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Production of a Soybean Meal with High-Protein and Low Anti-Nutritional Factors for Fish Feed

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Production of a Soybean Meal with High-Protein and Low Anti-Nutritional
Factors for Fish Feed

Production of a Soybean Meal with High-Protein and Low Anti-Nutritional
Factors for Fish Feed

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Food Science

by

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Universidad Nacional del Sur
Bioquímica, 2009

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This thesis is approved for recommendation to the Graduate Council.

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ABSTRACT

Aquaculture is one of the fastest growing areas of the food production system. According to the FAO, its rise is expected to continue through the year 2030 in order to maintain per-capita consumption levels required for the increasing population. Fishmeal—obtained from wild-harvested fish—has been the source of protein for fish feed. However, data indicates that these fish harvests are in decline, which could restrain that growth. The possibility of a shortage of fishmeal prompted the industry to look into possible alternatives. Soybean meal appears as a promising substitute since it is an affordable high quality source of protein. However, the presence of anti-nutritional factors—trypsin inhibitors, lectins, glycinin, β -conglycinin, saponins, phytates, and oligosaccharides—can negatively affect the growth and the general health of fish, limiting its inclusion as fish food. Several studies have been done in order to reduce these anti-nutritional factors. However, there is no method that eliminates all of them while preserving the protein content of the soybean meal. The aim of this work was to obtain a protein-rich soybean meal with low anti-nutritional factors and a greater protein digestibility to be used for fish food. To accomplish this, the deactivation kinetics (D and Z-values) of glycinin and β -conglycinin at different temperatures were studied using Differential Scanning Calorimetry (DSC). The reduction in the content of phytate was evaluated by pre-treatment of soybean meal with phytase. And lastly, Central Composite Rotatable Design (CCRD) was employed to determine the best combination of factors (temperature, time, pH, and ethanol concentration) that maximizes the extraction of soluble sugars, saponins, and phytate while increasing protein content and digestibility. Results indicated that the inclusion of phytase under different conditions reduced the phytate content. The CCRD determined that a pH of 4.5 at 59°C, 35% ethanol concentration for 65 minutes are the optimal conditions for the highest extraction of soluble anti-nutritional

factors, which increased the content of total protein and digestibility of the soybean meal.

However, according to the kinetics studies, the deactivation of glycinin—the more resistant of the two proteins—at this temperature is not complete.

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CHAPTER I

INTRODUCTION AND RESEARCH OBJECTIVES

The Food and Agriculture Organization of the United Nations (FAO) has estimated that the demand for aquaculture products will continue to rise through the year 2030 in order to maintain current per-capita consumption levels for the increasing global population. In order to accomplish that level, the aquaculture industry needs to expand to meet the demand for fish. However, the availability of fishmeal could restrain that growth (FAO 2012). In aquaculture feeds, protein is the most important and expensive and, at the same time, the most important component of the diet (Watanabe 2002). Traditionally, fishmeal and fish oil have been used as sources of proteins and lipids for fish feed, both obtained from wild-harvested fish. However, data indicate that these fish harvests are in decline. The possibility of a shortage of fishmeal compelled the industry to look into possible alternatives including both optimizing feed conversion ratios (FCRs) and reducing the proportion of fishmeal used for farmed fish feed. Although several plant protein meals are used to replace fishmeal, soybean meal is the most common source for herbivorous and omnivorous fish species (FAO 2012). Soybean meal has a well-balanced amino acid profile compared to other plant protein sources, is consistently available, and is economical (Watanabe 2002). Additionally, as long as fishmeal prices continue to rise, soybean protein concentrates will become increasingly important in the aquaculture industry (FAO 2012).

Soybeans are a rich source of proteins known for their high nutritional value and exceptional functional properties (Amadou and others 2010). A large portion of the soybean supply is used for oil production (Dixit and others 2011), which generates a residue—defatted soybean meal (less than 1% oil) (Jideani 2011)—which is often used in animal feed (Dixit and

others 2011). Soybean meal is used for fish, pig, and poultry feed (Dersjant-Li 2002). However, only a low inclusion level of soybean meal can be used as a fishmeal replacement because soy contains a variety of anti-nutritional factors (ANFs) that can negatively affect the growth and the general health of fish (Dersjant-Li 2002). Tilapia, carp, and mrigal fed with soybean showed reduced growth performance that was attributed to the anti-nutritional factors (Jana and others 2012).

Anti-nutritional factors in soybean meal include trypsin inhibitors (Van den Hout and others 1998, 1999; Machado and others 2008; Fasina and others 2003; Bajpai and others 2005), lectins (Machado and others 2008; Bajpai and others 2005; Fasina and others 2003), phytates (Storebakken and others 1998), oligosaccharides (Zdunczyk and others 2011; Gatlin III and others 2007), glycinin (Yang and others 2011; Kilshaw and Sissons 1979), and β -conglycinin (Yang and others 2011; Kilshaw and Sissons 1979). Additionally, Chen and others (2011) demonstrated that saponins cause negative effects in Japanese flounder when soybean meal is used as an alternative to traditional fish feed. In fact, morphological changes in the intestine of many fish species—rainbow trout, Atlantic salmon, Atlantic cod, and common carp—have been linked to inflammation of the small intestine (enteritis), associated with the presence of saponins in soybean meal (Knudsen and others 2008; The Research Council of Norway, 2011).

The pretreatment of soybean meal with phytase has been extensively studied in rainbow trout (Sugiura and others 2001; Cain and Garling 1995; Yang and others 2011), Nile Tilapia (Cao and others 2008), Korean rockfish (Yoo and others 2005), and Atlantic salmon (Storebakken and others 1998; Denstadli and others 2007). These studies concluded that phytase was able to reduce the phytic acid content in the soybean meal. Additionally, phytase treatment likely leads to improved mineral absorption (Obendorf and Kosina 2011). Trypsin inhibitors and

lectins can be reduced with heat treatment, which also enhances protein digestibility (Jana and others 2012). However, there is still no method that eliminates all the anti-nutritional factors while preserving the protein content of soybean meal.

The use of ethanol in the production of soy protein concentrates has been extensively studied since it allows the extraction of soluble sugars and saponins from the sample. However, ethanol is a flammable, volatile, colorless solvent with a slight odor that requires complex manipulation and more than one extraction to reduce the oligosaccharides content. In contrast, a single water extraction also allows the reduction of oligosaccharides and saponins making it a cheaper, simpler, and more sustainable alternative to ethanol extraction and therefore worthy of further investigation.

The goal of this research was to obtain a protein-enhanced soybean meal with enhanced nutritional value that can be used as a fishmeal replacement. The primary objective was to eliminate or minimize the anti-nutritional factors (galacto-oligosaccharides, phytates, glycinin, β -conglycinin, and saponins) present in the meal using aqueous buffer solutions or ethanol extractions while increasing the protein content and digestibility of the defatted soybean meal. To accomplish this, three specific objectives were established:

Specific objective 1: Study the deactivation kinetics of glycinin and β -conglycinin.

Specific objective 2: Evaluate the effect of the pre-treatment of soybean meal with phytase to reduce phytic acid content.

Specific objective 3: Evaluate the removal of oligosaccharides, saponins, and phytate with water or ethanol extraction.

CHAPTER II

LITERATURE REVIEW

1. SOYBEAN MEAL

Soybean is an extensively cultivated crop, with 83.18 million metric tons produced in the United States in 2011 (Soystats 2011). The United States is the largest producer, followed by Brazil, Argentina, and China (Soybeans and Oil Crops 2012). The bulk of soybean is used for soybean oil production, and the soybean meal residue is used for animal feed. A small percentage of this soybean meal is additionally processed into different food ingredients that include soy flour, concentrates, isolates and textured protein (Jideani 2011) (Figure 2.1). The composition of soybean meal may be influenced by the soybean variety and by the growing and processing conditions (Grieshop and others 2003).

2. MAJOR COMPONENTS OF SOYBEAN MEAL

2.1. Carbohydrates

Defatted soybean meal contains approximately 40% carbohydrates (Karr-Lilienthal and others 2005), which are present in a variety of forms—monosaccharides, oligosaccharides, polysaccharides, saponins, sterol glucosides, glycolipids, and isoflavones—(Eldridge and others 1979) (Table 2.1).

α -Galacto-oligosaccharides, or simply α -galactosides, are low molecular weight non-reducing sugars that are soluble in water and aqueous alcohol solutions. They have been characterized by the presence of $\alpha(1\rightarrow6)$ linkages between units of galactose linked by $\alpha(1\rightarrow3)$ linkages to a terminal unit of sucrose (Zdunczyk and others 2011). Two examples are stachyose, a tetraose with a galactose-galactose-glucose-fructose structure, and raffinose, a triose with a

galactose-glucose-fructose structure (Dixit and others 2011). During the production of soybean meal these oligosaccharides are not damaged or detached (Zdunczyk and others 2011).

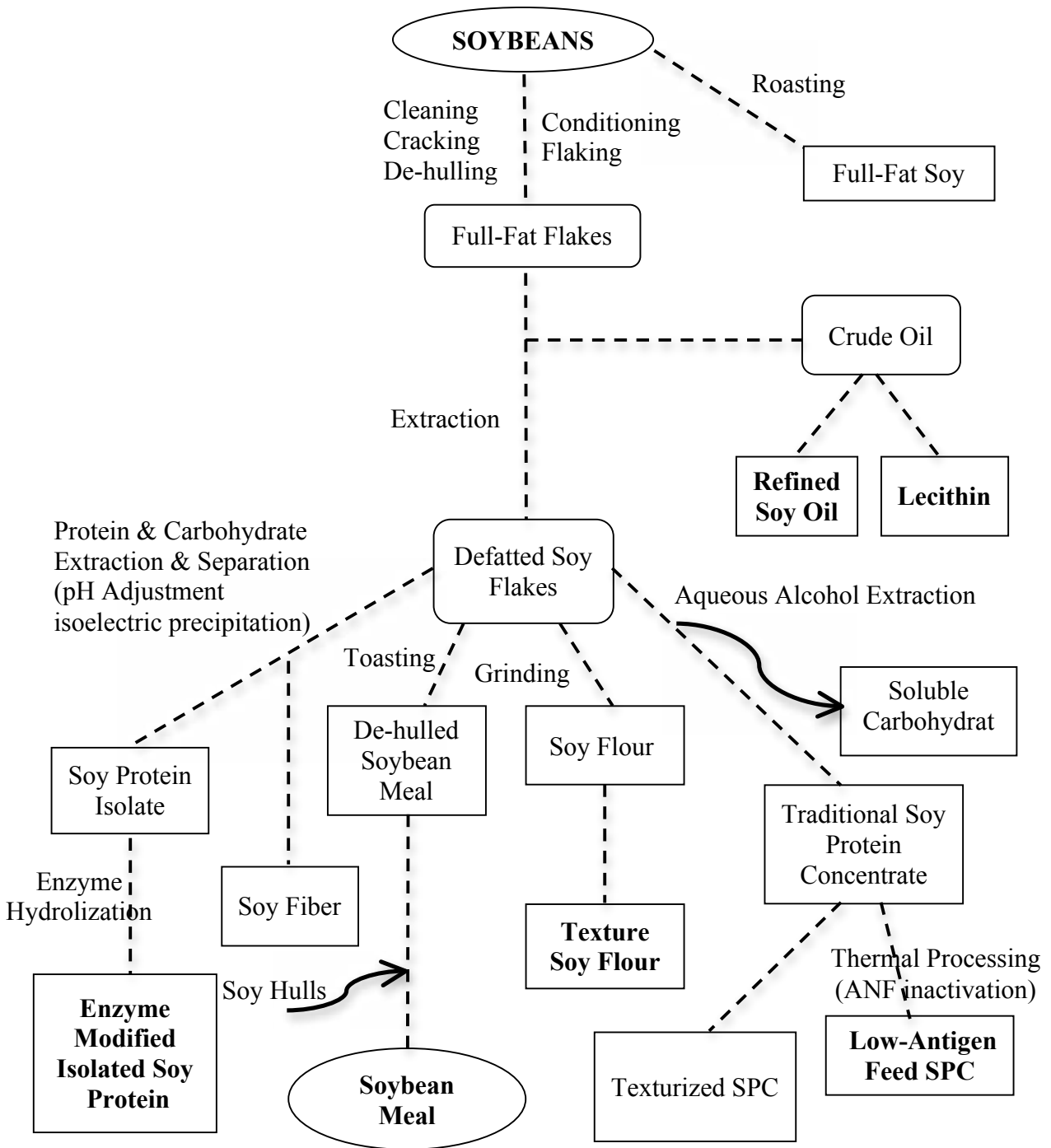


Figure 2.1: Soybean processing flow chart (From Soy Protein Concentrate for Aquaculture Feeds 2008).

Table 2.1: Classification of carbohydrates present in soybeans (From Karr-Lilienthal and others 2005, and Giannoccaro and others 2006).

	Classification	Examples	Soluble in H₂O
Non-Structural	Low molecular weight sugars	Glucose, galactose, fructose, sucrose	Yes
	Oligosaccharides	Raffinose, Stachyose, Verbascose	Yes
	Starch		No
Structural		Pectin, hemicellulose, cellulose	No

Soybean oligosaccharides are undesirable components present in food. They can cause excessive flatulence (Kim and others 2003) in monogastric animals (Zdunczyk and others 2011; Graham and others 2002) due to the absence of the enzyme α -galactosidase in the small intestine. Due to the α -galactoside linkage in their structure, the oligosaccharides present in soybean, stachyose and raffinose, are not digestible and are responsible for flatulence, nausea, and abdominal discomfort in animals (Karr-Lilienthal and others 2005; Bairy and others 2008). Even though they cannot be digested, they are fermented by the intestinal microflora producing short-chain fatty acids and various gases such as CO₂ and H₂ that can cause the aforementioned problems (Karr-Lilienthal and others 2005).

2.2. Proteins

Defatted soybean meal is comprised of 50% proteins (Fischer and others 2001), primarily globulins, which are classified according to their sedimentation coefficients (Hill and Breidenbach 1974) as 2S (22% of the total), 7S (37% of the total), 11S (31% of the total), and 15S (11% of the total) (Lusas and Rhee 1995). Soybean proteins contain all the amino acids needed for human health, making it the only vegetable food regarded as a complete protein

source for humans (Caprita and Caprita 2010). Thus, soy products are comparable in quality to proteins from animal sources with less saturated fat and no cholesterol (Dixit and others 2011).

Glycinin (11S) and β -conglycinin (7S) are the major storage proteins present in soybeans (Lusas and Rhee 1995; Moriyama and others 2005; Barač and others 2004; Guo and others 2012). These globulins account for about 65% of the total protein content (Delwiche and others 2007; Tukur and others 1996). Glycinin is composed of six subunits—300-380 kDa—each of them formed by an acidic and a basic polypeptide linked together by a single disulfide bond (Hou and Chang 2004; Tukur and others 1996; Guo and others 2012; Lakemond and others 2000). On the other hand, β -conglycinin is composed of three subunits: α (~67 kDa), α' (~71 kDa) and β (~50 kDa) (Moriyama and others 2005; Delwiche and others 2007; Tukur and others 1996; Guo and others 2012). Ionic strength and pH affect the structure of glycinin and β -conglycinin (Lakemond and others 2000), modifying the temperature of denaturation of both proteins (Koppelman and others 2004; Jiang and others 2010). Globulins are insoluble in water at their isoelectric point of 4.5 (Lusas and Rhee 1995; Barač and others 2004). They are most soluble at pH values of 1.5 to 2.5 and 7.0 to 12.0 and less soluble at pH in the range of 4.2 to 4.6 (Lusas and Rhee 1995). This behavior is related to the position of the acidic and basic polypeptides at pH 7.6 (Lakemond and others 2000). Additionally, protein solubility (%) decreases as the particle size of soybean meal increases. Thus, increasing the stirring speed and length of stirring time positively affects protein solubility (Parsons and others 1991).

Both globulins are considered allergens (Hei and others 2012; Ma and others 2010; Kilshaw and Sissons 1979; Rumsey and others 1994). Glycinin has been associated with intestinal damage, diarrhea, growth depression, and alteration of the immune function (Ma and others 2010; Rumsey and others 1994). Its prejudicial effects are lost by denaturation or

destruction of its quaternary structure (Koshiyama and others 1980-81). β -conglycinin has been associated with intestinal damage, protein digestibility, and allergenic symptoms (Hei and others 2012; Rumsey and others 1994).

The three main high protein soy products that are usually used for food are defatted soy flours, soy protein concentrates, and soy protein isolates. Defatted soy flours (52-54% protein content) are produced by grinding the dehulled, defatted soy flakes. Soy protein concentrates (65% minimum protein content) are made by extraction of the water or alcohol soluble components. Soy protein isolates (90% minimum protein content) are produced by extraction with water under alkaline conditions followed by acid precipitation (Lusas and Rhee 1995).

In the aquaculture industry, three different types of soy protein concentrates (SPC) are of interest: traditional SPC—produced by aqueous alcohol extraction of defatted soybean meal, texturized SPC—produced using extrusion on traditional SPC, and low-antigen SPC—produced by modification of temperature, aqueous alcohol proportion, and time of processing. Each technology accomplishes a reduction in the amount of anti-nutritional factors present in the final product. The low-antigen SPC has the lowest concentration of anti-nutritional factors and is therefore preferred for aquafeeds (Soy Protein Concentrate for Aquaculture Feeds 2008).

2.3. Phytochemicals

The major phytochemicals in soybean are: phytic acid (1.0-2.2%), sterols (0.23-0.46%), saponins (0.17-6.16%), isoflavones (0.1-0.3%), lignans (0.02%) sphingolipids, inositol, phenolic acids, and Bowman-Birk and Kunitz trypsin inhibitors (Luthria and others 2007; Choi and others 2002; Wolf 1976; Wu and Kang 2011). Anti-nutritional factors—trypsin inhibitors, lectins, phytates, and oligosaccharides—inhibit protein digestibility (Caprita and Caprita 2010), which negatively affects the nutritive value of the soybean meal (Kakade and others 1972; Charpentier

and Lemmel 1984). These anti-nutritional factors need to be minimized or inactivated in order to maximize the nutritional value of soybean meal (Caprita and Caprita 2010).

2.3.1. Trypsin inhibitors

Trypsin inhibitors—Bowman-Birk and Kunitz—are proteins that act as protease inhibitors and antigrowth factors while reducing the digestibility of other proteins in monogastric animals including carnivorous fish (Lusas and Rhee 1995; Refstie and Storebakken 2001). The Bowman-Birk inhibitor is stable to heat, acid, and proteolytic digestion because it has a rigid tertiary structure consisting of seven disulfide cross-linkages (Wolf 1976). However, both trypsin inhibitors can be inactivated using steaming and extrusion after the oil extraction process (Van den Hout and others 1999; Refstie and Storebakken 2001). Twenty percent of Bowman-Birk and Kunitz inhibitors remain active after heat treatment of soybean meal (Friedman and Brandon 2001); however this level is tolerable for carnivorous fish (Refstie and Storebakken 2001). Care must be taken when using heat in soybean processing. Even though undesirable substances may be eliminated, the functional and nutritional properties of other proteins may suffer damage (Kakade and others 1972). In general, the extent of protein damage is attributed to the temperature, moisture content, screw-speed, shear forces, and duration of heating during processing (Marsman and others 1997).

2.3.2. Phytates

Phytate (the salt of phytic acid) is a polyphosphorylated carbohydrate that serves as storage for phosphorus and minerals (Figure 2.2). It represents the major source of phosphorus in soy (Wu and Kang 2011), where it accounts for 70% of the total phosphorus (Smith and Rackis 1956). In people, it can contribute to mineral deficiencies since it acts as a strong chelator of calcium, magnesium, iron, and zinc (Wu and Kang 2011). For the same reason, these essential

cations appear to be unavailable to other monogastric animals (Okubo and others 1975) including fish (Yang and others 2011; Refstie and Storebakken 2001). In addition, phytate interacts with proteins forming phytate-mineral-protein complexes reducing the bioavailability of proteins (Morales and others 2012; Refstie and Storebakken 2001) in monogastric animals (Phumee and others 2011).

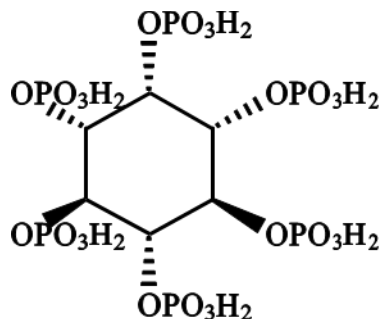


Figure 2.2: Phytic acid (From Wu and Kang, 2011).

Extraction with water at pH 5.0 removes about 75% of the phytate content (Lusas and Rhee 1995). Furthermore, the inclusion of phytase in the treatment of soybean meal can release phosphorus and chelated cations from the phytate-mineral-protein complexes, increasing both protein digestibility (Morales and others 2012) and the bioavailability of phosphorous (Imanpoor and Bagheri 2012). Experiments with rainbow trout have demonstrated that absorption and retention of phosphorus increase when soybean meal is supplemented with phytase in the diet (Phumee and others 2011).

2.3.3. Lectins

Lectins are glycoproteins with at least one non-catalytic site (or a site that binds to mono- or oligosaccharides in the cells). They have a great affinity for terminal N-acetyl-D-galactosamine and, to a lesser extent, D-galactose. Lectins are classified according to their degree of denaturation as agglutinating or non-agglutinating lectins. The former, with an intact

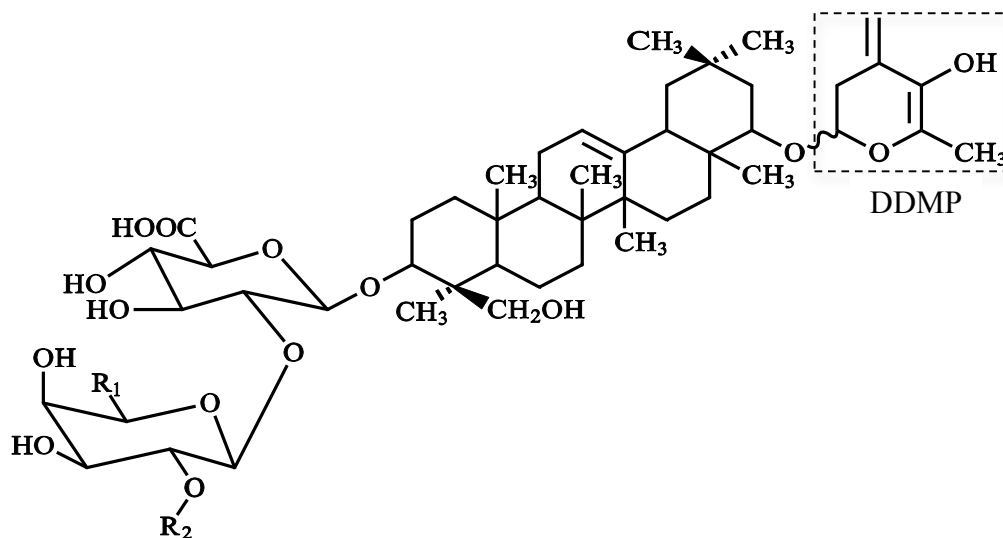
quaternary structure with multiple carbohydrate-binding sites, has the ability to bind to carbohydrates and agglutinate cell membranes. The latter has only one partially denatured carbohydrate-binding site and therefore binds to but does not agglutinate cell membranes (Fasina and others 2003). Given their carbohydrate binding ability, both groups of soybean lectins can attach to the enterocytes of the intestine of fish producing pathological changes. The concentration of lectins in soybean meal depends on the cultivar, the storage conditions, and the processing techniques and conditions used to produce it (Fasina and others 2003). Fortunately, lectins can be denatured by proper heat treatment (Buttle and others 2001) and are reduced to about 10% activity in defatted soybean meal as a consequence (Van der Ingh 1996).

2.3.4. Saponins

Saponins are triterpenoid or steroid aglycones linked to one or more units of sugars that occur naturally in plants (Knudsen and others 2008; Güçlü-Üstündağ and others 2007). Saponins are present in relatively high concentration in soybeans and soybean products (Hu and others 2002). A total of 30 soy saponins have been described (Dixit and others 2011). Their presence and quantity differ based on cultivar, age, physiological stage, geographical location, processing, and storage conditions. However, the total concentration, composition, and biological activity of saponins in soybean can change as a result of chemical modifications produced during processing and storage. Saponins are sensitive to thermal treatments (Mastrodi Salgado and Donado-Pestana 2011).

Saponins are amphiphilic compounds that have both a polar—one or more sugars chains (Mastrodi Salgado and Donado-Pestana 2011)—and a non-polar fraction—aglycone, triterpene or a steroid called sapogenin—(Mastrodi Salgado and Donado-Pestana 2011); thus, they are good emulsifiers and foaming agents (MacDonald and others 2005; Güçlü-Üstündağ and others

2007). Saponins are classified according to the number of sugar chains present in their structure (Güçlü-Üstündağ and others 2007) (Figure 2.3). Soybeans contain group A and B saponins (Knudsen and others 2008). Group B is the major saponin (Hu and others 2002) accounting for ~83% of the total saponins present in defatted soybean meal (Rickert and others 2004). Group A soyasaponins, associated with the bitter and astringent taste of soy products (Hubert and others 2005), are called bidesmosidic—two sugar chains (Güçlü-Üstündağ and others 2007; Gu and others 2002). Soyasaponins in group B are monodesmosidic—one sugar chain (Güçlü-Üstündağ and others 2007; Gu and others 2002)—and are the ones associated with the health benefits of soybean saponins (Hubert and others 2005). The monosaccharides that can be present in the structure of saponins include: glucose, galactose, glucuronic acid, rhamnose, arabinose, xylose, and fucose. The different aglycone moieties and sugars present in the structure vary significantly, making saponins a diverse group of compounds that have a great number of physical, chemical, and biological properties with only a few of them common to all compounds (Güçlü-Üstündağ and others 2007). It has been stated that only the DDMP-2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one- conjugated soybean saponins α g, β g, and β a are the real group B saponins present in soybean while the non-DDMP soyasaponins V, I, and II are products formed by heat exposure (Kudou and others 1994). It has been suggested that saponins may interact with the major storage proteins in soybeans, glycinin and β -conglycinin, through different types of interactions (Rickert and others 2004).



Saponin	R1	R2	DDMP
Bg	CH ₂ OH	α -L-Rhamnosyl	Yes
I	CH ₂ OH	α -L-Rhamnosyl	No
Ba	H	α -L-Rhamnosyl	Yes
II	H	α -L-Rhamnosyl	No
Γ g	CH ₂ OH	H	Yes
III	CH ₂ OH	H	No
Γ a	H	H	Yes
IV	H	H	No
Ag	CH ₂ OH	β -D-Glucosyl	Yes
V	CH ₂ OH	β -D-Glucosyl	No

Figure 2.3: Structure of saponins (From Hu and others 2002).

Saponins have been extensively used as surface active and foaming agents, but their use in foods has been limited because of their bitter taste. In addition, they have generally been regarded as “anti-nutritional factors” (Mastrodi Salgado and Donado-Pestana 2011; Güçlü-Üstündağ and others 2007). Saponins seem to have negative effects when present in animal diets (Chen and others 2011). To illustrate, they have been associated with lower feed intake, reduction in weight gain, and lower protein digestibility in tilapia (Francis and others 2001). They also have hemolytic and toxic effects in fish and invertebrates as a consequence of their ability to form foamy solutions in water (Mastrodi Salgado and Donado-Pestana 2011).

3. SOYBEAN AS A FISHMEAL REPLACEMENT

Fishmeal is the preferred protein ingredient for fish feed (Rawles and others 2011), particularly for carnivorous fish species (Dersjant-Li 2002). However, the rapid development of the aquaculture industry caused fishmeal prices to increase as supplies dwindled (Phumee and others 2011; Rawles and others 2011; Dersjant-Li 2002). For that reason, it is imperative to look for sustainable alternatives that allow the continued growth of aquaculture with lower production costs (Rawles and others 2011). Given the concurrent increase in the global production of soybeans (Biswas and others 2011) and the need for alternative protein sources of plant origin, soybean meal has become a potential source for the partial or total replacement of fishmeal (Phumee and others 2011).

Soybean meal is a rich source of protein, has a high nutritional value, is available in large quantities on the market, and costs less than fishmeal (Phumee and others 2011). However, the presence of anti-nutritional factors including trypsin inhibitors, lectins, phytate, saponins, oligosaccharides, glycinin, and β -conglycinin (Soy Protein Concentrate for Aquaculture Feeds 2008; Adelizi and others 1998) are an impediment for the use of soybean in fish diets (Chen and others 2011). The desolventizer-toaster process which is used to eliminate solvent following soybean oil extraction (Soybean Processing-Fact Sheet n.d.) also inactivates trypsin inhibitors and lectins, thereby improving the quality of the soybean meal as a fish feedstuff (Refstie and Storebakken 2001).

On the other hand, phytate cannot be inactivated, leading to a reduction in the bioavailability of mineral elements and proteins. This problem can be solved by the use of phytase as an additive in plant-based feeds, improving fish growth and mineral absorption (Yang and others 2011; Imanpoor and Bagheri 2012). Knudsen and others (2008) demonstrated in their

study that soybean saponins in combination with one or more unidentified components present in soybean induce enteritis in Atlantic salmon. Hillestad (The Research Council of Norway 2011) arrived at the same conclusion with salmon and rainbow trout. Furthermore, Sørensen and others (2011) demonstrated that raffinose and stachyose could also be involved in reduced feed utilization in Atlantic salmon. It is likely that the combination of saponins and oligosaccharides could be the source of enteritis in fish (Knudsen and others 2008) and it could also be involved in the reduction of gut length in crucian carp (Cai and others 2012). Both oligosaccharides and saponins should be removed from soybean meal in order to use it as fishmeal replacement. The negative effects produced by glycinin and β -conglycinin can be reversed by modification of the chemical structure of these antigens during processing (Rumsey and others 1994). Both of them can be inactivated using heat treatments.

4. REMOVAL OF ANTI-NUTRITIONAL FACTORS

Trypsin inhibitors and lectins can be inactivated during processing while phytate can be treated with phytase (Yang and others 2011). But the other anti-nutritional factors—saponins, oligosaccharides, conglycinin, and β -conglycinin—still present significant problems when using soybean meal in fish feed.

Various methods are used to produce soy protein concentrates (SPC) including aqueous alcohol, acid leaching, and hot-water leaching processes (Figure 2.4) (Lusas and Rhee 1995).

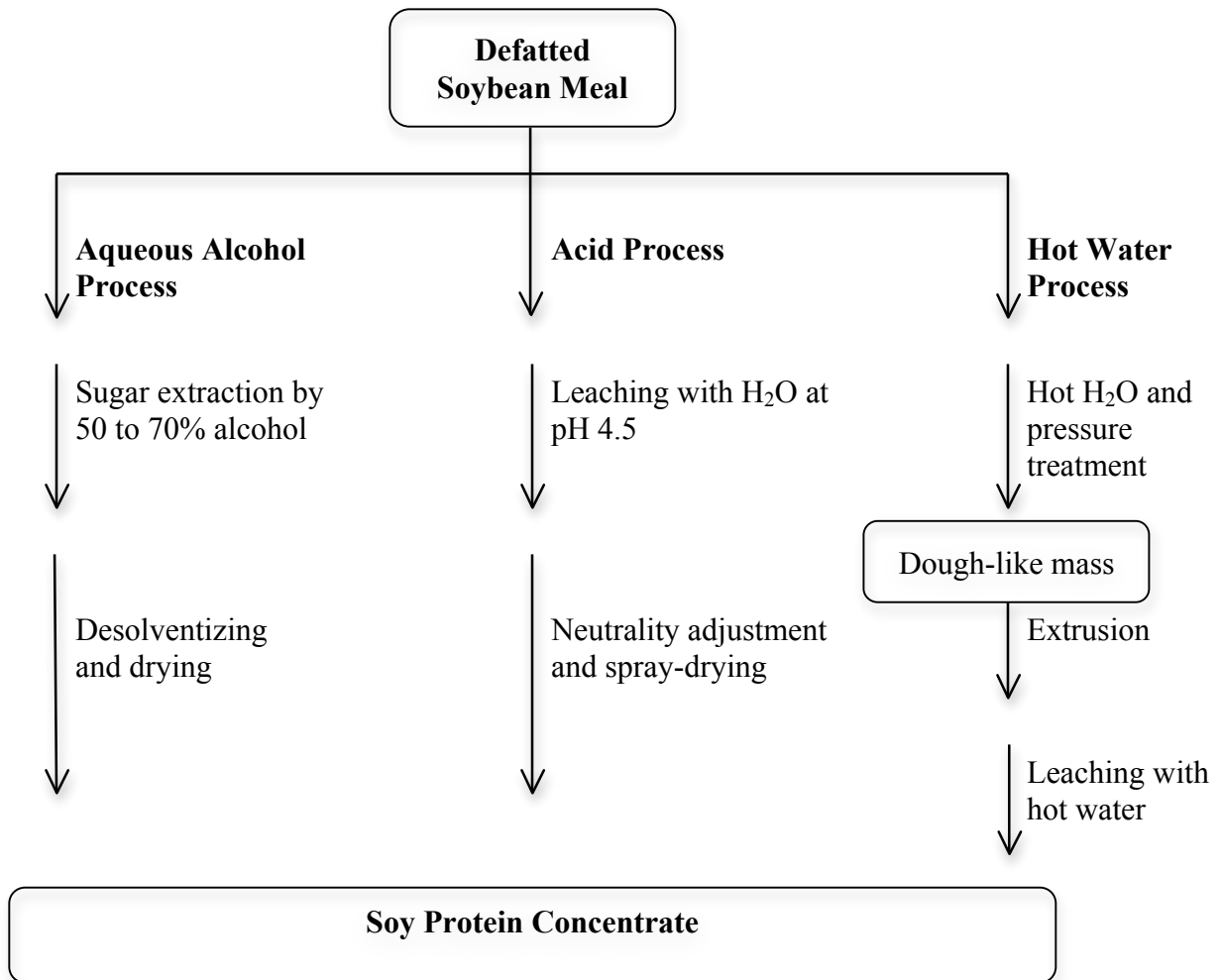


Figure 2.4: Soy Protein Concentrate Processing Methods (From Lusas and Rhee 1995).

Oligosaccharides and strong flavor components are removed during the SPC production. However, some minerals and other soluble components are also removed (Lusas and Riaz 1995).

CHAPTER III

KINETIC STUDIES ON GLYCININ AND β -CONGLYCININ

1. INTRODUCTION

Glycinin (11S) and β -conglycinin (7S) are the major storage proteins present in soybeans (Lusas and Rhee 1995), where they account for about 70% of the total protein content (Barac and others 2004). Both proteins are considered allergens for both humans and animals, because they are able to cause intestinal damage, diarrhea, growth depression, reduction of protein digestibility, and alteration of the immune function (Rumsey and others 1994; Ma and others 2010; Hei and others 2012). By denaturation or destruction of their quaternary structure, the harmful effects are lost (Koshiyama and others 1980-81). Heat denaturation of proteins is related to the disruption of the intramolecular hydrogen bonds (Nurul and Azura 2012), and can be affected by ionic strength and pH (Lakemond and others 2000; Koppelman and others 2004; Jiang and others 2010). The thermal stability of proteins can be studied by Differential Scanning Calorimetry (DSC) (Nurul and Azura 2012). DSC establishes the heat capacity (C_p) of the sample as a function of the temperature (Schön and Velázquez-Campoy 2005) and presents the information as an endothermic peak. The center of the peak corresponds to the maximum C_p . The integration of the area under the peak corresponds to the ΔH°_m (enthalpy change), which relates to the denaturation of the protein (Bruylants and others 2005). The changes produced in heat capacity are monitored as changes in heat flow (watts) (Perkin Elmer 2013).

The DSC equipment usually consists of two cells: a sample cell that contains the protein solution to be analyzed, and a reference cell that usually contains a buffer solution. The temperature is increased in both cells, and each cell temperature is monitored individually and continuously. Any difference in the heat capacity between the sample and the reference cells will

produce a temperature difference that will force the system to provide extra heat to the cell with the lower temperature. As a response, the system will provide the $\mu\text{J/s}$ or $\mu\text{cal/s}$ needed to maintain the temperature difference between the cells equal to zero. In the case of proteins, which require energy for the denaturation process, the system will provide the heat required to maintain the sample and reference cells at the same temperature until all the protein is denatured (Schön and Velázquez-Campoy 2005).

The objective of this work was to study the kinetics of deactivation of glycinin and β -conglycinin using the Decimal reduction time or D-value (time required to reduce 90% of the protein activity) and the thermal resistant constant or Z-value (temperature increase for one log reduction in D-value). To accomplish this, the remaining activity of both proteins after exposure to thermal treatments was determined by DSC. The technique relates the enthalpy of denaturation to the amount of active protein by comparing the heat capacity of a protein sample with the heat capacity of the untreated protein.

2. MATERIAL AND METHODS

2.1. Experimental design

The effect of temperature and time on the deactivation kinetics of each protein was studied with different combinations of temperature and time. The temperature levels were 40, 50, 65, 70, 75, 80, 85, and 90°C and time durations were 5, 10, 15, 20, and 30 minutes.

2.2. Soybean meal preparation

The soybean meal used in this study was provided by a soybean crusher in the state of Arkansas. The soybean meal was ground using a coffee grinder (Mr. Coffee, Rye, NY, USA),

and then sieved using a 60-mesh screen. The fraction of particles that passed the screen was used for the experiment.

2.3. Heat treatment of samples

Duplicates of five hundred milligrams of soybean meal were placed in a disposable culture tube (VWR borosilicate glass 16 x 100mm), and hydrated with 1.5 ml of distilled (DI) water. The tubes were slightly capped with Parafilm®—to avoid water evaporation and to prevent possible glass rupture when the tubes were immersed in the hot water bath—and left 1 hour at room temperature. The tubes were then put in a water bath at the specified temperatures of 40, 50, 65, 70, 80, 85, and 90 °C, and incubated for 5, 10, 15, 20, and 30 minutes. After the duration of the treatment was achieved, the tubes were removed and the heat treatment stopped by submerging the tubes in an ice bath. Sample pools for each treatment were generated for the DSC study.

2.4. Differential scanning calorimetry study

The DSC measurements were performed using a differential scanning calorimeter (Perkin Elmer, Norwalk, CT). Aluminum and stainless steel pans were used in the study. Approximately 20 mg of sample was weighed into stainless steel pans, or 4 mg of sample in the case of aluminum pans. The pans were then sealed. An empty pan was used as reference. The pans were heated at a scan rate of 10°C/min under nitrogen through the range of 20 to 120°C while data was collected. Transition temperatures (T_0 : onset temperature of denaturation, T_m : maximum temperature of denaturation, and T_E : end temperature), and enthalpy (ΔH : area under the curve in J/g) were determined with Pyris (v.3.52) (Perkin Elmer, Norwalk, CT).

2.5. Determination of D-value and Z-value

The rate of deactivation of glycinin (11S) and β -conglycinin (7S) as a function of temperature was studied using the concept of D- and Z- values. The results obtained for each treatment were plotted in an x-y scatter plot with a logarithmic scale for enthalpy and a regular scale for time. The data were fit with a linear regression line using the least-squares approach. D-values were calculated as the time needed to reduce 90% of the concentration of active protein. D-values were calculated as follows:

$$D - \text{value} = \frac{T_2 - T_1}{\log \Delta H_1 - \log \Delta H_2} \quad [\text{Eq. 3.1}]$$

Where:

T_i = temperature ($^{\circ}\text{C}$)

ΔH = enthalpy of denaturation in J/g

The Z-values were obtained by plotting D-values for each temperature in an x-y scatter plot with regular scales. The data was fitted to a linear regression by the least-squares method. Z-values were calculated as the temperature increase needed to reduce 1 logarithmic cycle the D-value. Z-values were calculated using the following equation:

$$Z - \text{value } (^{\circ}\text{C}) = \frac{T_2 - T_1}{\log D_1 - \log D_2} \quad [\text{Eq. 3.2}]$$

Where:

T_i = temperature ($^{\circ}\text{C}$)

D = D-value (min)

3. Results and Discussion

3.1. Decimal reduction time (D-value) of β -conglycinin and glycinin

Figure 3.1 shows a selected thermogram for the thermal denaturation of β -conglycinin (7S) and glycinin (11S) in the untreated sample. As seen in the graph, two thermal transitions at approximately 82.4°C and 102.8°C that correspond to the denaturation temperature of β -conglycinin and glycinin, respectively, are evident.

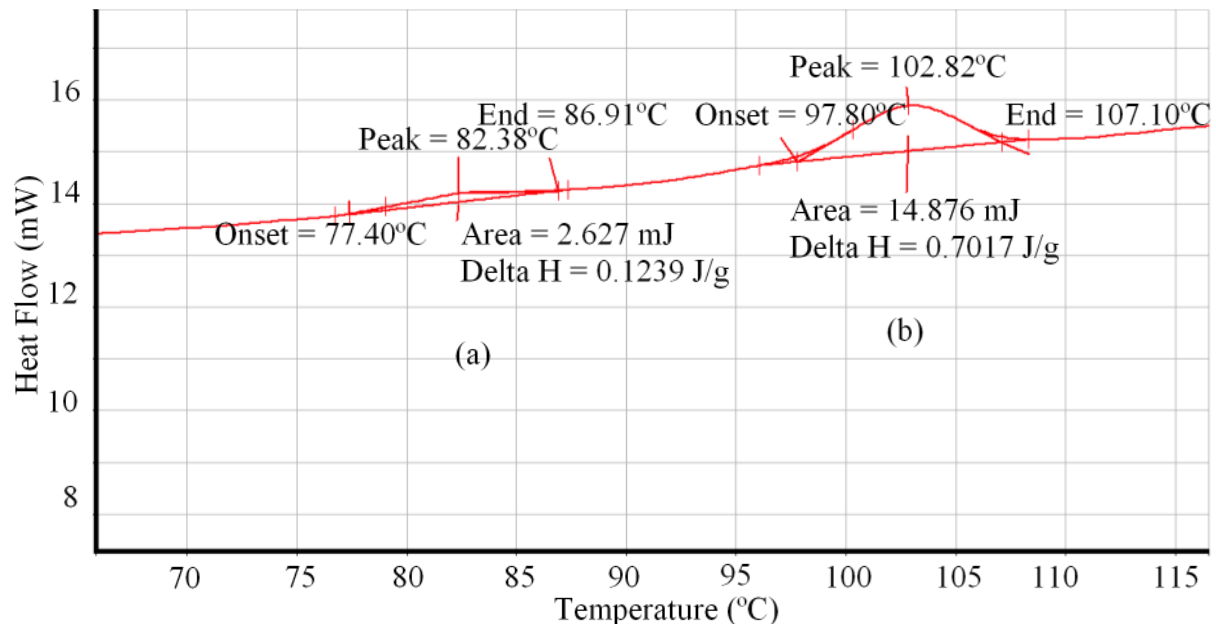


Figure 3.1: Thermogram showing the onset, maximum, end, and enthalpy of denaturation of a) β -conglycinin and b) glycinin in the untreated soybean meal.

The temperature of denaturation obtained for both proteins is higher than those reported by L'Hocine (2006), whose samples showed two different thermal transitions at approximately 75°C and 93°C corresponding to the denaturation temperature of β -conglycinin (7S), and glycinin (11S), respectively. The lower temperature reported by L'Hocine (2006) could be consequence of working with the isolated glycinin and β -conglycinin. This study indicated that

their denaturation requires a higher amount of energy when they are present within the matrix of the soybean meal when compared to the purified ones.

Figure 3.1 shows that denaturation of β -conglycinin in the untreated soybean meal starts at about 77°C, with maximum denaturation at 82.4°C, ending at approximately 87°C. Figure 3.2 shows the D-value plots for β -conglycinin within the soybean meal treated at different temperatures. ΔH (J/g) vs time (min) follows an approximate linear pattern (R^2 coefficients between 0.8996 and 0.9697). Table 3.1 displays the corresponding D-values calculated using the regression lines from graphs in Figure 3.2 and Eq. 3.1. It can be seen from the plots that there was deactivation of β -conglycinin at temperatures higher than 40°C. At lower temperatures, the deactivation occurred at a slower rate. D-value could not be determined at 40°C since the amount of protein at 0 and 30 minutes treatment remained the same and the small differences found could be attributed to experimental error. There was no detectable protein after either the 20 minutes treatment at 65°C or after 15 minutes at 70°C. As long as treatment time increased at a specified temperature, the concentration of active protein decreased, which is indicated by the lower ΔH (J/g). In the case of temperature, as long as treatment temperature increased, the time required to deactivate the protein declined as indicated by the lower D-values (Table 3.1).

Denaturation of glycinin in the untreated soybean meal occurs in the range of 98 to 107°C, with maximum denaturation temperature at approximately 103°C (Figure 3.1). Figure 3.3 shows the D-value graphs for temperature treatments ranging from 40 to 90°C. D-values follow a linear regression pattern with R^2 coefficients ranging from 0.8664 to 0.9968. Table 3.2 displays the D-values calculated using the linear regression lines from Figure 3.3 and Eq. 3.1. Denaturation of glycinin occurred in the whole range of temperatures studied, except at 40°C. D-value could not be determined in the 40°C treatment since there was no deactivation of glycinin in the period of

time analyzed. The other temperature treatments revealed how ΔH (J/g)—related to the amount of remaining active protein—decreased as long as time and temperature increased. As time increased during a specified temperature treatment, the remaining active glycinin decreased. The same occurred when the temperature of the treatment increased.

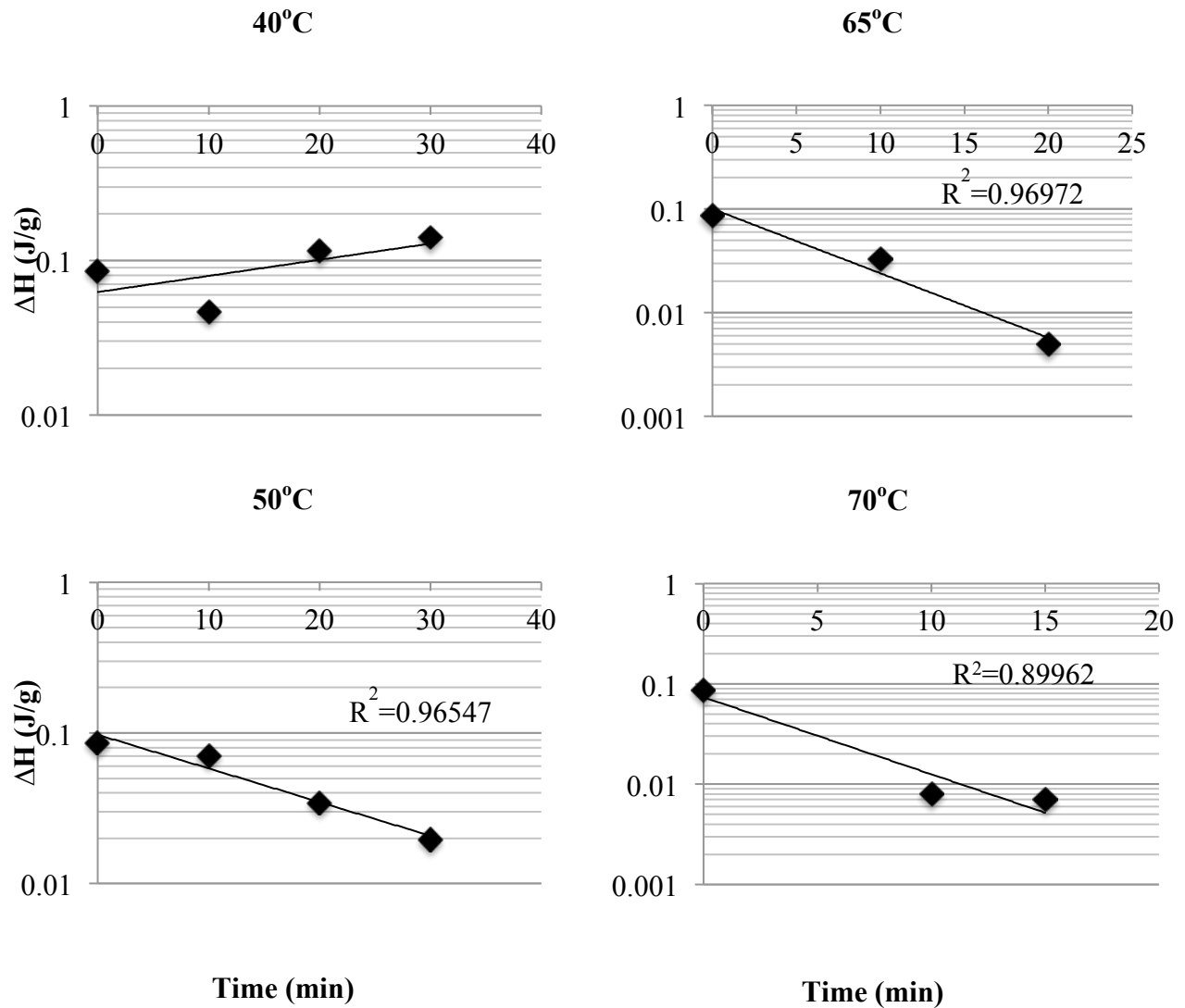


Figure 3.2: ΔH (J/g) vs time (min) of β -conglycinin at different temperatures.

Table 3.1: D-values of β -conglycinin at different temperatures.

Temperature ($^{\circ}$C)	D-value (min)
40	-
50	47.2
65*	21.9
70	14.9

*Aluminum pans

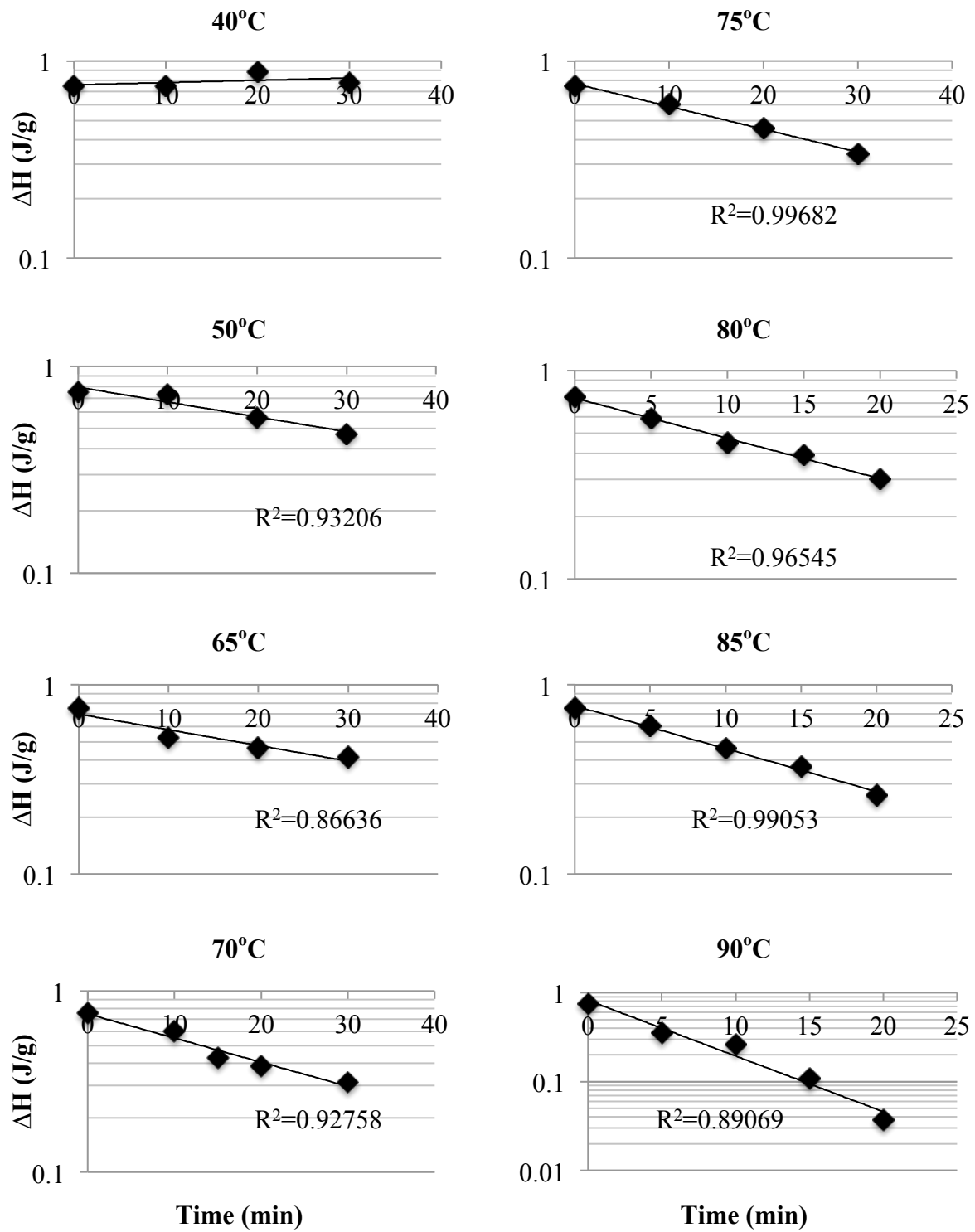


Figure 3.3: ΔH (J/g) vs time (min) of glycinin at different temperatures.

Table 3.2: D-values at different temperatures of glycinin.

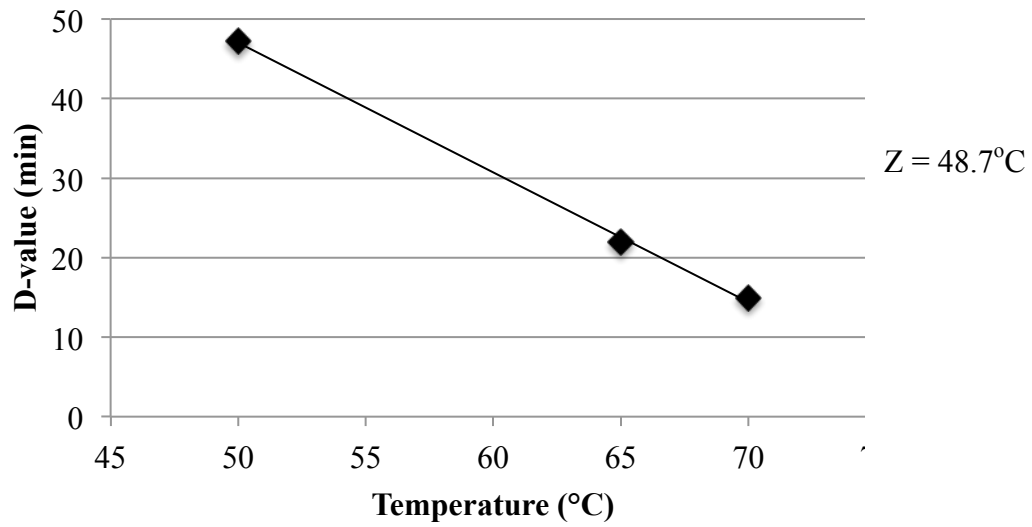
Temperature (°C)	D-value (min)
40	-
50	133.3
65*	112.4
70	73
75*	75.2
80	55.6
85*	47.6
90	16.3

* Aluminum pans

3.2. Thermal resistant constant (Z-value) of β -conglycinin and glycinin

Z-values were determined using Figure 3.4 and Eq. 3.2. The Z-value obtained for β -conglycinin was 48.7°C, which means that an increase in 48.7°C is needed to reduce 1 log of the D-value. In the case of glycinin, an increase of 70.9°C is needed to reduce 90% of the D-value.

(a) β -conglycinin



(b) Glycinin

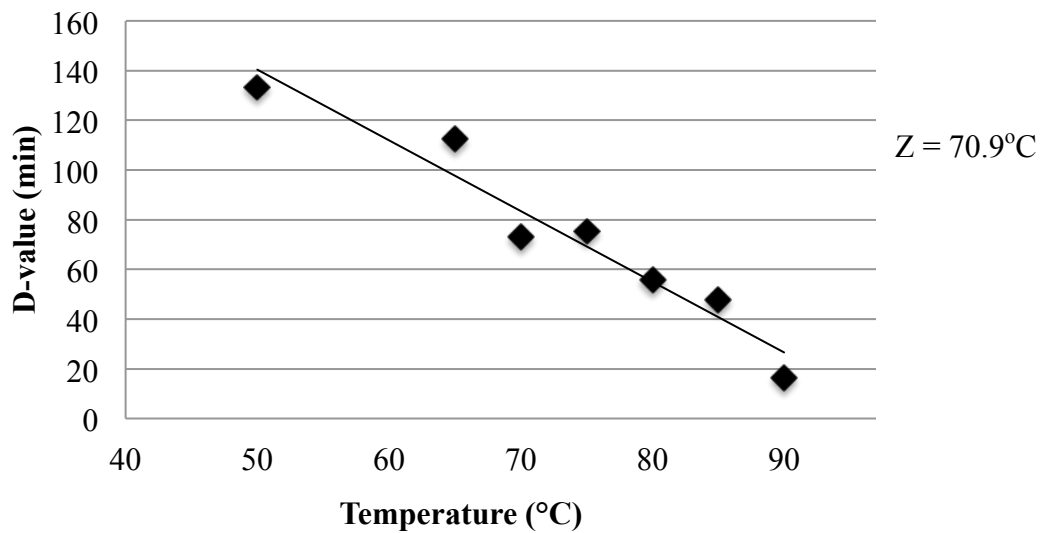


Figure 3.4: D-value (min) vs temperature (°C) of (a) β -conglycinin, and (b) glycinin.

4. CONCLUSIONS

The results of this study indicated that both glycinin and β -conglycinin are resistant to temperature, and that glycinin is the most resistant. D-values—time needed to reduce 90% of the protein activity—of glycinin were higher compared to those of β -conglycinin for the same temperature treatment. The same occurred with the thermal resistant constant (*Z*-value); the temperature increase needed to reduce 1 log of the D-value was also higher for glycinin. According to this study, an efficient heat treatment based on the deactivation characteristics of glycinin could be employed in order to reduce the content of active protein present in the sample, yielding a soybean meal with a superior nutritional value.

CHAPTER IV

EFFECT OF PRETREATMENT OF SOYBEAN MEAL WITH PHYTASE

1. INTRODUCTION

The presence of phytate—the indigestible form of phosphate—in soybean meal is one of the limiting factors for its inclusion in fish food. Phytate is a poly-phosphorylated carbohydrate that represents about 70% of the total phosphorus present in soybean (Smith and Rackis 1956). Phytate cannot be digested because of the lack of an intestinal phytase in monogastric animals, resulting in phosphorus deficiencies in the diet and also in contamination of water bodies from excreted phosphorus (Cao and others 2008). Phosphorus deficiency can cause problems in bone mineralization and impair weight gain (Cain and Garling 1995). Also, phytate forms complexes with some proteins and with minerals such as zinc, magnesium, and calcium, thus reducing their bioavailability (Denstandli and others 2007). Therefore, a process to reduce or eliminate the content of phytate from the meal could be of importance, for instance by pre-treating the meals with phytase.

Phytase is an enzyme that has the ability to hydrolyze phytate (Cao and others 2008). Pre-treatment or dephytinization of feedstuffs and spraying phytase onto pellets are the two treatments used to study the role of phytase (Cao and others 2007). Working on carp, Schäfer and others (1995) found that the addition of 500 and 1000 U/kg of phytase, delivered on sprayed pellets, was able to release 20 and 40% of phosphate, respectively, from the phytic acid present in the soybean meal diet. Lanari and others (1998) and Tudkaew and others (2008) reported that the inclusion of phytase in diets for rainbow trout increased the availability of dietary phosphorus, while lowering the release of phosphorus into the environment. Additionally, the pretreatment of soybean meal diets with phytase made the inorganic phosphate from phytic acid available to rainbow trout (Cain and Garling 1995, Sugiura and others 2001, Yang and others 2011). Cao and

others (2008), in their work with Nile tilapia, also found that the pretreatment of plant ingredients with phytase effectively transformed the phytate present in the sample into available phosphate. The apparent digestibility of phosphorus also increased in Korean rockfish (Yoo and others 2005). In their study with Atlantic salmon, Storebakken and others (1998) reported that the pretreatment of soy protein concentrate with phytase reduced the concentration of phytic acid by about 94%. Studies using soy protein concentrate also showed a reduction of 66% in phytic acid in the samples treated with phytase (Denstadli and others 2007). All authors, although working under different experimental conditions, concluded that either the supplementation or the pretreatment of the samples with phytase was effective in hydrolyzing phytic acid and making inorganic phosphate available. These techniques can replace the supplementation of inorganic phosphorus in the diets, thus reducing costs (Cao and others 2008) and also the phosphorus content of aqueous effluents (Cain and Garling 1995).

The objective of this study was to determine the effectiveness of a microbial phytase derived from *Aspergillus niger* (American Laboratories Inc, Omaha, NE) in reducing the content of phytate in soybean meal. To accomplish this, the sample was pretreated with the enzyme under two different experimental conditions. Enzyme concentration, incubation time, and sample-to-buffer ratios were studied in order to determine if the hydrolysis of phytate could be affected by any of these factors. The ratio of soybean meal to citrate buffer used was 1:1 and 1:15 (w/v). The ratio 1:1 is usually employed in the pre-treatment of soybean meal with phytase. However, since other anti-nutritional factors—oligosaccharides and saponins—can be reduced in the sample using a higher amount of buffer, this study attempted to determine if the effectiveness of phytase could be disturbed by the new ratio employed. Additionally, this new approach was useful to determine if the buffer played an important role by itself in the extraction of phytate,

while giving the correct pH to the enzyme. After treatment, the efficiency of the enzyme was evaluated by measuring total phosphate. This determination is more straightforward than determining the remaining phytate in the treated soybean meal and provides comparable results. Since phytate represents about 70% of the total phosphorus in soybean meal, the determination of total phosphorus can be used to estimate the remaining phytate after treatment. The quantification of total phosphorus was performed for both the solid fraction and the washing liquid, from now on referred to as supernatant.

2. MATERIALS AND METHODS

2.1. Materials

Defatted soybean meal, phytase (1500 U/g, American Laboratories Inc, Omaha, NE), citric acid monohydrate, sodium citrate, trichloroacetic acid solution (TCA), iron (II) sulfate heptahydrate, ammonium molybdate tetrahydrate, and phosphorus standard solution, all obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Treatments

Two different approaches were followed.

2.2.1. Treatment 1

The experiment was performed following the procedure described by Cao and others (2008) with some modifications. Soybean meal was treated with microbial phytase at 0, 750, or 1500 U/Kg. The enzyme was dissolved in 0.2M citrate buffer pH 5.5 using a magnetic stirrer for 30 min. Twenty five grams of soybean meal were then added to the buffer at 1:1 (w/v) ratio, and heated with constant stirring to 50-55°C on a hot plate (Super-Nuova, Barnstead International, Dubuque, IA). The mixture was covered with aluminum foil and incubated (Thermo Scientific

MAXQ 4450, Dubuque, IA) at 55°C for 6 hours. After treatment, the solid fraction was washed twice with 75 ml distilled H₂O to separate any phosphorus hydrolyzed by the enzyme from any phytate that might be present in the treated soybean meal. The solid fraction was dried in an oven (VWR model # 1310) at 60°C for 24 hours. The liquid fraction was clarified by centrifugation (Beckman Coulter Allegra X-22R) at 3900 x g for 30 minutes.

2.2.2. Treatment 2

This experiment followed the same steps as Treatment 1; however, the enzyme concentrations, incubation time, and sample to buffer ratio employed were different. Phytase at 0, 50, 100, 150, 300, or 450 U/g of soybean meal was dissolved in 150 ml of 0.2M citrate buffer pH 5.5 using a magnetic stirrer for 10 minutes. Then, 10 g of soybean meal was added to the buffer. The mixture was heated to 55°C with constant stirring at 300 rpm in a hot plate. The heated mixture was covered with aluminum foil and incubated at 55°C for 3 and 6 hours. To deactivate the enzyme, the mixture was then heated for 5 minutes at 95°C on a hot plate. After treatment, the supernatant was separated from the solid fraction and centrifuged at 3900 x g for 30 minutes. Both fractions were dried in an oven at 60°C for 24 hours.

2.3. Determination of total phosphorous

Quantification of total phosphorus in the treated soybean meal (solid fraction) was conducted by a contract lab (Agricultural Diagnostic Laboratory, Fayetteville, AR, USA). The method consisted of a wet digestion using HNO₃ and H₂O₂ on a heated block, and analyzed by a Spectro Arcos Inductively Coupled Plasma (ICP) (Ametek, Kleve, Germany).

Total phosphorus in the supernatant was analyzed using the Molybdate-Blue Method. All determinations were performed in triplicate. Two milliliters of sample were mixed with 2 ml of deionized water, 1 ml of 10% (v/v) Trichloroacetic acid solution (TCA), and 5 ml of Tausky-

Shorr color reagent (TSCR). Absorbance was read at 660nm with a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Columbia, MD, USA) and compared to a standard of phosphate. The standard curve of phosphate was prepared following the procedure for the enzymatic assay of phytase (EC 3.1.3.26) with concentrations ranging from 1 to 5 μ moles of phosphate.

3. RESULTS AND DISCUSSION

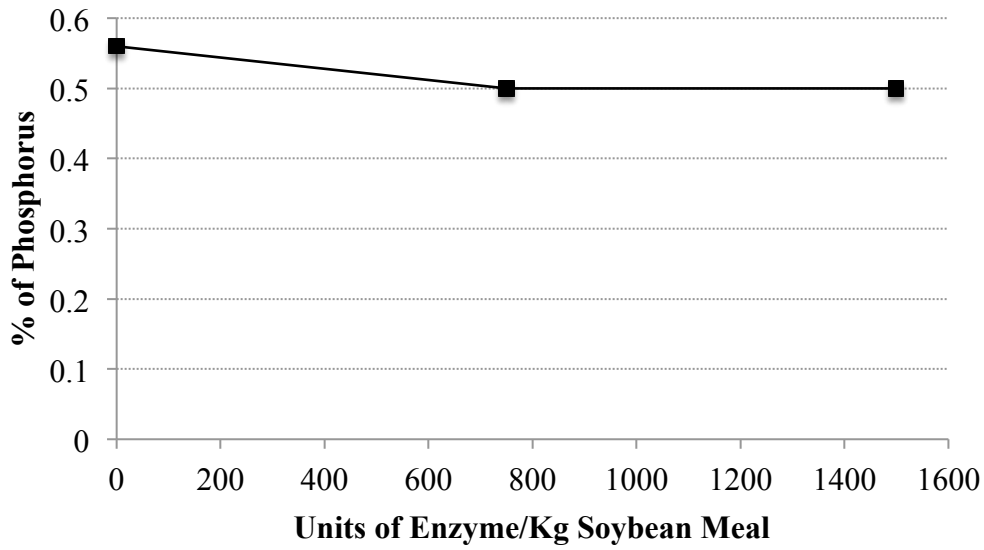
3.1. Treatment 1

Figure 4.1 (a) shows the remaining content of phosphorus in the solid fraction at the two different concentrations of phytase employed and the control (without the presence of enzyme) test. It appears that the pH of the buffer utilized in the experiment was enough to extract the phytic acid from the soybean meal since the amount of total phosphorus in the control is almost the same as the content in the experimental trials. The enzyme did not seem to have any effect on the reduction of the phytate present in the sample. However, interesting results were found when the supernatant was analyzed for total phosphorus. In this case, as shown in figure 4.1 (b), the total content of phosphorus in the supernatant for the control test was almost zero. That little amount could be attributed to the inorganic phosphorus originally present in the soybean meal that was rinsed with H₂O during the last step of the experiment. On the other hand, the 750 and 1500 U/Kg of soybean meal tests showed a high concentration of phosphorus. Additionally, the 750 U/Kg test exhibited the highest amount of total phosphorus. The concentration was approximate 38% greater than the total phosphorus in the 1500 U/Kg test. The determination of total phosphorus in the supernatant showed that the enzyme was able to release phosphate from the phytic acid present in soybean meal. If the samples were not washed during the experimental procedure, that phosphorus could be available for fish if the soybean meal pretreated with phytase was used as fish feed.

Cao and others (2008) found that 1000 U of phytase was the optimal dose needed to transform the phytate present in 1 kg of plant ingredients into inorganic phosphate. He reported that about 70% and 89% of phosphate were released from the phytate present in soybean meal when doses of 750 and 1500 U/kg were used in a proportion of 1:1 (w/v) soybean to buffer. He also reported that the citrate buffer used in the experiment could also help in the transformation of phytate into available phosphate to some degree.

The content of total phosphorus in the untreated soybean meal used in this study was 0.81% (Data not shown). About 70% of that content corresponds to phytate (Smith and Rackis, 1956). According to this work, about 38% of the total phosphorus was removed from the sample. Moreover, the enzyme was able to hydrolyze the phosphorus from the phytate present, thus increasing the amount of free phosphorus. These results are to a certain point comparable to the ones described by Cao and others (2008), even though he worked with phytate determination and available phosphorus instead of total phosphorus.

(a)



(b)

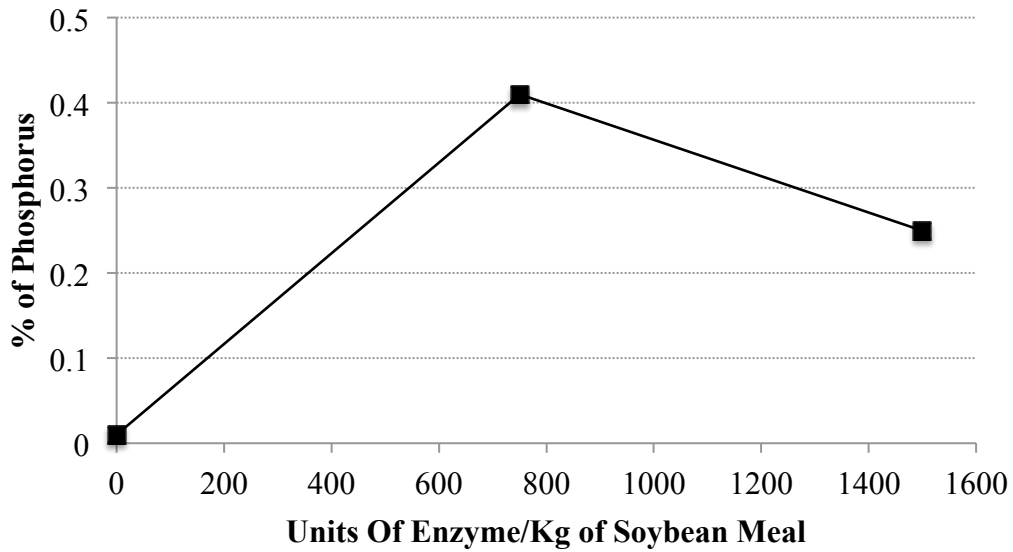
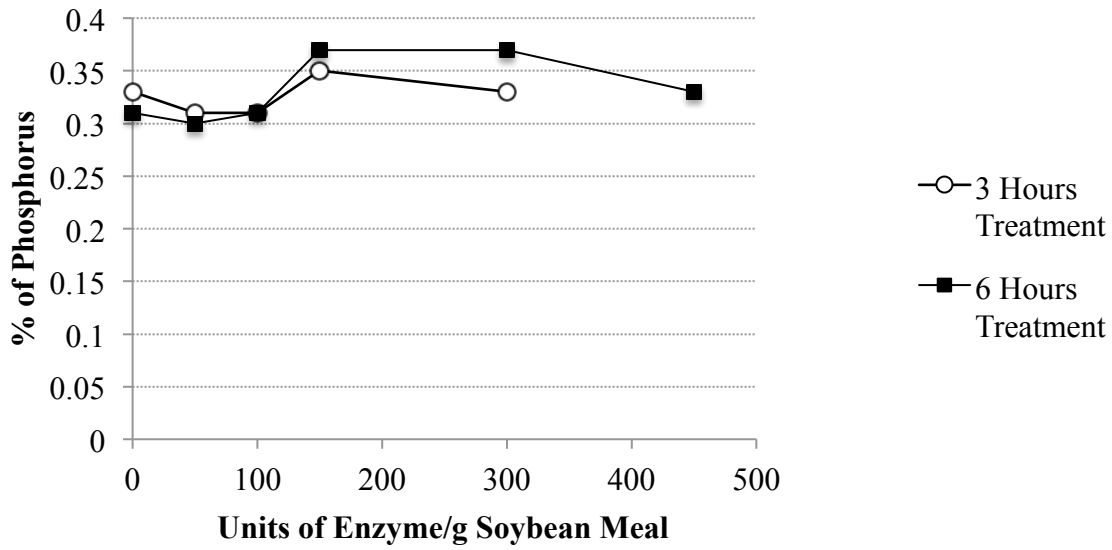


Figure 4.1: Total phosphorus (%) in (a) solid fraction, and (b) supernatant after 6 hours treatment with phytase.

3.2. Treatment 2

Figure 4.2 displays the results of total phosphorus in the solid fraction and supernatant at 3 and 6-hour treatments. The content of total phosphorus in the solid fractions was similar in all samples (Figure 4.2 (a)). No meaningful differences were found between the samples treated only with buffer at pH 5.5 and the samples treated with buffer at pH 5.5 and different concentrations of phytase (50, 100, 150, or 300 U/g of soybean meal) during the 3 and 6 hours treatments. The treatment with buffer alone seemed to remove most of the phytate present in the soybean meal as well as the content of inorganic phosphate. On the other hand, the concentration of total phosphorus in the supernatant showed a completely different pattern (Figure 4.1 (b)). The concentration in the controls was almost zero. Since the Molybdate-Blue method only quantifies inorganic phosphate, even though the phytate released from the soybean meal by the action of the buffer was present in the supernatant it could not be quantified. That was the reason that explained the lower values. On the other hand, the concentration of total phosphate in the samples treated with the enzyme at lower concentrations (50 and 100 U/g of soybean meal) for both 3 and 6 hour treatments were similar, and higher compared to the results obtained for the experimental trials using 150 and 300 units of enzyme per gram of soybean meal. That amount represents the inorganic phosphate liberated from the phytic acid in addition to the phosphate already present in the sample and liberated by the buffer.

(a)



(b)

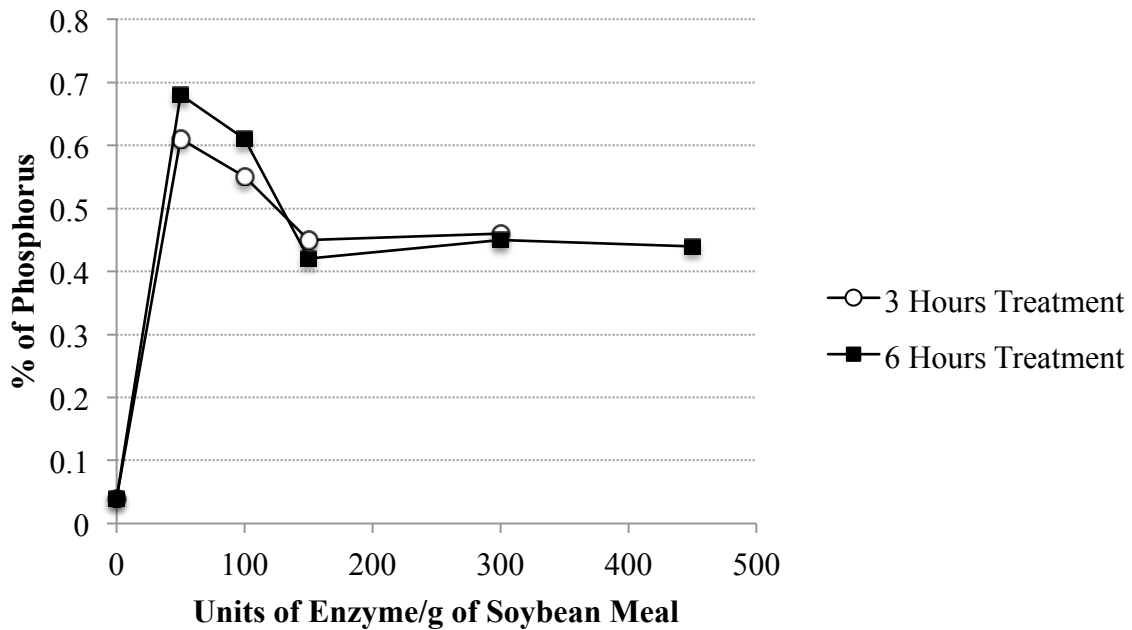


Figure 4.2: Total phosphorus in (a) solid fraction, and (b) supernatant after 3 and 6 hours treatment with phytase.

4. CONCLUSIONS

Results from Treatment 1, where a soybean meal to citrate buffer ratio of 1:1 (w/v) was employed, showed that the microbial phytase successfully released phosphate from the phytic acid present in the sample. The same result was attained from Treatment 2 using a 1:15 (w/v) proportion of soybean meal to buffer. Both trials provided positive results, but the applicability of each of them is different. Each test can be practical under different conditions. The pretreatment with the enzyme using a 1:1 sample to buffer ratio (Treatment 1) is a good alternative if the purpose is to improve the availability of phosphorus for fish feed, and also minimize its release to the environment. However, if the idea is to reduce the phytic acid content while concurrently reducing other soluble anti-nutritional compounds, the second treatment is a better choice. Even though the latter reduces the content of phosphorus that could be necessary for a balanced fish diet, it also reduces the content of the indigestible phytate to a greater extent than treatment 1. Additionally, it reduces the presence of other factors that could cause digestive problems in fish.

CHAPTER V

REMOVAL OF OLIGOSACCHARIDES, SAPONINS, AND PHYTATE BY EXTRACTION WITH WATER- ETHANOL SOLUTIONS

1. INTRODUCTION

The presence of anti-nutritional factors—oligosaccharides, saponins, and phytate—in soybean meal limits its use as a substitute for fishmeal. Oligosaccharides have been associated with several digestive problems—flatulence, nausea, and abdominal discomfort—when present in animal diets (Karr-Lilienthal and others 2005, Bairy and others 2008). These problems appear to be exacerbated when in the presence of saponins (Knudsen and others 2008). Saponins also reduce weight gain by decreasing feed intake and reducing protein digestibility (Francis and others 2001). Lowered protein digestibility is also a consequence of phytate in the sample (Refstie and Storebakken 2001). Phytate can form complexes with proteins, which makes them less suitable for digestion (Morales and others 2012). Furthermore, phytate contributes to water contamination because of its lack of absorption during the normal digestive process (Cao and others 2008). Elimination or reduction of these anti-nutritional factors in soybean meal will increase the likelihood of using soybeans as alternative feed ingredients to replace fishmeal.

The objective of this work was to reduce the content of soluble anti-nutritional factors—oligosaccharides, saponins, and phytate—from soybean meal while boosting the protein content and its digestibility using hot water-ethanol extractions. The central composite rotatable design (CCRD) was used to determine the effect of temperature, time, pH, and ethanol concentration that optimized the extraction of the aforementioned anti-nutritional factors, and increased the protein concentration and digestibility of the soybean meal.

2. MATERIALS AND METHODS

2.1. Materials

Commercial defatted soybean meal (SBM) used in the experiments was obtained from a soybean crusher in the state of Arkansas. The main reagents employed were citric acid monohydrate (99-102%), sodium phosphate dibasic (99%), formononetin, and methanol, all obtained from Sigma-Aldrich (St. Louis, MO, USA). Sulfuric acid (96.5%), phenol, and glucose were obtained from J.T. Baker (Phillipsburg, NJ, USA); urea from Omni-Pur EMC (Darmstadt, Germany); monobasic and dibasic potassium phosphate from VWR (West Chester, PA), and ethanol from EMD Millipore (Billerica, MA).

2.2. Methods

Extraction of anti-nutritional factors from defatted soybean meal was performed using different combinations of citrate-phosphate buffer and ethanol concentrations as a solvent at pHs in the range of 4.5 to 7, and temperatures between 25 to 75°C for a period of 5 to 65 minutes. According to the literature and preliminary studies, only one extraction is necessary to remove all the soluble sugars when water is used as a solvent. For that reason, one extraction was performed in successive experiments. Preliminary studies have also shown that the best ratio of water to soybean meal (SBM) that maximizes the amount of soluble sugars extracted is 15:1. Therefore this ratio was utilized. Experiments were accomplished using the central composite rotatable design (CCRD) to establish the best extraction conditions.

2.2.1. Experimental design

The effect of temperature, time, pH, and ethanol concentration for the extraction of soluble sugars, saponins, and phytate was studied using a central composite rotatable design (CCRD) with four factors, five levels, and 31 runs (Table 5.1). The responses obtained

experimentally were fitted to a quadratic polynomial equation (Eq. 5.1) and the significance of the terms determined using analysis of variance. The response surface was analyzed with Minitab version 15.1.30.0. (Minitab Inc., State College, PA) using full quadratic models for each response.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_1^2 + \beta_6 X_2^2 + \beta_7 X_3^2 + \beta_8 X_4^2 + \beta_9 X_1 X_2 + \beta_{10} X_1 X_3 + \beta_{11} X_1 X_4 + \beta_{12} X_2 X_3 + \beta_{13} X_2 X_4 + \beta_{14} X_3 X_4 \quad [\text{Eq. 5.1}]$$

Table 5.1: Central Composite Rotatable Design for the extraction of soluble sugars, saponins, and phytate using ethanol-water extractions.

Std Order	Run Order	Codified Factors				Real Factors			
		X ₁	X ₂	X ₃	X ₄	Temp. (°C)	pH	Time (min)	[EtOH] (%)
27	1	0	0	0	0	50.0	5.75	35.0	35.0
3	2	-1	1	-1	-1	37.5	6.38	20.0	17.5
21	3	0	0	-2	0	50.0	5.75	5.0	35.0
22	4	0	0	2	0	50.0	5.75	65.0	35.0
31	5	0	0	0	0	50.0	5.75	35.0	35.0
8	6	1	1	1	-1	62.5	6.38	50.0	17.5
17	7	-2	0	0	0	25.0	5.75	35.0	35.0
18	8	2	0	0	0	75.0	5.75	35.0	35.0
20	9	0	2	0	0	50.0	7.00	35.0	35.0
24	10	0	0	0	2	50.0	5.75	35.0	70.0
1	11	-1	-1	-1	-1	37.5	5.13	20.0	17.5
16	12	1	1	1	1	62.5	6.38	50.0	52.5
23	13	0	0	0	-2	50.0	5.75	35.0	0.0
4	14	1	1	-1	-1	62.5	6.38	20.0	17.5
10	15	1	-1	-1	1	62.5	5.13	20.0	52.5
7	16	-1	1	1	-1	37.5	6.38	50.0	17.5
5	17	-1	-1	1	-1	37.5	5.13	50.0	17.5
14	18	1	-1	1	1	62.5	5.13	50.0	52.5
28	19	0	0	0	0	50.0	5.75	35.0	35.0
12	20	1	1	-1	1	62.5	6.38	20.0	52.5
6	21	1	-1	1	-1	62.5	5.13	50.0	17.5
9	22	-1	-1	-1	1	37.5	5.13	20.0	52.5
30	23	0	0	0	0	50.0	5.75	35.0	35.0
26	24	0	0	0	0	50.0	5.75	35.0	35.0
13	25	-1	-1	1	1	37.5	5.13	50.0	52.5
11	26	-1	1	-1	1	37.5	6.38	20.0	52.5
29	27	0	0	0	0	50.0	5.75	35.0	35.0
19	28	0	-2	0	0	50.0	4.50	35.0	35.0
25	29	0	0	0	0	50.0	5.75	35.0	35.0
2	30	1	-1	-1	-1	62.5	5.13	20.0	17.5
15	31	-1	1	1	1	37.5	6.38	50.0	52.5

Note: Factors were codified as follows: $X_1 = \frac{T-50}{12.5}$, $X_2 = \frac{pH-5.75}{0.625}$, $X_3 = \frac{t-35}{15}$, $X_4 = \frac{[EtOH]-35}{17.5}$, where T = temperature, and t = time.

2.2.2. Sample preparation

Samples were prepared as follows: 450 ml of citrate-phosphate buffer/ethanol, in the proportions described by the experimental design, were added to 30g of SBM and stirred at 300 rpm on a Super-Nuova SP131825 hot plate (Barnstead International, Dubuque, IA, USA), at the temperature, time, and pH specified by the CCRD. After treatment, the supernatant was separated from the treated soybean meal by centrifugation with a Beckman Coulter Allegra X-22R centrifuge (Palo Alto, CA) for 30 minutes at 3900 x g. The insoluble solids were dried at 25°C until constant weight using a VWR oven (Cornelius, OR).

2.2.3. Analytical methods

2.2.3.1. Moisture content

Moisture content was determined by oven drying 15g of SBM at 115°C until constant weight (18-24 hours) in a VWR #1310 oven (VWR, West Chester, PA).

2.2.3.2. Ash content

Ash was determined according to the AOAC 923.03 method. Approximately 1 g of SBM was put into a porcelain crucible and placed in a muffle furnace at 550°C for 24 hours. After treatment, the crucible was removed from the furnace and placed in a closed desiccator to allow the container to cool before weighing the ashes. The ash content was calculated using the following equation:

$$\% \text{ ash} = \frac{\text{weight of ashes} - \text{tare of crucible}}{\text{SBM sample weight} - \text{tare of crucible}} \times 100 \quad [\text{Eq. 5.2}]$$

2.2.3.3. Total carbohydrate content

Total percentage of carbohydrates was calculated by subtracting protein, ash, and crude lipids from the initial sample weight on a dry basis (Kim and others 2003).

2.2.3.4. Total soluble sugars

Extraction of soluble sugars from the defatted soybean meal sample was performed following the procedure described by Giannoccaro and others (2006). Triplicate samples of 1g of soybean meal were added to 5 ml of distilled water aliquots and stirred at 50°C for 15 minutes. Then, the supernatant was separated from the solids by centrifugation at 3900 x g for 15 minutes and soluble sugars were measured in the supernatant by the phenol-sulfuric acid method (Dubois and others 1956). For this procedure, five hundred microliters of supernatant were mixed with 500 µl of 5% phenol solution and 2.5 ml of concentrated sulfuric acid. Following a 30-minute incubation at room temperature, absorbance was read at 490 nm using a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Columbia, MD, USA) and compared to a 5-point glucose standard curve.

2.2.3.5. Total fiber

Total fiber content was determined by subtraction of ash, proteins, lipids and soluble sugars from the initial sample on a dry basis (Giannoccaro and others 2006).

2.2.3.6. Crude protein content

Crude protein content was determined by the Agricultural Diagnostic Laboratory, University of Arkansas (Fayetteville, AR), measuring total nitrogen combustion with Elementar Variomax (Elementar Americas, Inc. Mt. Laurel, NJ, USA). Total crude protein was determined by multiplying total nitrogen content by 6.25.

2.2.3.7. Urease assay

Potential residual lectin and trypsin inhibitor activity was evaluated using the urease activity assay (Official Method Ba 9-58, American Oil Chemists Society 1968) as an indirect marker of activity. Approximately 0.2 g of finely ground soybean meal was placed into a test tube and 10 mL of buffered urea solution were added. The content was mixed and placed in a water bath at 30°C. In a second test tube, 0.2 g of soybean meal was added to 10 ml of 0.05M phosphate buffer solution (blank). The content was mixed and placed in a water bath at 30°C. The content of both test and blank tubes were mixed every five minutes during the 30-minute incubation period. Then, the tubes were removed from the water bath and allowed to stand for five minutes at room temperature (approximately 25°C). Approximately 5 ml of the supernatant was transferred to a new test tube and the pH measured in both the blank and the treated sample. The difference in pH between the treated sample and the blank was an index of urease activity. Activities higher than 0.15 were indicative of a high level of urease as a result of under-processing, while activities lower than 0.05 pH units were indicative of over-processing.

2.2.3.8. Saponins

Extraction of saponins was performed following the procedure described by Rupasinghe and others (2003) with some modifications. Five hundred milligrams of soybean meal was weighed directly into a 50 ml conical bottom flask and the exact weight was recorded. Five hundred microliters of formononetin (1.5 $\mu\text{mol/ml}$) were then added as an internal standard. Ten milliliters of 70% ethanol and a stir bar were added to the flask, stirred at room temperature on a magnetic stirrer for at least 2.5 hours, then centrifuged at 3900 x g for 10 minutes and the supernatant was filtered through a Whatman #1 filter paper, using a glass funnel. Ten milliliters of 100% ethanol was added to the residue. After centrifugation at 3900 x g for 10 minutes, the

supernatant was filtered, and the residue discarded. The supernatant was rotary evaporated to dryness at 30°C (Rotavapor® R II, Buchi UK Ltd., Lancashire OL9 9QL, United Kingdom) and 2.4 ml of 100% methanol were added to the residue. The sample was transferred to a conical bottom tube and centrifuged at 3900 x g for 10 minutes. From the tube, 1.6 ml of supernatant was removed and 0.4 ml MQ water was added. The final sample was put into a mini-centrifuge tube and allowed to stand overnight. The sample was filtered with a 0.45 µm PFTE membrane filter (VWR, West Chester, PA) into a sample vial to be analyzed by HPLC.

Quantification of saponins was performed by the Nutrition Laboratory at the Food Science Department of the University of Arkansas (Fayetteville, AR). HPLC analysis was done with an RP-18, YMC-Pack-ODS-AM (250mm x 4.6mm) L x ID column (YMC America Inc., Allentown, PA). Saponins were separated with a gradient of 0.05% trifluoroacetic acid in water (solvent A) and 100% acetonitrile (solvent B) at a total flow rate of 1 mL/min. The gradient of elution was as follows: before injection the column was stabilized for 9 min with 63%A and 37% B. After injection, B was increased from 37% to 40% in 12 min and then ramped to 48% in 25 min. Solvent B was then incremented to 100% in 1 min, held for 2 min, and then returned to 37% in 1 min. The temperature of the column was 25°C (room temperature), and the injection volume 35 µl. The flow rate used was 1.0 ml/min during a 50-minute cycle. Compounds were monitored with a 168 Beckman Photodiode Array detector at a wavelength range between 200 and 600 nm. Data was analyzed using 32 Karat (v.8.0) software (Beckman Coulter Inc., Brea, CA, USA). Concentrations of saponins were calculated using standard calibration curves prepared with purified standards of the individual saponins.

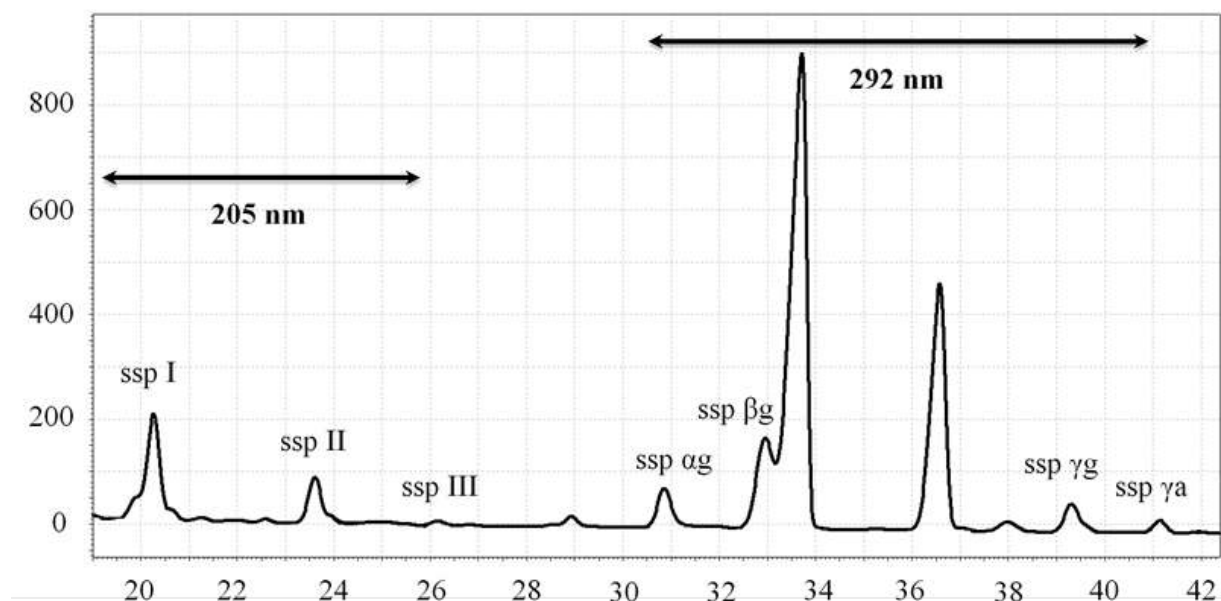


Figure 5.1: Monitored absorbance of saponins at 205 and 292 nm.

2.2.3.9. Phosphate

Total phosphate was quantified by the Agricultural Diagnostic Laboratory, University of Arkansas (Fayetteville, AR) using wet digestion with H₂O₂ and HNO₃, followed by analysis with a Spectro Arcos Inductively Coupled Plasma (ICP) (Ametek, Kleve, Germany).

3. RESULTS AND DISCUSSION

3.1. Chemical composition of the untreated soybean meal

The defatted soybean meal sample contained $9.10 \pm 0.09\%$ moisture content, $7.93 \pm 0.06\%$ dry basis ash, $34.67 \pm 0.27\%$ d.b. total carbohydrate content ($9.40 \pm 1.56\%$ soluble sugars plus $25.27 \pm 1.29\%$ total fiber), and a crude protein concentration of $57.40 \pm 0.21\%$ d.b..

The initial concentration of phosphate and saponins were $0.81 \pm 0.03\%$ d.b. and $0.103 \pm 0.002\%$ d.b., respectively. The protein digestibility of the untreated sample was $72.2 \pm 0.97\%$. The results obtained for moisture content, crude protein, ash, phosphorus, and total carbohydrates are comparable to those reported by Lusas and Rhee (1995) and Tudkaew (2008).

However, they are higher than those reported by Kim and others (2003), Da Silva (2009), and Lujan-Rhenals (2013). The differences found in composition could be related to different cultivars, storage, and processing conditions of the soybean meal samples utilized by the different authors.

3.2. Residual trypsin inhibitors and lectins

Trypsin inhibitors and lectins are two important anti-nutritional factors present in soybean (Machado and others 2008; Bajpai and others 2005; Fasina and others 2003). However, according to the literature, both of them are inactivated during the desolventizer-toaster process employed to eliminate the residual solvent following the oil extraction from the soybean (Refstie and Storebakken 2001). Nevertheless, it was decided to analyze if there was any residual activity of these two anti-nutritional factors using the urease activity test. According to this assay the residual level was 0.085 ± 0.02 units, meaning no lectins and trypsin inhibitors were active in the sample.

3.3. Extraction of soluble sugars

Total soluble sugars response fitted a quadratic equation that contained the linear terms temperature (X_1), time (X_3), and ethanol concentration (X_4), the quadratic term time (X_3^2), and the time-ethanol interaction ($X_3 X_4$) (Eq. 5.4). The analysis of variance (Table 5.2) indicates the significance of the terms of Eq. 5.4 and the goodness of the statistical fit of the model that can be judged by a “lack-of-fit” p-value of 0.903. These findings are in agreement with those published by Kim and others (2003) and Giannoccaro and others (2006), whose works described a strong relationship between temperature, time, and aqueous alcohol solutions with the extraction of soluble sugars. The magnitude of the coefficients of each term described the importance of each

factor involved in the extraction. This is particularly important in the case of temperature, where the coefficient is suggestively large.

$$Y = 11.8839 + 1.0738 X_1 + 0.6054 X_3 - 0.5037 X_4 - 0.6805 X_3^2 + 0.7431 X_3 X_4 \quad [\text{Eq. 5.4}]$$

Table 5.2: Analysis of variance (ANOVA) for total soluble sugars.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	65.017	65.017	13.0035	12.00	0.000
Linear	3	42.558	42.558	14.1859	13.09	0.000
Square	1	13.624	13.624	13.6241	12.57	0.002
Interaction	1	8.836	8.836	8.8358	8.15	0.009
Residual Error	25	27.094	27.094	1.0837		
Lack-of-fit	9	5.214	5.214	0.5793	0.42	0.903
Pure Error	16	21.880	21.880	1.3675		
Total	30	92.111				

The response surface of the extraction of soluble sugars shows a twisted shape (Figure 5.2). At short treatment time, extraction was favored at low ethanol concentration. However, the opposite was true at longer treatment times, where the extraction was enhanced at high ethanol concentrations. The concentration of soluble sugars extracted steadily increased with treatment time up to the 35-40-minute treatment at low ethanol concentration, after which the concentration started to decrease as the time of treatment increased. This could be attributed to the polarity of the solvent used. Sugars, because of their structure, are more soluble in water than in alcohols, which could explain why less time is necessary to remove the sugars from the sample when a higher concentration of water is used. However, at higher ethanol concentrations the extraction of sugars progressively increased with treatment time. It was also noticed that concentrations of ethanol in the range of 50% to 100%, significantly favored the extraction of sugars. The maximum amount of soluble sugars that were extracted under the conditions described by Eq. 5.4 was 14.03% using the combination of 50°C, pH 5.75, an ethanol concentration of 35% during a period of 35 minutes. The minimum concentration determined

was 7.95% following the combination of 75°C, pH 5.75, 35% ethanol concentration during a 35-minute treatment. The content of total soluble sugars quantified in the untreated soybean meal ($9.40 \pm 1.56\%$) by the technique described by Giannoccaro and others (2006) using water as the extracting solvent is low compared to the concentrations obtained using citrate-phosphate buffer/ethanol as a solvent. This study shows that the use of citrate-phosphate buffer/ethanol under controlled pH and temperature can be successfully employed to optimize the extraction of soluble sugars.

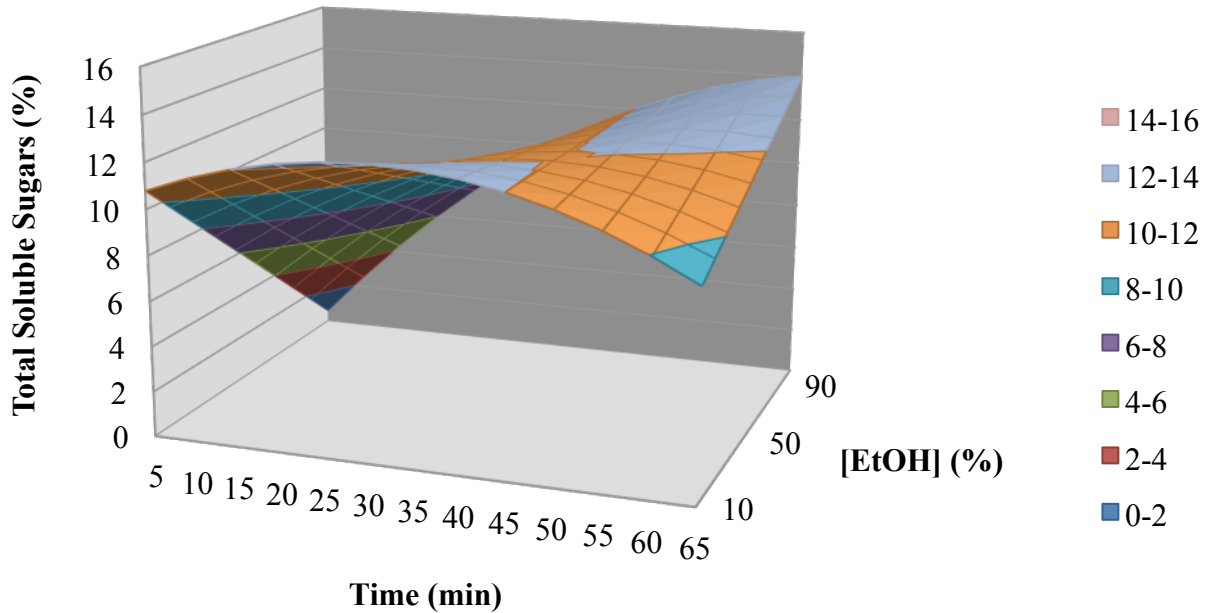


Figure 5.2: Total soluble sugars (%) extracted as a function of time (min) and [EtOH] (%) at 50°C.

3.4. Extraction of phosphate

The coefficients for the quadratic equation that represent the response of remaining total phosphorus in the sample are described in Eq. 5.5. The significance of the terms of the equation was determined by analysis of variance (ANOVA) (Table 5.3), and the “lack of fit” p-value of 0.111. Ethanol concentration seemed to be the factor that played the most important role in the extraction of phosphate since it is has the largest coefficient.

$$Y = 0.859737 + 0.0220833 X_1 + 0.0354167 X_2 + 0.06875 X_4 + 0.0236732 X_4^2 + 0.025625 X_1 X_2 - 0.026875 X_2 X_4 \quad [\text{Eq. 5.5}]$$

Table 5.3: Analysis of variance (ANOVA) for remaining total phosphorus.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	6	0.19380	0.19380	0.032299	13.44	0.000
Linear	3	0.15525	0.15525	0.051749	21.53	0.000
Square	1	0.01649	0.01649	0.016487	6.86	0.015
Interaction	2	0.02206	0.02206	0.011031	4.59	0.021
Residual Error	24	0.05769	0.05769	0.002404		
Lack-of-fit	8	0.02894	0.02894	0.003617	2.01	0.111
Pure Error	16	0.02875	0.02875	0.001797		
Total	30	0.25148				

The extraction of phosphate described by Eq. 5.5 was favored at low pH, and also low ethanol concentration (Figure 5.3). As long as the pH of the solvent, and the concentration of ethanol increased, the remaining content of phosphate in the sample increased, meaning the extraction was not efficient under the circumstances described by the objective of the experiment, which was to reduce the remaining content of phosphate. The maximum extraction of phosphorus, according to Eq. 5.6, occurred at 62.5°C, a pH of 5.13, and a 17.5% ethanol concentration during a 20 or 50-minute treatment, leaving 0.75% of total phosphorus in the treated sample. On the other hand, the least effective treatment occurred at 50°C, pH 5.75, with an ethanol concentration of 70% during a treatment of 35 minutes yielding a sample with 1.09% of remaining total phosphorus. The results obtained in this work are in accordance with the findings of Lusas and Rhee (1995) who found that phytate is effectively extracted with water at pH 5.0. These results also confirm that the ethanol concentration and pH, both described by the model employed, play an important role in the extraction of phytate.

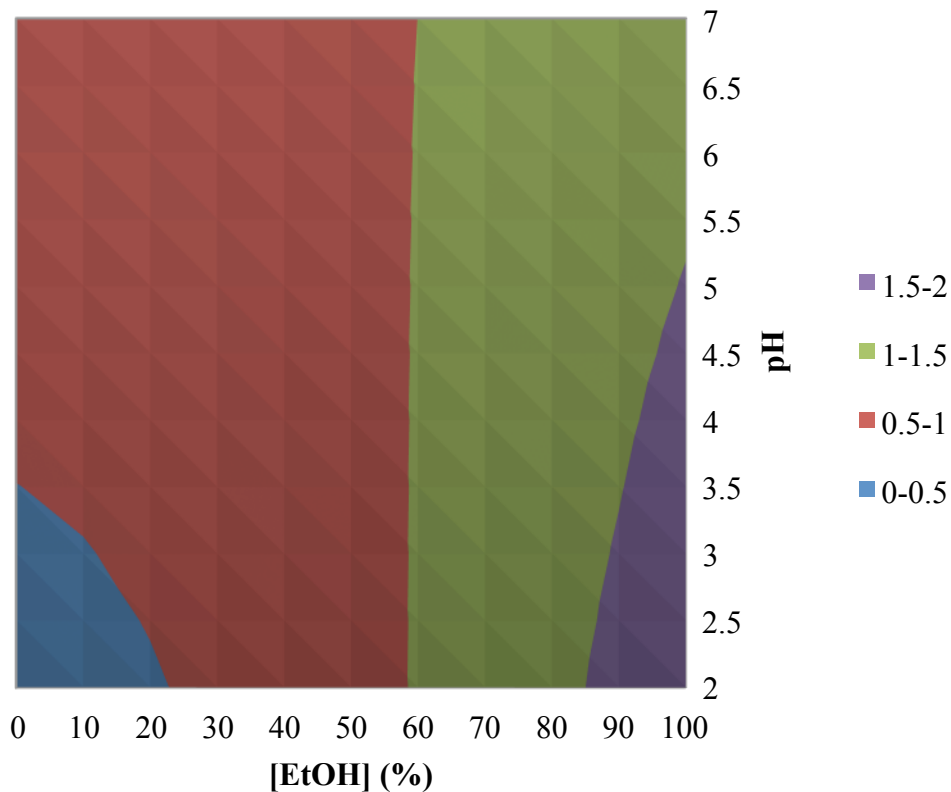


Figure 5.3: Remnant phosphate (%) as a function of [EtOH] (%) and pH at 50°C.

3.5. Extraction of saponins

The study of total saponins was focused on Group B saponins, which account for approximately 83% of the total saponins present in defatted soybean meal. At the same time, the behavior of DDMP— α g, β g, β a, γ g, and γ a—and non-DDMP—I, II, and III—saponins within Group B was studied. Quantification of saponins was performed according to Figure 5.1. The significant terms with the corresponding coefficients for remaining total Group B saponins in the treated soybean meal sample are shown in Eq. 5.6 and Table 5.4. Ethanol concentration exhibited the largest coefficient, meaning it could be the most powerful factor in the extraction of saponins. Ethanol concentration and temperature are two factors known for playing an important role in the extraction of saponins. Temperature is an important factor since non-DDMP saponins are

products formed by heat exposure (Kudou and others 1993), therefore affecting the final content of saponins present in the samples. Additionally, saponins are amphiphilic compounds readily soluble in aqueous alcohol solutions, which favors their extraction from the soybean meal sample.

$$Y = 0.052125 - 0.003768 X_1 - 0.000425 X_2 - 0.005429 X_3 - 0.035465 X_4 + 0.003023 X_1^2 + 0.003020 X_3^2 + 0.015524 X_4^2 + 0.004376 X_2 X_3 \quad [\text{Eq. 5.6}]$$

Table 5.4: Analysis of variance (ANOVA) for total saponins in treated soybean meal.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	8	0.038641	0.038641	0.004830	132.68	0.000
Linear	4	0.031238	0.031238	0.007810	214.52	0.000
Square	3	0.007096	0.007096	0.002365	64.97	0.000
Interaction	1	0.000306	0.000306	0.000306	8.42	0.008
Residual Error	22	0.000801	0.000801	0.000036		
Lack-of-fit	16	0.000658	0.000658	0.000041	1.73	0.259
Pure Error	6	0.000143	0.000143	0.000024		
Total	30	0.039442				

The extraction of total saponins increased as the time of treatment increased, in all ethanol concentrations tested (Figure 5.4). However, it is hard to perceive this behavior from the graph since the starting and extracted concentrations of saponins were extremely small. The extraction was greatest at 50-60% ethanol concentration. The highest concentration of total saponins described by Eq. 5.6 under the experimental conditions was 0.19% employing the combination of 50°C, pH 5.75, citrate-phosphate buffer (no ethanol) for a 35-minute treatment. On the other hand, the lowest concentration of saponins, which corresponds to the most efficient treatment, was 0.025% following treatment at 62.5°C, pH 5.13, 52.5% ethanol for 50 minutes. The comparison of the concentration of Group B saponins in the untreated soybean meal sample (0.103 ± 0.002%) with the minimum concentration described by Eq. 5.6 (0.025%) shows that an

effective treatment could lead to a sample with reduced concentration of saponins more suitable to be used as fishmeal replacement. However, the highest concentration described (0.19%) is higher than the concentration of the untreated sample demonstrating that some treatments can also lead to harmful results.

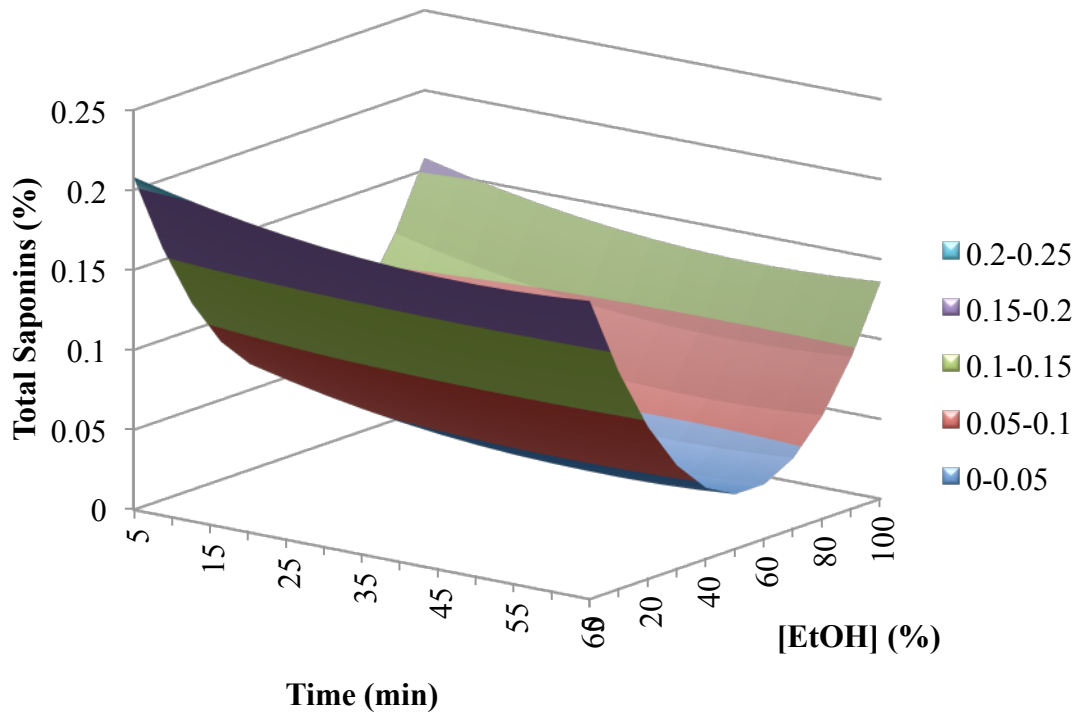


Figure 5.4: Total saponins content (%) as a function of time (min) and [EtOH] (%) at 50°C and pH of 5.75.

3.5.1. 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one saponins

In the DDMP saponins study, the linear model was the highest order model with significant terms (Eq. 5.7 and Table 5.5). Ethanol concentration is critical since, as already mentioned, the structure of saponins makes them soluble in aqueous alcohol solutions. Temperature could play a role by transforming the DDMP saponins into non-DDMP saponins, thus reducing the DDMP content in the sample as the temperature of the treatment increases. In addition, as the time of the treatment increases, the remaining content of saponins decreases.

$$Y = 0.000113 - 0.000018 X_1 - 0.000012 X_3 - 0.000048 X_4 \quad [\text{Eq. 5.7}]$$

Table 5.5: Analysis of variance (ANOVA) for DDMP saponins in treated soybean meal.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	3	0	0	0	49.31	0.000
Linear	3	0	0	0	49.31	0.000
Residual Error	27	0	0	0		
Lack-of-fit	11	0	0	0	0.97	0.510
Pure Error	16	0	0	0		
Total	30	0				

The extraction of DDMP saponins, (Figure 5.5), was extremely dependent on time and ethanol concentration. The extraction improved as treatment time and ethanol concentration increased. The highest concentration of DDMP saponins in the treated soybean meal described by Eq. 5.7 was 0.000209% and occurred with the following combination of factors: 50°C, pH 5.75, 0% ethanol concentration for a 35-minute treatment. On the other hand, the lowest concentration of remaining DDMP saponins was 0.000017% following treatment at 50°C, pH 5.75, 70% ethanol concentration for a period of 35 minutes. The concentration of DDMP

saponins in all treated samples was lower—even the highest concentration (0.000209%) found in the least effective treatment—than the concentration present in the untreated sample (0.00022%, data not shown).

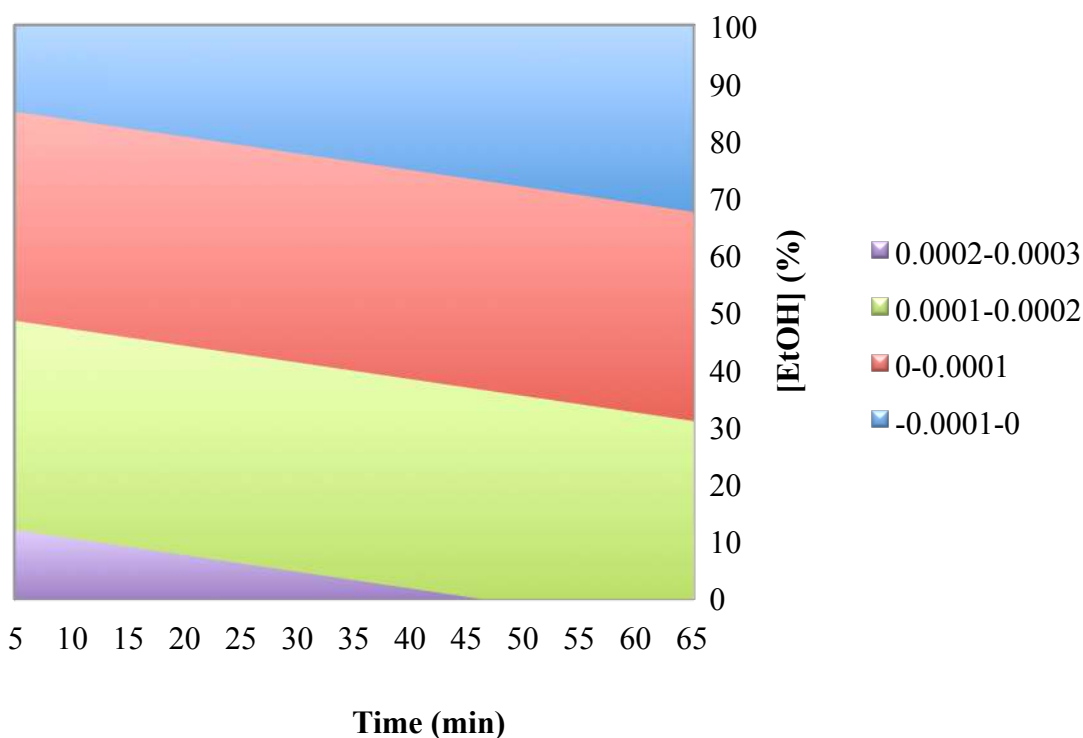


Figure 5.5: Total DDMP saponins (%) as a function of time (min) and [EtOH] (%) at 50°C.

3.5.2. Non-2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one saponins

The content of non-DDMP saponins in the treated soybean meals followed the same pattern as the total Group B saponins. The concentration of DDMP saponins was negligible and did not have any impact on the total concentration of saponins. The magnitudes of the coefficients for all significant terms are extremely low (Eq. 5.8 and Table 5.6). The coefficient corresponding to ethanol concentration is the largest one, meaning it could have a greater role in the extraction of non-DDMP saponins. The extraction of non-DDMP saponins improved as the

period of time of treatments increased for the ethanol concentrations examined (Figure 5.6). At 50-60% ethanol concentration, the extraction was heightened. The lowest concentration of non-DDMP saponins in the treated soybean meals was 0.025% following treatments at 62.5°C, pH 5.13, 52.5% ethanol concentration, for 50 minutes. On the other hand, the highest amount of non-DDMP saponins was 0.18% following treatment at 50°C, pH 5.75, in the presence of only citrate-phosphate buffer, for a treatment time of 35 minutes. The highest concentration of non-DDMP saponins present after treatments (0.19%) almost double the concentration found in untreated soybean meal (0.102% data not shown). Non-DDMP saponins are products formed by heat exposure of the DDMP saponins, which can explain this unfortunate result. Excessive temperature used during the removal procedure could affect the production of these compounds. Optimization of the extraction procedure can lead to significant improvement in the elimination of these harmful components for fish diets.

$$Y = 0.052021 - 0.003750 X_1 - 0.000417 X_2 - 0.005417 X_3 - 0.035417 X_4 + 0.003019 X_1^2 + 0.003019 X_3^2 + 0.015519 X_4^2 + 0.004375 X_2 X_3 \quad [\text{Eq. 5.8}]$$

Table 5.6: Analysis of variance (ANOVA) for non-DDMP saponins in treated soybean meal.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	8	0.038547	0.038547	0.004818	132.24	0.000
Linear	4	0.031150	0.031150	0.007787	213.73	0.000
Square	3	0.007091	0.007091	0.002364	64.87	0.000
Interaction	1	0.000306	0.000306	0.000306	8.41	0.008
Residual Error	22	0.000802	0.000802	0.000036		
Lack-of-fit	16	0.000659	0.000659	0.000041	1.73	0.258
Pure Error	6	0.000143	0.000143	0.000024		
Total	30	0.039348				

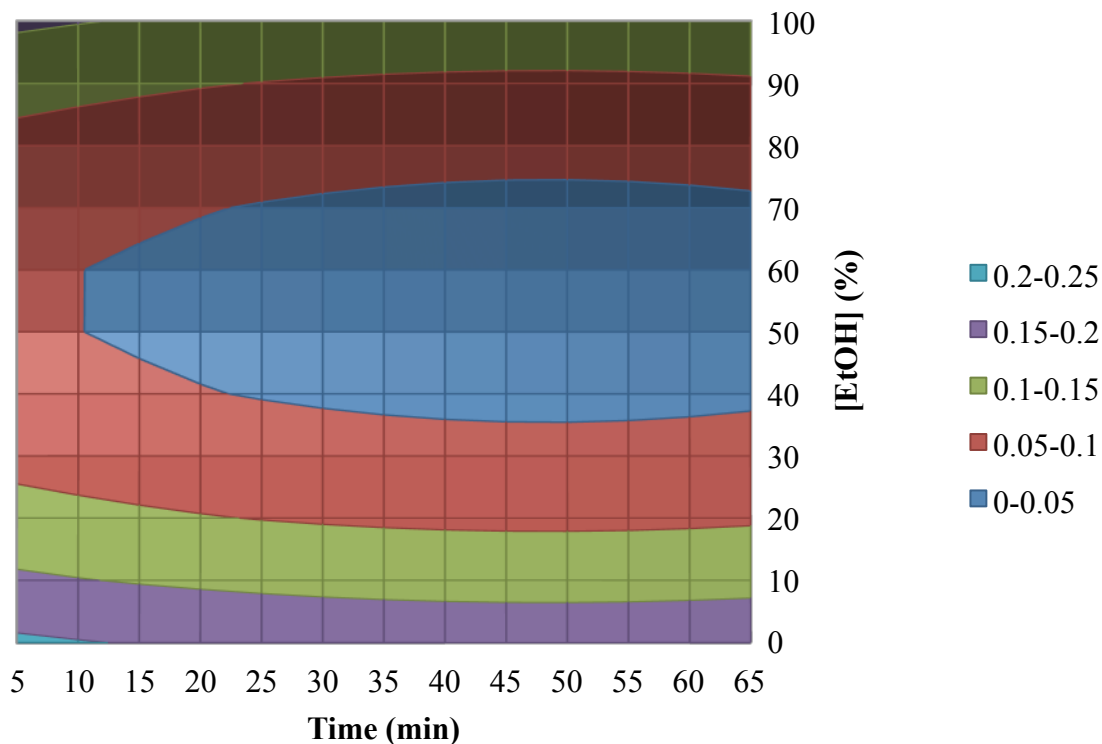


Figure 5.6: Total Non-DDMP saponins (%) as a function of time (min) and [EtOH] (%) at 50°C and pH of 5.75.

4. CONCLUSIONS

The analysis of the individual extractions showed that it is possible to obtain a soybean meal with reduced content of anti-nutritional factors to be used in animal feed. The method used was able to lower the content of soluble sugars, saponins, and phytate producing comparable results to those published by the aquaculture industry. However, the technique developed in this work presents the advantage of being a one-step process compared to the manufacturing industry where anti-nutritional factors are removed using 60-70% alcohol solutions and additional exposure to thermal processes. It also has the improvement of using less ethanol concentration. Still, there was not a unique treatment that could eliminate or minimize the three anti-nutritional

compounds studied at the same time. The treatment that was efficient for the extraction of one compound did not produce the same effect on the extraction of a different one. This was the case for the extraction of phytate and DDMP saponins. The most efficient treatment for the extraction of DDMP saponins (50°C, pH 5.75, 70% ethanol concentration, for a 35 minute treatment) produced the lowest extraction of phosphate. The quadratic pH and the interactions temperature-time, and temperature-ethanol were not significant in any of the extractions studied. The optimization of the factors temperature, pH, time, and ethanol concentration that produces the highest extraction of soluble sugars, saponins, and phytate, in addition to protein concentration and digestibility improvement, in a unique treatment will be discussed in the next chapter.

CHAPTER VI

PROTEIN CONTENT AND DIGESTIBILITY, AND RESPONSE OPTIMIZATION

1. INTRODUCTION

This chapter is a continuation of the work presented in Chapter V. Here, the treated soybean meals obtained according to the conditions presented in the previous chapter were analyzed for protein content and digestibility. An optimization response was also evaluated considering all the factors studied in order to obtain the one that best fitted the objective of this work, which was to reduce the anti-nutritional factors present in soybean meal while increasing the protein content and digestibility to be used as fish feed.

Protein digestibility is a measure of the protein quality. There are in-vivo and in-vitro methods to study digestibility. The in-vivo methods are time-consuming and very expensive to perform. In-vitro methods have the advantage of being cheaper and faster (Fenerci and Şener 2005) and therefore were used in this work. Protein digestibility was studied by comparing the drop in the pH of casein (considered to be 100% digestible) treated with three digestive enzymes—trypsin, α -chymotrypsin, and peptidase—to the drop in pH of samples treated with the same enzymes (Hsu and others 1977). The use of a multi-enzyme system reduces the variability that can be found using a single-enzyme system (Hsu and others 1977).

As observed in the previous chapter, there was not a unique treatment that could eliminate all the anti-nutritional factors at the same time. The process optimization previously discussed allows maximizing or minimizing the desired responses based on the factors studied. In this work, the optimization response focused on the minimization of the remaining content of soluble sugars, saponins, and phytate, while maximizing the crude protein content and

digestibility of the treated soybean meal samples. As a result, the temperature, time, pH, and ethanol concentration at which the responses are optimized are obtained.

2. MATERIALS AND METHODS

2.1. Materials

The chemicals employed were trypsin from porcine pancreas, Type IX-S, 13,000-20,000 BAEE U/mg (Sigma Aldrich, St Louis, MO, USA), chymotrypsin from bovine pancreas, Type II, ≥ 40 U/mg protein (Sigma Aldrich), peptidase 100,000 HUT/g (American Laboratories Inc., Omaha, NE, USA), and casein from bovine milk (Sigma Aldrich).

2.1.1. Treated soybean meals

The starting materials were the 31 treated soybean meals obtained in Chapter V after treatment of the defatted soybean meal samples under the conditions described by the CCRD experimental design (Table 5.1).

2.2. Methods

2.2.1. Experimental design

The responses of the effect of temperature, time, pH, and ethanol concentration on the protein content and digestibility, performed according to the CCRD experimental design described in Chapter V, were fitted to the quadratic polynomial equation (Eq. 5.1) with Minitab version 15.1.30.0 (Minitab Inc., State College, PA).

2.2.2. Response optimization

The optimization of soluble anti-nutritional factors extraction (oligosaccharides, saponins, and phytate), protein content, and digestibility were evaluated using Minitab version 15.1.30.0 (Minitab Inc., State College, PA). Optimization was conducted using the response optimizer

provided by Minitab 15.1.30.0, which gives the optimal solution to the desired responses for the combination of factors studied, and also an optimization plot of the results. The response optimization provides the overall desirability (D)—a number in the range from 0 (one or more responses are not within the acceptable limits) to 1 (ideal situation)—that describes how well the responses fit the proposed goals, and the optimal desirability for each response. The response optimization also provides the maximal composite desirability obtained by the combination of the individual desirabilities, and identifies the optimal condition of the factors that led to the results.

2.2.3. Analytical methods

2.2.3.1. Crude protein content

Crude protein content was determined as described in Chapter V.

2.2.3.2. Protein digestibility

Protein digestibility was determined by the method described by Hsu and others (1977) with some modifications. All treated soybean meal samples from chapter V were finely ground using a coffee grinder (Mr. Coffee, Rye, NY, USA), and then sieved with a US standard 60-mesh screen. Fifty milliliters of 1% NaCl solution was added to each soybean meal sample previously weighed to produce a protein suspension with a final concentration of 6.25 mg protein/ml. The samples were adjusted to pH 8.0 with 0.1 N HCl and/or 0.1 N NaOH, while stirring in a water bath at 37°C. At the same time, a 1% NaCl solution containing the following solid enzymes was prepared: 1.6 mg trypsin, 3.1 mg chymotrypsin, and 1.3 mg peptidase/ml, and maintained in an ice bath and adjusted to pH 8.0 with 0.1 N HCl and/or NaOH. Five milliliters of the multi-enzyme solution were then added to the protein suspension, while stirring at 37°C. The pH drop of the samples was recorded automatically over a 10-minute period using a pH meter (VWR

Symphony SP70P). The activities of the individual enzymes were determined using casein from bovine milk (6.25 mg/ml). Protein digestibility was calculated as follows:

$$\text{Protein Digestibility (\%)} = \left(\frac{-\Delta\text{pH treated soybean meal}}{-\Delta\text{pH casein}} \right) \times 100 \quad [\text{Eq. 6.1}]$$

3. RESULTS AND DISCUSSION

3.1. Crude protein

The significant terms with their corresponding coefficients in the analysis of crude protein content were the linear terms temperature (X_1), time (X_3), and ethanol concentration (X_4), the quadratic terms for temperature (X_1^2) and time (X_3^2), and the interaction temperature-ethanol concentration (X_1X_4) (Eq. 6.2, Table 6.1). The interaction temperature-ethanol concentration seemed to be the most important term in the behavior of crude protein content since it is the factor with the largest coefficient. Time is the next significant factor, while temperature and ethanol concentration appeared to have a comparable influence.

$$Y = 64.6483 - 0.4004 X_1 + 0.6946 X_3 + 0.3804 X_4 - 0.4626 X_1^2 - 0.4976 X_3^2 + 0.7881 X_1 X_4 \quad [\text{Eq. 6.2}]$$

Table 6.1: Analysis of variance (ANOVA) for total crude protein content in treated soybean meal.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	6	41.232	41.232	6.8720	5.64	0.001
Linear	3	18.900	18.900	6.3000	5.17	0.007
Square	2	12.394	12.394	6.1968	5.09	0.014
Interaction	1	9.938	9.938	9.9383	8.16	0.009
Residual Error	24	29.244	29.244	1.2185		
Lack-of-fit	8	15.215	15.215	1.9019	2.17	0.089
Pure Error	16	14.029	14.029	0.8768		
Total	30	70.476				

The content of crude protein increased with treatment time, reaching the maximum at a 45-minute treatment (Figure 6.1). Beyond that point, concentration started to decrease as treatment time increased. A similar behavior followed the factor temperature. The protein content increased with increasing temperature, reaching maximum concentrations at 40-50°C, after which point concentration started to decrease with increasing temperature. The maximum concentration of protein in treated soybean meal described by Eq. 6.2 was 65.41% following treatment at 50°C, pH 5.75, with a concentration of ethanol of 70% during a 35 minute-treatment. The pH of this treatment is close to the isoelectric point of proteins, which could explain the highest amount of protein found in the sample. According to Lusas and Rhee (1995), the result obtained categorizes the sample as soy protein concentrate. Moreover, this crude protein level is desired for aquaculture feeds since it is similar to the protein content of fishmeal (Soy Protein Concentrate for Aquaculture Feeds 2008). The minimum concentration occurred with treatment at 50°C, pH 5.75, 35% ethanol concentration for 5 minutes, yielding a soybean meal sample with 61.27% protein content. The protein concentration of all samples, even in the less efficient treatment, was higher compared to the untreated soybean meal ($57.40 \pm 0.21\%$). The optimum result is comparable to those of Lujan-Rhenals (2013) who found that treatments with 0.25 to 1.7% of H₂SO₄ for 0.5 to 2.5 hours at 80°C increased the protein content from 48% to 58% d.b.

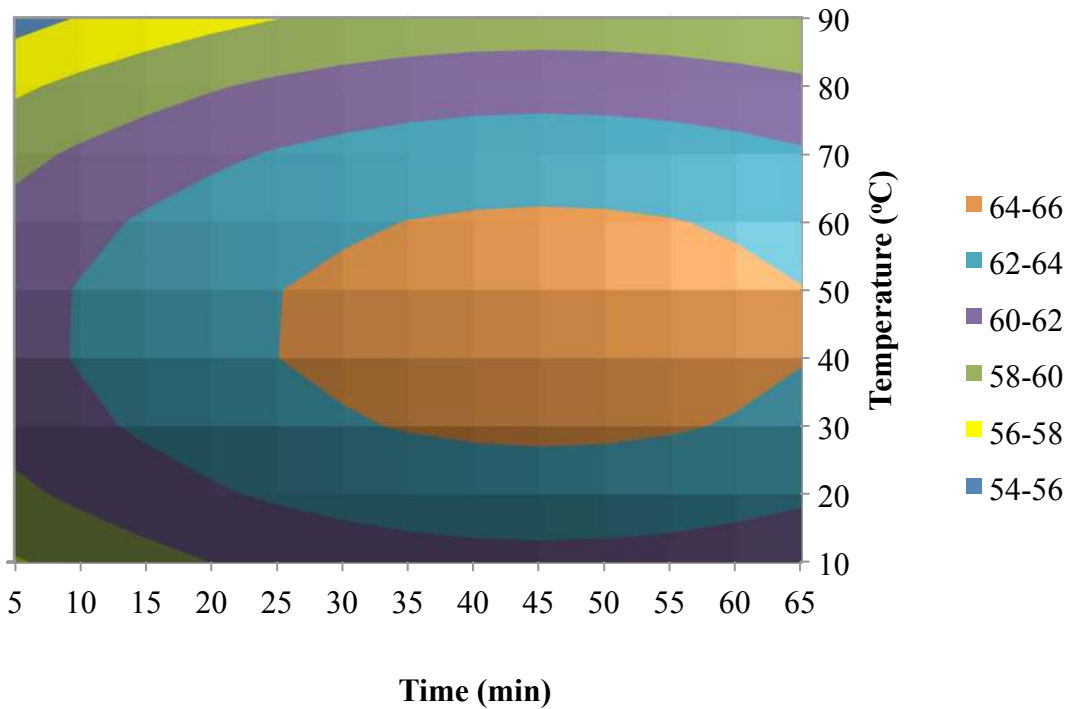


Figure 6.1: Crude protein content (%) as a function of time (min) and temperature (°C) at a concentration of ethanol of 35 %.

3.2 Protein digestibility

3.2.1. Calibration curves of digestive enzymes

The calibration curves were studied in order to determine the behavior of different digestive enzymes in the presence of casein, and additionally, to determine if the behavior of the new source of peptidase employed was similar to the one used in the technique described by Hsu and others (1977). The pH drop curves obtained by incubation of casein with both the individual digestive enzymes and the multi-enzyme technique are shown in Figure 6.2 and demonstrate that all the enzymes added to the casein solution were able to drop the pH of the mixture. The starting pH of the solution was 8 in all cases, and the pH drop was measured every minute during a 10-minute period. The highest drop in pH after 10 minutes of incubation was obtained when casein

was in the presence of the enzymatic cocktail, followed by trypsin, α -chymotrypsin, and finally peptidase, the one with the lowest pH decline. These results are in agreement with those presented by Hsu and others (1977), even though the source of peptidase employed was different. The enzyme used in Hsu and others (1977) technique was a porcine intestinal peptidase (Grade III), 40 units per g powder from Sigma Chemical Company. However, that enzyme has been discontinued. American Laboratories Inc., (Omaha, NE, USA) offers the enzyme from a different source. Their peptidase is produced by *Aspergillus melleus*. According to the results presented in this study, this new source of enzyme can replace the porcine intestinal peptidase originally employed in the protein digestibility technique without compromising the results.

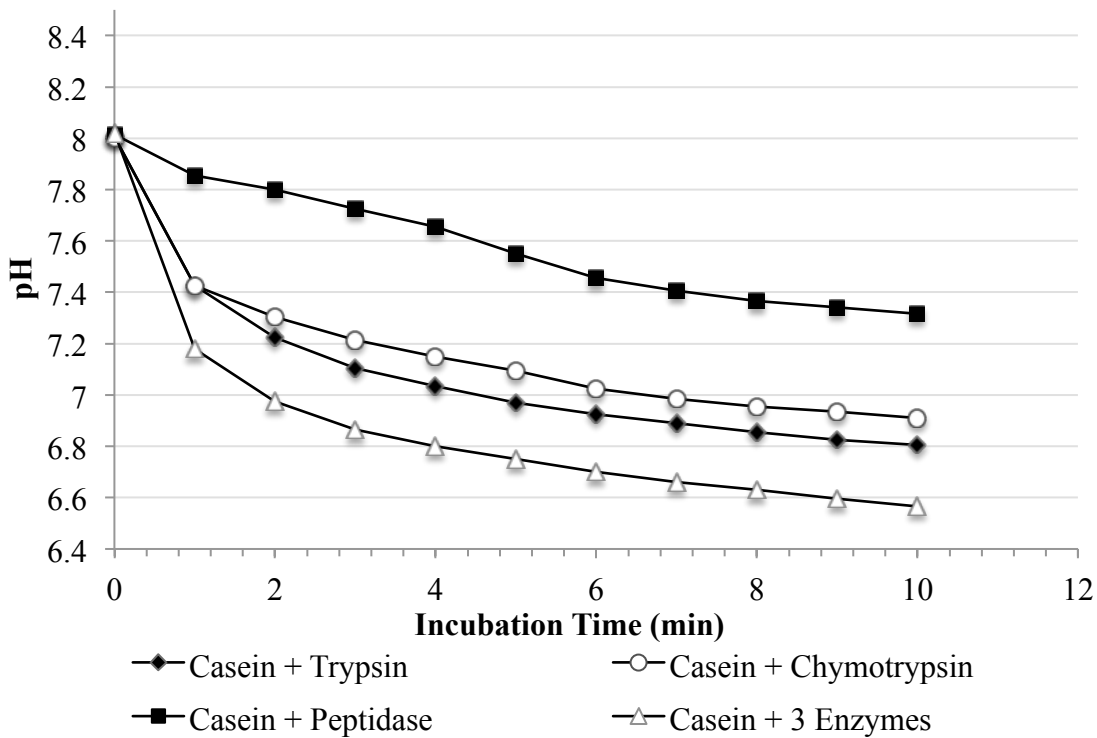


Figure 6.2: Calibration curves of digestive enzymes in the presence of casein.

3.2.2. Digestibility of samples

The coefficients for the quadratic equation that represent the response of protein digestibility are shown in Eq. 6.3 and Table 6.2. Interactions between factors were not significant. The three significant terms appear to play an important role in protein digestibility. Ethanol likely aids the removal of oligosaccharides and saponins, an optimal pH prevents loss of protein during the extraction process thus increasing the protein concentration, and the ideal temperature prevents protein damage while assisting in the removal of soluble sugars, saponins, and phytate. Ethanol concentration seemed to be the factor with the greatest influence in protein digestibility since it is the term with the largest coefficient.

$$Y = 83.140 + 1.604 X_1 - 2.154 X_2 + 4.729 X_4 - 1.170 X_1^2 - 2.457 X_4^2 \quad [\text{Eq. 6.3}]$$

Table 6.2: Analysis of variance (ANOVA) for protein digestibility in treated soybean meal.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	912.96	912.963	182.593	25.62	0.000
Linear	3	709.89	709.891	236.630	33.20	0.000
Square	2	203.07	203.072	101.536	14.25	0.000
Residual Error	25	178.18	178.185	7.127		
Lack-of-fit	9	89.03	89.030	9.892	1.78	0.152
Pure Error	16	89.15	89.155	5.572		
Total	30	1091.15				

Protein digestibility considerably increased up to 50% ethanol concentration, after which point digestibility started to decrease (Figure 6.3). The behavior of pH in protein digestibility was interesting since as long as the pH increased, the protein digestibility decreased, over the range of ethanol concentrations tested. The largest protein digestibility in the treated samples was 88 % following treatment at 62.5°C, pH 5.13, 52.5% ethanol concentration for a 20-50-minute treatment. This result is greater than the protein digestibility of soy concentrate (87.2%) reported

by Hsu and others (1977), and even greater than the protein digestibility of fishmeal ($78.08 \pm 0.36\%$) reported by Ali and others (2009). The lowest protein digestibility was 63.85% after treatment at 50°C, pH 5.75, 0% ethanol concentration for 35 minutes. The uppermost protein digestibility obtained with treatments was approximately 22% higher than the protein digestibility of the untreated sample ($72.2 \pm 0.97\%$). However, the less efficient treatment produced a sample with a protein digestibility lower than the untreated soybean meal.

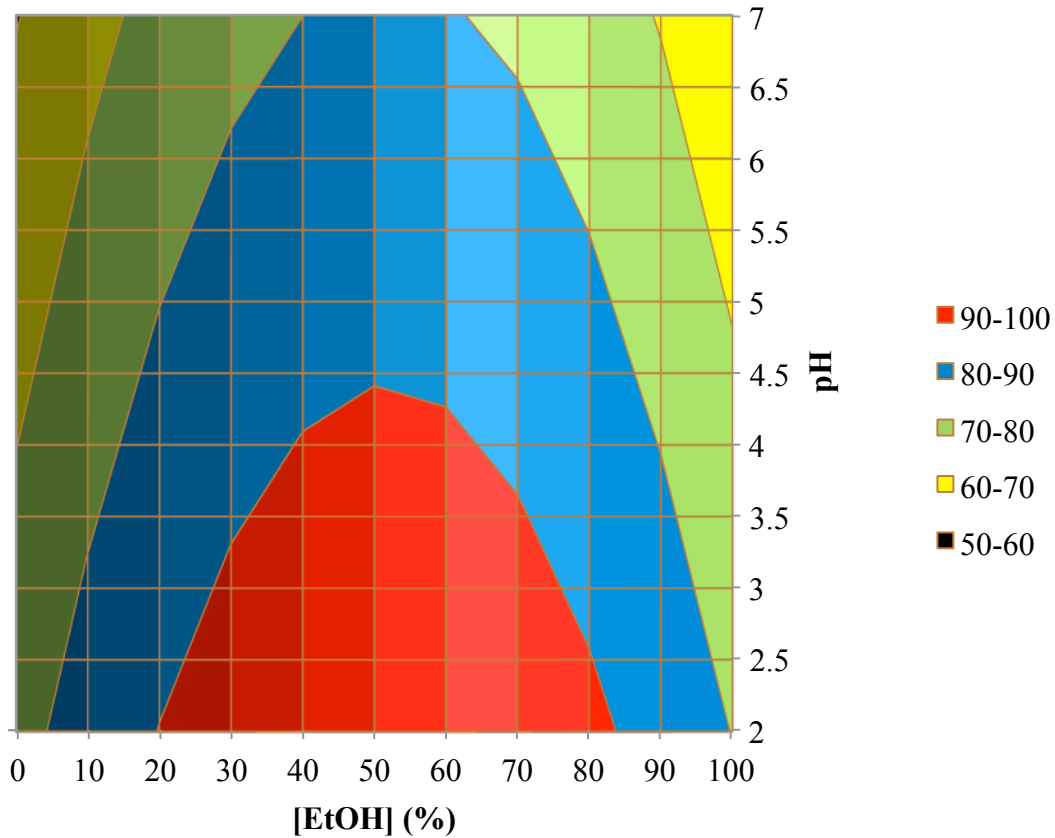


Figure 6.3: Protein digestibility (%) as a function of [EtOH] (%) and pH at 50°C.

3.3. Response optimization

The objective of the optimization process was to obtain a final product with low concentrations of oligosaccharides, saponins, and phosphate while concurrently maximizing the protein content and digestibility. The response optimization that best represents the objective of this work was the one that optimized protein digestibility (Figure 6.4). At this level the content of total saponins and phosphate in the treated sample were minimized to 0.036% and 0.77%, respectively. The desirability of both responses was low, especially in the case of remaining total phosphate. The optimization also maximized the content of total sugars extracted to 11.10%, the crude protein content to 64.23%, and the protein digestibility of the samples to 87.90%. with optimal desirabilities, indicating that ideal results were obtained according to the proposed goals. The response optimization's composite desirability was 0.71, meaning that one or more responses were not within the suitable limits. In this particular case, the content of remaining phosphate, and to a lesser extent, the saponins content, affected the overall response. The best conditions that led to the aforementioned results were a consequence of working with a temperature of 59°C, a pH of 4.5, and a 35% ethanol concentration for a period of 65 minutes.

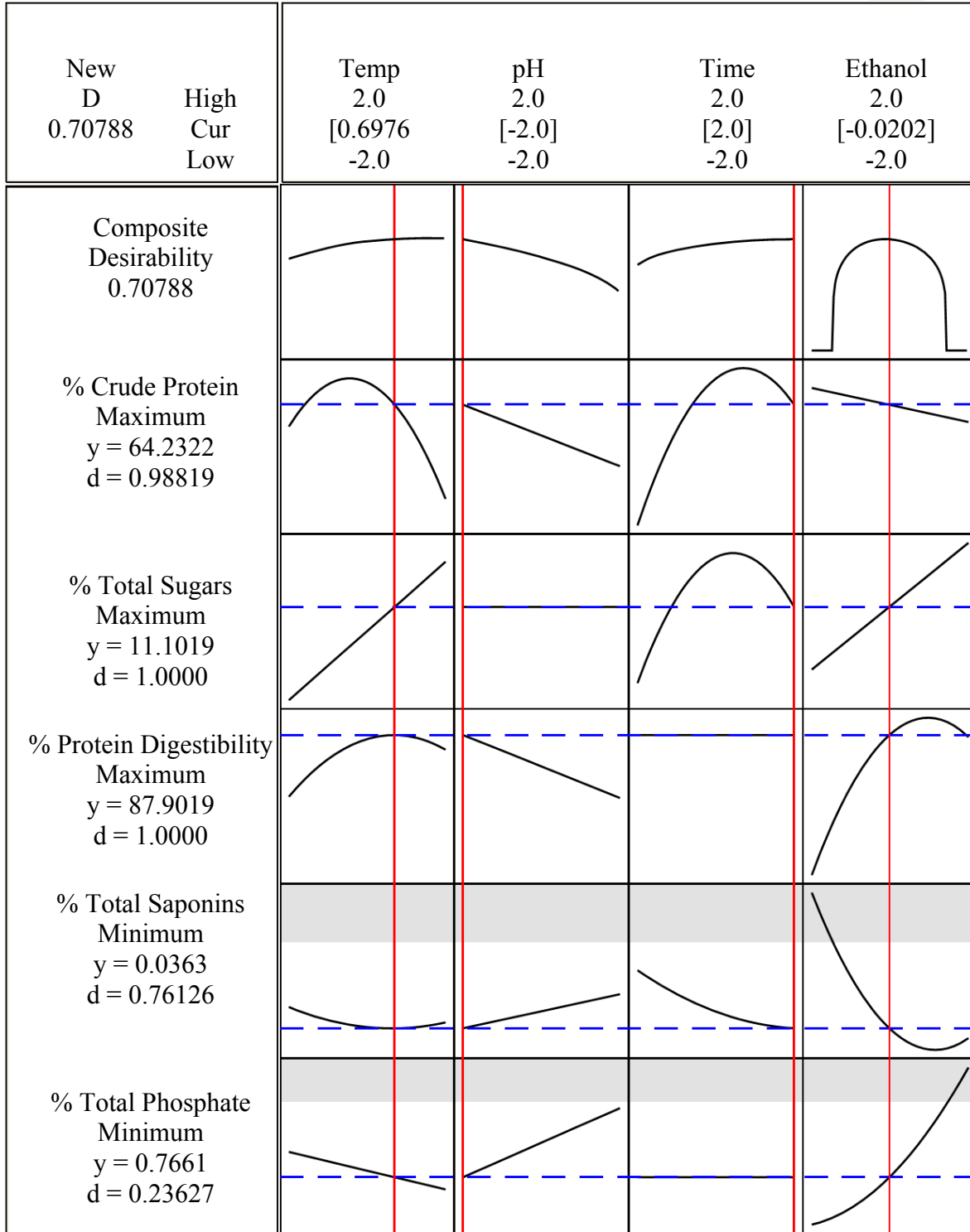


Figure 6.4: Optimization plot with maximized protein digestibility.

4. CONCLUSIONS

The different treatments performed on soybean meal were able to increase both the protein content and digestibility of the sample. The most efficient treatments were able to produce a soybean meal with an increase in protein content from 57.4% to 65.41%, and a protein digestibility increase from 72.2% to 88%, making the final product a good alternative for fish feed. The technique used in this work is simple and economical. However, similar to the analysis of soluble sugars, saponins, and phytate, there was not one treatment that could increase both protein content and digestibility at the same time. Nonetheless, the optimization response can be employed to define the optimal solution based on the desired responses for the combination of factors studied. The optimization of protein digestibility demonstrated that it is possible to work at relatively low temperature, pH, and ethanol concentration for a short processing time, and obtain a pronounced reduction in soluble sugars and saponins content while increasing the protein content and digestibility.

CHAPTER VII

CONCLUSIONS AND FUTURE RESEARCH

This research established the deactivation kinetics of β -conglycinin and glycinin using D-values and Z-values, and evidenced the resistance to temperature of both proteins when present within the matrix of the soybean meal. It concludes that effective heat treatments could be employed to reduce the content of active protein based on the deactivation characteristics of glycinin, the more resistant of the two proteins. The deactivation of these proteins produces a soybean meal with a greater nutritional value that could be used for fish feed.

Reduction of the phytic acid content present in the soybean meal sample with microbial phytase was confirmed with this study. Depending on the characteristics of the final product desired two different treatments could be employed using different citrate buffer proportions. The 1:1 (w/v) treatment showed that phytase is able to make phosphorus available, which could be of nutritional importance for fish feed, and also reduces its release to the environment. On the other hand, the 1:15 (w/v) treatment is a good alternative if the objective is to reduce the phytic acid content while reducing the content of other soluble anti-nutritional compounds present in the sample that could also cause damage to fish.

Optimization of the extraction of soluble sugars, saponins, and phosphate while increasing the protein content and digestibility of soybean meal was achievable in this work using the optimization response of protein digestibility. The optimal solution based on the factors studied allowed us to reduce the content of saponins in the sample to 0.036%, the phosphate content to 0.77%, increase the extraction of soluble sugars to 11.1%, and improve the protein content and digestibility to 64.23% and 87.90%, respectively, while working at 59°C, pH of 4.5, 35% ethanol concentration for 65 minutes. These results are comparable to those published by

the aquaculture industry; however, the important advantage is that the results were obtained using a one-step process, which makes it simple and also economical since it uses a lower ethanol concentration.

Further studies in connection with this research could include an economic analysis of the method employed for the extraction of the anti-nutritional factors to enhance the protein content and digestibility of the soybean meal. Also, it could be of importance to evaluate the in-vivo protein digestibility of the sample obtained under this work, and to do the in-vivo test in different species—omnivores (catfish) and carnivores (bass, trout). Additionally, it would be useful to investigate the advantages/disadvantages of the product obtained when nutritionally enhanced soybean meal is used as a fishmeal substitute.

CHAPTER VII

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APPENDICES

APPENDIX I

Experimental results of total soluble sugars (%), phosphorus (%), non-2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (non-DDMP) saponins (%), 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) saponins (%), and total saponins (%) in soybean meal obtained after treatment of defatted soybean meal under the CCRD conditions.

Run Order	Soluble sugars	Phosphorus	Non-DDMP	DDMP	Total saponins
1	9.95	0.88	0.05	0.000069*	0.050069
2	10.61	0.91	0.11	0.000189	0.110189
3	7.73	0.87	0.08	0.000135	0.080135
4	10.76	0.83	0.05	0.000073	0.050073
5	11.66	0.86	0.06	0.000095	0.060095
6	13.30	0.93	0.11	0.000120	0.110120
7	8.89	0.88	0.08*	0.000154	0.080154*
8	13.30	0.97	0.05	0.000077	0.050077
9	11.82	0.87	0.05	0.000075	0.050075
10	11.04	1.07	0.04	0.000051	0.040051
11	10.45	0.78	0.12	0.000209	0.120209
12	13.30	1.10*	0.03	0.000037	0.030037
13	13.49	0.86	0.19	0.000189	0.190189
14	12.75	0.88	0.10	0.000174	0.100174
15	11.04	0.96	0.04	0.000063	0.040063
16	10.45	0.79	0.11	0.000207*	0.110207
17	10.10	0.72	0.09*	0.000165	0.090165*
18	11.35	0.94	0.03	0.000047	0.030047
19	10.92	0.80	0.05	0.000103	0.050103
20	10.80	0.99	0.04	0.000050	0.040050
21	11.74	0.69	0.10	0.000177*	0.100177
22	7.65	0.88	0.05	0.000108	0.050108
23	10.49	0.82	0.05	0.000099	0.050099
24	12.21	0.91	0.05	0.000114	0.050114
25	11.43	0.95	0.04	0.000059	0.040059
26	8.27	0.89	0.04	0.000091	0.040091
27	12.91	0.86	0.06	0.000113	0.060113
28	13.88	0.84	0.05	0.000146	0.050146
29	14.43*	0.85	0.05	0.000107	0.050107
30	13.49	0.71	0.12	0.000156	0.120156
31	11.86	0.93	0.04	0.000065	0.040065

*Unusual observations

APPENDIX II

Experimental results of total crude protein (%) and digestibility (%) obtained after treatment of soybean meal under the CCRD conditions.

Run Order	Crude Protein (%)	Protein Digestibility (%)
1	64.75	82.8
2	63.00	71.1
3	61.10	77.3*
4	65.81*	82.5
5	64.88	82.2
6	60.25*	73.2
7	63.31	74.6
8	63.88*	80.4
9	63.94	79.0
10	65.81	86.3
11	65.31	76.6
12	64.75	83.2
13	63.50	58.4*
14	61.69	80.8*
15	63.06	87.3
16	63.56	72.9
17	66.00	76.3
18	63.25	85.9
19	64.44	82.5
20	63.69	82.8
21	63.06	81.8
22	62.06	81.8
23	65.69	84.5
24	65.31	84.9
25	64.94	86.3
26	62.69	81.1
27	65.25	83.8
28	65.50	90.4
29	65.25	80.8
30	61.19	78.4
31	64.13	80.4

* Unusual observations