20 min, 4,500x*g*).

Then the mixture of the protein and enzyme was centrifuged at 12,000×*g* for 15 min at room temperature. The supernatants were collected and kept frozen (-20 ^{o}C) for further analysis. Protein content of each gluten sample was assessed using the Lowry method with BSA as the standard (Lowry et al., 1951). The process productivity expressed as mg of obtained protein divided by the added enzyme activity units in the system and reaction time. The initial reaction rate (r, h^{-1}) was calculated as follows:

$$r = \left(\frac{dDH}{dt}\right)_{t \to 0} \quad (4)$$

where *DH* is the degree of hydrolysis (%) at time *t* (h). The DH was determined by pH stat method as described below.

6.2.2. Determination of the degree of hydorolysis

The number of peptide bonds cleaved is directly proportional to the base consumption. The degree of hydrolysis, *DH* (%) was calculated according the following equation:

$$DH(\%) = \frac{h \cdot 100}{h_{tot}} = \frac{N_b \cdot B \cdot 100}{\alpha \cdot m_p \cdot h_{tot}} \quad (5)$$

where B is the consumption of the base in mL, N_b is the normality of the base, m_p is the mass of protein in g, h is the number of equivalents of peptide bonds hydrolysed at the time per weight unit; h_{tot} is the total amount of peptide bonds per weight unit of a protein and can be calculated from its amino acid composition (for wheat gluten proteins h_{tot} is 8.38 mmol/g protein), α is the degree of dissociation of the α-amino groups (α = 0.926 at 60 °C and pH 8.0).

The α value is pH dependent and can be estimated as follows:

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \quad (6)$$

where *p*H is the value at which enzymatic hydrolysis is performed and p*K* is the average p*K* of the α-amino groups liberated during the hydrolysis. The correct p*K* value is a mean of the values of the different α-amino groups released during the hydrolytic process, changing according to the pH and temperature of the reaction conditions (Camacho et al., 2001). For parameter α, the values presented in the literature (Adler-Nissen, 1986) were adopted and under reaction conditions given bellow were in the range from 0.33 to 0.99 (Table 6).

Table 6. The calibration factor (1/α) for pH stat at various temperatures(Adler-Nissen, 1986)

Calibration factor (1/α)*			
pH	Temperature (pK for amino groups)		
	40 °C	50 °C	60 °C
	(7.3)	(7.1)	(6.9)
7	3.00	2.27	1.79
8	1.20	1.13	1.08
9	1.02	1.01	1.02

*Calibration factor for the pH-stat is the reciprocal of the degree of dissociation of α-amino groups

Note that for the range of pH considered here, the method has proved to be reliable and accurate. However, to eliminate any uncertainty, the *DH* for several hydrolytic runs (especially at pH 7 and higher gluten concentration of 9%, $w_{protein}/v$) was also measured at different time intervals by the spectrophotometric determination of the chemical complex formed by the α-amino groups released during the hydrolysis with 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS) following Adler-Nissen protocol (Adler-Nissen, 1979). Good correlations between this TNBS method and pH stat method show that the pH stat method is sufficiently accurate to be used as a rapid method for the determination of the reaction progress in enzymatic hydrolysis for the range of pH, temperature and protein concentration considered here.

6.2.3. Thermal pretreatment

Prior to enzymatic reaction, the wheat gluten proteins were pretreated by conventional thermal treatment in order to compare the impact of thermal pretreatment on enzymatic hydrolysisof wheat gluten. Thermal treatment was carried out by heating wheat gluten solution at 75 °C for 30 min. After that, the pretreated protein solution was

subjecting to the hydrolysis by alcalase as described. The samples were prepared in a beaker of 600 mL capacity where the working volume (~200 mL) was kept constant for all experimental sets. The control was submitted to the same process and performed at the same conditions but without thermal pretreatment.

6.2.4. Protein determination by Lowry method

The Lowry method has been the most widely used method to estimate the amount of proteins in biological samples. First the proteins are pre-treated with copper ion in alkali solution and then aromatic amino acids in the treated sample reduce the phosphor molybdate phosphor tungstic acid present in the Folin Reagent. The end product of this reaction has a blue color. The amount of protein in the sample can be estimated via reading the absorbance (at 500 nm) of the end product of the folin reaction against a standard curve of a selected protein solution (in our case; bovine serum albumin(BSA) solution).

A standard curve was prepared as follows: bovine serum albumin (BSA) powder was dissolved in distilled water and diluted to a concentration of 1 μg/mL. A series of dilutions (0, 0.1, 0.2, 0.3, 0.4, 0.5) were made with a final volume of 1mL. Samples were diluted such that they would fall within the BSA standard range and 1 mL placed in each tube. After standards and samples were diluted and transferred to the test tubes, 2 mL of biuret reagent was added to each tube and mixed thoroughly with repeated pipeting. Biuret reagent was prepared by mixing 0.5 mL of 1% cupric sulfate with 0.5 mL of 2% sodium potassium tartrate, followed by the addition of 50mL of 2% sodium carbonate in 0.1 N NaOH. The mixture was then allowed to incubate at room temperature for 10 min prior to the addition of 0.2 mL Folin & Ciocalteu's reagent. Samples were mixed immediately with repeated pipeting with each addition. Color was allowed to develop for 30 min at room temperature in the dark and the absorbance measured at 500 nm against the blank.Typically obtained standard curve for determining protein content by the Lori method is shown in Fig.21.

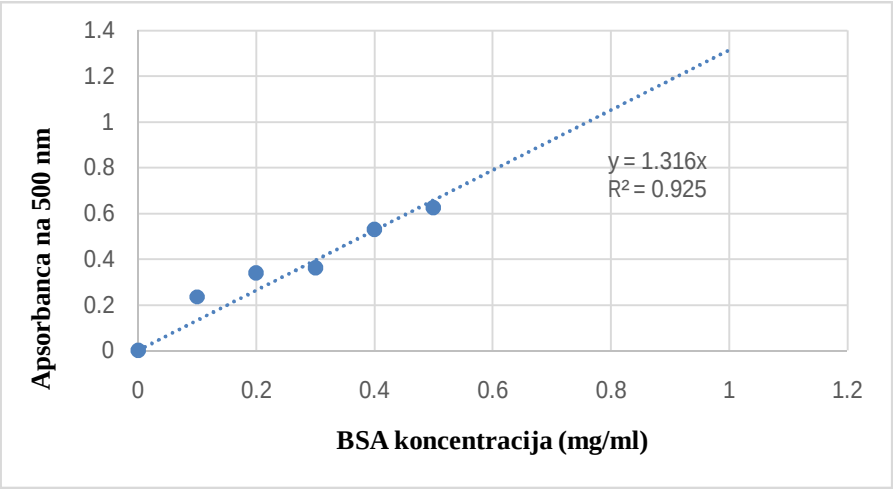


Figure 21. Standard curve for determination of protein content

6.2.5. Functional properties of wheat gluten hydrolysates

6.2.5.1. Protein solubility

Protein solubility of non-pretreated, heat-pretreated and hydrolyzed wheat gluten was determined at different pH. Samples were suspended in water (1 % w/w), and pH was adjusted to 2, 4, 6, 8, 10, and 12using 0.5M HCl or 0.5 M NaOH while stirring at room temperature for 1 h. The samples were then centrifuged at 12,000×*g* for 10 min. The protein contents in the supernatant were determined using the Lowry method using BSA as the standard. Solubility was expressed as the percentage of protein remaining in the supernatant as compared to the to the total protein contentin the sample after solubilization.

6.2.5.2. Determination of emulsification activity and stability

Wheat glutendispersions were analyzed by the turbidimetric technique for emulsion activity index and emulsion stability index as described by Pearce and Kinsella (1978) except for the noted changes in fewcases. Emulsions of the each wheat gluten dispersion (1 % w/w) were prepared with sunflower oil in molar ratio 1:2 and mixing for 90 s with a laboratory homogenizer.

The absorbance of the diluted emulsions was measured by a UV-Vis spectrophotometer (Ultrospec 3300 pro, Amerischam Bioscience) at 500 nm. The absorbance was read initially and turbidity and was calculated by the following formula:

$$T = 2.303\frac{A}{l} \quad (7)$$

where T is turbidity, A is absorbance at 500 nm and l is a path length (m).
The emulsion activity index (*EAI*) was then calculated as:

$$EAI = 2 \cdot T\frac{r}{c \cdot \theta} \quad (8)$$

where T is turbidity (calculated from above equation), θ isvolume fraction (mL), c is the weight of protein per unit volume of aqueous phase before emulsion is formed (g) and r is dilution factor.

For determining emulsion stability, the wheat gluten dispersions were kept at 4 °C for 24 h and analysed for emulsion activity as described previously. Theemulsion stability index (*ESI*) was calculated by the following formula:

$$ESI = \frac{T \cdot \Delta t}{\Delta T} \quad (9)$$

where$T$ is turbidity value at 0 h, ΔT is change in turbidity during 24 h period and Δt is time interval (24 h).

6.2.5.3. Determination of foam capacity and stability

The wheat gluten protein hydrolysates were diluted to 1% (w/w) to prepare foams. The initial volume of 50 mL (20 ºC) foaming solution was placed in the plastic beaker of 700 mL (diameter 7.2 cm) and whipping for 4 min with a laboratory homogenizer at a speed of 9.500 rpm and ambient temperature (Yellowline, DI 25 basic, Ica Works Inc., Wilmington, 600 W, 50 V, 8000-24000 rpm. Foam capacity (*FC*) was expressed as foam expansion at 0 min, which was calculated according to the following equation:

$$FC\ (\%) = \frac{A-B}{A} \cdot 100 \quad (10)$$

where *A* is the volume after whipping (mL) and *B* is the volume before 4 min of whipping (mL). The foam stability (*FS*) was defined as the percentage of liquid still present in the foam after 30 min compared to the solution at 4 min after whipping:

$$FS\ (\%) = \frac{A-B}{A} \cdot 100 \quad (11)$$

A is the volume of foam after 30 min standing (mL) and *B* is the volume before 4 min whipping (mL).

6.2.5.4. Fat-binding capacity

The ability of the wheat gluten hydrolysates to binding fat was measured as follows. Samples of non-treated and hydrolyzed gluten protein (0.5 g) weremixed with 10 mL of corn oil in 50 mL centrifuge tube. The mixturewas then kept for 30 min at room temperature of (25^{o}C) whilemixing every 10 min for 30 s. Afterward, the mixture was centrifugedfor 25 min at 2,000 x*g* and the volume of the supernatants wasweighed. Fat adsorption assay was performed in triplicate and absorptionwas expressed as the volume (mL) of fat absorbed by 1 g ofprotein hydrolysate. Fat adhesion to the walls in the tube was estimatein a blank tube.

6.2.5.5. Water-holding capacity

The ability of the wheat gluten hydrolysates to absorb water was determined as follows. Samples (1 g) were stirredwith 10 mL of destilled water in 12 mL centrifuge tube. The mixturewas then kept for 30 min at room temperature and afterward was centrifugedfor 30 min at 2,200 x*g*. The volume of supernatant wasweighed. The water-holding capacities were expressed as the numberof grams of water retained by 100 g of protein.

6.2.6. Determination of antioxidant activity

6.2.6.1. DPPH radical scavenging activity

The scavenging activity of wheat gluten protein hydrolysates against the DPPH radical was determined based on the method described previously with only slight protocol modification. Briefly, a 0.5 mL aliquot of theobtained hydrolysate was mixed with 0.5 mL of 0.15 mM DPPH solution in methanol. After mixing vigorously for 2 min, the mixture was allowed to stand at room temperature in the dark and after 30 min absorbance was measured at 517 nm using UV-Vis spectrophotometer (Ultrospec 3300 pro, Amerscham Bioscience). DPPH radical scavenging activity (RSA, %), expressed as the percentage of inhibition, was calculated according next equation:

$$RSA\ (\%) - \left[1 - \frac{A_s \quad A_b}{A_c}\right] \times 100$$

where A_s is the absorbance of the tested wheat gluten hydrolysate (WGH), A_b is the absorbance of the WGH in methanol and A_c is the absorbance of the DPPH solution without the sample.

6.2.6.2. ABTS radical scavenging activity

The ABTS radical scavenging activity of prepared hydrolysates was measured using the decolorization assay (Stefanović et al., 2014). This method is based on the ability of antioxidant molecules to quench the stable bisradical cation, $ABTS^{\bullet+}$, a blue-green chromophore with characteristic absorption at 734 nm. In this research, the $ABTS^{\bullet+}$ solution was prepared by reaction of 5 mL of a 7 mM aqueous ABTS solution and 0.088 mL of a 140 mM (2.45 mM final concentration) potassium-persulfate solution. After storage in the dark for 16 h, the radical cation solution was further diluted with 5 mM phosphate-buffered saline (PBS, pH 7.4) until the initial absorbance value of 0.7 ± 0.05 at 734 nm was reached. Solutions of each sample under study were prepared in water so that their final concentration after the addition of 0.01 mLthe radical solution (2 mL) was 0-15

μM and a 20-80 % decrease in the initial absorbance of the reaction solution was achieved. Absorbance was measured at 734 nm after 5 min and ABTS radical scavenging activity (%) was calculated using following equation:

$$ABTS\ (\%) = \frac{A_s - A_b}{A_c} \times 100 \quad (12)$$

where A_s represents the absorbance of the sample solution in the presence of the $ABTS^{\bullet+}$, A_b is the absorbance of the sample solution without $ABTS^{\bullet+}$ and A_c is the absorbance of the control solution with $ABTS^{\bullet+}$.

6.2.6.3. Determination of antioxidant capacity by superoxide radical scavenging (SRS) method

The SRS assay was accomplished using spectrophotometric monitoring of the inhibition of pyrogallol autoxidation as formerly described by Xie et al. (2008) with slight modifications. Briefly, non-hydrolyzed gluten, gluten hydrolysates or their fractions were diluted with TRIS-HCl buffer (50 mM, pH 8.3, containing 1 mM EDTA) to a final concentration of 0.8 mg/mL and a diluted aliquot(0.16 mL) was convey into a clear microplate well. A prepared solution of 1.5 mM pyrogallol (0.16 mL) was put into each well and plate was incubated at room temperature in the dark during a period of 8 min. The course of the reaction was followed by measuring the absorbance at 320 nm immediately after addition of pyrogallol over a period of 8 min using a miroplate reader (Multiscan GO, Thermo Fisher Scientific, Waltham, MA, USA). The SRS activity was calculated by the following Equation:

$$\text{SRS}\ (\%) = 100 \cdot \left(\frac{(\Delta A/min)_{\text{b}} - (\Delta A/\text{min})_{\text{S}}}{(\Delta A/\text{min})_{\text{b}}}\right) \quad (13)$$

where *b* and *s* are blank and sample, respectively.

6.2.6.4. Fractionation of gluten hydrolysate using membrane ultrafiltration

The selected hydrolysate has been further separated by sequential ultrafiltration into three major gluten fractions (GF), GF I (10–30 kDa), GF II (3–10 kDa) and GF III (<3 kDa). The ultrafiltration was performed using a Millipore ultrafiltration stirred cell unit (model 8050 1 unit, Millipore Corporation, Bedford, MA, USA) through cellulose membranes. During the ultrafiltration process, the pressure was applied with nitrogen, as indicated by the manufacturer of the membranes. The protocol was as previously described (Jovanović et al., 2016). Each retentate or permeate was collected and stored in the freezer until required for further analysis. After that, both protein content and the antioxidant capacity were determined in the fraction.

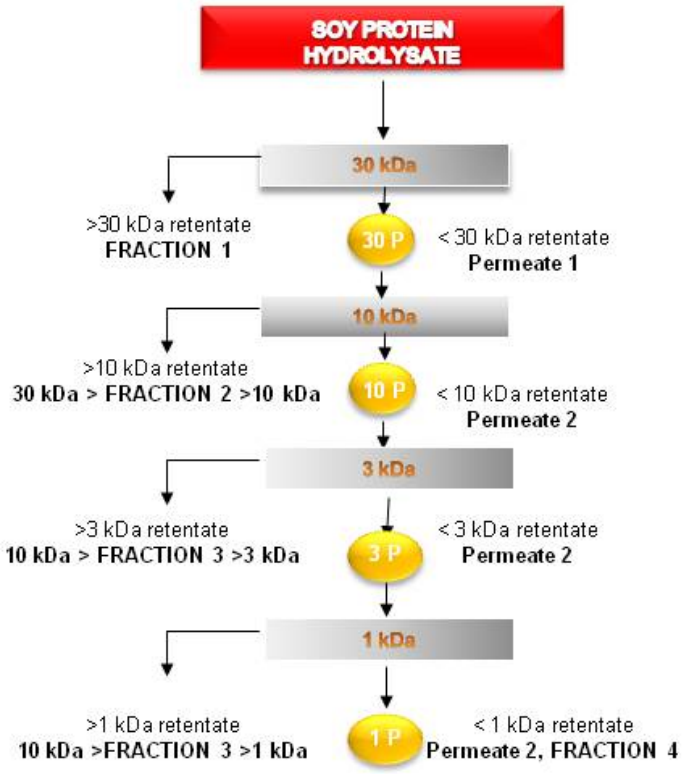


Figure 22. Schematic representation of the separation of the hydrolysates on fractions

6.2.7. Optimization study

6.2.7.1. The effects of process parameters on functional properties by the means of an experimental design

The effects of some relevant process parameters on selected functional properties of hydrolysates obtained in the alcalase-catalyzed wheat gluten hydrolysis such as gluten concentration (X_1; 1-9% w/v), temperature (X_2; 40-60 ^{o}C), pH (X_3; 7-9) and enzyme/substrate ratio, [*E*]/[*S*] ratio (X_4; 0.25– 0.75 AU g^{-1} of protein) were investigated by the means of an experimental design. These variables were chosen based on the results obtained in a preliminary study and are the most commonly used for modeling enzymatic hydrolytic reactions. The degree of hydrolysis and several functional properties like solubility, foam capacity (*FC*) and foam stability (*FS*) as well as emulsion activity index (*EAI*) and emulsion stability index (*ESI*) were taken as the response variable. The design of experiments employed as well as the variables and their levels selected for developing the model are presented in Table7. To avoid bias, 29 runs were performed in a totally random order.

Table 7. Experimental setup for three-level, four-factor Box-Behnken experimental design with 29 experimental points in terms of coded and actual values of variables.

Factors	Symbol	Level		
		-1	**0**	**1**
Gluten concentration, %	$\boldsymbol{X_1}$	0.1	0.5	0.9
Temperature, °C	$\boldsymbol{X_2}$	40	50	60
pH	$\boldsymbol{X_3}$	7	8	9
[*E*]/[*S*] ratio, AU/g gluten	$\boldsymbol{X_4}$	0.25	0.50	0.75

The data obtained were fitted to a second-order polynomial equation:

$$y = \beta_{k0} + \sum_{i=1}^{4} \beta_{ki} X_i + \sum_{i=1}^{4} \beta_{kii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{kij} X_i X_j \qquad (14)$$

here β_{k0}, β_{ki}, β_{kii} and $\beta_{kij}$are regression coefficients for intercept, linear, quadratic and interaction terms, respectively and X_i and $X_j$are independent variables.

The coefficients of the response function and their statistical significance were evaluated by theresponse surface regression analysis, using the softver. Non-significant terms ($p \geq 0.05$) weredeleted from the second order polynomial and a new polynomial has been recalculated. The Fisher test (F value) was used to determine whether the second-order model was adequate todescribe the obtained data while the goodness of fit of the model was evaluated by the determination coefficient (R^2).

6.2.7.2. The effects of process parameters on antioxidant properties by the means of an experimental design

In this optimization study the investigated parameters for the alcalase-catalyzed gluten hydrolysis were also gluten concentration, temperature, pH and *E*/*S* ratio which affect the predicted response y (y_1 and y_2- antioxidant capacity measured by DPPH and ABTS methods, respectively. The second-order polynomial equation was considered for prediction of the antioxidant activity measured by two methods for alcalase-catalyzed gluten hydrolysis as a function of independent variables (i= 4) of gluten concentration (x_1), temperature (x_2), pH (x_3) and *E*/*S* ratio (x_4) as coded valuesalready shown in Table 7. The stirring rate (200 rpm) and running time (2 h) were kept constant. RSM analysis of experimental data with a three-dimensional response surface and contour plots of independent variables and their interactions were applied in order to predict and optimizeinfluence of parameters on alcalase-catalyzed gluten hydrolysis.

The four-factor Box-Behnken experimental design with 29 experimental points (5 central points) is presented in Table 8.

The collected experimental data were analyzed by using the statistical Design Expert Statistical 8.0.7.1 (Stat Ease Inc., Minneapolis, USA) software package to fit to a second order (quadratic) multiple regression model for each response. The coefficients of the response function and their statistical significance were evaluated by theresponse

surface regression analysis, using the softver. Non-significant terms ($p \geq 0.05$) weredeleted from the second order polynomial and a new polynomial has been recalculated. The Fisher test (F value) was used to determine whether the second-order model was adequate todescribe the obtained data while the goodness of fit of the model was evaluated by the determination coefficient (R^2).

Table 8. Box-Benken experimental design with real values for the variables for degree of hydrolysis (*DH*%) and DPPH and ABTS antioxidant activities of gluten hydrolysates.

Run No.	**RSM experimental variables**			
	X_1	X_2	X_3	X_4
1	1	40	8	0.5
2	9	40	8	0.5
3	1	60	8	0.5
4	9	60	8	0.5
5	5	50	7	0.25
6	5	50	9	0.25
7	5	50	7	0.75
8	5	50	9	0.75
9	1	50	8	0.25
10	9	50	8	0.25
11	1	50	8	0.75
12	9	50	8	0.75
13	5	40	7	0.5
14	5	60	7	0.5
15	5	40	9	0.5
16	5	60	9	0.5
17	1	50	7	0.5
18	9	50	7	0.5
19	1	50	9	0.5

20	9	50	9	0.5
21	5	40	8	0.25
22	5	60	8	0.25
23	5	40	8	0.75
24	5	60	8	0.75
25	5	50	8	0.5
26	5	50	8	0.5
27	5	50	8	0.5
28	5	50	8	0.5
29	5	50	8	0.5

6.2.8. Statistical analysis

In this thesis, all experiments were carried out in triplicates and expressed as means withstandard deviation. The effects of different parameters under the significance level of $p<0.05$ were examined using one-way analysis of variance (ANOVA) and Student *t* test.Analysis of variance, followed by the Tukey test was performed to examine the effects of different pretreatments under the significance level of $p<0.05$. All statistical analyses including calculations were conducted using OriginPro 8.5 (OriginLab Corp., Mass., U.S.A.).

7. RESULTS AND DISCUSSION

7.1. The effect of the heat pretreatment of WGPs onenzymatic hydrolysis induced by alcalase

7.1.1. The effect of the heat pretreatment on degree of hydrolysis

The WGPs were subjected to the heat treatment at 75 °C during 30 min and then hydrolysed by alcalase. The DH is a measurement of the extent of hydrolysis degradation of a WGPs and generally used as a proteolysis monitoring parameter. The resulted DH, obtained from this experimental set, depending on the hydrolysis time was presented in Fig.23.

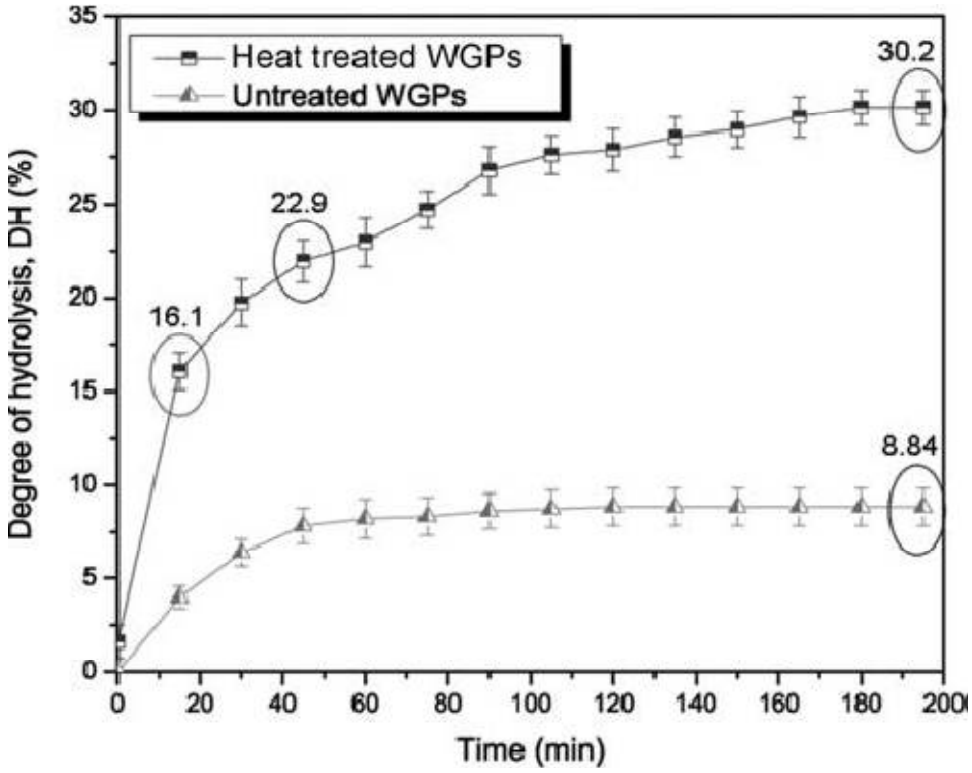


Figure 23. Effect of heat treatment on time-course ofalcalase-catalyzed wheat gluten hydrolysis. Heat treatment—glutentreated at 75 ^{o}C for 30 min. WGPs were hydrolyzed using alcalaseat the followingconditions (pH 8, 60 ^{o}C, *E*/*S* 0.5 AU/g gluten).

The results obtained forthe enzymatic hydrolysis of untreated and heat-pretreated WGPs (Figure 23) implied that the heat treatment significantly improved susceptibility of WGPs to alcalase. Namely, DH achieved in the hydrolysis of WGPs varied from 2 to 30% over the 195 min time period. The hydrolysis proceeded at a rapid rate during the initial 45 min of the reaction and recorded DH was about 22%. Afterward, the enzymatic hydrolysis proceeded with a slow increase in hydrolysis rate, for the next 150 min, and then entered the steady state. It appeared that the heat-pretreated WGPs substrate hadthe considerable susceptibility by alcalase and thus prepared hydrolysate was used to identify functional and antioxidant characteristics. The represented DH profile with time is similar to the typical hydrolysis curve reported by Klompong et al. (2007).

7.1.2. The effect of the heat pretreatment on selected functional properties

7.1.2.1. Emulsifying and foaming ability

The main objective was tofind the adequate enzymatic hydrolysis conditions (hydrolysis timewith a high DH) that would lead to improving functionality of the hydrolysates.The aim wasto obtain highly hydrolyzed WGPs, but also an end hydrolysate withimproved functionality.It is known that specified functional properties of the protein hydrolysates play a more dominant role than others and order the choice of hydrolysates for a specific end use. The range of desirable and attractive functional properties that should be looked for is almost as broad as the range of foods themselves. On the basis on the above, the main objective in this research was to determined functional properties such as foaming and emulsifying ability and solubility. Emulsifying and film forming ability of plant proteins is essential for those proteins to perform well in some food systems. Also a protein's ability to form emulsions is a critical issue for their application in mayonnaise, salad dressing, milks, and frozen desserts (Vodjdani and Whitaker, 1989).

In order to find an optimal DH values, three DH values (approximate 16%, 22%, and 30%) were identified and selected from the hydrolysis time curveand then thesethree hydrolysates wereprepared for the further analysis of functional properties.For comparison

purpose, the untreated sample with DH of 8.84% has also been tested for selected functional properties. Emulsifying and foaming ability have been tested first. The results were summarized in Fig. 24.

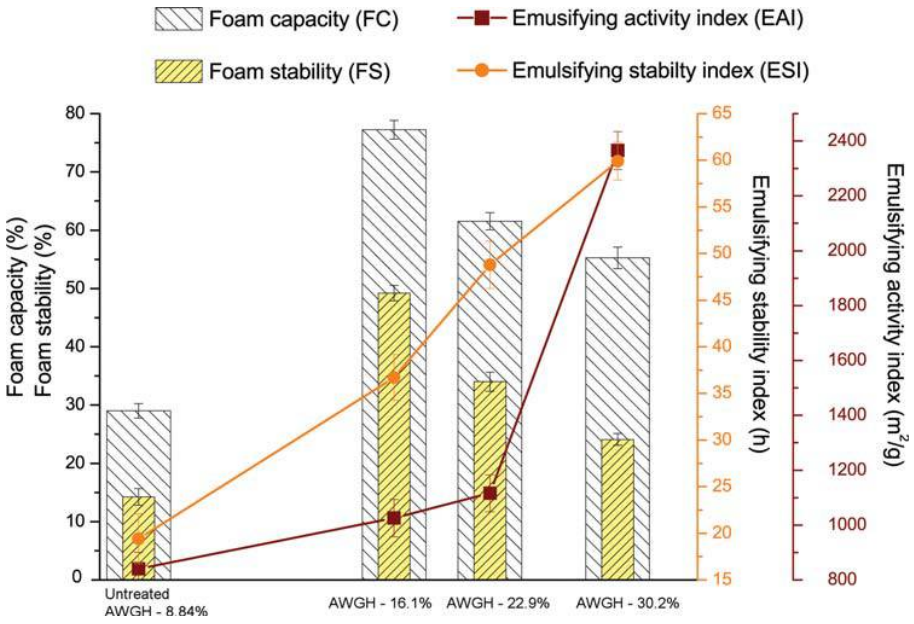


Figure 24. Changes in foaming and emulsifying ability of heat pretreated samples of alcalase hydrolysates as a function of DH. The values in this figure are means of triplicates. The conditions of the heat pretreatment and enzymatic hydrolysis of wheat gluten are as described in Fig.23.

According to the results presented in Fig.24, it appearedthat both *EAI* and *ESI* showed an improvement when the highest DH of 30.1% was achieved compared with the untreated hydrolysate and the hydrolysates with smaller DH. It can be noted that the *ESI* significantly increased during hydrolysis time in comparing to the untreated hydrolysate. The enhanced emulsifying ability could be described by the factthat hydrolysis liberated medium-molecular-weight peptides from the untreated WGPs resulting in a larger surface area and consequently a greater emulsion formation (Linares et al., 2000). The researchers explained the observed improvement in the emulsifying properties upon very limited hydrolysis that it could be probably attributed to exposure of the hydrophobic protein

interior which enhanced adsorption at the interface, forming a cohesive interfacial film, with the hydrophobic residues interacting with oil and hydrophylic residues with water (Phillips and Beuchat, 1981). Opposite to our results, Klompong et al. (2007) reported that the *EAI* decreased with increasing protein hydrolysis and that this decrease could be ascribed to the presence of smaller peptides which were less beneficial in stabilizing emulsions. Fig.24also showed that the heat pretreated and hydrolyzed wheat gluten exhibited improved foaming properties in terms of foam capacity (*FC*) and stability (*FS*) compared to the untreated one. A significant increase in the *FC* for the DH 16.1% (77.3%) was observed. This can be explained with an increase in surface activity, likely because of the initially greater number of polypeptide chains that arose from partial proteolysis, which allowed more air to be incorporated. However, *FC* decreased notably with increasing protein hydrolysis and the more extensively hydrolysed WGPs, DH 22.9% (61.5%) and DH 30.2% (55.3%) showed reduction in the *FC* compared to the hydrolysate with a smaller *DH*. Evidently, extensive hydrolysis could reduce *FC* due to the production of amphiphilic peptides with a reduced molecular weight. These peptides are less flexible and capable to form a stable interfacial layer which is liable for increasing the rate of diffusion to the interface, resulting in improved foam capacity (Kong et al., 2007). Furthermore, such peptide layer can stabilize gas bubbles in dispersions by preventing bubbles from approaching each other and eventually merging (Wouters et al., 2016). This occurrence could also have an opposite effect on the surface activity, probably due to the lower surfactant activity of smaller polypeptide chains from extensive hydrolysis. Klompong et al. (2007) reported good foam capacity for yellow stripe trevally protein hydrolysates at a rather low DH (5%).

Similarly, the *FS* decreased as the extent of hydrolysis increased, thus it is assumed that stable protein foams were obtained due to presence of some larger protein components and partially hydrolysed protein (Bombara et al., 1994). A similar effect of DH over *FC* and *FS* was reported by Kong et al. (2007) where the lowest *FC* and *FS* values were reported for the largest DH. The effect of enhanced foaming properties caused by enzymatic hydrolysis seemed to strongly dependent on the applied treatment before

hydrolysis and thisimprovement in foaming properties of food proteins was reported by several authors (Kong et al., 2007; Popović et al., 2013).

7.1.2.2. Solubility

Good solubility of proteins is essential in many functional applications, especially for emulsions, foams, and gels purposes. The limited solubility of wheat gluten in aqueous solvent has commonly been ascribed to bothlarge molecular size and intermolecular aggregation, arising fromstrong noncovalent interactions, involving hydrogen bonds and hydrophobic interactions (Weegels et al., 1994). The pH dependence of the solubility of theobtained wheat gluten hydrolysates is presented in Fig. 25.

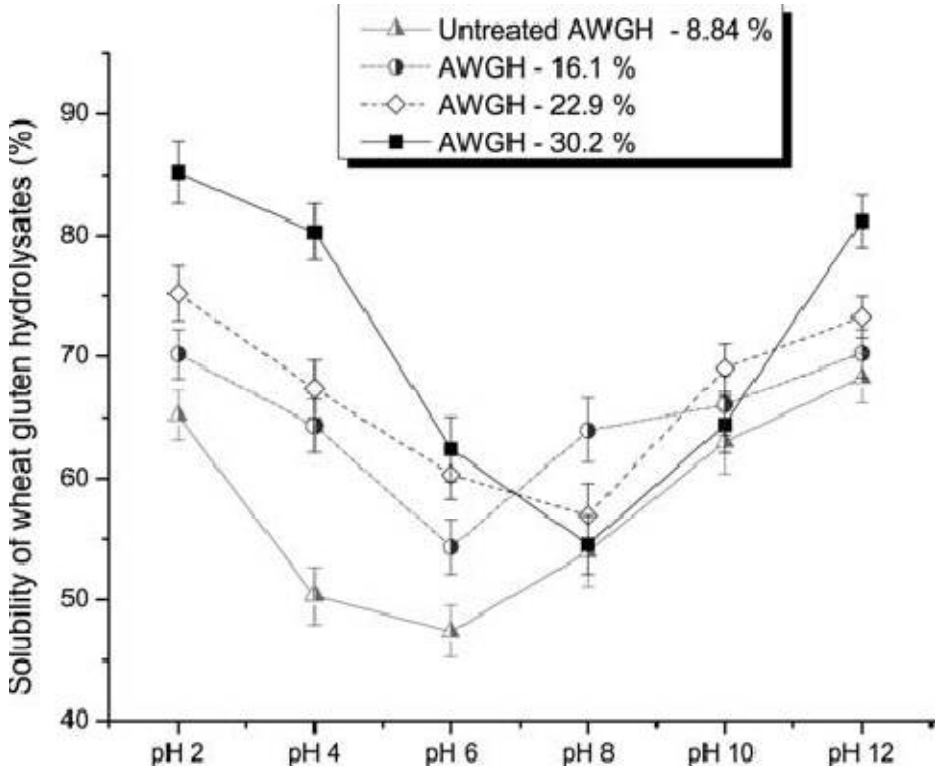


Figure 25. Influence of DH on solubility profiles of heat-treated wheat gluten hydrolysates under different pH value.

As shown, the solubility was markedly improved by a heat treatment followed by limited enzymatic hydrolysis which may be explained by the increase of cleavable peptide bonds. All wheat gluten hydrolysatespresented a similar pattern of solubility, which could be due to the fact that the specificity of the enzyme used was the same in all these cases. The pH-solubility profile of the untreated wheat gluten hydrolysate exhibited a typical bell shaped curve, with minimum solubility of 47.4% at the pH 6 (isoelectric point). In addition, the isoelectric point of the heat-treated hydrolysates shifted to about pH 8. With the increase of DH, the solubility of the heat-treated hydrolysates was gradually increased and was pH-dependent over the studied range. Decreasing the pH from 6 to 2 increased the water solubility in all samples that might be related to slight degradation of the gluten proteins in the acetic condition. Furthermore, at above or below the isoelectric point of the gluten, the water solubility increased (at pH 4, 10 and 12). This trend in solubilities pattern was in agreement with data reported by Hardt et al. (2013) and Wang et al.(2006), who observed a similar trend. Klompong et al. (2007) also support a finding that protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) meat had an excellent solubility at high DH. However, theresults were in contrast to Kong et al. (2007) who reported pH- independent solubilities over the studied pH range for wheat gluten hydrolysates. The improvement in heat-treated WGPs solubility by enzymatic hydrolysis can be attributed to the release of soluble peptides from insoluble aggregates or precipitates, which increase the number of exposed ionisable amino and carboxyl groups (Day, 2011). Thus, a combination of heat treatment and limited hydrolysis could be used as an effective alternative to improve the solubility of wheat gluten proteinsleading to potential applications in formulating food systems.

7.1.2.3. Fat-binding and water-holding capacity

Fat-binding capacity represents a major functionality of ingredients used in the food and confectionery industries. The binding affinity of hyrolysates to fat depends upon several factors such as bulk density of the protein (Kinsella, 1976), degree of hydrolysis (Kristinsson and Rasco, 2000) and enzyme-substrate specificity (Haque, 1993). All wheat

gluten hydrolysates exhibited remarkable binding affinity to fat and results are represented in Fig.26.

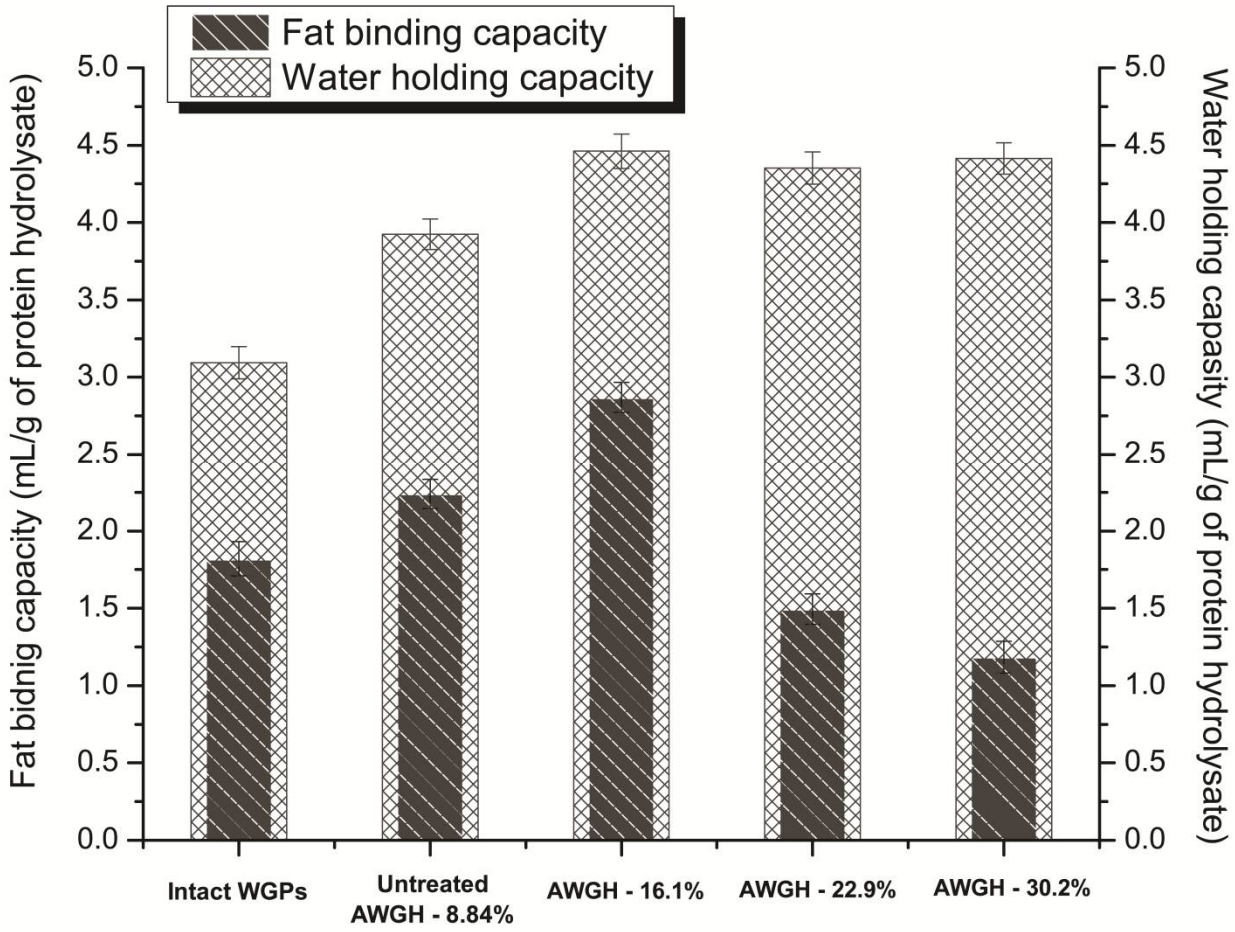


Figure 26. Water-holding capacity and fat-binding capacity of heat-treated wheat gluten hydrolysates as a function of DH.

As shown, the wheat gluten hydrolysates (WGHs)withDH of 16.1% had the highest binding affinity to fat (2.87 mL/g), while the hydrolysate with DH of 30.2% had lower binding affinity to fat (1.12 mL/g) in comparation to intact wheat gluten proteins and untreated wheat gluten hydrolysates. Both hydrolysates, AWGH with DH of 22.9% and AWGH with DH of 30.2% had a smaller affinity (p<0.05) to bind corn oil which was higher than intact wheat gluten proteins (WGPs) and hydrolysate obtained withouth heat

pretreatment (AWGH) used as a control. Based on a review of the literature data and by comparing the results, it can be concluded that the fat-binding capacity of wheat gluten hydrolysates was in the analogous value range with grass carp skin hydrolysate (2.2–3.6 mL/g) (Wasswa et al., 2007) but superior than those of shark muscle hydrolysates (0.3–0.5 mL/g) (Diniz and Martin, 1997) and whey protein hydrolysates (0.16–0.34 mL/g) (Sinha et al., 2007). These represented results could be explainedby the fact that the synergistic effect of heat treatment and enzyme hydrolysis can liberate some peptides from theintact wheat gluten proteins, which would improve the flexibility of the peptides ofAWGH with DH of 16.1%. Also, the decrease in binding affinity to fat might bedue to hydrolytic degradation of the protein structure (Wasswa et al., 2007). On the other hand, the extensive enzyme hydrolysis would break many peptide bonds, thus contributing to the decrease of thecorn oil binding properties shown in AWGH with DH of 22.9% and AWGH with DH of 30.2%.According to presented results, it can be emphasized that AWGH with DH of 16.1% contained larger peptides and more medium-hydrophobicpeptides than the other prepared AWGHs. In contrast, other studies indicated that enzyme hydrolysis produced a significant increase in oil capacity of bean protein concentrate (Mune, 2015a), cowpea protein concentrate (Mune, 2015b), and soy protein isolate (Achouri et al., 1998).

Also, in food applications, the water-holding capacity of the intact proteins and their hydrolysates represents an important factor which usually improves the food textures. The relationship of water-holdingcapacity and DH ofWGP was determined and the all prepared AWGHsexhibited a remarkable water uptake capacity. The results are shown inFig. 26. In this research, the water uptake capacity significantly (p<0.05) increased with increasing DH.The hydrolysate with DH of 30.2% had the highest water-holding capacity (4.42 mL/g). Also, both AWGH with DH of16.1% (4.46 mL/g) and22.9% (4.35 mL/g) had greater ability to uptake water than untreated AWGH (3.92 mL/g) and intact WGPs (3.09 mL/g) used as controls. These results may be attributed to the high solubility and accordingly, the protein dissociation into minor subunits, which increased the functional groups, especially hydrophilic with more water binding sites as a resultof synergistic effect of heat treatment and controlled enzymatic hydrolysis. The water-holding capacity of AWGH with DH of 16.1% was significantly higher compared to those withDH of 22.9%

and 30.2%. Likewise, there were significant differences in the water-holding capacitybetween AWGHs withdifferent DH and untreated AWGH. This result indicated that the high solubility of AWGHs led to the increaseing water holding capacity of wheat gluten. Alike dependence in water-holdingcapacity and DH was observed for grass carp skin hydrolysate (Wasswaet al., 2007). However, the water-holding capacity results of grass carp skin hydrolysate alsocontradicted with other fish protein hydrolysates studies. Diniz and Martin (1997) observed that the water-holding capacity was negatively influenced by extensive hydrolysis for shark muscle hydrolysates. The reduction of water-holding capacity might have been due to the hydrolytic degradation of the protein structure where physical entrapment plays an important role in the uptake of water. Generally, the results showed that AWGHs prepared by synergistic effect of heat treatment and controlled enzymatic hydrolysis exhibited good fat and water adsorption and could be emphasized that they arevery useful in the food and confectionery industries.

7.1.3. The effect of the heat pretreatment on selected antioxidant properties

The antioxidant activity of protein hydrolysates and peptideshas has been widely studied and well documented. When discussing their antioxidant activity, the application of at least two methods is recommended due to the differences between the test systems investigated (Schlesier et al. 2002). Therefore, the antioxidant capacity of alcalase wheat gluten hydrolysates (AWGHs) was evaluated by measurement ABTS and DPPH radical scavenging activities.

ABTS and DPPH radical scavenging activities of AWGHs produced by synergistic effects of heat treatment and controlled enzymatic hydrolysis with different DHs are presented in Fig. 27.

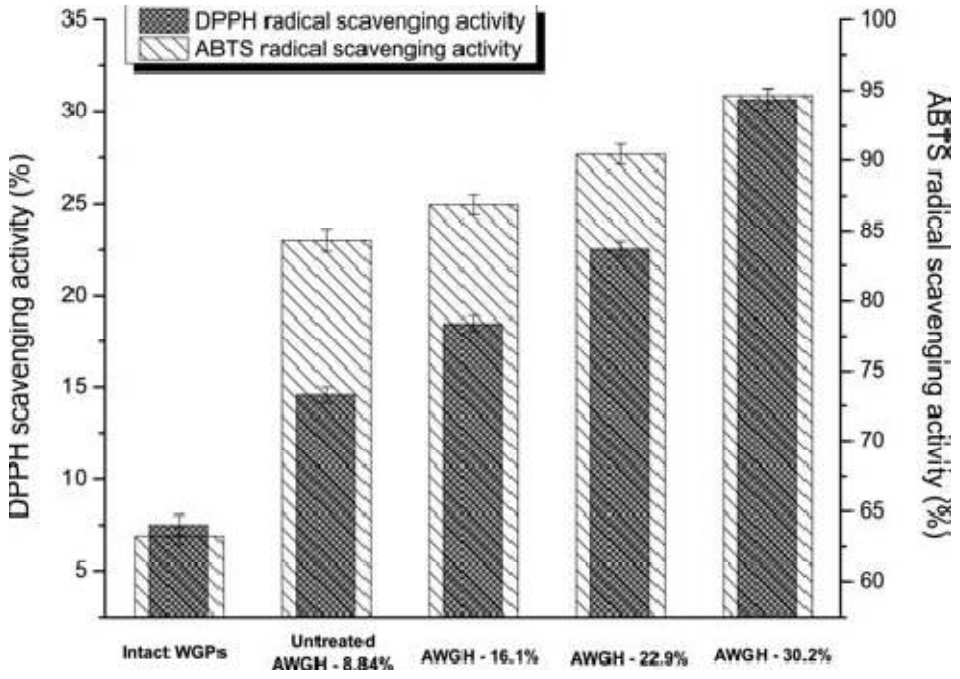


Figure 27. Evaluation of free radical scavenging activities of heattreated AWGHs at different DH.

The ABTS radical scavenging assay represents a good method for determining the antioxidant activity, in which ABTS activity is established on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radical (Binsan et al., 2008). As shown, with the increasing DH, ABTS radical scavenging activities significantly increased (p<0.05). Both hydrolysates with higher DH exhibited the greatest activities in which the activities of 90.4% and 94.6% were obtained for AWGH with DH of 22.9% and AWGH with DH of 30.2%, respectively. It was noticed that there were no differences in ABTS radical scavenging activity between untreated AWGH with DF of 8.84% and heat-treated AWGH with DH of 16.1% (p>0.05). Likewise, the synergistic effects of heat treatment and hydrolysis were upgraded the ABTS activity compared to the intact WGPs. These results suggested that more antioxidant peptides were produced when WGPs were more cleaved at higher DH. It is known that all protein hydrolysates contained peptides or smaller protein

fractions were hydrogen donor and could react with radicals to convert them to more steady products, thereby finalizing the radical reaction (Kittiphattanaba-Won et al., 2012).

The DPPH radical scavenging assay measures the radical scavenging activity of antioxidants toward DPPH radical and has been used largely as verification for novel natural antioxidants (Zhang et al., 2015). Fig. 27 shows that DH has been correlated with the antioxidant activity of AWGHs and has shown that the DPPH radical scavenging activity increases with an increase in DH. The similar results were obtained by Liu et al. (2010). According to the results, the DPPH radical scavenging activity significantly increased (p>0.05) with the progress of wheat gluten hydrolysis because the opening and expositionof active amino acid residues, which could react with oxidants or reactive oxygen species. Between the different hydrolysates, AWGH with DH of 30.2% had the highest value of DPPH radical scavenging activity and it was 30.6%. Compared with the intact WGPs, the all alcalase hydrolysates had possessed significantly higher value of DPPH radical scavenging activity. Despite the aforementioned, it is knownthat the comercial food-grade enzyme alcalase acts as an endopeptidase, accordingly cleaving peptide bonds at the interior of the polypeptidechain. Therefore, alcalase mainly produces small and medium-sized oligopeptides or polypeptides, some of which were antioxidative (Liuet al., 2010). In general, the results of the strong antioxidant activity for the allprepared AWGHs were in agreement with the results of the otherresearchers who were also demonstrated that the heat treated gluten hydrolysates possess antioxidant ability (Park et al., 2008; Seung et al., 2014; Wang et al., 2007).

7.2. Impact of reaction conditions on reaction progress and degree of hydrolysis

The enzymatic hydrolysis of wheat gluten proteins was performed in thestirred tank reactor consisted of a 600 mL glass vessel with a water jacket and equipped with a pH electrode and an impeller as described in the subchapter 6.2.1. The conventional batch process is industrially advantageous as it can be easily controlled with mild reaction conditions in terms of process control. However, the batch process has limited productivity

and yield, due to the unfavorable reaction equilibrium, product inhibition, and enzyme inactivation to the enzyme autolysis and/or hydrodynamic stresses. Especially, the system tested in this thesis is very complicated, since gluten dispersions are highly viscous and exhibit non-Newtonian flow properties (Song and Zheng, 2009). This creates problems with both mass and heat transfer within the batch reactor as well as mechanical problems related to pumping and efficient mixing of the reaction mixture. Previous studies in the literature showed that outcomes of a protein hydrolysis in the batch reactor system depend in a great deal upon several key operating factors, such as substrate concentration, *E*/*S* ratio, permeate flow and impeller speed (Nouri et al. 1997; Shi et al. 2009).Thus, the effects of these parameters have been tested in the thesis.

The effect of critical operational parameters suchas impeller geometry and agitation speed was explored first in this system at 50 ^{o}C using an initialgluten concentration of 5% (*w/v*), *E*/*S* ratio 0.5 AU/g protein and pH 8. The results are presented graphically in Figs. 28A and 28B.

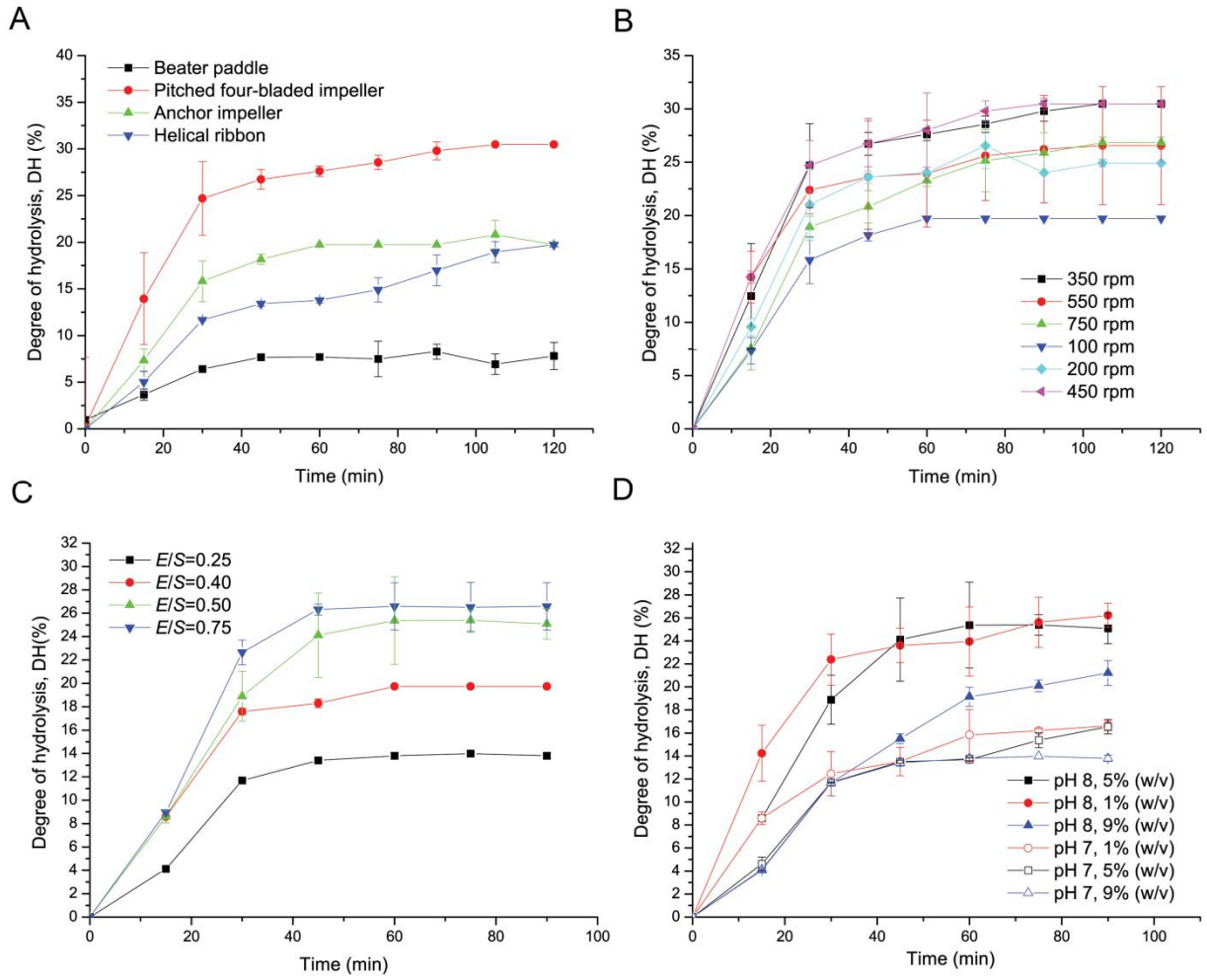


Figure 28. The effects of critical operational and reaction parameters such as (A) impeller geometry; (B) agitation speed; (C) *E*/*S* ratio and (D) gluten concentration at pH 7 and 9; on degree of hydrolysis (DH).

It appeared that the degree of hydrolysis was strongly affected by impellergeometries. Native gluten has poor water dispersibility and normally forms aggregates inwater (Peterson et al., 2011). Thus, problems could arise related to poor mixing of thisdispersion, such as poor distribution of the enzyme and/or difficulties with temperature andpH control, resulting in a possible reaction rate decrease. Axial flow impellers like helicalribbon impeller seem to be suitable for the dispersion mixing processes since they provideaxial discharge of gluten particles by producing strong top to bottom motion and have theability to keep the entire vessel contents circulating (Delaplace

et al., 2000). Furthermore, anchor impeller generates both tangential and axial motion, which enhances the mixingefficiency and avoids the stagnation of the products at the vessel walls, since the anchor bladeworks as a scraper. This shear near the vessel wall reduces the formation of stagnant zonepromoting heat and mass transfer. On the other hand, beater paddle provides radial flow andhigh shear conditions, which could cause low reaction rate and an extensive enzymedeactivation (Jakovetić et al., 2015). In this study, the pitched four-blade impeller appeared to be the most favorable impeller amongst compared for the gluten hydrolysis since thehighest DH of 30.47 was achieved after 105 min. On the other hand, the achieved DH was only 7.82±1.44% after 120 min with the paddle. Although large-diameter impellers likeanchor and helical impellers (diameters are 90 and ≈94% of the interior vessel diameter,respectively) have been successfully used to reduce shear damage and improve mixing inviscous suspensions, the highest initial reaction rate (0.84 min^{-1}) here in was achieved with thepitched four-blade impeller. This impeller pumped in a radial and axial direction with a wide discharge steam suitable for mixing of off-bottom suspension of solid particles, providing theshear conditions necessary to disperse gluten particles. It seemed that it provided properbalance between requirements for adequate mass/heat transfer and low shear stress, allow in a higher degree of hydrolysis for shorter reaction time compared to other impellers. Because no studies have been found that considered the effect of different impeller geometries one nzymatic gluten hydrolysis it was difficult to compare results of mixing studies with otherresults in the literature. The effect of the impeller speed was further studied in the batch stirred tank with thepitched blade impeller. As shown, the hydrolysis of gluten was strongly affected by agitation speed, with the degree of hydrolysis after 120 minutes reaching 19.73, 24.93, 30.47, 30.47,26.56±5.53, and 26.85±0.50 for 100, 200, 350, 450, 550, and 750 rpm respectively (Fig. 28B). When the agitation speed increased from 350 to 450 rpm, the DH and reaction rate increasednon-significantly ($p \geq 0.05$). At impeller speeds lower than 350 rpm, an increase in agitationspeed apparently improved the efficiency of gluten hydrolysis. Insufficient mass and/or heattransfer as well as inhomogeneous enzyme distribution associated with poor mixing couldexplain the decrease in DH at lower agitation speed. Furthermore, intensive mixing could cause the particle size reduction and thus, an increase of surface area, which

was accompanied with an increased adsorption of alcalase on gluten microparticles suspended inwater solution. However, the DH decreased at higher agitation speed, which may be connected to the rapid initial foaming. As previously observed for the hydrolysis of gluten fraction with pepsin in a batch stirred reactor, the DH was also proportional to the impellerspeed at a low range of stirring speed (Nouriet al., 1997). However, the DH was affected by the formation of foam generated by the high impeller rotational speed (750 rpm) and favored by foaming properties of the peptides obtained by enzymatic hydrolysis.

Several experiments have also been carried out to analyze the influence of the initial*E*/*S* ratio, gluten concentration and pH of the reaction mixture on the hydrolytic reaction andthe results are presented in Figs. 28C and 28D. The *E*/*S*ratio in the range from 0.25 to 0.75 AU/gwas varied first while all other conditions (agitation speed of 200 rpm, gluten concentrationof 5% ($w_{protein}/v$), pH 8, 50 ^{o}C) were unaltered. Unlike typical hydrolysis time courses, asigmoid curve was obtained at almost all *E*/*S* ratio. The positive influence of the *E*/*S* ratio onthe gluten hydrolysis was apparent, but enzyme cost and process productivity requiredfinding the optimal *E*/*S* ratio, which was the subject of future research. It appeared that therate, extent, and pattern of gluten hydrolysis also varied according to the gluten concentration (Fig. 28C). The reaction with the lowest gluten concentration of 1% followed the typicalhyperbolic hydrolysis time courses at both pH 7 and 8. Namely, the reaction proceeded at arapid rate during the initial 30 min with a slow increase in hydrolysis rate for the next 60 min, and then entered the steady state. However, in the case of higher gluten concentrations, thetime courses curves were of sigmoid form (Fig. 28C) as a sharp increase in the degree ofhydrolysis was observed after 15 min. This increase may be apparent due to an increase in thesolubility of gluten as the reaction was extended and similar findings were reported by Wanget al. (2007), where gluten hydrolysis was performed using papain. The highest DH was achieved with the lowest gluten concentration and higher pH value. These results may be connected to an increased product and/or substrate inhibition, insufficient mixing, sterich indrance and lack of cleavage sites at higher gluten concentration. The low solubility of gluten at neutral pH might have contributed to low DH obtained and this was pronounced athigher gluten concentration (Takeda et al., 2001).

7.3. Optimization of the process parameters regarding functional properties of wheat gluten hydrolysates

Usually, the optimization of enzymatic reactions involves varying one parameter at a time, while keeping the others constant. Such approach does not offer insight in the existence and the nature of interactions between factors. On the other hand, statistical tools including response surface methodology (RSM) and experimental design are very useful, not only in process optimization, but also in explaining qualitatively and quantitatively the relationship between the important reaction parameters.

In the thesis, the effects of some relevant process parameters for the alcalasecatalyzed gluten hydrolysis and their interactions were investigated by the means of the four-factor Box-Behnken experimental design with 29 experimental points (5 central points) as show in Table 9. Independent variables and their range that have been considered include gluten concentration (1–9% $w_{protein}$/w), temperature (40–60 °C), pH (7–9), and *E*/*S* ratio (0.25–0.75 in AU activity units per g protein). The stirring rate (200 rpm) and running time (2 h) were kept constant. The degree of hydrolysis and several functional properties including solubility, foam capacity and stability as well as emulsifying activity and stabilitywere set as response variables.

Table 9. Experimental setup for three-level, four-factor Box-Behnken design in terms of coded, actual values of variables and experimental data of degree of hydrolysis, emulsion activity index and emulsion stability index, solubility, foam capacity and stability.

No.	Nivoi promenljivih				*DH*, %	*EAI*, m^2/g	Solu-bility	*ESI*, h	*FC*, %	*FS*, %
	X_1 **A**	X_2 **B**	X_3 **C**	X_4 **D**						
1.	1(-1)	40(-1)	8(0)	0.5(0)	27.32	2245.20	86.69	19.42	**80.31**	7.407
2.	9(1)	40(-1)	8(0)	0.5(0)	19.29	1670.07	85.59	43.42	50.98	0.000
3.	1(-1)	60(1)	8(0)	0.5(0)	25.41	786.51	97.83	45.73	78.26	13.79

4.	9(1)	60(1)	8(0)	0.5(0)	28.34	763.81	77.43	36.71	68.94	29.55
5.	5(0)	50(0)	7(-1)	0.25(-1)	09.29	1000.58	88.92	55.62	69.69	15.25
6.	5(0)	50(0)	9(1)	0.25(-1)	24.75	826.53	85.18	43.02	67.53	**35.06**
7.	5(0)	50(0)	7(-1)	0.75(1)	15.51	1509.56	94.44	61.73	71.59	15.25
8.	5(0)	50(0)	9(1)	0.75(1)	29.52	1140.53	93.40	76.05	69.69	33.33
9.	1(-1)	50(0)	8(0)	0.25(-1)	25.16	2236.84	92.47	54.35	37.50	13.79
10.	9(1)	50(0)	8(0)	0.25(-1)	21.16	1115.96	77.02	48.80	67.10	34.21
11.	1(-1)	50(0)	8(0)	0.75(1)	27.21	2737.97	55.75	**86.07**	34.21	10.71
12.	9(1)	50(0)	8(0)	0.75(1)	26.60	1366.36	84.42	83.32	57.62	19.35
13.	5(0)	40(-1)	7(-1)	0.5(0)	8.85	1550.32	**98.85**	46.33	59.01	24.24
14.	5(0)	60(1)	7(-1)	0.5(0)	17.14	1525.68	89.98	77.64	62.12	7.40
15.	5(0)	40(-1)	9(1)	0.5(0)	25.16	840.67	85.18	59.90	34.21	16.66
16.	5(0)	60(1)	9(1)	0.5(0)	31.01	1050.91	89.99	48.89	37.50	21.87
17.	1(-1)	50(0)	7(-1)	0.5(0)	16.32	**2782.92**	78.69	67.64	32.43	10.71
18.	9(1)	50(0)	7(-1)	0.5(0)	13.60	1233.65	86.35	53.01	67.53	10.71
19.	1(-1)	50(0)	9(1)	0,5(0)	**31.50**	2038.42	94.82	60.83	32.43	7.40
20.	9(1)	50(0)	9(1)	0,5(0)	28,72	1129.60	83.74	77.05	39.02	24.24
21.	5(0)	40(-1)	8(0)	0.25(-1)	17.20	1795.31	90.78	45.28	24.24	7.40
22.	5(0)	60(1)	8(0)	0.25(-1)	19.90	798.37	86.38	59.68	39.02	7.40
23.	5(0)	40(-1)	8(0)	0.75(1)	22.04	1137.33	87.32	58.09	56.14	26.47
24.	5(0)	60(1)	8(0)	0.75(1)	27.20	1352.42	81.90	60.95	43.18	19.33
25.	5(0)	50(0)	8(0)	0.5(0)	24.76	1281.71	91.41	71.09	45.24	24.14
26.	5(0)	50(0)	8(0)	0.5(0)	25.16	970.57	86.42	21.49	44.86	24.67
27.	5(0)	50(0)	8(0)	0.5(0)	24.93	1124.35	88.94	54.23	44.97	23.34

28.	5(0)	50(0)	8(0)	0.5(0)	25.01	1058.32	89.41	67.52	45.64	23.38
29.	5(0)	50(0)	8(0)	0.5(0)	24.75	991.46	90.08	70.21	45.11	25.09

*Experiments from 26-29 presented central points.

7.3.1. Response surface modeling for *DH*

Based on the results from the RSM analysis, the final second-order polynomial model wasdeveloped to describe the degree of hydrolysis (DH) by considering only the significantcoded terms:

$$Y_1 = 24.92 - 1.36 \cdot x_1 + 2.33 \cdot x_2 + 7.50 \cdot x_3 + 2.55 \cdot x_4 + 3.02 \cdot x_1x_2 + 1.54 \cdot x_1^2 - 1.18 \cdot x_2^2 - 3.51 \cdot x_3^2 - 1.74 \cdot x_4^2 \quad (15)$$

Significant model terms included gluten concentration, temperature, pH and *E*/*S* ratio in theirlinear and quadratic terms. It appeared that only gluten concentration and temperatureshowed strong interaction effect whereas the interactions among other parameters wereinsignificant. The coefficient of determination (R^2) is of 0.981, which indicated an adequateadjustment of the experimental data, showing that more than 98% of the data variability wasexplained in the proposed empirical equation.

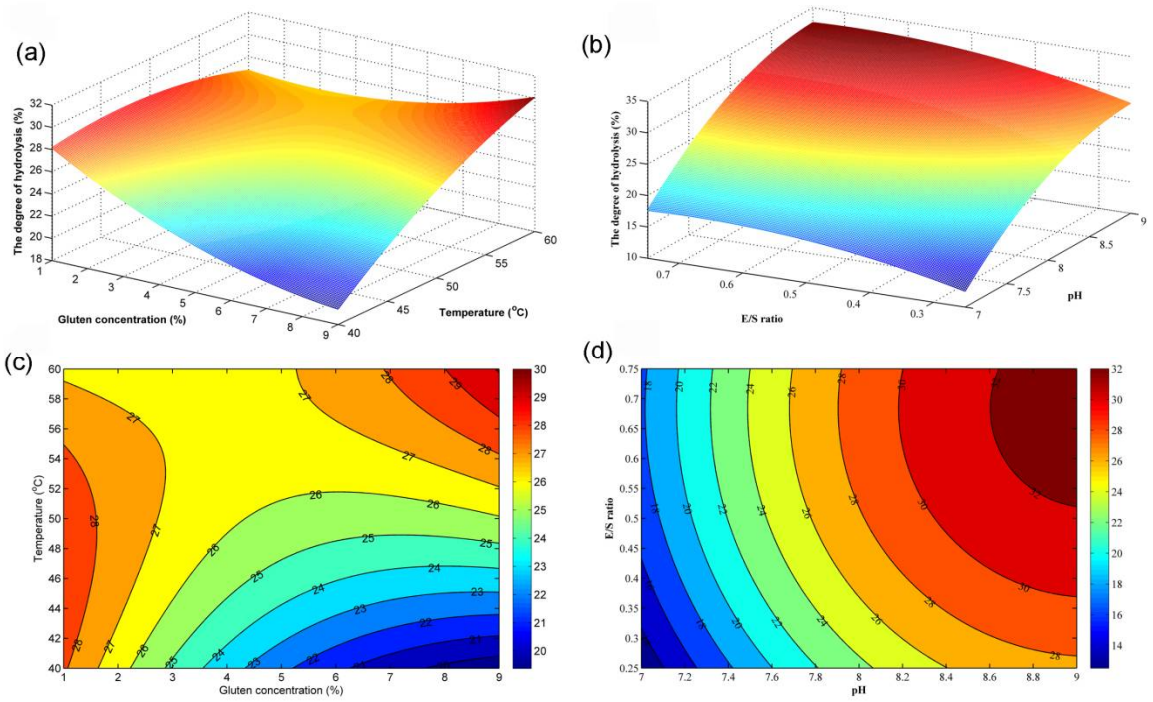


Figure 29. Response surface and contour plots showing the effect of (a) and (c) gluten concentration, $x_1$and temperature x_2, respectively as well as (b) and (d) *E*/*S* ratio, $x_4$and pH, $x_3$on the degree of hydrolysis.

The shape of the three-dimensional surface-representing degree of hydrolysis versusgluten concentration and temperature is shown in Fig. 29a. It is apparent an increase/decreasein one axis and decrease/increase in the other axis, revealing that both gluten concentration and temperature may affect reaction rate in opposite ways. The DH gradually increased astemperature increased at higher gluten concentrations, suggesting that at those conditions thekinetic effect was predominant. However, at lower gluten concentration, as the temperature increased, the expected increase in reaction rate resulting from more productive molecule collisions per unit time was offset by the increasing rate of enzyme denaturation. At glutenconcentration of 1%, the DH initially slightly increased with the temperature, passing througha maximum at around 50 ^{o}C and then decreased. This was probably due to the enzymedenaturation at higher temperature which was pronounced at lower substrate concentration. The highest degree of hydrolysis of 30.08% was achieved at

60^{o}C and high level of glutenconcentration of 9% (Figs. 29a and 29c). On the other hand, the DH increased with an increase in pH at any given temperature (data not shown) or *E*/*S* ratio (Figs. 29b and 29d). Negative signs of regression coefficients of quadratic terms of pH and *E*/*S* ratio emphasized the existence of local maximum of DH. Asexpected, higher degrees of hydrolysis were achieved at higher studied [*E*]/[*S*] ratio.However, when the process productivity was analyzed (mg of obtained protein divided by theadded enzyme activity units in the system and reaction time), it seemed that the highest productivity of 25.44 mg/(AU h) was obtained at the lowest *E*/*S* ratio of 0.25 (data not shown).

On the other side, the influence of pH was much greater than the influence of the *E*/*S*ratio. For an illustration, at pH 9, the DH varied from 27.2 to 32.3% on increasing the *E*/*S* ratio from 0.25 to 0.75 AU/g. On the other side, at *E*/*S* ratio of 0.5, it even increased from13.9 to 28.9% with pH increase from 7 to 9, indicating that a rater high DH and processproductivity were possible with small amounts of alcalase at high pH value, which could bebeneficial from the economic viewpoint (Fig. 29b). The obtained degrees of hydrolysis after 90 min varied from 8.85 to 31.01 depending on reaction conditions, as shown (Table 9).

This data was similar or higher than the results reported previously by several reports, in which authors reported DH values widely ranging from 4.7 to 26% after 3.5 to 24 h for alcalase-catalyzed reaction, depending on reaction system, operating and reaction conditions (Gottardi et al., 2014; Kong et al., 2007; Koo et al., 2014). For example, Zhang et al. (2010) found that the batch hydrolysis of gluten by alcalase was considerably enhanced by the addition of very small amounts of cysteine. This might be explained by the effect of the cysteine on the structural and rheological properties of wheatgluten, altering a typical gluten viscoelastic behaviour (ranging from more solid-like to more fluid-like) and increasing its solubility. The increase in DH% observed herein can be attributed to a properly designed reactorsetup and efficient mixing. Namely, the batch-wise enzymatic hydrolysis of wheat gluten often carried out in the absence of mechanical agitation, resulting in substrate and product concentration gradients and mass transfer limitations.

7.3.2. Response surface modeling for functional properties

The results of the study of the influence of the four selected process parameters of the wheat gluten hydrolysis reaction on the functional properties of the gluten protein hydrolysate (solubility, foam capacity (*FC*) and stability (*FS*) as well as emulsion activity index and emulsion stability index) are shown in Table 9. The results of the second-order response surface modelsobtained by analysis of variance (ANOVA) representing the empirical relation between these functional properties (responses: Y_1-solubility; Y_2-emulsion activity index; Y_3- emulsion stability index; Y_4- foam capacity and Y_5-foam stability) and variables like gluten concentration, x_1, temperature x_2, *E*/*S* ratio, x_4 and pH, x_3 are presented in Table 10. Hydrolysis of wheat gluten by alcalase was optimized regarding several functional properties and five response equations were obtained, making it possible to predict selected functional properties from known values of the four main factors.

Table 10. Response equationsfor five selected functional properties

Response functions	**The empirical second-order polynomial equations with significant factors**
Solubility	$\boldsymbol{Y_1} = 89.33 - 0.98 \cdot A - 0.91 \cdot B - 0.41 \cdot C - 1.96 \cdot D + 11.03 \cdot A \cdot D - 5.93 \cdot A^2$
Emulsion activity index, *EAI*	$\boldsymbol{Y_2} = 1151{,}54 - 622{,}34 \cdot A - {,}123 \cdot B - 208{,}27 \cdot C + 18{,}05 \cdot D + 301{,}60 \cdot A \cdot B + 457{,}11 \cdot A^2 + 229{,}57 \cdot D^2$
Emulsion stability index, *ESI*	$\boldsymbol{Y_3} = 46{,}72 + 1{,}91 \cdot A + 5{,}13 \cdot B - 0{,}11 \cdot C + 9{,}13 \cdot D - 8{,}26 \cdot A \cdot B + 7{,}71 \cdot A \cdot C + 4{,}37 \cdot A \cdot D - 10{,}58 \cdot B \cdot C - 4{,}00 \cdot B \cdot D + 6{,}51\, C \cdot D - 2{,}82 \cdot A^2 - 5{,}12\, B^2 + 11{,}79\, C^2 + 9{,}98\, D^2$

Foam capacity, *FC*	$\boldsymbol{Y_4} = 45{,}16 + 4{,}67 \cdot A + 2{,}01 \cdot B - 6{,}83 \cdot C + 2{,}28 \cdot D + 5{,}00 \cdot A \cdot B - 7{,}13 \cdot A \cdot C - 1{,}55 \cdot A \cdot D + 0{,}046 \cdot B \cdot C - 6{,}93 \cdot B \cdot D - 0{,}068\ C \cdot D + 4{,}87 \cdot A^2 + 3{,}31\ B^2 + 4{,}42\ C^2 + 3{,}76\ D^2$
Foam stability, *FS*	$\boldsymbol{Y_5} = 24{,}23 + 4{,}64 \cdot A + 1{,}57 \cdot B + 4{,}58 \cdot C + 0{,}70 \cdot D + 5{,}45 \cdot A \cdot B + 4{,}21 \cdot A \cdot C - 2{,}94 \cdot A \cdot D + 5{,}51 \cdot B \cdot C - 2{,}53 \cdot B \cdot D - 0{,}0{,}43\ C \cdot D - 6{,}53 \cdot A^2 - 6{,}20\ B - 1{,}68\ C^2 - 0{,}63\ D^2$

A: X_1- substrate concentration (gluten); *B*: X_2-temperature of the hydrolysis reaction; C: X_3-pH reaction of hydrolysis; D: X_4-enzyme-substrate ratio [*E*]/[*S*].

The results of the second-order response surface model were examined by analysis of variance (ANOVA) and Fischer's *F*-test. Based on the obtained response surfaces (Figures 30-34), it is easier to analyze the effect of temperature, pH of hydrolysis, as well as the concentration of gluten and *E*/*S* ratio on the observed responses. The fit of the models was checked by the R^2, which was calculated to be in the range of 84.4 to 98.1, indicating that 84.4-98.1% of the variability in the response could be explained by the model (Table 11). The models also showed statistically insignificant lack of fit, as is evident from the lower calculated *F* values than the theoretical *F* value at 5% level. Results obtained by the statistical analyses are shown in Table 11.

Table 11. Results obtained by the statistical analyses (ANOVA)

Response functions	Determination coefficient R^2	Probability (p-value)	Fisher test, F - value	Coefficient of variation (CV)	Lack of fit	Adequate precision
Y_1 – Solubility	0.9440	<0.0001	2.93	7.78	0.0679	7.82
Y_2 –Emulsion activity index (EAI)	0.9915	<0.0001	46.3	4.42	0.0676	36.214
Y_3 –Emulsion stability index(*ESI*)	0.9651	<0.0001	23.76	4.31	0.4824	41.497
Y_4 –Foam capacity (*FC*)	0.8440	<0.0001	4.64	4.64	0.0529	17.232
Y_s –Foam stability (*FS*)	0.9812	<0.0001	6.25	3.71	0.0517	18.416

7.3.2.1. Influence of process parameters on the solubility

The solubility is an important aspect that needs to be considered due to its considerable effect on other techno-functional properties and quality of the end hydrolysate. For example, to obtain optimum functionality in foods that requires gelation, emulsifying, and foaming properties, a highly soluble protein is desirable.

Figure 30 shows the statistically analyzed influence of gluten concentrations, *E*/*S* ratio and pH in 3-dimensional graph. The obtained solubility model, characterized by the coefficient of determination R^2 = 0.9440, proved to be adequate (p<0.05), with only 5.6% of the total number of variations that can not be explained by the model.

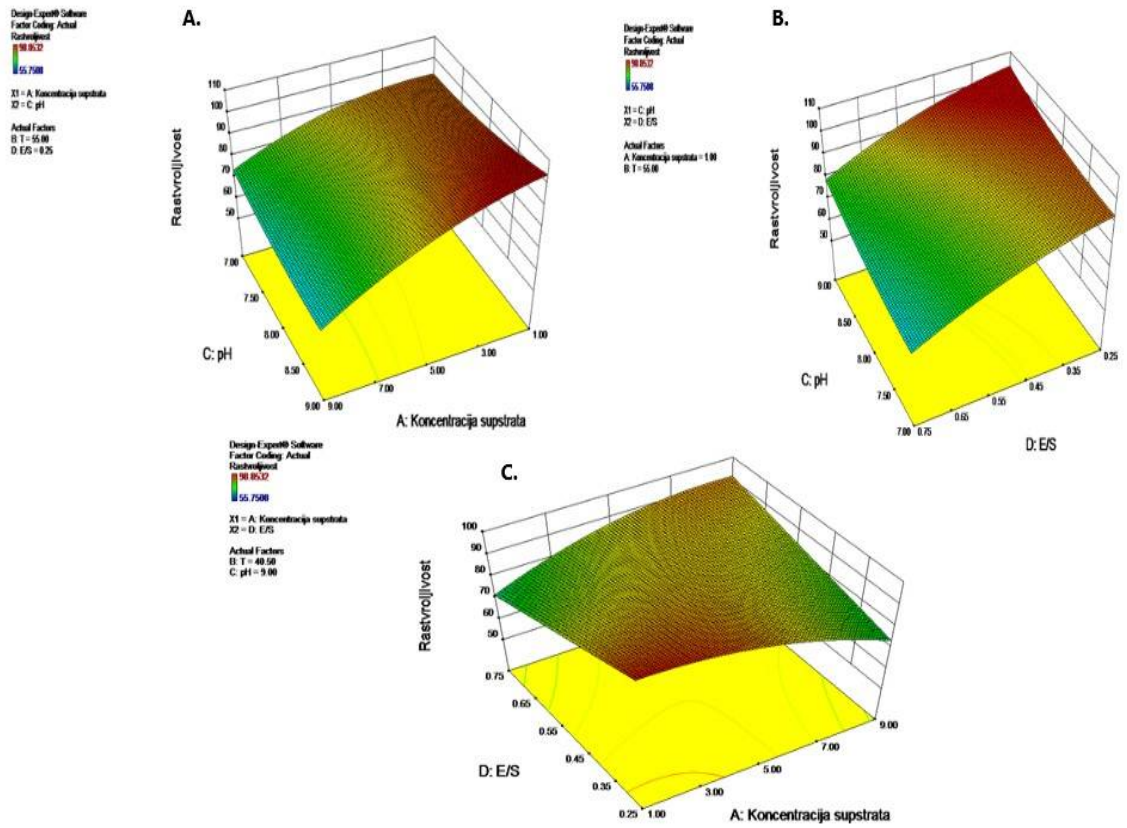


Figure 30. Response surface and contour plots showing the effect of A) gluten concentration and pH, B) pH and *E*/*S* ratio and C) *E*/*S* ratio and gluten concentration on the solubility.

The analysis reveals that all tested variables have a significant effect on the solubility, within the experimentally tested ranges. The most relevant variable seems to be *E*/*S* ratio, but the effects of gluten concentration, pH, temperature and gluten concentration and *E*/*S* interactions are also significant. The quadratic term of gluten concentration is also significant, indicating that a response is a quadratic function with a local maximum.

From Fig. 30A, it can be noticed that the highest solubility of the hydrolysate (~ 100%) is achieved at a pH of 9.0 and the substrate concentration of 1%. The results are verified at an elevated temperature of 55 ° C and a low ratio [*E*]/[*S*] (0.25 AU / g of gluten). From Fig.30B it can be noticed that the highest solubility of the hydrolyzate (~ 100%) is achieved at a high pH value of 9.0 and a low [*E*]/[*S*] ratio (0.25 AU / g of gluten).

It can be concluded that the high solubility of the wheat gluten hydrolysates is achieved at a low temperature of the reaction of enzymatic hydrolysis (40 ° C) and a pH of 9, using minimal amounts of substrate and a rather low amount of enzymes, since the optimal [*E*]/[*S*] ratio seems to be 0.5 AU/g of gluten. Based on both results obtained for DH and presented results (Fig. 30), it is obvious that the enzymatic hydrolysis of wheat gluten has improved gluten solubility since the positive correlation between the DH and solubility is observed. Thus, the enzymatic hydrolysis influenced the reduction of the molecular weight and the hydrophobicity of wheat protein as well as the increased content of polar and ionizing groups, resulting in a significant increase in the solubility. In general, it is apparent that an increase in the degree of hydrolysis has been influenced by the appearance of smaller gluten peptides with the content of a large number of polar amino acid residues relative to non-hydrolyzed gluten, and consequently stronger hydrogen bonds with water molecules could be formed and become more soluble in aqueous solutions.

7.3.2.2. Influence of process parameters on the emulsion activity index and emulsion stability index

The influence of the independent variables (gluten concentration, temperature and pH reaction, [*E*]/[*S*] ratio) on the emulsifying properties of wheat protein hydrolysates prepared by alkalase is given in Figs. 31 and 32. The presented results are given as emulsion activity index (*EAI*) and emulsion stability index (*ESI*). The activity of creating protein emulsions is the ability of proteins to participate in the process of emulsion generation, and *EAI* represents the size of the surface of an intermediate oil-water that can stabilize one gram of protein, while *ESI* represents the time during which the protein can continue on the surface of the oil-water and affects the maintenance of stability of the formed emulsions.

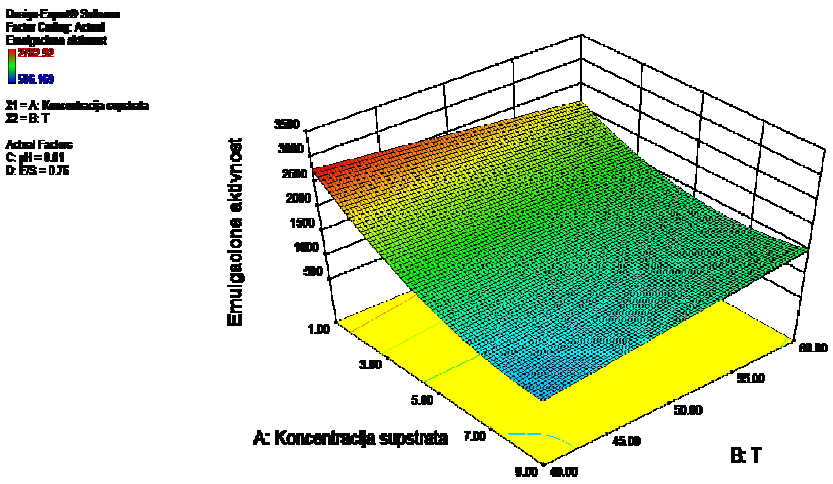


Figure 31. 3-D response surface showing the effect of temperature, x_2 and gluten concentration, x_1 on the emulsion activity index.

Fig. 31 shows the response surfaces of the effect of temperature, x_2 and gluten concentration, x_1 on the emulsion activity index. The obtained model, characterized by the coefficient of determination R^2 = 0.9915, proved to be adequate (p<0.01), with only 0.85% of the total number of variations that can not be explained by the model. The analysis reveals that all tested variables have a significant effect on the *EAI*, within the experimentally tested ranges. The most relevant variable seems to be interaction of the gluten concentration and temperature (p<0.0001), thus this interaction is graphically presented (Fig. 31). The quadratic terms of gluten concentration and temperature are also significant, indicating that a response is a quadratic function with a local maximum.

It appears that the highest *EAI* of 2782.9 m²/g is achieved at pH 8.0 and at a temperature of 50 °C, [*E*]/[*S*] of 0.75 AU /g gluten and a substrate concentration of 1%. It is generally accepted that the protein hydrolysates, also gluten hydrolysates, exhibit better emulsifying properties than the native proteins, due to higher exposure of hydrophobic amino acid residues to the surface of protein molecules, but this depends on the size of protein chains. For emulsifying activities and the possibility of protein migration to the oil-water surface, protein chains with a molecular weight greater than 10 kDa are needed, as they contribute to the production of better emulsions than chains of less molecular weight.

Fig. 32 shows the effect of the influence of the tested independent variables on the emulsion stability index. The high value of the coefficient of determination of 0.9651 indicates that the second-order polynomial model perfect fits the experimental results for the *ESI*, since only 3.49% of the variation could not be desribed by the model. Parameters X_1, X_2, X_3, X_4 and their interactions like X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4, as well as X_1^2, X_2^2, X_3^2, X_4^2 are significant at the level of 0.05 (p<0.05).

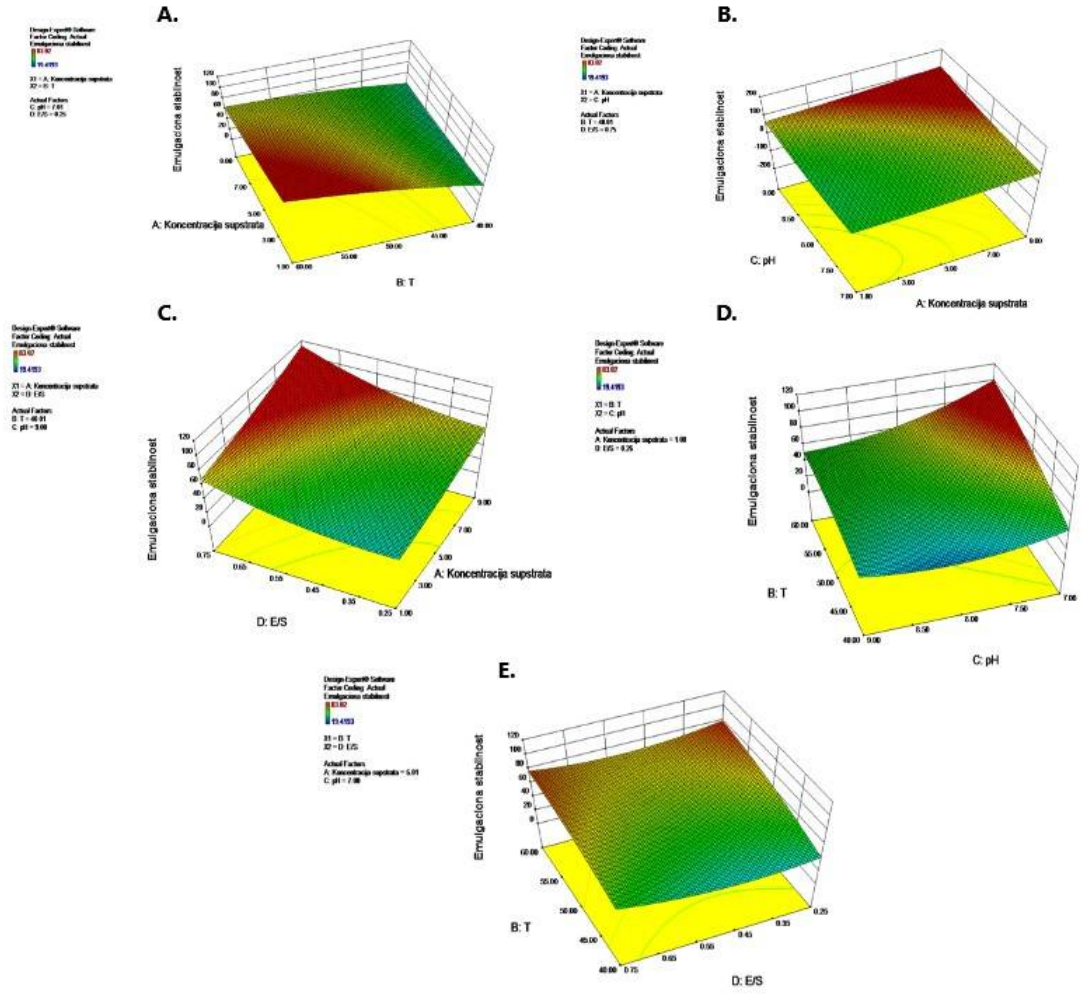


Figure 32. 3-D response surfaces showing the effect of A) temperature, x_2 and gluten concentration, x_1; B) pH, x_3 and gluten concentration, x_1; C) *E*/*S* ratio, x_4 and gluten concentration, x_1; D) pH, x_3 and temperture, x_2; and E) *E*/*S* ratio, x_4 and temperturex_2 on the *ESI*.

Fig. 32A shows that the greatest emulsifying stability of protein hydrolyzate of 70% was achieved at t = 58 °C, substrate concentration of 5.2%, and at [*E*]/[*S*] of 0.25 AU/ g of gluten and pH 7.0. It was also apparent from Fig. 32B that the emulsifying stability of protein hydrolysates was in almost linear functional dependence of pH and substrate concentration, wherein the maximum of *ESI* of 105 h was achieved at pH 9.0 and the substrate concentration of 9.0%.

Overall, it can be concluded that after enzymatic hydrolysis, the peptides "unfold" and hydrophobic regions become more accessible, and consequently facilitated better orientation towards the oil/water interface improving the emulsifying properties of the gluten hydrolysates. In addition, the increased hydrophobicity due to enzymatic hydrolysis could be also a reason for the high stability of hydrolysates, since the hydrophobic interactions play an important role in the formation of the adsorption layer at the phase boundary, whose strength depends on interaction (hydrophobic and electrostatic interaction) of protein molecules and oil droplets. As more protein molecules are adsorbed at the boundary surface, the interactions of the oil droplets which contribute to the collapse of the emulsion structure are less likely.

Previous studies have shown that the controlled and mild enzymatic hydrolysis could be successfully applied to improve and upgrade the functional properties of gluten. However, excessive hydrolysis affects the functional properties of the hydrolysates negatively and may reduce their application for human consumption. For example, it has been reported that the solubility of the hydrolysates obtained with a fungal protease from *Aspergillus oryzae* increases as the DH increases, but foaming properties seem to be impaired beyond a certain DH (14%) (Drago et al., 2001). The authors concluded that a minimum protein structure has to be retained, in order to keep good foaming properties. In another study, the authors have stated that the DH should be even lower, <5% in order to obtain desirable emulsifying and foaming properties (Kong et al. 2007). Thus, the degree of hydrolysis should be controlled because the *ESI* or *EAI* increase with increasing the degree of hydrolysis (decreasing peptide chain length) up to a critical value after which the efficiency decreases.

7.3.2.3. Influence of process parameters on the foam capacity and stability

The foaming ability of the proteins and their hydrolysates is one of the most important technological-functional properties. The basic preconditions that a protein and/or protein hydrolysates have the ability to form a foam is to quickly adsorb during the mixing process at the water-air interface, then quickly change their conformation and the distribution of the functional groups on the surface of the molecules, as well as having the possibility of forming a cohesive viscous-elastic film using intermolecular interactions. The obtained results of the foaming ability of the wheat gluten hydrolysate prepared by alkalase are presented as foam capacity (*FC*) and foam stability (*FS*) and are presented in Figs. 33 and 34.

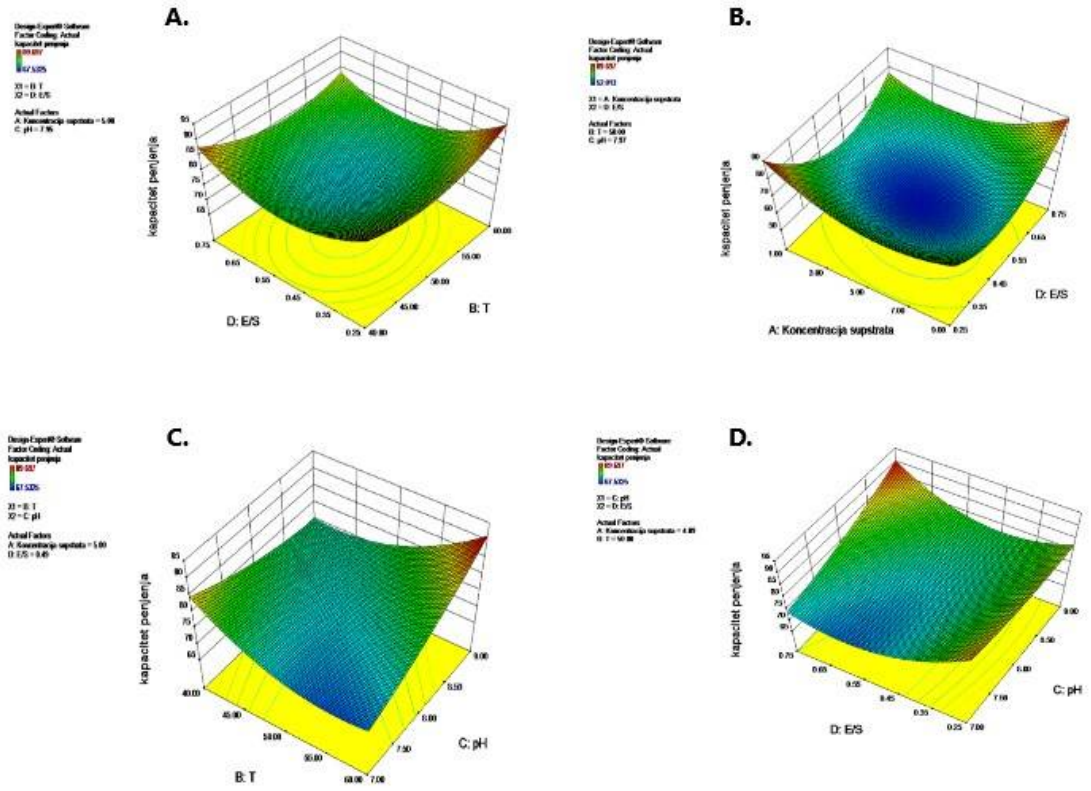


Figure 33. Response surface showing the effect of A) temperature and *E*/*S* ratio; B) *E*/*S* ratio and gluten concentration; C) pH and temperature; and D) pH and *E*/*S* ratio on the foam capacity.

The obtained results reveal that the foam capacity range of the wheat gluten hydrolysates are in the range of 24.2-80.3% (Table 9), depending on the independent variables that were tested. Overall, it is apparent that the gluten hydrolysates have a relatively good foam capacity, since in a large number of experiments this ability ishigher than 50%.

Fig. 34 shows the effect of the tested independent variables on the stability of the gluten hydrolysate foam. The value of the coefficient of determination of 0.9812 indicates that the fitted second-order polynomial well approximates the experimental results for the interval tested since only 1.88% of the variation could not be ascribed by the model. Also, the goodness of fit and significance of the model (p <0.05) are confirmed. All parameters, X_1, X_2, X_3, X_4 and interactions like X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4, as well as quadratic terms $X_1^2X_2^2$, X_3^2, $X_4^2$are significant at the level of 0.05 (p<0.05).

It seems that the most relevant variables for the *FSI* are gluten concentration and pH with estimated effects of 4.64 and 4.58, respectively. The effects of temperature and *E*/*S* ratio are also significant (p<0.05). It appears that while substrate concentration and all other parameters have a positive effect, the gluten concentration and *E*/*S* ratio interaction has a significant negative influence on *FSI*.

According to the statistical analysis, the maximum *FSI* can be obtained at high level of gluten concentration and pH value. Among the various treatments, the highest *FSI* of 35.06% is achieved in run no. 6 (gluten concentraton of 5%, 50^{o}C, pH 9 and *E*/*S* ratio 0.25). Experimental values for FSI are found to be in good agreement with predictions.

The effects of parameters on the *FSI* and their interactive effectsare shown in Fig. 34.

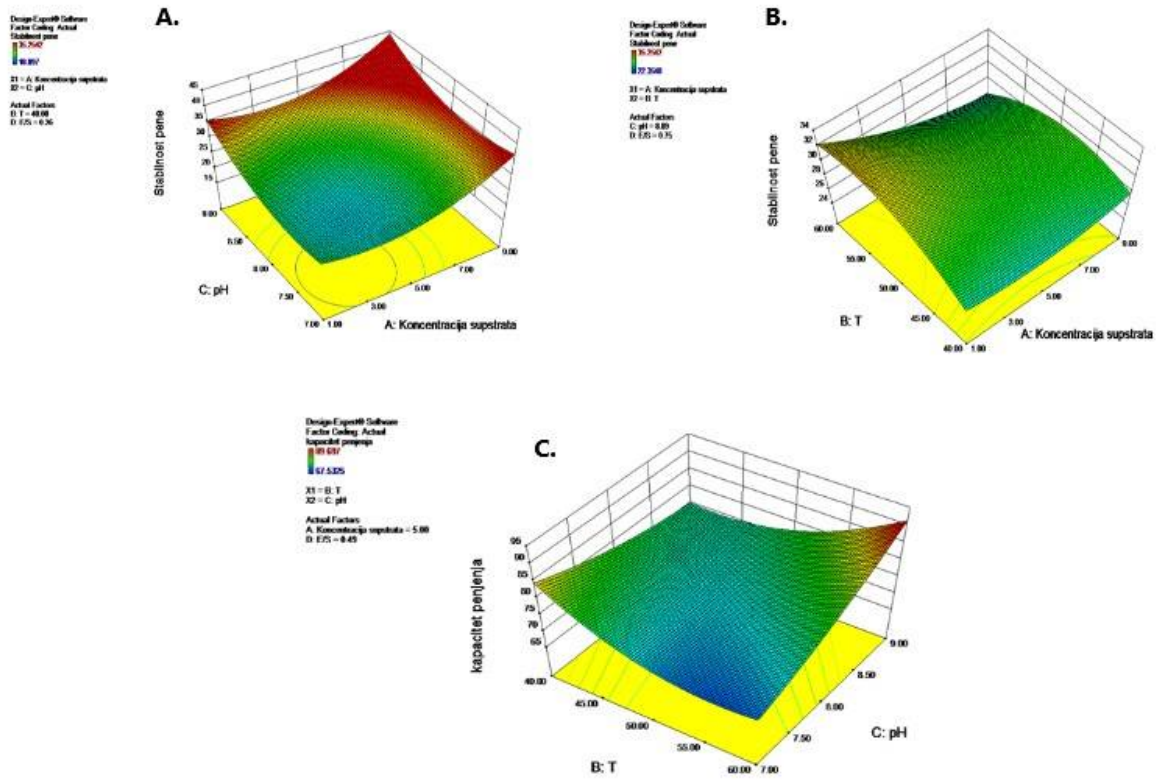


Figure 34. Response surface showing the effect of A) gluten concentration and pH; B) gluten concentraton and temperature;and C) pH and temperature on the foam stability.

Temperature showed an interactive effect with pH. The shape of the three-dimensional surface-representing *FSI versus* temperature and pH is shown in Fig. 34C. It appears that the surface is smooth showing increase/decrease in one axis and decrease/increase in the other axis, which reflect that the temperature may affect reaction rate in opposite ways. Specifically, as the temperature increases, the expected increase in reaction rate resulting from more productive molecule collisions per unit time is offset by the increasing rate of enzyme denaturation at higher pH value. At intermediate and low levels of pH, however, a different behavior is observed as the surface increses when reaction temperature increases. This could be result of a negative temperature-pH interaction, probably caused by thermal inactivation of enzyme which is more pronounced

at higher pH value. The maximum yield of *FSI* could be obtained when working at low temperatures and high level of pH.

7.4. Response surface modeling for antioxidant properties

The aim of this thesis was to achieve a high degree of hydrolysis but also to establish an efficient process for production ofan end gluten hydrolysate with improved antioxidant capacity. Thus, the effect of operating parameters: gluten concentration, temperature, pH and [*E*]/[*S*] ratio on the antioxidant properties of the hydrolysates was also investigated using Box-Benken experimental design and response surface methodology. To our knowledge, this is the first study focusing on the relationship between reaction conditions, DH and antioxidant properties of the gluten hydrolysates, measured by two methods.The data showing the predicted and experimental DH as well as antioxidant activity measured by two methods for the 29 experiments of the statistical design are presented in Table 12.

The coefficients of the response function and their statistical significance were evaluated by the response surface regression analysis, using the softver. Non-significant terms ($p \geq 0.05$) were deleted from the second order polynomial and a new polynomial has been recalculated. The Fisher test (F value) was used to determine whether the second-order model was adequate to describe the obtained data while the goodness of fit of the model was evaluated by the determination coefficient (R2). The results of the response surface regression analysis are presented in Table 13.

Table 12. Box-Benken experimental design with real values for the variables and responses for degree of hydrolysis (DH%) and DPPH and ABTS antioxidant activities of gluten hydrolysates.

Run No.	RSM experimental variables				Experimental responses		
	X_1	X_2	X_3	X_4	DH (%)	DPPH (%)	ABTS (%)
1	1	40	8	0.5	28.45	47.61	74.09
2	9	40	8	0.5	19.29	82. 71	89.73
3	1	60	8	0.5	25.41	35.96	61.65
4	9	60	8	0.5	28.34	56.35	91.47
5	5	50	7	0.25	9.29	89.89	95.73
6	5	50	9	0.25	24.75	48.23	87.99
7	5	50	7	0.75	15.51	87.23	95.58
8	5	50	9	0.75	29.52	54.43	99.42
9	1	50	8	0.25	25.16	37.41	70.46
10	9	50	8	0.25	21.16	82.27	98.89
11	1	50	8	0.75	27.21	55.14	92.73
12	9	50	8	0.75	26.6	64.36	99.53
13	5	40	7	0.5	8.85	82.99	95.26
14	5	60	7	0.5	17.14	88.12	84.11
15	5	40	9	0.5	25.16	71.10	78.67
16	5	60	9	0.5	31.01	18.08	92.96
17	1	50	7	0.5	16.32	52.84	85.47
18	9	50	7	0.5	13.60	94.50	97.16
19	1	50	9	0.5	31.5	35.22	74.41
20	9	50	9	0.5	28.72	49.38	97.79
21	5	40	8	0.25	17.2	72.69	91.94
22	5	60	8	0.25	19.9	53.41	77.09
23	5	40	8	0.75	22.04	82.00	92.68
24	5	60	8	0.75	27.20	58.16	99.37
25	5	50	8	0.5	24.76	67.91	98.26
26	5	50	8	0.5	25.16	64.89	98.42
27	5	50	8	0.5	24.93	65.13	97.96
28	5	50	8	0.5	25.01	64.97	98.02
29	5	50	8	0.5	24.75	65.83	97.84

*Experiments from Run No 26 to 29 present central points

Table 13. ANOVA of the regression models for variables of the wheat gluten protein hydrolysates.

Variables	Coefficient of determination (R^2)	*P*-value	*F* -test	Coefficient of variation	Lack of fit	Adequate precision
DH	0.9810	<0.0001	109.6	4.40	0.0790	37.20
DPPH	0.9905	<0.0001	103.84	4.11	0.0576	40.497
ABTS	0.9651	<0.0001	23.76	3.34	0.4644	17.454

7.4.1. The DPPH radical scavenging activity

Different techniques have been applied to study the antioxidant potential of proteinhydrolysates. DPPH method is a powerful experimental technique to obtain meaningful dataabout the ability of compounds to act as free radical scavengers or hydrogen donors. The following regression equation, representing empirical relation between DPPHscavengingactivity, Y_2 and gluten concentration, x_1,temperature, x_2 pH, x_3 and [*E*]/[*S*] ratio, x_4 wasobtained:

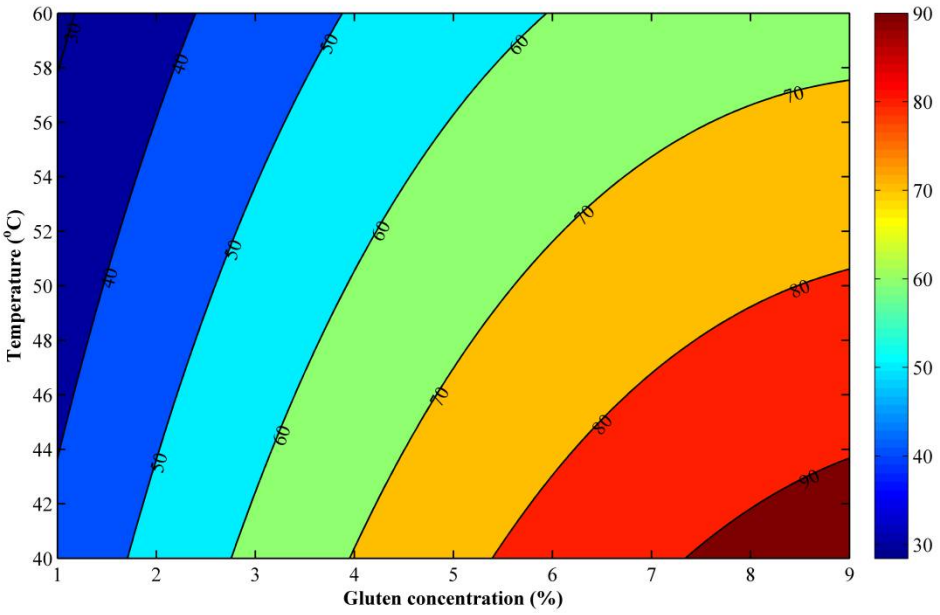
$$Y_2 = 65.42 + 13.78 \cdot x_1 - 10.75 \cdot x_2 - 18.26 \cdot x_3 + 1.45 \cdot x_4 - 3.68 \cdot x_1 \cdot x_2 - 3.68 \cdot x_1 \cdot x_3 - 8.91 \cdot x_1 \cdot x_4 - 14.54 \cdot x_2 \cdot x_3 - 8.56 \cdot x_1^2 + 2.78 \cdot x_4^2 \quad (16)$$

Results revealed that the hydrolysis favored the antioxidant capacity of obtainedhydrolysates (18.08-94.50) as compared to the control non-hydrolysed gluten (7.51%) butthis effect significantly depended on process conditions. The maximum scavenging activityof 94.5% was achieved in the exp. No 18, when the process parameters were as follows: gluten concentration 9%, temperature 50 °C, pH 7 and [*E*]/[*S*] ratio 0.5 AU enzyme per g of protein. The analysis of variance showed that the DPPH activity was significantly (p<0.05) dependent on the linear terms of all tested variables as well as of quadratic terms of gluten concentration and *E*/*S* ratio. The most significant were the

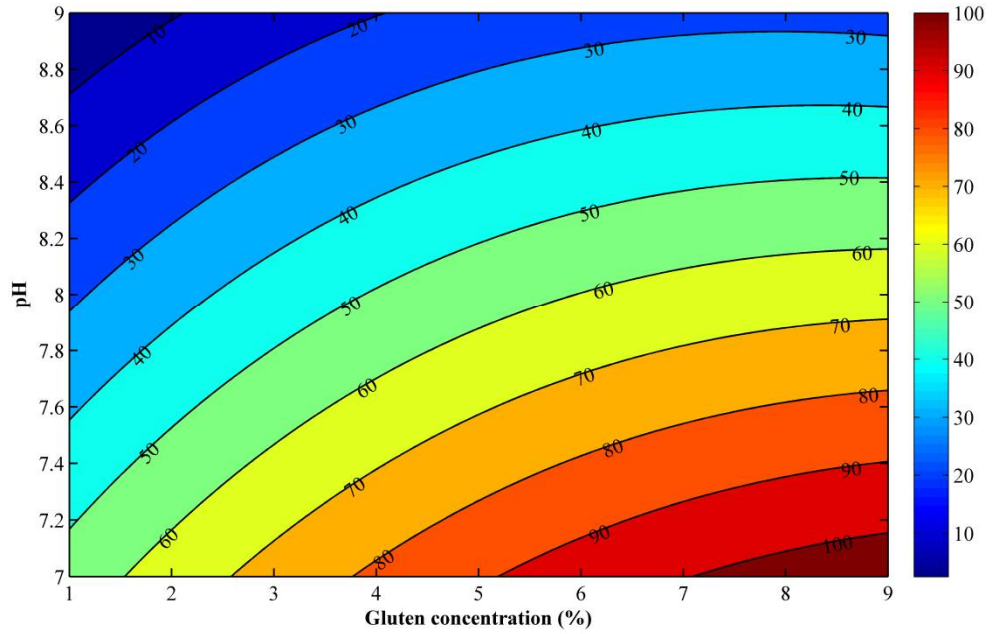
negative influence of the pH and its interaction with temperature, followed with the positive influence of gluten concentration.

The regression model showed a good fit with the experimental data with an R^2 value of0.9905. Fig. 35 displays the combined effect of process parameters on DPPH scavengingactivity. It was easily observed that the highest DPPH activity was achieved with the highest gluten concentration and the lowest value of temperature and/or pH (Figs. 35a and 35b).

A)



B)



C)

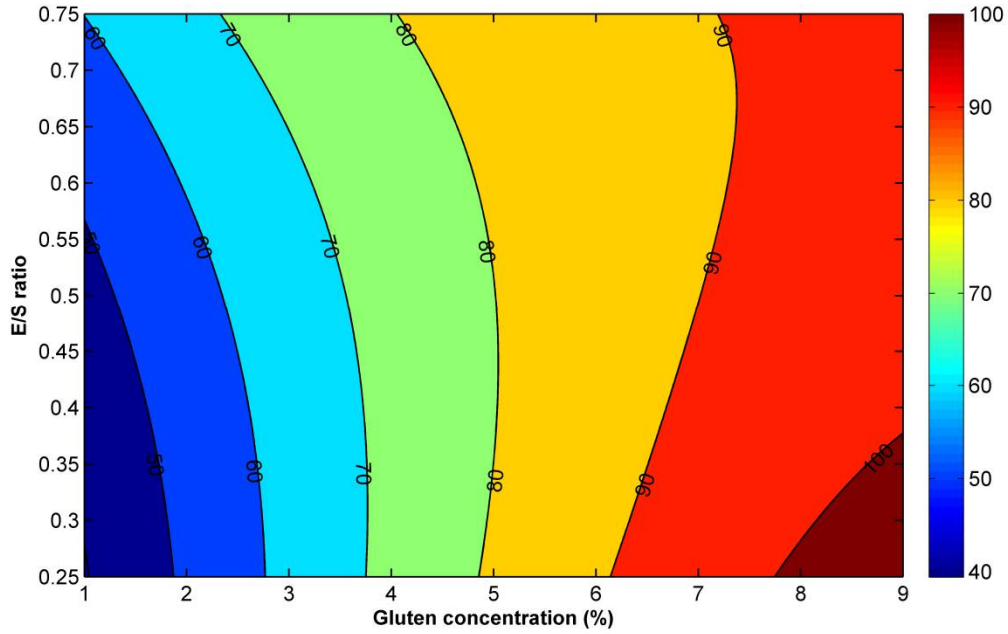


Figure 35. Contour plots for DPPH radical scavenging activity of gluten hydrolysates as a function of the A) gluten concentration, x_1 and temperature, x_2 B) gluten concentration, x_1 and pH, x_3 and C) gluten concentration, x_1 and *E*/*S* ratio, x_4.

At all gluten concentrations, an increase of temperature caused decrease in DPPH activity, and a rather similar trend was obtained in the case of pH. On the other hand, Fig. 29 previously revealed that the highest DH was achieved with the highest pH and temperature level. These results that high DPPH values were associated with lower DH, suggested that there were peptides with considerable size presenting a good DPPH activity. This would be in line withthe report of Valdez-Flores et al. (2016) showing that high antioxidant peptide capacity couldbe associated with low DH%. The DPPH activity was related to the DH(%) and the results have been presented in Fig. 36. It appeared that the DH followed an equation regressed by the second order. Hydrolysis up to around DH(%) 15 improved DPPH radical scavenging activity, while excessive hydrolysis worsened it, suggesting the necessity for finding a reasonable compromise between achieved DH (peptide yield or process productivity) and DPPH activity. Furthermore, high peptide concentration might also contribute for improving the antioxidant activity of hydrolysates which was closely related with substrate concentration.

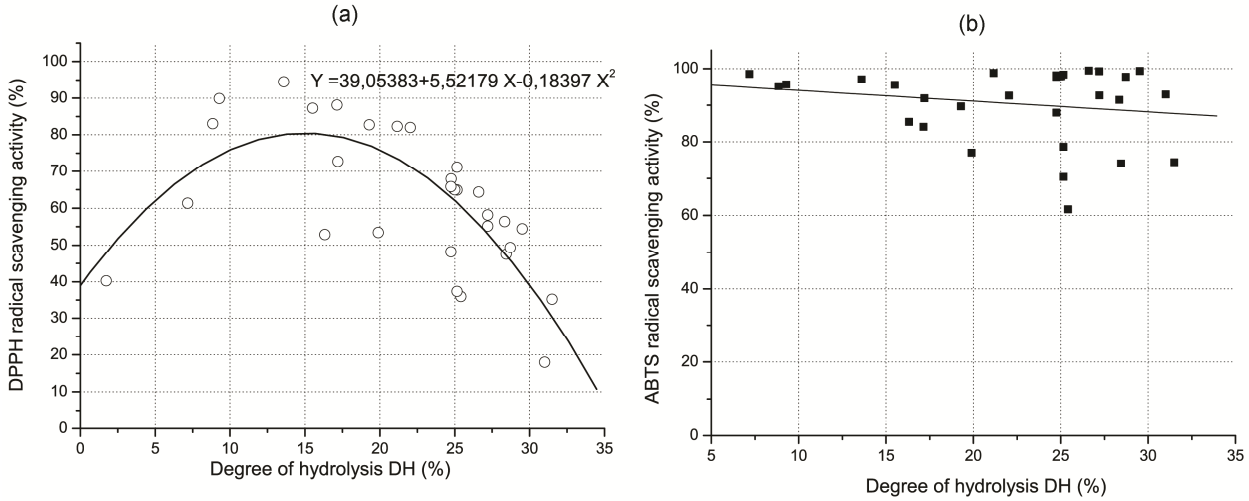


Figure 36. Correlation between the antioxidant activity and degree of hydrolysis measured by (a) DPPH method and (b) ABTS method.

7.4.2. The ABTSscavenging activity

Since the performance of antioxidants often varies against different free radicals, $ABTS^{\cdot+}$radical quenching activity has been used as a second measure for assessing the antioxidantcapacity of the gluten hydrolysates. ABTS activity ranged from 61.65 to 99.53 depending onprocess conditions (Table 12).

The highest ABTS scavenging activity of 99.53% was achieved in the exp. No 12, when the process parameters were as follows: gluten concentration 9%, temperature 50 ^{o}C, pH 8, *E*/*S* ratio 0.75 AU/g. Contrary to DPPH, the highest values of ABTS antioxidant activity was detected in the hydrolysates obtained athigh DH of 26.6%. However, there was apparent similarity between the obtained response functions for different antioxidant test systems, revealing that the operating condition comparably affect antioxidant activity measured by DPPH, and ABTS methods. The following model was developed to describe the response (*Y*) surfaces:

$$Y_3 = 98.10 + 9.65 \cdot x_1 - 1.31 \cdot x_2 - 1.84 \cdot x_3 + 4.77 \cdot x_4 + 3.54 \cdot x_1 \cdot x_2 + 2.92 \cdot x_1 \cdot x_3 - 5.41 \cdot x_1 \cdot x_4 + 6.36 \cdot x_2 \cdot x_3 - 5.38 \cdot x_2 \cdot x_4 - 8.39 \cdot x_1^2 - 8.93 \cdot x_2^2 - 1.99 \cdot x_3^2 + 0.12 \cdot x_4^2 \quad (17)$$

Similar to the DPPH scavenging activity, all of the tested variables showed significant effect on the ABTS quenching ability. The most significant was the positive influence of the gluten concentration, followed with the quadratic term of temperature and interaction between pH and temperature, as was previously observed for DPPH activity. Nevertheless, the effects of temperature and pH were some what different, since the positive quadratic coefficients were significant and the positive interaction with gluten concentration was apparent. Furthermore, the ABTS values did not show a linear logarithmic relation with DPPH values (y=0.155x+3.86; R^2=0.20), what was expected, since ABTS and DPPH assays were performed in different reaction systems, as previously described. For example, the hydrolysate obtained in exp. No 16 was not particularly good quencher of DPPH radical butwas exceptional in quenching ABTS radical, revealing that not all antioxidant peptides behaved the same toward different radical sources. Fig. 36b

shows the correlation between the ABTS activity and the degree of hydrolysis (DH%). Although the ABTS activity even slightly decreased with an increase in DH, theclear correlation between ABTS and DH was not apparent. This was not in accordance with several studies reporting a strong correlation between antioxidant activity and degree ofhydrolysis in terms of reduced peptide sizes. However, some studies have also reported decrease of antioxidant activity with an increase in DH (Castro et al., 2015). For example, the authors reported higher ABTS radical scavenging activities in hydrolysates prepared by pepsin (5% DH) and *Virgibacillus* sp. SK33 proteinase (13% DH), than those prepared by alcalase (27% DH) and trypsin (11% DH) (Wiriyaphan et al., 2012). Lin et al. (2013) have also reported that the antioxidant activity may not be a direct function of the DH, and the proper hydrolysis conditions to produce antioxidant activity peptides were different from the proper hydrolysis conditions to reach high DH. In this context, Cian et al. (2015) reported that peptides of medium size (5.5 kDa) were primarily responsible for ABTS scavenging activity indicating that the sequence of peptides and peptide conformation, are mainly responsible for antioxidant activity. These results suggested that a certain retained protein structure was needed in order to have good ABTS activity. Gluten concentration is possibly one of the most important factors to be considered. As expected, the ABTS activity generally increased at higher gluten concentration as seen in Fig. 37.

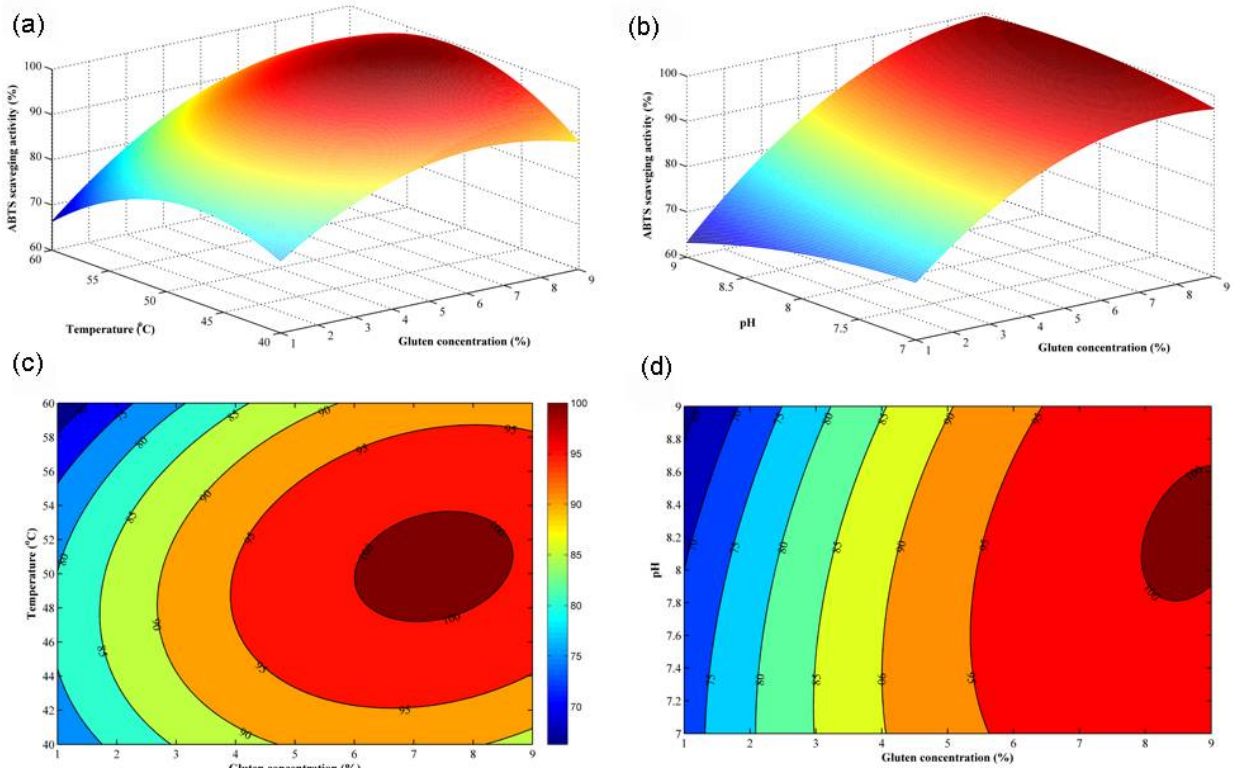


Figure 37. (a) 3-D response surface and (c) 2-D contour plots showing the effect of temperature, x_2 and gluten concentration, x_1 on the ABTS radical scavenging activity of hydrolysate; (b) 3-D response surface and (d) 2-D contour plots of pH and gluten concentration (x_3 and x_1) effects on the ABTS activity.

However, this parameter influences the enzyme reaction in several ways: through achange in the degree of hydrolysis and/or through a change in the bioactive peptideconcentration. Besides this, an excess of substrate actually protected enzyme from denaturation and auto-cleavage. On the other hand, for higher solid concentrations, wheat gluten formed a suspension with high viscosity. Thus, it was expected that it showed interactive effects with all other parameters.

7.5. Molecular weight distribution profile of the gluten hydrolysate fraction and their antioxidant capacity

The hydrolysate obtained in exp No 18 showing the highest DPPH and ABTS radical quenching ability, respectively, has been further separated by sequential ultrafiltration into three major peptide fractions, GF I (10–30 kDa), GF II (3–10 kDa) and GF III (<3 kDa) and fractions were compared based on their protein content and antioxidant activity. As peptide fractions may respond in a different manner to different radical or oxidant sources, different methods including DPPH, ABTS and superoxide radical scavenging effects were used to evaluate the antioxidant capacities. The results are presented in Fig. 38.

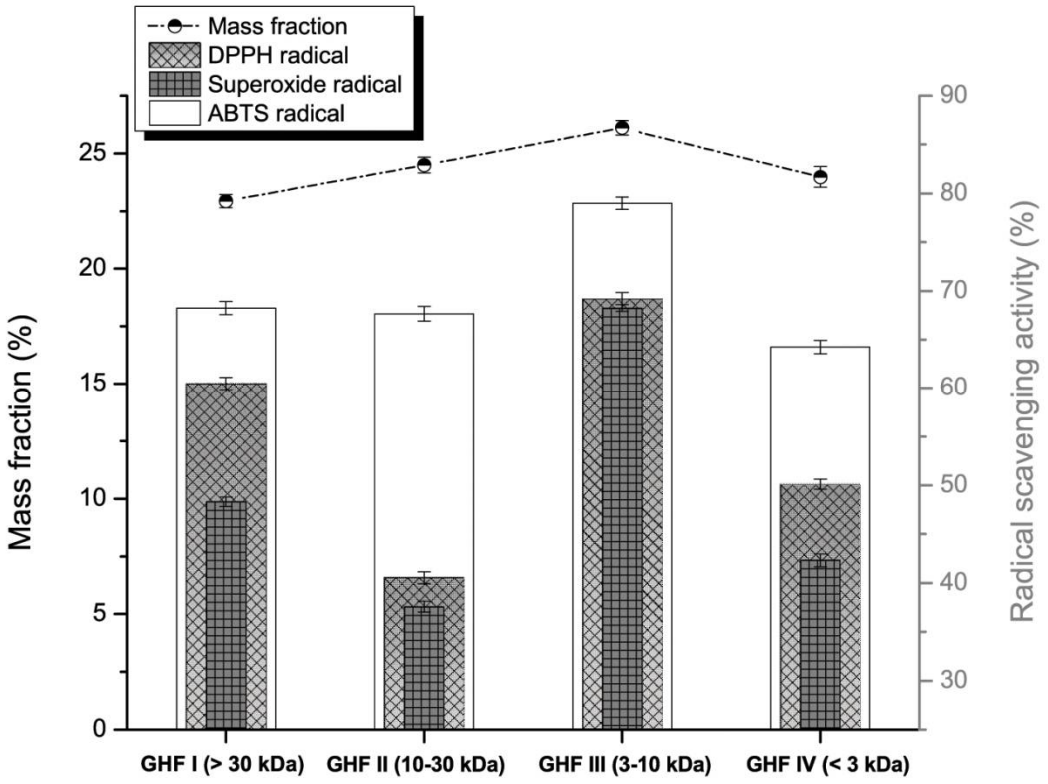


Figure 38. The molecular weight distribution profile of ultrafiltered fractions of wheat gluten hydrolysate and their radical scavenging activity against DPPH, ABTS and superoxide radical

It appeared that the hydrolysate showing the highest DPPHand ABTSactivity (DH 13.6%) had the highest percentage of peptides with medium molecular masses (3–10 kDa).Furthermore, this peptide fraction seemed to be primarily responsible for all three scavengingactivities. These results were also similar to those found by Cian et al. (2015) for wheat gluten, who also reported that peptides of medium size (5.5 kDa) were primarily responsible for ABTSscavenging activity, indicating that the sequence of peptides and peptide conformation, were mainly responsible for antioxidant activity. These results suggested thata certain retained protein structure was needed in order to have good antioxidant activity.

8. CONCLUSION

The aim of the study was to find the optimal operational and process parameters for the enzymatic hydrolysis of wheat gluten in a batch stirred bioreactor regarding both functional properties and antioxidant capacity of the obtained hydrolysates. In the first part, the effect of heat pretreatment on alcalase-catalyzed wheat gluten hydrolysis and functional and antioxidant properties of the obtained hydrolysates were investigated. Based on the obtained results, the following conclusions can be made:

1) The wheat gluten was heat-treated during 30 min at 75 ^{o}C and intensively hydrolyzed with alcalase at degree of hydrolysis (DH) 16.1%, 22.9%, and 30.2%. All the hydrolysates had excellent solubility over a pH range of 2–12. Emulsifying activity and stability were also improved, while proteolysis was deleterious to foam capacity and stability, water-holding capacity, fat-binding capacity and did not show improvement at higher DH (22.9% and 30.2%). As well, controlled hydrolysis of heat-treated gluten resulted in a remarkable improvement in antioxidant activities. The results show that the heat-treated AWGHs were superior to the untreated hydrolysate in the functional and antioxidant properties tested.

2) The effect of several process parameters includingimpeller geometry, agitation speed, gluten concentration, temperature, pH and enzyme-gluten (*E/S)* ratio on initial reaction rate and degree of hydrolysis was investigated in the batch reactor. It appeared that impeller geometry and agitation speed influenced the mass transfer resulting in enhanced gluten hydrolysis. The highest initial reaction rate (0.83±0.02 min^{-1}) and degree of hydrolysis (30.47%) were achieved with the pitched four-bladed impeller and agitation speed of 350-450 rpm, conditions which provided proper balance between requirements for adequate mass/heat transfer and low shear stress. The highest DH was achieved with the lowest gluten concentration and higher pH value. These results may be connected to an increased product and/or substrate inhibition, insufficient mixing, steric hindrance and lack of cleavage sites at higher gluten concentration. The low solubility of gluten at neutral pH might have contributed to low DH obtained and this was pronounced athigher gluten concentration.

3) The impact of selected process conditions includinggluten concentration, temperature, pH and enzyme-gluten (*E*/*S)* ratio on the enzymaticreaction was further investigated by applying a Box-Behnken experimental design from the viewpoint of the degree of hydrolysis (DH). The coefficient of determination (R^2) is of 0.981, which indicated an adequate adjustment of the experimental data, showing that more than 98% of the data variability was explained in the proposed empirical equation. The statistical analysis showed that each variable had a significant effect on degree of hydrolysis. It appeared that only gluten concentration and temperatureshowed strong interaction effect whereas the interactions among other parameters were insignificant.

4) The impact of process conditions includinggluten concentration, temperature, pH and enzyme-gluten (*E*/*S)* ratio on the enzymaticreaction was also investigated by applying a Box-Behnken experimental design from the viewpoint of the selected functional properties like solubility, foam capacity, foam stability,emulsion activity index and emulsion stability index.

- The analysis revealed that all tested variables had a significant effect on the solubility, within the experimentally tested ranges. The most relevant variable seemedto be *E*/*S* ratio, but the effects of gluten concentration, pH, temperature and gluten concentration and *E*/*S* interactions were also significant. The quadratic term of gluten concentration was also significant, indicating that a response was a quadratic function with a local maximum. It can be concluded that the high solubility of the wheat gluten hydrolysates was achieved at a low temperature of the reaction of enzymatic hydrolysis (40 ° C) and a pH of 9, using minimal amounts of substrate and a rather low amount of enzymes, since the optimal [*E*]/[*S*] ratio seemed to be 0.5 AU/g of gluten.

- The analysis revealed that all tested variables had a significant effect on the *EAI*, within the experimentally tested ranges. The most relevant variable seemed to be interaction of the gluten concentration and temperature (p<0.0001). The quadratic terms of gluten concentration and temperature were also significant, indicating that a response was a quadratic function with a local maximum. It appeared that the highest *EAI* of 2782.9 m²/g was achieved at pH 8.0 and at a temperature of 50 °C, [*E*]/[*S*] of 0.75 AU /g gluten and a substrate concentration of 1%.

- The obtained results revealed that the foam capacity range of the wheat gluten hydrolysates were in the range of 24.2-80.3%, depending on the independent variables that were tested. Overall, it was apparent that the gluten hydrolysates had a relatively good foam capacity, since in a large number of experiments this ability was higher than 50%.

- It seemed that the most relevant variables for the foam stabilitywere gluten concentration and pH with estimated effects of 4.64 and 4.58, respectively. The effects of temperature and *E*/*S* ratio were also significant ($p< 0.05$). It appeared that while substrate concentration and all other parameters had a positive effect, the gluten concentration and *E*/*S* ratio interaction had a significant negative influence on foam stability. According to thestatistical analysis, the maximum foam stability can be obtained at high level of gluten concentration and pH value.

5) The further aims of this thesis were to explore the effects of process parameters including gluten concentration, temperature, pH and enzyme/substrate, *E*/*S* ratio on antioxidant capacity of the obtained wheat gluten hydrolysates and to relate both DPPH and ABTS scavenging activity to the degree of hydrolysis. The statistical analysis showed that each variable had a significant effect on degree of hydrolysis and the antioxidant capacity of both tested systems. A suitable degree of hydrolysis is desirable for the achievement of high DPPH radical scavenging activity. It seemed there was not a correspondence between degree of hydrolysis and ABTS activity at different protein concentrations, suggesting that there were peptides with considerable size presenting a good antioxidant capacity.

Overall, results are relevant to the protein ingredient industry because of the economic importance of novel gluten–based bioactive products and can provide useful information for the design an efficient enzymatic process for their production in high yield and with high antioxidant properties.

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10. BIOGRAPHY

Mohamed Bashir Elmalimadi was born on 26. June 1967. at Misurata- Libya, where he finished basic and high school. He graduated Bachelor of Chemistry at University of Misurata, Science Faculty on 1990/1991. He finished Master degree in Scientific Research, at University of Misurata. Science Faculty, Chemistry Department, at year 2010.

He was hired as head of Training Departmen at Misurata Soap Factory, and member of Purchases community, during period from March 1999 up to June 2006. In meantime started as a lecturer at Education Faculty - University of Misurata since October. 2011. He was teaching vary of course in Chemistry department such as: Biochemistry I, Biochemistry II and Biochemistry Lab.

He attended a course in the field of powder soap manufacturing from 17 March to 17 April 2003. He approved Intermediate English language courses during the year 2009. Improved Microsoft Windows Courses for computer and internet applications at year 2000. Attended educational Seminar “Diplomatic Protocol” during 19 Febuary to 15 April 2016. Major skills are in controlling of soap raw materials in production process of soap powder. Started doctoral dissertation at Faculty of Technology and Metallurgy in Belgrade, Serbia at November in 2013, at Department of Biochemistry, by advisor of mentor full professor Zorica Knežević-Jugović.

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