DEAMIDATION OF SOY PROTEIN BY PROTEIN-GLUTAMINASE: PROCESS EVALUATION AND EFFECT OF DEAMIDATION ON PROTEIN FUNCTIONAL PROPERTIES AND FLAVOR-PROTEIN INTERACTIONS

BY

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DISSERTATION

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ABSTRACT

Flavor is a major determinant of the consumer acceptance of a food product. The availability of a flavor compound for sensory perception is greatly influenced by its interaction with non-volatile food constituents including fats, carbohydrates and proteins. The binding of flavor compounds to soy protein can be problematic since it can lead to flavor fade (loss of flavor or lowering flavor intensity) and hence a decline in product quality. These flavor-protein binding interactions can be altered by changing the conformation of the proteins. While chemical deamidation of soy protein isolate was previously found to decrease flavor-protein binding, the use of an enzymatic method for deamidation is generally more desirable since it is substrate specific, can be conducted under mild reaction conditions, and is perceived as natural and safe.

Optimization of the enzymatic deamidation of soy protein isolate (SPI) by protein-glutaminase (PG) was successfully carried out using response surface methodology (RSM) to obtain a deamidated SPI with high degree of deamidation (DD) and an acceptably low degree of hydrolysis (DH). The deamidated SPI had enhanced solubility in both acidic and neutral conditions, improved emulsification properties, increased foaming capacity, but decreased foaming stability over the resting time.

The effects of PG deamidation on flavor binding properties of SPI under aqueous conditions were evaluated by a modified equilibrium dialysis technique. It was found that partial deamidation (43.7% DD) decreased overall binding affinity for selected carbonyl containing flavor compounds (vanillin and maltol). The thermodynamic parameters of binding indicated that the flavor-protein interactions were spontaneous and that the nature

of the interactions shifted from entropy to enthalpy driven after deamidation. Deamidation of soy protein appears to change the mechanism of binding from hydrophobic interactions and/or covalent bonding (Shiff-base formation) to weaker van der Waals forces or hydrogen bonding.

The effect of PG deamidation on protein solubility and flavor binding potential of soymilk was studied. The sensory characteristics on aroma of deamidated soymilk (DSM) did not differ from those of the control soymilk (treated without PG; CSM). Protein solubility in the DSM was enhanced at weakly acidic conditions (pH 5.0). DSM had lower flavor binding potential than the CSM as evidenced by the fact that the odor detection thresholds for the flavor compounds vanillin and maltol were approximately 5 and 3 fold lower, respectively, in DSM than in CSM. The sigmoidal relationship of dose-response curves relating concentration of flavor compounds to aroma intensity demonstrated that DSM had lower flavor binding potential than CSM. The *n* exponents from Stevens's power law indicated that vanilla and cotton candy intensities increased, as a function of vanillin or maltol concentration, at a higher rate in DSM than in CSM.

The findings of this study can lead to the development of technology to produce proteins with improved functional properties and potentially decreased problems associated with flavor-protein interactions, especially with carbonyl containing flavor compounds. The information about binding mechanisms caused by modification of binding sites in protein by PG will allow the food industry to produce protein ingredients, from soybean as well as from other sources, with improved functional properties and potentially decreased flavor fade problem, especially for use in acidic protein-fortified foods and beverages.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BET	Best estimate threshold
BSA	Bovine serum albumin
β-lg	β-lactoglobulin
CD	Circular dichroism
CSM	Control soymilk (treated without PG)
DD	Degree of deamidation
DH	Degree of hydrolysis
DSM	Deamidated soymilk
DSPI	Deamidated soy protein isolate
EAI	Emulsion activity index
E/S	Enzyme:substrate ratio
ESI	Emulsion stability index
f_i	Response factor
FC	Foaming capacity
FDA	Food and Drug Administration
FS	Foaming stability
FSM	Untreated soymilk
FT-IR	Fourier transform infrared
ΔG	Free energy of binding
GC	Gas chromatography

ΔH	Enthalpy of binding
HP	High pressure
HPLC	High performance liquid chromatography
IGC	Inverse gas chromatography
K	Equilibrium binding constant
L	Single ligand
LSD	Lease significant difference
MS	Mass spectrometry
MSD	Mass selective detector
n	Number of binding site
NMR	Nuclear magnetic resonance spectroscopy
NOE	Nuclear Overhauser effect
P	Protein molecule
PP	Pentyl pyridine
PAGE	Polyacrylamide gel electrophoresis
PFG	Pulsed field gradient
PG	Protein-glutaminase
pI	Isoelectric point
r^2	Coefficients of determination
RH	Relative humidity
RSM	Response surface methodology
ΔS	Entropy of binding
SDS	Sodium dodecyl sulfate

SPC	Soy protein concentrate
SPI	Soy protein isolate
SPME	Solid-phase microextraction
TCA	Trichloroacetic acid
TGase	Transglutaminase
TSP	Total soluble protein
UV	Ultraviolet
VIS	Visible
WPC	Whey protein concentration
WPI	Whey protein isolate

CHAPTER 1

INTRODUCTION

1.1 IMPORTANCE OF FLAVOR IN FOOD PRODUCT QUALITY AND ACCEPTANCE

Flavor is an important quality factor influencing the consumer's decision to purchase or consume a food product (Plug and Haring, 1994; Guichard, 2002; 2006; Kühn et al., 2006) and is, therefore, a major determinant of the commercial success of any newly launched food product (Plug and Haring, 1993; 1994; Preininger, 2006). In addition, product shelf life is often dictated by flavor quality deterioration. Thus, food manufacturers are interested in maintaining the desired flavor profile and minimizing off-flavor development during processing and storage, and in delivering a final product with a predictable and desirable flavor release profile during consumption (Zhou, 2005).

1.2 FLAVOR CHALLENGES ASSOCIATED WITH SOY FOODS

Soy protein is a popular food ingredient because of its good functionality and potential health benefits (Liu, 2005; Cadwallader and Chang, 2010). It has become an important ingredient in the formulations of many food products, such as beverages, dietary snack bars, and meat substitutes (Preininger, 2006). However, despite these benefits the consumption of soy foods in the United States and Europe is limited due to the presence of undesirable flavors and flavor-matrix interactions.

There are two main flavor challenges facing soy foods manufacturers. The first is the off-flavor problem itself. This is caused by the presence of volatiles inherent to soy. These compounds impart undesirable "green", "beany", and "grassy" aroma attributes to

soy (Boatright and Crum, 1997; Wilson, 1985; Zhou et al., 1999). Generally, it is believed that off-flavors result from lipoxygenase activity or oxidation of unsaturated lipids in the raw, crushed soybean or full-fat soy flours. These compounds are naturally present in soy products and are resistant to removal during processing due to their interaction with soy proteins (Aspelund and Wilson, 1983; Kinsella et al., 1985; Wilson, 1985; MacLeod and Ames, 1988; Preininger, 2006). A second flavor challenge facing soy manufactures is also related to the above mentioned flavor-protein interactions, but in this case pertains to the selective binding of added flavor compounds (Gremli, 1974; Kinsella et al., 1985). This can deleteriously affect the flavor quality of a food product due to the development of an unbalance flavor profile, loss of key flavor compound(s), or lowering of flavor intensity, so called "flavor fade" (Gremli, 1974; Kinsella et al., 1985; Zhou and Cadwallader, 2008). The selective binding of flavor compounds by soy proteins has important practical implications in the food industry since the binding of added flavorings to soy proteins can cause a number of problems related to formulation soy food products. In particular, it makes it difficult to choose proper flavorings and dosages needed to create the desired flavor profile in the final food product (Glemli 1974; Zhou and Cadwallader, 2006).

In recent years, new and novel technologies have been developed to decrease inherent soy-associated off-flavors in popular soy products like soymilk (Maheshwari et al., 1995; Yuan et al., 2008); however, the flavor fade problem is still unresolved. In order to solve the flavor fade problem it is necessary to fully understand the mechanisms involved in flavor-soy protein binding interactions. Such knowledge can be used to

develop methods or to optimize processes to counteract the effects of the flavor-protein binding interactions leading to more acceptable high protein food products.

1.3 INFLUENCE OF PROTEIN-FLAVOR INTERACTIONS ON FOOD FLAVOR QUALITY AND PERCEPTION

As mentioned earlier, flavor perception is one of the most important factors affecting consumer acceptance of a food product. At the same time the demand for low-calorie healthy foods containing high protein content and reduced fat, sugar and sodium is on the rise (Plug and Haring, 1993; Guichard, 2002). Unfortunately, these "healthy" foods often do not satisfy the consumer due to inferior flavors. In particular, although high protein foods are a popular choice, the addition of protein to a food product may alter its flavor either by imparting undesirable off-flavors or by changing the food's flavor release/flavor perception profile due to flavor-protein binding interactions (Zhou and Cadwallader, 2008).

In fact, selective flavor binding is commonly encountered in various foods in which flavorings are intentionally added. This has caused difficulties for food manufacturers with respect to the selection and application of flavors for use in these types of products (Gremli, 1974; Aspelund and Wilson, 1983; Kinsella et al., 1985; Damodaran, 1996). Furthermore, the flavor binding capacity of the protein used in a food formulation can make it difficult to achieve the targeted flavor profile of the finished product (Kinsella et al., 1985). To make matters more complicated, flavor-protein binding interactions are affected by many factors including temperature, structure and

chemical nature of the flavor compounds and the processing history of the food protein (O'Neill, 1996).

1.4 PROTEIN MODIFICATION AND FLAVOR BINDING

Beside the nature of flavor compounds themselves, the conformation of protein is another important factor affecting flavor-protein binding interactions (Damodaran and Kinsella, 1980; Suppavorasatit and Cadwallader, 2010). Therefore, any factors that alter protein conformation would also affect the flavor binding properties of proteins. These include temperature, pH, ionic strength, and chemical or enzymatic modification of the protein (O'Neill and Kinsella, 1988; Li et al., 2000; Chobpattana et al., 2002; Kühn et al., 2007; Lozano and Cadwallader, 2009).

Deamidation is a type of protein modification which alters the primary, secondary and tertiary structures of protein by converting amide groups in glutamine and asparagines residues into acid residues (carboxyl groups). The removal of amide residues from the protein could reduce flavor-protein binding interactions by decreasing the protein's ability to react with carbonyl-containing flavor compounds via Shiff-base reactions. Lozano and Cadwallader (2009) demonstrated that chemical deamidation, accomplished by the use sodium dodecyl sulfate (SDS), decreased the overall binding of carbonyl containing flavor compounds to soy protein isolate (SPI). Protein deamidation also can done by enzymatic means, which is generally more desirable than chemical methods because it is substrate specific, can be conducted under mild reaction conditions, and is perceived as natural and safe (Hamada, 1991; Shih, 1996). Several enzymes can be used for protein deamidation, including transglutaminase (Tgase), protease,

peptidoglutaminase, and protein-glutaminase (PG) (Hamada, 1994; Yamaguchi et al, 2001). PG has been recently affirmed as GRAS (Generally Recognized as Safe) (GRAS Notice No. GRN 000267; FDA, 2008) and is commercially available as a food grade product from Amano Enzyme, Inc.

Enzymatic deamidation has excellent potential to reduce the flavor-protein binding interactions and reduce flavor fade in soy foods. This knowledge will be beneficial to soy protein and soy foods manufacturers and will enable them to develop soy-containing products, especially beverages, with optimum flavor profiles.

1.5 STUDY OBJECTIVES

The ultimate goal of this study was to develop a technology to produce proteins with decreased potential for flavor-protein interactions. This project assessed the effect of enzymatic deamidation by protein-glutaminase (PG) on the flavor binding affinity/capacity of soy protein in an aqueous environment. The central hypothesis of this study was that enzymatic deamidation of soy protein by PG will remove amide groups from the protein and thus reduce the protein's affinity/capacity to bind with carbonyl-containing flavor compounds. Three specific tasks were completed to test this hypothesis:

(1) Optimal conditions for the enzymatic deamidation of soy protein isolate (SPI) by PG were determined by using response surface methodology. Selected functional properties of the deamidated SPI were compared with those of the untreated SPI.

(2) Effect of enzymatic deamidation by PG of SPI on the binding characteristics of the protein against selected carbonyl containing flavor compounds (vanillin and maltol) under aqueous conditions was evaluated by use of an equilibrium dialysis

technique. The flavor binding properties of deamidated SPI and untreated SPI were compared.

(3) Effect of enzymatic deamidation of soymilk by PG on protein solubility and flavor binding potential was evaluated. The protein solubility was compared between deamidated soymilk and untreated control soymilk. Change in flavor binding potential due to deamidation was assessed by comparing the odor detection thresholds of selected carbonyl-containing flavor compounds (vanillin and maltol) in deamidated and control soymilks. Additional sensory studies measured the dose-(odor intensity) response behavior of vanillin and maltol in deamidated and control soymilks.

This work is creative and original in that it is the first study to evaluate the use of enzyme deamidation for the reduction of the flavor binding potential of a protein. In addition, this study was first to optimize the enzymatic deamidation of soy protein isolate and to assess the effects of the process on the functional properties of the protein. During the course of this study a novel and more convenient technique was developed for the measurement of protein binding constants and thermodynamic parameters in aqueous conditions. This study was also first to make use of odor detection threshold and dose-response measurements to evaluate the effects of flavor binding in a real food system. Finally, this study was first to assess the protein binding characteristics of maltol.

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CHAPTER 2

LITERATURE REVIEW *

2.1 BINDING OF FLAVOR COMPOUNDS BY FOOD PROTEINS

In food systems, interaction between flavor (aroma) compounds and food matrix components (e.g., lipids, carbohydrates, and proteins) can affect flavor perception. One critical factor that influences flavor perception is the flavor release rate. Lipids in food have the greatest impact on flavor perception since they can act as solvents for lipophilic flavor molecules and thus reduce the rate of flavor release during food consumption (O'Neill, 1996). Carbohydrates can bind to flavor compounds, especially polar molecules, and form dipole-dipole interactions and hydrogen bonds. Thus, it can cause the change in flavor release and perception (Plug and Haring, 1993; 1994). Beside lipids and carbohydrates, proteins can also influence flavor perception. In particular the binding interaction of flavor molecules with protein can be especially problematic in protein-enriched foods leading to decline or loss of flavor (flavor fade) and hence a decline in product acceptability (Kühn et al., 2006).

2.2 GENERAL FLAVOR-PROTEIN INTERACTIONS

Protein on its own does not impart much flavor, but it can alter flavor perception by binding with flavor compounds. Protein can bind with off-flavors or bind selectively with desirable flavors, and hence change the flavor profile of the food. When flavor compounds are added to food products containing proteins, the retention of flavors during

*Adapted with permission from Suppavorasatit, I.; Cadwallader, K. R. Flavor-soy protein interactions. In *Chemistry, texture, and flavor of soy*.; Cadwallader, K. R. and Chang, S. K. C. Eds. American Chemical Society: Washington, DC., 2010; pp 339-359. Copyright (2010) American Chemical Society.

processing and storage will be altered, thus making it difficult for food manufacturers to choose and control the proper level of flavoring necessary to achieve the desired flavor intensity in the food (Gremli, 1974; Schutte and van den Ouweland, 1979; Aspelund and Wilson, 1983).

Changing the amino acid sequence can alter the chemical characteristics of a protein for several reasons. The binding properties of a protein is strongly influenced by the its three-dimensional structure, which is formed as a result of disulfide bridges and hydrogen bonds between amino acids (Plug and Haring, 1993). Many studies have attempted to relate binding to the molecular structures of flavor compounds and proteins, but the results have been inconsistent due to the differences among proteins, flavor compounds and experimental conditions (Kühn et al., 2006). In general, the type of interaction depends on the nature of proteins and flavor compounds and can be reversible (physicochemical) or irreversible (chemical) (Kühn et al., 2006; Preininger, 2006). Reversible binding includes hydrogen bonding, hydrophobic interactions and ionic bonds. In contrast, irreversible binding occurs when flavor compounds, especially aldehydes (such as vanillin) form covalent bonds (Schiff-bases) with the amide side chains of proteins (Damodaran, 1996; Mottram et al., 1996; Meynier et al., 2004; Kühn et al., 2006). Gremli (1974) suggested that reversible interactions may not necessarily be negative, in that this type of binding might protect flavor compounds during food processing so that they can be later released from the food during consumption.

Most of studies related to flavor-protein interactions have been conducted with milk proteins (Damodaran and Kinsella, 1980; O'Neill and Kinsella, 1987a, 1988; Dufour and Haertlé, 1990; McNeill and Schmidt, 1993; Fares et al., 1998; Sostmann and

Guichard, 1998; Guichard and Langourieux, 2000; Li et al, 2000; Marin and Relkin, 2000; Fabre et al., 2002; Chobpattana et al., 2002; Lübke et al., 2002; Miranov et al., 2003; Yang et al., 2003; Meynier, et al., 2004; Considine et al., 2005; Liu et al., 2005a; Gkionakis et al, 2007; Kühn et al., 2007, 2008; Tavel et al., 2008). Other proteins studied include soy proteins (Damodaran and Kinsella, 1981a, b; Aspelund and Wilson, 1983; O'Neill and Kinsella, 1987b; O'Keefe et al., 1991a, b; Li et al, 2000; Zhou et al., 2002; Zhou and Cadwallader, 2004, 2006, 2008; Zhou et al., 2006; Gkionakis et al, 2007; Lozano and Cadwallader, 2009), fababean protein (Ng et al., 1989a, b), ovalbumin (Adams et al., 2001; Grinberg et al., 2002), myofibrillar proteins (Pérez-Juan et al., 2006), broad bean protein (Semenova et al., 2002 a, b, c) and 13lavor13angea protein (myoglobin) (Gianelli et al., 2005).

2.3 SOY PROTEINS

Soybean (*Glycine max*) has been cultivated about 5,000 years according to Chinese written records (Watanabe and Kishi, 1984). It is a good source of protein because of its high protein content, which is constitutes about 40% protein of the seed (Endres, 2001a). Soy proteins are popular food ingredients because of their nutritional benefits and excellent functional properties.

2.3.1 Soy Nutrients

Soybean differs from other beans and grains in its composition. It contains about 40% protein, 20% lipid and 30% carbohydrate (Watanabe and Kishi, 1984). Nutritional quality, essential amino acid composition, amino acid requirements, and digestibility are

the factors affecting protein quality (Endres, 2001b). Compared with other vegetable proteins, soy proteins contain more essential amino acids; however, they are relatively low in sulfur-containing amino acids such as methionine and 14lavor14a (Watanabe and Kishi, 1984; Endres, 2001b). This imbalance of essential amino acids is not a serious limitation because the human diet usually contains various protein sources such as animal proteins, legumes, and cereals. In addition, about 92-100% of soy protein can be digested, which is not different from other high quality protein sources including meat, milk, fish and egg (Riaz, 1999; Endres, 2001b). Moreover, soy proteins are good source of dietary fiber, which can help control blood cholesterol (Endres, 2001c). The health claims authorized by the Food and Drug Administration (FDA) conclude that consuming foods containing soy protein may reduce the risk of coronary heart disease by lowering blood cholesterol (FDA, 1999).

2.3.2 Functional Properties of Soy Proteins

In general, proteins from animal sources including milk, egg and meat are usually used in most food systems. In addition, plant proteins are limited because of lack of some desirable functional properties (Hettiarachchy and Kalapathy, 1997). Soy proteins are used primarily for their functional properties food applications to achieve consumer acceptability of the product. These functionalities, which describe the behavior of proteins in food systems including sorption, solubility, gelation, surfactancy, ligand-binding (such as flavor) and film formation are influenced by food composition, structure, and conformation of protein itself (Kinsella, 1979; Endres, 2001d). A summary of the functional properties of soy protein is provided in **Table 2.1**.

Table 2.1 Functional properties of soy proteins in food systems [a]

Functional Property	Mode of action	Food system
solubility	protein solvation, pH dependent	beverages
water absorption and binding	hydrogen bonding and entrapment of HOH	meats, meat products, bake goods
viscosity	thickening	soups
gelation	protein matrix formation and setting	curds, cheeses
cohesion-adhesion	acts as adhesive material	meat products, bake goods
elasticity	disulfide links in gels deformable	meats, bakery products
emulsification	formation and stabilization of fat emulsion	meat products, soup, cakes
fat adsorption	binding of free fat	meat products
flavor-binding	adsorption, entrapment, release	beverages, bake goods
foaming	form stable film to entrap gas	whipped cream, sponge cakes
color control	bleaching of lipoxygenase	breads

[a] Source: adapted from Kinsella (1979).

2.3.3 Commercial Soy Protein Products

Various commercial soybean products are produced both in the United States and internationally. Beside oil products, there are three major types of soybean products made from the original soybean including soy flours and grits, soy protein concentrate (SPC), and soy protein isolate (SPI). These three types differ in fat, carbohydrate and especially protein content, which could affect their functionality (Cowan et al., 1973; Lusas and Riaz, 1995). Soy flours and grits contain around 40-54% protein, while SPC and SPI contain about 65-70% and more than 90% protein, respectively (Lusas and Riaz, 1995; Endres, 2001a). The composition of soy protein products is shown in **Table 2.2**.

Table 2.2 The composition of soy protein products [a] (%)

constituent	defatted soy flours and grits	SPC	SPI
protein	52-54	62-69	86-87
fat	0.5-1.0	0.5-1.0	0.5-1.0
crude fiber	2.5-3.5	3.4-4.8	0.1-0.2
soluble fiber	2	2-5	<0.2
insoluble fiber	16	13-18	<0.2
ash	5.0-6.0	3.8-6.2	3.8-4.8
moisture	6-8	4-6	4-6
carbohydrates	30-32	19-21	3-4

[a] Source: adapted from Endres (2001a).

Before or after oil removal, soybean flakes are used to produce flours and grits (full-fat or defatted) by grinding. Soy flours and grits are the least refined form of soy for human consumption. The fat content, particle size and degree of heat treatment of flakes may vary due to processing conditions. SPC are produced by removing most of water-soluble and non-protein constituents from dehulled and defatted soybeans. Acid leaching, extracting with aqueous alcohol and using moist heat to denature protein before water extraction are the three basic processes to produce SPC. Among soy protein products, SPI is the most refined form. It is also produced by from dehulled and defatted soybeans by removing non-protein components to reach the desire protein content (Endres, 2001a). SPI is typically bland in flavor and light in color due to its production. SPIs from different manufactures are similar in chemical composition; however, there might be differences in physical and functional properties among them because of manufacturing process variation (Kinsella, 1985; Hettiarachchy and Kalapathy, 1997).

2.4 BINDING OF FLAVOR TO SOY PROTEINS

Similar to other proteins, soy protein can bind with flavor compounds in foods and cause flavor problems, resulting in a decrease in product acceptability. An understanding of this interaction and mechanisms that govern it can help to solve these problems by development of better processing methods or alternative flavoring strategies (Aspelund and Wilson, 1983). With respect to flavor-soy protein interactions, useful information has been obtained from the study of both solid state (low-moisture) and aqueous model systems.

2.4.1 Binding of Flavor Compounds to Soy Proteins in the Solid State

Soy proteins are important ingredients in many low-moisture food products such as snack bars, baked goods and cereal-based products. Understanding flavor-protein interaction in low-moisture foods is particularly important because low-moisture foods have a long shelf-life, and thus even slow reactions can lead to decline in product quality. During storage, the flavor components of low-moisture foods can migrate into and out of the product leading to adsorption (either desirable or undesirable depending on the characteristics of the flavor compound) and a decline in flavor intensity (flavor fade) (Hau et al., 1998). The relative humidity (RH) level is an important factor affecting the shelf-life of low-moisture food products. In addition, when the moisture migrates into and out of the food product, it can change the flavor retention/release properties of the food system (Kinsella, 1989). Consequently, to help control and maintain the desirable flavor of low-moisture products during storage, it is necessary to maintain the appropriate storage conditions.

Aspelund and Wilson (1983) used thermodynamics as a tool to study the adsorption of selected off-flavor compounds, including homologous series of alcohols, aldehydes, ketones, hydrocarbons and methyl esters, onto dry soy protein isolate (SPI). They found that hydrocarbons were bound most weakly and alcohols most strongly. Their results demonstrated that the functional group of a flavor compound plays an important role on its binding to soy protein under dry conditions. They also found that the binding of flavor compounds to soy protein is driven by the enthalpy of adsorption in the gaseous system. Furthermore, it was hypothesized that the binding of SPI with flavor compounds occurs by the combination of nonspecific van der Waals forces and hydrogen

bonding (Wilson, 1985). The effect of processing parameters (temperature and moisture content) on the binding of off-flavors with soy was studied by Crowter and others (1980). These researchers determined the heat of adsorption and adsorption coefficients of the binding of alcohols, aldehydes and ketones to soy protein. They found that moisture and temperature affected the binding of flavor compounds due to protein denaturation.

Recently, Cadwallader and coworkers developed an inverse gas chromatography (IGC) technique to study flavor-soy protein binding interaction under controlled relative humidity. They found that increasing RH from 0 to 30% caused a reduction in binding between flavor compounds (1-hexanol and hexanal) and SPI due to competition between water and the flavor molecules at the binding sites on the protein surface (Zhou and Cadwallader, 2004). In agreement with results of Aspelund and Wilson (1983), they also found that chemical structure of flavor compounds greatly affects binding. For the non-polar flavor compounds (hydrocarbons), the main binding forces are nonspecific van der Waals dispersion forces, which were not affected by adsorbed water. On the other hand, both specific (hydrogen bonding and dipole forces) and nonspecific interactions were involved in the binding of more polar flavor compounds, including esters, ketones, aldehydes and alcohols. Also, binding of these flavor compounds is weakened if water is adsorbed onto the dehydrated SPI (Zhou and Cadwallader, 2006). Evaluation of the binding of selected butter flavor compounds to soy proteins was investigated in a wheat soda cracker system (Zhou et al., 2006). There was no effect on the binding of diacetyl and hexanal to the crackers due to the presence of soy proteins, but binding of γ-butyrolactone and butyric acid were strongly affected. These researchers suggested that the stronger binding observed for the soy cracker might be due to the greater polarity of

this matrix (Zhou et al., 2006). Furthermore, the competitive binding of selected volatile compounds (hexanol, hexanal, hexane, and I-2 hexenal) by dehydrated SPI under controlled RH using IGC coupled with atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) was studied (Lozano and Cadwallader, 2009). The results showed strong competition of unsaturated and saturated aldehydes on binding interaction with SPI when alcohols were present. In contrast, there was no significant effect on binding of alcohols with SPI when with the presence of aldehydes or alkanes. In addition, alkanes also showed no effect over the binding affinity of alcohols or aldehydes (Lozano and Cadwallader, 2009).

2.4.2 Binding of Flavor Compounds to Soy Proteins in Aqueous Model Systems

Flavor changes due to binding are known to occur under ambient conditions in aqueous media containing soy, for example, in soymilk during storage. These changes may be caused by release of previously bound "beany" flavor compounds of the soy protein itself, leading to off-flavors; or by binding interactions between the protein and added flavorings, thus causing flavor fade (Gremli, 1974). Researchers have investigated the binding interactions of flavor compounds and soy proteins in aqueous model systems (Gremli, 1974; Damodaran and Kinsella, 1981a, b; O'Neill and Kinsella, 1987b; Chung and Villota, 1989; O'Keefe et al., 1991a, b; Li et al., 2000; Zhou et al., 2002; Gkionakis et al., 2007). The interaction of flavor compounds with soy protein in an aqueous system was initially studied using static headspace-gas chromatography (GC) (Gremli, 1974). It was reported that alcohols underwent weak interactions with the soy protein, while unsaturated aldehydes, and to a lesser extent saturated aldehydes, strongly interacted. It

was concluded that both reversible and irreversible reactions were involved (Gremli, 1974).

Damodaran and Kinsella (1981b) used an equilibrium dialysis method to study the binding interaction of ketones (2-heptanone, 2-octanone, 2-nonanone and 5-nonanone) and nonanal with soy proteins in an aqueous model system. They found that the binding constants increased with an increase in the chain length of the flavor molecule. The hydrophobic free energy of binding also decreased when the position of carbonyl group was shifted from the terminal end to the interior of the flavor molecule. They also concluded that the binding interaction of carbonyls with soy protein was relatively weak under aqueous conditions. Further studies by Damodaran and Kinsella (1981a) focused on the effect of the conformation of soy proteins on their flavor binding properties. Glycinin (11S) and β-conglycinin (7S) protein fractions were found to bind differently with 2-nonanone. Glycinin had almost no binding affinity to 2-nonanone, while β-conglycinin did not differ from whole soy protein. In addition, presence of urea or chemical modification (succinylation) of the protein also decreased the binding affinity of the protein to 2-nonanone. These results were in agreement with those of O'Neill and Kinsella (1987b), who demonstated that the binding affinity of β-conglycinin was around five-fold greater than glycinin. However, these results contradicted the results of O'Keefe and others (1991b), who investigated the influence of temperature on the binding properties of flavor compounds to soy proteins. These researchers found that the binding affinities of various flavor compounds, including aldehydes (butanal, pentanal, hexanal, and octanal), ketones (2-hexanone, 3- hexanone, 2-nonanone and 5-nonanone), hexanol and hexane, were much higher for glycinin than for β-conglycinin. They also found that

for aldehydes an increase in chain length caused an increase in their affinity to glycinin, while it had no effect relative to β-conglycinin binding. In 2002, Zhou and others performed a comparison study of the binding affinities of β-conglycinin, glycinin and SPI with 2-pentyl pyridine (2PP). Their results showed that the binding affinity of 2PP to glycinin was the greatest, followed by β-conglycinin and then SPI. Binding was greater under alkaline condition than under neutral or acidic conditions. Greater binding of 2PP occurred at high temperature (74 °C) than at lower temperatures (4 or 25 °C). This might be because of thermal denaturation, which can increase the ability of proteins to bind with 2PP (Zhou et al., 2002). In contrast, the binding affinity at 4 °C was higher than at 25 °C, which agrees with previous studied by Damodaran and Kinsella (1981b) where they demonstrated that the protein hydrophobicity was greater at 5 °C than at 20 °C. Furthermore, the binding of alcohols by soy protein in aqueous solutions was studied using equilibrium dialysis method. The researchers found that the binding of alcohols to soy protein might involve hydrophobic interaction and hydrogen bonding. They also concluded that increasing in level of denaturation by heat treatment affected alcohol-protein binding by limiting hydrogen bond formation (Chung and Villota, 1989).

A comparison study of the binding of selected flavor compounds, such as vanillin, to soy and dairy proteins (casein and whey protein) was conducted by Li and others (2000). They found that whey protein demonstrated the strongest binding affinity towards vanillin. Moreover, the binding of vanillin to dairy proteins was driven by the enthalpy, which might be due to the interaction of the carbonyl and hydroxyl groups of vanillin with the proteins. On the other hand, the binding of vanillin to soy protein was driven by entropy, which means that the conformation of soy protein could affect binding.

Therefore, any parameter that could influence the conformation of soy protein, such as denaturation by heating, could also influence the binding of lactones (γ-9, γ-10, δ-10, and δ-11) with SPI, amino acids or casein. The results showed that there was no difference in the degree of binding for the lactones on SPI. Study of the competitive binding of two lactones with similar stuctures, δ-10 and γ-11, to soy protein showed that there was some competition between these two lactones for the available binding sites on the protein molecule (Gkionakis et al., 2007).

2.5 TECHNIQUES FOR MEASURING FLAVOR-PROTEIN INTERACTIONS

The molecular study of the interactions between flavor compounds and macromolecules, such as proteins has been conducted by different approaches, including instrumental techniques and sensory analyses (Kühn et al., 2006). Instrument techniques have been used as a popular option for studying flavor-protein interaction for decades. However, instrumental results do not directly relate to consumer perception of flavor in a real food system. Therefore, sensory analyses are necessary to correlate or relate instrumental measurements with consumer acceptance (Reineccius, 2006).

2.5.1 Conventional Techniques

The two main methods commonly used to study flavor-protein interactions are static headspace and equilibrium dialysis (Damodaran and Kinsella, 1981a, b; O'Keefe et al., 1991a, b; O'Neill, 1996). Both methods are conducted under equilibrium conditions and the systems are often considered to be simple, since they are liminted to the study of the binding of a single flavor compound to a single protein (Kühn et al., 2006).

Headspace analysis techniques are based on measurement of the vapor-liquid partition equilibrium in a well-defined system (Kühn et al., 2006). These methods measure the change in the partition coefficient by directly determining the change in volatile concentration in the headspace above the food or model system at equilibrium (O'Neill, 1996). In a headspace analysis technique, a known amount of flavor compound is added to a buffered protein solution, the mixture is allowed to reach equilibrium, and then the volatile concentration in the headspace is measured by GC. The difference between the volatile concentration above the protein solution and the blank buffer solution (control) is then compared (O'Keefe et al., 1991a, b; O'Neill, 1996). This technique provides a simple and straightforward means to measure the impact of flavor-protein interactions, especially in liquid products. However, headspace techniques are unsuitable for semi-volatile compounds. In this case, a large amount of sample is needed for adequate detection. In addition, this technique is also limited since it does not provide kinetic information, thus making it difficult define which binding mechanisms are involved (O'Neill, 1996; Kühn et al., 2006). To resolve the problem of poor sensitivity splitless or on-column GC techniques can be used. Furthermore, increasing the equilibrium temperature can help, but thermal reactions may occur (Kühn et al., 2006).

A popular alternate to increase the sensitivity and utility of the headspace technique has been application of solid-phase microextraction (SPME) (Adams et al., 2001; Fabre et al., 2002; Jung and Ebeler, 2003a; Gianelli et al., 2005; Kühn et al., 2008). It was found that headspace SPME is good for both static and dynamic headspace analysis for measurement of milk protein-flavor interactions (Fabre et al., 2002). In addition, SPME can also be used to measure the flavor concentration using an

equilibrium dialysis technique (Zhou et al., 2002). Pawliszyn and coworkers developed SPME around 1990 by using fused silica fiber coated with thin layer of a selective coating to adsorb (or absorb) the volatile flavor compounds from the headspace above the sample. Then the adsorbed (or absorbed) volatile flavor compounds can be analyzed by thermally desorption into the GC for analysis (Arthur and Pawliszyn, 1990; Zhang and Pawliszyn, 1993; Jung and Ebeler, 2003a). SPME is a sensitive, rapid, inexpensive, selective and solvent-free sampling technique, and is suitable for automation. In addition, it can be used with many separation methods including GC, GC-MS, and high performance liquid chromatography (HPLC) (Zhang et al., 1994). However, SPME is difficult to use with external standards in some complex matrices and the bias in quantitative analysis can be caused by the competition of flavor compounds for the fiber. Therefore, it can be concluded that SPME is more suitable for simple systems than complex ones (Yang and Peppard, 1994; Grote and Pawliszyn, 1997; Robert et al., 2000; Kühn et al., 2006).

Equilibrium analysis is another technique that has been commonly used for studying flavor-matrix binding under equilibrium conditions (O'Neill, 1996; Kühn et al., 2006). This method is based on liquid-liquid partition equilibrium. The ligands (flavor compounds), which are not bound with the specific food component (such as protein) at equilibrium, are measured. In a dialysis cell system, the protein solution and the solution containing the flavor compound of interest are separated by a semi-permeable membrane in a twin chambered cell. Then the cell is shaken at constant temperature until equilibrium is reached. The flavor compounds in the solutions are extracted and the concentrations are determined by GC (Damodaran and Kinsella, 1981a, b; O'Neill,

1996). For a flavor compound with low volatility, such as vanillin or benzaldehyde, HPLC or UV-VIS methods can be used to determine free (unbound) flavor compound after equilibrium with the protein (Ng et al., 1989a; McNeill and Schmidt, 1993; Li et al., 2000; Chobpattana et al., 2002; Kühn et al., 2006).

As described above, equilibrium dialysis can be widely used for the study of flavor-protein interaction. It can provide useful information including equilibrium binding constant, number of binding sites and useful thermodynamic parameters can be calculated to show the nature of binding. However, this method is very time consuming and the flavor compounds might be lost during testing (Kühn et al., 2006). There are some other factors that can alter the binding including pH, reducing agents contained in the buffer, solvent extraction procedures, and the dialysis membrane might be plugged or bind with ligands (Wilson, 1985). Furthermore, this method is not suitable for the study of solid (dry) system.

2.5.2 Inverse Gas Chromatography Technique

Inverse gas chromatography (IGC) has been widely used for the characterization of surface physicochemical properties of solid substances based on gas-solid adsorption chromatography theory (Greene and Pust, 1958; Gale and Beebe, 1964). It has been applied for the characterization of the surface of polymers and their interactions with fragrance molecules (Cantergiani and Benczédi, 2002). In contrast to conventional GC, the roles of the stationary phase and mobile phase are inverted. In IGC, the subject of interest is the non-volatile substance. The GC column is prepared by packing the non-volatile substance (stationary phase), then injecting known amounts of volatile

compounds (volatile probles), which are have known structures and physical properties, into the GC. The surface chemistry, such as surface sorption and phase transition, of the non-volatile solid substance (stationary phase) can be obtained by IGC based on the partitioning of the volatile probes between the mobile and stationary phases. In addition, the thermodynamic properties of the sorbate-sorbent system can be measured at the same time (Greene and Pust, 1958; Gale and Beebe, 1964; Zhou and Cadwallader, 2004).

IGC has been mostly used in the material science and chemical engineering fields. However, IGC has been applied in the food science discipline, mainly for the determination of water sorption isotherms for dry foods (Helen and Gilbert, 1985). IGC technique can be used as a tool to study binding properties of small ligands (flavor compounds) to food substances (such as proteins) under dry (moisture free) or low moisture conditions (Aspelund and Wilson, 1983; Zhou and Cadwallader, 2004, 2006, 2008; Zhou et al., 2006; Lozano and Cadwallader, 2009). There are some advantages of IGC over the conventional methods including its simplicity, speed and accuracy. In addition, it is suitable for the study of dry and semidry food materials (Zhou and Cadwallader, 2004).

2.5.3 Sensory Analysis

Instrumental analysis of flavor-protein interactions provides useful information on the mechanisms involved, which is important for understanding the nature of the binding. However, the results obtained from a model system may not be directly applicable to real foods, since the instrumental technique cannot give the actual impact of flavor-protein interaction when the food is consumed. The use of sensory analysis can provide

additional information related to the effect of binding on flavor perception and product acceptability. Correlation of the instrumental and sensory data can then provide a better understanding of the cause-and-effect relationship of the flavor-protein interactions. The knowledge obtained from the combined studies can help food producers develop improved food formulation with more acceptable flavors (Kühn et al., 2006; Zhou et al., 2006). However, to obtain precise sensory results, intensive training of the panelists is often necessary. Also, sensory analysis can be expensive and time consuming (Kühn et al., 2006).

One of the methods used for sensory study is sniffing (odor evaluation), which is the perception of aroma intensity of volatile flavor compounds present in the gas phase (headspace) above the food. The amount of flavor compounds in the headspace is determined by the distribution the volatile components between the headspace and food matrix, which is affected by flavor-matrix interactions (Taylor, 1998; Marin et al., 1999). Zhou and others (2006) evaluated flavor binding of selected volatile butter flavor compounds (diacetyl and butyric acid) onto soy containing crackers using IGC and sensory techniques. They found the general agreement between the IGC and sensory evaluation data. There are some other sensory studies that examine the effect of flavor-protein interaction on flavor perception (Ng et al., 1989b; Hansen and Heinis, 1991, 1992; McNeill and Schmidt, 1993; Reiners et al., 2000). Ng and others (1989b) compared the sensory perception of vanillin versus free vanillin measured instrumentally (HPLC) in a fababean protein model system. They showed that free vanillin contributed to perceived flavor, and concluded that it is possible to use instrumental results to predict human perception of specific flavor compounds in the food system containing both flavorant and

protein. Hansen and Heinis (1991) using flavor perception studies found that vanillin flavor intensity declined in solutions containing either sodium caseinate or whey protein concentrate. Similar to vanillin, *d*-limonene intensity was also decreased in the present of proteins in the solution. For benzaldehyde, the flavor intensity declined only in the presence of whey protein concentrate (Hansen and Heinis, 1992). Another study concerned with the effect of heat and emulsifier addition on the interaction of vanillin with milk proteins made use of sensory and HPLC techniques. A reduction of flavor perception was observed, but there was no correlation between sensory and HPLC results (McNeill and Schmidt, 1993).

2.5.4 Other techniques

The equilibrium methods are suitable for the the study molecular interactions between volatile compounds and proteins or other ingredients in food matrices. However, they do not provide enough information about the nature of these interactions. Spectroscopic techniques can be used to obtain more information about the nature of the interactions by providing conformational changes while proteins are modified (Kühn et al., 2006).

Fluorescence spectroscopy is one of the tools for the investigation of the structure, function and reactivity of biological molecules, including proteins. This technique is fast and simple. In addition, as compared to light absorption techniques, fluorescence spectroscopy has 100 to 1000 times greater sensitivity. Information about the local interactions can be acquired by investigation of wavelength shifts and fluorescence emission intensity of tryptophan residues in the proteins. For example, when the binding

between flavor compounds and protein molecule occurs, the conformation of the protein itself might change. Binding constants and also number of binding site can be described and interpreted in terms of the change in fluorescence intensity (Dufour and Haertlé, 1990; Marin and Relkin, 2000; Liu et al., 2005a). However, the environmental effects (such as pH) can interfere with the optical effects, leading to inaccurate results. Also, the protein studied must contain at least one tryptophan residue (Muresan et al., 2001).

Nuclear magnetic resonance (NMR) spectroscopy is a suitable technique for the study of conformation changes at the atomic level. It is a useful technique to investigate intra- and intermolecular interactions. NMR spectroscopy has been used for the study of milk protein conformational changes as affected by temperature, pH and high pressure treatments (Belloque and Ramos, 1999; Jung et al., 2002). Therefore, NMR spectroscopy is potentially very useful for the study of mechanisms involved in protein-flavor interactions. Lübke and others (2002) studied the conformation changes of β-lactoglobulin caused by the binding of flavor compounds. They found that the binding mechanisms were disclosed by using two-dimensional (2D) NMR technique. NMR data provided the precise information of binding location and confirmed findings from previous studies, which were done by fluorometry, affinity chromatography and infrared spectroscopy studies. Furthermore, diffusion-based NMR techniques, which are fast and easy to perform, were proposed as rapid screening techniques in the study of molecular interactions between flavor compounds and biological macromolecules, but the methods lack sensitivity. The diffusion-based nuclear Overhauser effect (NOE) pumping method, which is the combination of pulsed field gradient nuclear magnetic resonance spectroscopy (PFG-NMR) and a NOE experiment, can be used to screen and identify

which flavor compounds in the mixture are selectively bound to proteins. However, these diffusion-based NMR methods can be more precise if the experiments are combined with 2D-NMR techniques to give more information about specific binding sites and also mechanisms involved (Jung et al., 2002; Jung and Ebeler, 2003b).

Fourier transform infrared (FT-IR) spectroscopy is another technique that has been used to investigate changes in the secondary and tertiary structures of biological macromolecules. It was recently used for the study of the aggregation and structural properties of SPI as affected by high pressure treatment (Tang and Ma, 2009). Along with NMR, FT-IR spectroscopy has been used to discriminate or screen aroma compounds. The discrimination is based on the protein spectral changes in the amide I band (1700-1600 cm^{-1}), which is from the amide bonds that link the amino acids and the secondary structure of proteins. When FT-IR results are combined with the specific binding site information (for example, strand β-G, α helix, and strand β-I) obtained from NMR techniques, the relationship between the ligand structure and their binding behaviors can be obtained (Tavel et al., 2008).

2.6 DETERMINATION OF BINDING PARAMETERS

Characterization of the parameters involved flavor-protein binding is important for the understanding of the mechanisms involved. The most frequently used technique assumes that there is equilibrium between a protein molecule (*P*) and single ligand (*L*). This simplest case can be described as follow (Steinhardt and Reynolds, 1969; O'Neill, 1996; Price et al., 2001a):

$$P + L \leftrightarrows PL$$

The equilibrium binding constant, *K*, for this reaction is defined by:

$$K = \frac{[PL]}{[P][L]} \quad \text{or} \quad [PL] = K[P][L]$$

From [*P(total)*] = [*PL*] + [*P*], therefore [*PL*] = *K*[*L*]([*P(total)*] – [*PL*]), and then

$$\frac{[PL]}{[P(total)]} = \frac{K[L]}{1 + K[L]}$$

Since υ is the number of moles of ligand bound per mole of total protein, which equal to $\frac{[PL]}{[P(total)]}$, then:

$$\upsilon = \frac{K[L]}{1 + K[L]}$$

If one type of ligand can bind to more than one site of a protein (*n* binding sites), the equation will be *n* times that of one binding site, with the same equilibrium binding constant, *K*, therefore:

$$\upsilon = \frac{nK[L]}{1 + K[L]} \quad \text{or} \quad \frac{\upsilon}{[L]} = Kn - K\upsilon$$

This equation can be rearranged into a Scatchard plot, which is the plot between $\frac{\upsilon}{[L]}$ vs υ.

The slope of the plot will give the value of –*K*, and y-intercept gives the value of *nK*. In addition, the above equation can be rearranged to a form most commonly used in ligand-protein binding studies (Scatchard equation) (Scatchard, 1949; O'Neill, 1996):

$$\frac{1}{\upsilon} = \frac{1}{n} + \frac{1}{Kn[L]}$$

The plot between $\frac{1}{\upsilon}$ vs $\frac{1}{[L]}$ gives a double reciprocal plot, or Klotz plot (Klotz et al., 1946; Klotz and Urquhart, 1949). The slope of the plot gives the value of $\frac{1}{Kn}$, while the y-intercept gives the value of $\frac{1}{n}$. The equation parameters for both Scatchard and Klotz plots is summarized in **Table 2.3**.

Table 2.3 Equations and parameters commonly used in ligand-protein binding studies

equation	plot	binding constant (*K*)	number of binding sites (*n*)
Scatchard plot $\frac{\upsilon}{[L]} = Kn - K\upsilon$	$\frac{\upsilon}{[L]}$ vs υ	slope = -*K*	y-intercept = *nK*
Klotz plot $\frac{1}{\upsilon} = \frac{1}{n} + \frac{1}{Kn[L]}$	$\frac{1}{\upsilon}$ vs $\frac{1}{[L]}$	slope = $\frac{1}{Kn}$	y-intercept = $\frac{1}{n}$

As the measurement is conducted at constant temperature, the value of the binding constant can be used to determine the thermodynamic parameters that relate to binding including free energy of binding, enthalpy of binding and entropy of binding as follow (O'Neill, 1996):

The free energy of binding (ΔG)

$$\Delta G = -RT\ln K$$

The enthalpy of binding (ΔH)

$$\Delta H^\circ = \frac{-R \cdot d \ln K}{d(1/T)}$$

The entropy of binding (ΔS)

$$\Delta S = \frac{\Delta H° - \Delta G°}{T}$$

As mentioned above, Scatchard and Klotz plots are the models usually used. The assumption of both models is that protein must have equal and independent binding sites. If the binding sites in protein are not equal and dependent, they can cause either Scatchard or Klotz plots to be non-linear. Another plot that can be used in when these two plots are non-linear is a Hill plot. The Hill equation is as follow:

$$\upsilon = \frac{n}{\frac{1}{(K[L])^h} + 1}$$

Where *h* is the Hill coefficient, which reflects the cooperation between binding sites. The Hill equation can be rewritten in double-reciprocal form as follows:

$$\frac{1}{\upsilon} = \frac{1}{n(K[L])^h} + \frac{1}{n}$$

Similar to the Klotz plot, the Hill plot is the plot between $\frac{1}{\upsilon}$ vs $\frac{1}{[L]}$ (Guichard and Etiévant, 1998; Yven et al., 1998; Kühn et al., 2006).

Besides the thermodynamic parameters, protein hydrophobicity is also considered in many flavor-protein binding studies. When a protein is unfolded, the non-polar groups in the protein will be exposed to the environment. These non-polar groups are responsible for the hydrophobic binding of proteins with other ligands (O'Keefe et al., 1991a, b). Also, there might be some correlation between hydrophobicity and either entropy or enthalpy of binding. In case of the headspace technique, the concentration in the headspace is one of the parameter that is considered (Pérez-Juan et al., 2006). In addition, partition coefficient, which is the ratio of the concentration of volatile compound in gas

phase to its concentration in liquid phase, can also be calculated (Jung and Ebeler, 2003a; Meynier et al., 2003).

2.7 COMPARISON OF FLAVOR BINDING CAPACITIES OF SOY PROTEINS WITH OTHER FOOD PROTEINS

The binding parameters determined by different research groups for selected flavor compounds (aldehydes and ketones) with various proteins (such as soy proteins and dairy proteins) are shown in **Table 2.4**. The techniques used in these studies differed, which could explain why there are differences among the results for even the same protein type, flavor compound and experimental conditions.

Carbonyl compounds (ketones and aldehydes) are the flavor compounds most often chosen for study. Researchers tend to use the same flavor compounds to study flavor binding properties of different proteins. For example, the Kinsella research group used 2-nonanone as a model flavor compound to compare binding properties of whole soy protein, SPI, soy protein fractions (7S and 11S) and β-lactoglobulin (β-lg). They selected equilibrium dialysis as a tool for their study, which makes it possible to compare the binding parameters determined in each study (Damodaran and Kinsella, 1981b; O'Neill and Kinsella, 1987a, b). Other researchers also used 2-nonanone to study other types of proteins including whey protein isolate, whey protein concentrate (WPC), β-lg, and bovine serum albumin (BSA) (Jung et al., 2002; Liu et al., 2005a; Kühn et al., 2007); however, those results cannot be readily compared because different techniques and experimental conditions were employed by the various groups.

Table 2.4 Binding parameters for selected flavor compounds (aldehydes and ketones) with various food proteins

Protein	*Flavor compound*	*n* [a]	*K* [a]	*ΔG* [a]	*Tech.*[b]	*T (K)*	Ref [c]
soy protein (whole)	2-Nonanone	5.5	570		1	-	1
SPI	2-Heptanone	4	110	-2781	1	298	2
	2-Octanone	4	310	-3395	1	298	2
	2-Nonanone	4	930	-4045	1	298	2
	5-Nonanone	4	541	-3725	1	298	2
	Hexanal	-	-	-825	2	363	3
	Hexanal	-	-	-1386	2	313	4
	Nonanal	4	1094	-4141	1	298	2
	Vanillin	3.18	683.5	-3696	1	285	5
β-Con-	2-Nonanone	1.8	3050	-	1	-	1
glycinin	Hexanal	23	1437	-	3	293	6
(7S)	Hexanal	32	256	-3440	3	-	7
Glycinin	2-Nonanone	3.1	540	-	1	-	1
(11S)	Hexanal	84	483	-	3	293	6
	Hexanal	96	270	-3690	3	-	7
Casein	Vanillin	0.66	352.66	-3322	1	285	5
WPI	2-Nonanone	1.1	370		4	-	8
	Vanillin	0.67	1713.04	-4217	1	285	5
WPC	Heptanone	0.24	$4x10^7$	-	3	310	9
	Octanone	0.21	$4.5x10^7$	-	3	310	9
	2-Nonanone	8	130	-	4	-	8
	Benzaldehyde	0.2	$3.7x10^7$	-	3	310	9
β-lacto-	2-Heptanone	-	150	-2980	1	-	10
globulin	2-Octanone	-	480	-3660	1	-	10
	2-Nonanone	-	2440	-4620	1	-	10
	2-Nonanone	1.1	2700	-	4	-	8
	2-Nonanone	0.2	$5.3x10^7$	-	3	310	9
	β-Ionone	1.08	$1.7x10^6$	-	5	-	11
	β-Ionone	0.8	$1.9x10^6$	-	5	-	12
	β-Ionone	0.85	15015	-	1	-	12
	Benzaldehyde	1	$6.3x10^6$	-	5	358	13
	Vanillin	1	17000	-	6	-	14
BSA	Vanillin	2	4600	-	1	298	15
	Vanillin	0.72	310000	-	6	-	14
Ovalbumin	Vanillin	0.24	4500	-	6	-	14

[a] n = number of binding sites, *K* = binding constant (M^{-1}), ΔG = free energy (cal/mol).
[b] Applied technique, 1 = equilibrium dialysis, 2 = IGC, 3 = headspace analysis, 4 = headspace-SPME, 5 = fluorescence spectroscopy, 6 = UV-VIS spectroscopy.
[c] 1= O'Neill and Kinsella, 1987, 2 = Damodaran and Kinsella, 1981b, 3 = Aspelund and Wilson, 1983, 4 = Zhou and Cadwallader, 2004, 5 = Li et al., 2000, 6 = O'Keefe et al., 1991a, 7 = O'Keefe et al., 1991b, 8 = Kühn et al., 2007, 9 = Liu et al., 2005a, 10 = Schutte and van den Ouweland, 1979, 11 = Dufour and Haertlé, 1990, 12 = Muresan et al., 2001, 13 = Marin and Relkin, 2000, 14 = Mikheeva et al., 1998, 15 = Burova et al., 2003.

To compare binding capacities of soy proteins with other food proteins, results for the same flavor compounds, same techniques and same experimental conditions should only be considered. Parameters for the binding interaction of 2-nonanone with various proteins based on the equilibrium dialysis technique are compared in **Table 2.4**. Only a slight difference exists for the number of binding sites (n) and the binding constants (*K*) among whole soy protein (5.5 and 570 M^{-1}), soy protein isolate (SPI) (4 and 930 M^{-1}) and glycinin (11S) fraction (3.1 and 540 M^{-1}). However, n and *K* differed between β-conglycinin (7S) fraction (1.8 and 3050 M^{-1}) and the aforementioned proteins (Damodaran and Kinsella, 1981b; O'Neill and Kinsella, 1987). It is therefore concluded that β-conglycinin has higher affinity for 2-nonanone than does whole soy protein, SPI or glycinin.

Based on results of O'Neill and Kinsella (1987a), the binding constant of β-lg with 2-nonanone is 2440 M^{-1}, which is greater than what was observed for soy protein and its fractions. To compare the affinity, the negative free energy of binding (ΔG) should also be considered. The negative ΔG values for β-lg and SPI were 4620 and 4045 cal/mol, respectively. Based on these values it can be concluded that β-lg has a greater affinity for 2-nonanone than does SPI. The data in **Table 2.4** should be interpreted cautiously, since they differ from results from binding studies conducted by other researcher groups which used different method. For example, Liu and others (2005a), using a headspace dialysis technique, reported values that were over 20000-fold higher than what was reported by O'Neill and Kinsella (1987a) eventhough the same protein was studied. Therefore, it can be concluded that data generated from different techniques should not be directly compared.

Another flavor compound that is of great interest is vanillin. This popular flavor compound is commonly added into soy-based beverages. Li and others (2000) compared the binding of vanillin to three types of protein, including SPI, sodium caseinate and WPI. They found that the number of binding sites for SPI was higher than for the other two proteins which contained about the same number of binding sites. The binding constant for WPI was 1713 M^{-1}, which was higher than SPI (683.5 M^{-1}) and sodium caseinate (352.7 M^{-1}). The negative free energy of binding (ΔG) for WPI was highest, followed by SPI and sodium caseinate (4217, 3696, and 3322 cal/mol, respectively). From both binding constant and ΔG, it was indicated that the affinity of WPI for vanillin was higher than those of SPI and sodium caseinate. In addition, these data can be compared with those Burova and others (2003), who studied the affinity of BSA for vanillin using an equilibrium dialysis technique. Number of binding sites for BSA was 2, which was not much different from other proteins, while the dissociation constant for BSA was much higher than what was measured for WPI, SPI and sodium caseinate. Based on these findings, BSA appears to have a higher affinity towards vannilin as compared with the above three proteins. However, there were no data on ΔG and the temperature used in the study was 298K (13K higher). Moreover, the data obtained (showed in **Table 2.4**) cannot be compared with those of Mikheeva and others (1998) who investigated the binding of vanillin with β-lg, BSA and ovalbumin because that research group used UV-VIS in their study. In addition, the study from Li and others (2000) reported changes in enthalpy (ΔH) and entropy (ΔS), which are not shown in **Table 2.4**. They concluded that interaction of vanillin with sodium caseinate and WPI was driven by enthalpy because their ΔH and ΔS values were negative (-1264 cal/mol and

-32.70 cal/K·mol, respectively, for sodium caseinate and -8495.76 cal/mol and -15.01 cal/K·mol, respectively for WPI). In contrast, interaction of vanillin with SPI was driven by entropy due to the highly positive in enthalpy (7424 cal/mol), which was endothermic. The binding, which happened naturally due to entropy change, was high (39.02 cal/K.mol), which also resulted in a negative ΔG. This result is in good agreement with the work from Aspelund and Wilson (1983) who also found entropy drove the interaction of SPI with hexanal and hexanone. Therefore, it can be concluded that a change in conformation of SPI due to protein unfolding, and which is confirmed by a high entropy value, plays a key role in the interaction of soy protein with vanillin.

2.8 FACTORS AFFECTING FLAVOR-PROTEIN BINDING

In the food industry, proteins play a major role in determining the sensory and textural, as well as nutritional characteristics, of various food products. Proteins have the ability to interact with water, lipids, sugars, flavors and other ingredients. With respect to their flavor binding properties, conformation state of protein could have the greatest impact on flavor-protein binding (Damodaran and Kinsella, 1980). Thus, all the factors that can alter protein conformation would affect binding, including temperature, pH (acid/basic), ionic strength, presence/concentration of certain chemicals, and protein modification (Damodaran and Kinsella, 1981b; O'Neill and Kinsella, 1988; Li et al., 2000; Chobpattana et al., 2002; Zhou et al., 2002; Pérez-Juan et al., 2006; Kühn et al., 2007).

In general, heat and high pressure treatments result in changes in secondary and tertiary structure of native proteins without breaking covalent bonds (Hettiarachchy and

Kalapathy, 1997). Heat treatment is one of the most important food processing methods that causes a change in functionality (Boye et al., 1997). Heat treatment can cause protein denaturation, which includes protein unfolding and aggregation of unfolded protein molecules (Kühn et al., 2006). Effect of heat treatment (75 °C) on the binding of 2-nonanone to β-lagtoglobulin B was studied by O'Neill and Kinsella (1988). They found that an increase in heating time lead to a further decrease in the binding constant. Conformation changes due to protein-protein interactions (aggregations) were indicated by changes in florescence spectra and results of non-denaturating polyacrylamide gel electrophoresis, which showed an increase in higher molecular weight proteins after heating. Chobpattana and others (2002), who studied the effect of denaturation on the binding of vanillin binding to milk protein, showed that the amount of free vanillin increased significantly upon heating of bovine serum albumin (BSA) solutions (68 °C for 30 minutes and 75 °C for 15 minutes) as compare to non-heated BSA. The increase in free vanillin content was due to a decrease in binding affinity to vanillin caused by heat-induced structural changes in the protein.

In addition to heat treatment, low temperature can also affect flavor-protein binding. At 5 °C, tertiary and quaternary structures of soy protein can be induced to change. The binding constant of 2-nonanone to SPI at 5 °C (2000 M^{-1}) was higher than at 25 or 40 °C (930 M^{-1}) (Damodaran and Kinsella, 1981b). This might be because hydrophobic interactions within protein structure were weakened at 5 °C. The protein subunits can become reorganized within protein molecule, thus changing the hydrophobic binding site and resulting in higher binding affinity (Damodaran and Kinsella, 1981b). Li and others (2000) also found that decreasing temperature from 12 to 4°C resulted in an

increase in the number of binding sites in sodium caseinate, WPI, and SPI and increased the binding constants for sodium caseinate and WPI.

High pressure (HP) treatment can be used for protein modification. Recently, HP has been used for improving the functional properties of soy proteins and other food proteins (Liu et al., 2005b; Tang and Ma, 2009). The results of these studies, which focused on flavor-protein interactions, were in general agreement in that the flavor compound structure determines its binding affinity to proteins under HP (Yang et al., 2003; Liu et at., 2005a; Kühn et al., 2008). Yang and others (2003) modified β-lg by HP and found that the affinity of capsaisin was decreased after treated with HP at 600 Mpa and 50 °C for 32 min, while HP did not alter the binding of α-ionone, β-ionone, cinnamaldehyde, and vanillin with β-lg. This might be because HP can cause β-lg unfolding, but may not cause an increase in surface hydrophobicity. Therefore, the binding affinity towards hydrophobic flavor compounds might not change. Later, Liu and others (2005a) studied the effect of HP on flavor-binding of WPC. Benzaldehyde and methyl ketones were the flavor compounds selected for study. They found that the number of binding sites and the binding constant of WPC changed after HP treatment (600 Mpa at 50°C). They concluded that binding was depended on type and concentration of the flavor compound, and also holding time during HP treatment. The effect of HP (250 versus 600 Mpa) on binding of selected flavor compounds (2-nonanone, 1-nonanal, and *trans*-2-nonenal) with WPI (Kühn et al., 2008) was studied using the three stage model developed by Considine and others (2005). At stage I (0.1-150 Mpa), the native structure of β-lg was stable; at stage II (200-450 Mpa), the native monomer was 40lavor40angeable (reversible) with the non-native monomer and

disulfide-bonded dimmers; and at stage III (>500 Mpa), high molecular weight aggregates of β-lg were produced. The authors found that the binding of *trans*-2-nonenal to WPI increased when after treatment at 250 Mpa, while the binding of 1-nonanal and 2-nonanone were not altered. For the 600 Mpa treatment, the binding of *trans*-2-nonenal continuously increased, the binding of 2-nonanone decreased, and there was no effect on the binding of 1-nonanal. They concluded that HP affected protein-flavor interactions in accordance with flavor compound structure and suggested that hydrophobic interactions were weakened, while covalent interactions were strengthened by HP.

pH can be related to flavor-protein binding because it can induce conformation changes in protein. At neutral pH, most proteins are stable due to a small net electrostatic repulsive energy. However, the swelling and unfolding of protein molecule can occur at extremes in pH causing strong intramolecular electrostatic repulsion. Disulfide bonds in the protein molecule can be broken at alkaline pH, causing protein unfolding, which usually results in an increase in flavor binding (Damodaran, 2008). Zhou and others (2002) varied the pH (4.5, 7, and 9) in the study of binding properties of 2-pentyl pyridine (2PP) to soy protein. They found that 2PP bound more strongly to soy proteins (SPI, β-conglycinin, and glycinin) under basic conditions followed by neutral and then acidic conditions. Furthermore, ionic strength also affects protein conformation and thus its flavor binding ability. Guichard (2002) found that a "salting out" effect caused a decrease in retention of benzaldehyde by β-lg. Besides, Zhou and others (2002) also found that binding of 2PP decreased when the concentration of NaCl was increased due to the destabilization of electrostatic interactions.

Chemical modifications such as ethylation, glycosylation, and deamidation have been used to improve the functional properties of proteins. When protein side groups are modified, the generally result will be a change in the polarity and/or net charge of the protein. Therefore, protein conformation may change due to folding, unfolding, and aggregation with other protein molecules (Hettiarachchy and Kalapathy, 1997). O'Neill and Kinsella (1987a) studied the binding of 2-nonanone with native β-lg B versus β-lg B modified by ethylation (ethyl-esterification) and reduction of disulfide bonds with sodium disulfite. Binding decreased after modification due to changes in protein conformation caused by the destabilizing effects of the esterified free carboxylic groups, thus the native form of protein unfolded and underwent hydrophobic interactions with other protein molecules (O'Neill and Kinsella, 1987a). Effect of the modification of sodium caseinate by glycosylation using galactose, maltose, glucose, lactose, and fructose on flavor binding was studied by Fares and others (1998). They found that increasing of degree of modification could decrease the binding of diacetyl.

2.9 DEAMIDATION AND FLAVOR BINDING

Deamidation is a modification method that can be used for improving the solubility and other functional properties of food proteins (Hamada, 1994). Hydrolysis by deamidation can alter secondary and tertiary structures of proteins by removing amide groups in the glutamine and asparagines residues. Amide groups are converted into acid residues (carboxyl groups) with the release of ammonia. The reduction in pH leads to a decrease in the isoelectric point (pI) due to the increase in number of negatively charged

carboxyl groups. Therefore, the deamidated proteins should be more soluble under weakly acidic conditions (Hamada and Marshall, 1989).

Deamidation can be conducted both enzymatically and non-enzymatically. Enzymatic deamidation has several advantages over chemical methods. This includes mild reaction condition, high specificity, and perceived safety (Hamada, 1994). Several enzymes can be used for protein deamidation, including transglutaminase (Tgase), protease, peptidoglutaminase, and protein-glutaminase (PG) (Hamada, 1994; Yamaguchi et al, 2001).

In terms of flavor binding, Lozano and Cadwallader (2009) studied the effect of non-enzymatic deamidation of SPI on the binding of selected flavor compounds using the IGC technique. They found that using sodium dodecyl sulfate (SDS) for deamidation could reduce the overall flavor binding affinity of SPI. The binding affinity of deamidated SPI depended somewhat on the chemical characteristics of the flavor compound. Binding potentials of carbonyl containing flavor compounds to deamidated SPI were significantly decreased due to the reduction of imide formation and change in binding mechanism to mainly hydrogen bonding.

2.10 ENZYMATIC DEAMIDATION BY PROTEIN GLUTAMINASE

The enzyme protein-glutaminase (PG; EC 3.5.1) catalyzes the deamidation of protein. It was first isolated in 2000 from a bacterium (*Chryseobacterium proteolyticum,* strain 9670) isolated from soil (Yamaguchi and Yokoe, 2000). It catalyzes the deamidation of proteins at glutamine residues and releases ammonia (as shown in **Figure**

2.1) in both short peptide chains and proteins, but not at asparaginyl residues or free glutamines (Yamaguchi et al., 2001).

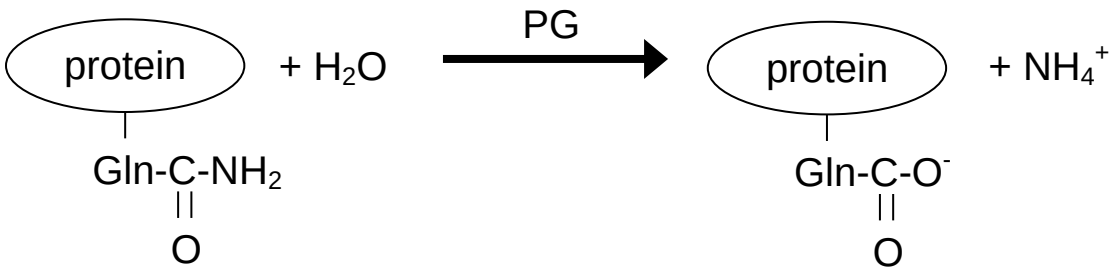

Figure 2.1 Schematic of the deamidation of protein by protein-glutaminase (from Miwa et al., 2010).

PG is different from other enzymes because it does not cause any side reactions, such as cross-linking by Tgase, peptide hydrolysis by protease, and is not limited to the deamidation of glutamine in short peptide chains only as with peptidoglutaminase. In addition, the effects of deamidation of several proteins, including α-lactalbumin, α-zein, wheat gluten, and skim milk, on structural and functional properties have been studied and it was found that solubility and some functional properties could be improved (Gu et al., 2001; Yong et al., 2004, 2006; Miwa, et al., 2010).

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CHAPTER 3

OPTIMIZATION OF THE ENZYMATIC DEAMIDATION OF SOY PROTEIN BY PROTEIN-GLUTAMINASE AND ITS EFFECT OF THE FUNCTIONAL PROPERTIES OF THE PROTEIN [*]

3.1 ABSTRACT

The effects of enzymatic deamidation by protein-glutaminase (PG) on the functional properties of soy protein isolate (SPI) were studied. Deamidation conditions were evaluated by means of response surface methodology (RSM). Optimal conditions based on achieving a high degree of deamidation (DD) with a concurrently low degree of hydrolysis (DH) were 44 °C, enzyme:substrate ratio (E/S) of 40 U/g protein and pH 7.0. Under optimal conditions, both DD and DH increased over time. SDS-PAGE results indicated that lower molecular mass subunits were produced with increasing DD. Far-UV circular dichroism spectra revealed that the α-helix structure decreased with higher DD, while the β-sheet structure increased until 15 min of deamidation (32.9% DD), but then decreased at higher DD. The solubility of deamidated SPI was enhanced under both acidic and neutral conditions. SPI with higher DD showed better emulsifying properties and greater foaming capacity than SPI, while foaming stability was decreased. It is possible to modify and potentially improve the functional properties of SPI by enzymatic deamidation using PG.

[*] Adapted with permission from Suppavorasatit, I.; de Mejia, E. G.; Cadwallader, K. R. Optimization of the enzymatic deamidation of soy protein by protein-glutaminase and its effects on the functional properties of the protein. *J. Agric. Food Chem*. **2011**, *59*, 11621-11628. Copyright (2011) American Chemical Society.

3.2 INTRODUCTION

Soy protein is widely used in the food industry due to its excellent nutritional and functional properties. It is possible to further improve the solubility and functional properties of soy proteins, especially for specific end uses, by physical, chemical and/or enzymatic modification. Deamidation is one type of modification that can improve solubility and other functional properties of food proteins (Hamada, 1994). Hydrolysis by deamidation can alter secondary and tertiary structure of proteins by removal of amide groups from glutamine and asparagines residues. During deamidation amide groups are converted into acid residues (carboxyl groups) with the subsequent release of ammonia. This leads to a decrease in the isoelectric point (pI) of the protein due to the increase in number of negatively charged carboxyl groups. As a consequence, deamidated proteins are more soluble under weakly acidic conditions (Hamada and Marshall, 1989). Deamidation can be conducted both enzymatically and non-enzymatically (chemically). Enzymatic deamidation has several advantages over chemical methods, including mild reaction conditions, higher specificity and greater safety (Hamada, 1994). Enzymes that have been used for protein deamidation include transglutaminase, protease, peptide-glutaminase and protein-glutaminase (PG) (Hamada, 1994; Yamaguchi et al., 2001).

PG was first isolated in 2000 from the bacterium *Chryseobacterium proteolyticum* (Yamaguchi and Yokoe, 2000). This enzyme catalyzes the deamidaton of proteins at glutamine residues in both short peptide chains and proteins, but does not deamidate asparaginyl residues or free glutamines (Yamaguchi et al., 2001). The specific activity of PG for various protein substrates, including soy protein isolate (SPI), has been previously reported (Yamaguchi et al., 2001). With respect to deamidation PG differs from other

enzymes in that it does not cause side reactions, such as cross-linking (transglutaminase), peptide hydrolysis (protease) or the deamidation of glutamine residues in short peptide chains (peptidoglutaminase). Studies on the deamidation by PG of some proteins and food materials, including α-lactalbumin, α-zein, wheat gluten and skim milk, have demonstrated that protein solubility and various functional properties can be improved (Gu et al., 2001; Yong et al., 2004, 2006; Miwa et al., 2010).

The most important factors in enzymatic hydrolysis are enzyme concentration, reaction temperature, reaction time, pH and the nature of the protein substrate (Adler-Nissen, 1986; Lahl and Braun, 1994). Since there are several factors affecting enzymatic hydrolysis, the optimization of the process parameters is essential in order to achieve an economical and optimal process. Response surface methodology (RSM) has been used for process optimization. RSM is a mathematical and statistical technique used for modeling and analysis of complex reactions or processes, in which the response of interest (dependent variable) is influenced by several independent variables (Montgomery, 1996). Various researchers have used RSM to study enzymatic hydrolysis of various types of protein such as crayfish processing by-products (Baek and Cadwallader, 1995), fish protein (Nilsang et al., 2005), chicken meat (Kurozawa et al., 2008), and mussel meat (Silva et al., 2010).

To our knowledge there are no detailed reports on the use of PG for deamidation of soy protein to modify its conformation, which in turn can affect its solubility and other functional properties. Hence, the objective of this study was to employ RSM in order to optimize process parameters for the PG deamidation of soy protein isolate (SPI) and to

evaluate the effect of deamidation on solubility and functional properties, including emulsifying and foaming properties, compared to untreated SPI.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Soy protein isolate (SPI; Profam 974) was obtained from Archer Daniels Midland Company (Decatur, IL). Protein-glutaminase "Amano" 500 (500 U/g) was obtained from Amano Enzyme, Inc. (Elgin, IL).

3.3.2 Methods

3.3.2.1 Enzymatic deamidation

Deamidation of SPI was performed in 0.01 M citrate-phosphate-borate buffer (Östling and Virtama, 1946) containing 20 mg/mL SPI and incubated for 90 min. The experiments were conducted in 50 mL test tubes with Teflon lined caps under different conditions with respect to E/S (5-50 U/g protein), temperature (40-60 °C), and pH (5-9) (Adler-Nissen, 1986; Gu et al., 2001; Yong et al., 2004, 2006; Miwa et al., 2010). The enzymatic activity was stopped by increasing temperature to 80 °C for 10 min (Yamaguchi et al., 2001).

3.3.2.2 Optimization of PG deamidation

A central composite design was used to determine the optimum condition for deamidation using three independent variables including E/S, temperature, and pH. The selection of the ranges of these factors was based on available literature (Gu et al., 2001;

Yong et al., 2004, 2006; Miwa et al., 2010) and on preliminary studies of enzyme properties conducted by the enzyme manufacturer. The experimental plan with the total of 17 combinations is shown in **Table 3.1**.

Table 3.1 Experimental design (coded and actual values) for the degree of deamidation (DD, %) and degree of hydrolysis (DH, %) for the enzymatic deamidation of soy protein isolate (SPI) using protein-glutaminase (PG)

design point	independent variables						dependent variables	
	coded			actual (uncoded)			DD (%)	DH (%)
	x_1	x_2	x_3	E/S	T (°C)	pH		
1	-1	-1	-1	14	44	5.8	24.9	4.54
2	1	-1	-1	41	44	5.8	28.9	2.45
3	-1	1	-1	14	56	5.8	28.7	3.16
4	1	1	-1	41	56	5.8	39.2	3.39
5	-1	-1	1	14	44	8.2	25.4	3.26
6	1	-1	1	41	44	8.2	33.4	4.10
7	-1	1	1	14	56	8.2	30.7	3.19
8	1	1	1	41	56	8.2	34.9	2.96
9	-1.68	0	0	5	50	7	24.4	3.90
10	1.68	0	0	50	50	7	33.2	4.13
11	0	-1.68	0	22.5	40	7	33.4	3.09
12	0	1.68	0	22.5	60	7	35.2	2.82
13	0	0	-1.68	22.5	50	5	29.2	1.94
14	0	0	1.68	22.5	50	9	19.5	2.28
15	0	0	0	22.5	50	7	33.9	3.49
16	0	0	0	22.5	50	7	34.9	3.76
17	0	0	0	22.5	50	7	33.9	3.90

The estimated response surface $\hat{y}$ (dependent variable) can be described as a second-order mathematical model:

$$\hat{y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \quad (3.1)$$

where β_0 is the constant term; β_1, β_2, β_3 are linear terms; β_{12}, β_{13}, β_{23} are interaction effect terms; β_{11}, β_{22}, β_{33} are quadratic terms of the model, while x_1, x_2, x_3 represent the

independent variables in coded values. The predicted regression coefficients of the model were calculated by using SPSS Statistics software version 17.0 (SPSS Inc., Chicago, IL). The surface plots were produced by Statistica software version 8.0 (StatSoft, Inc., Tulsa, OK).

The following deamidation conditions were selected from RSM and used in additional time dependent experiments: reaction temperature of 44 °C, E/S ratio of 40 U/g protein and pH of 7.0. The enzymatic deamidation was performed in triplicate at the indicated conditions for various reaction time periods until 24 h. A control sample of SPI was treated under the same conditions without addition of PG for 24 h. Degree of deamidation (DD) and degree of hydrolysis (DH) were measured at each individual period of time.

3.3.2.3 Determination of degree of deamidation (DD)

The DD was determined according to the methods of Yong et al. (2006) and Cabra et al. (2007) with some modifications. The amount of ammonia released from deamidated glutamine residues was determined by using an ammonia assay kit (Sigma-Aldrich, Inc., St. Louis, MO). The DD was expressed as the ratio (in percentage) of the amount of released ammonia by PG reaction and the total glutamine residues of proteins, which was determined by the released ammonia when protein was treated with 2 N sulfuric acid at 100 °C for 4 h.

3.3.2.4 Measurement of degree of hydrolysis (DH)

DH was performed as described by Cabra et al. (2007) with some changes. The DH is expressed as the percentage of the dissolved protein in the deamidated soy protein samples after precipitation with 0.2 N trichloroacetic acid (TCA), compared to the total dissolved protein (100%), which was obtained after complete hydrolysis with 2 N sulfuric acid at 100 °C for 4 h.

3.3.2.5 Determination of total soluble protein (TSP)

TSP was determined by using the DC Protein Assay^{TM} (Bio-Rad Laboratories, Inc.; Hercules, CA) according to the method described by Dia et al. (2009). The absorbance was measured at 630 nm. Total soluble protein concentration was quantified using a bovine serum albumin (BSA) standard curve ($r^2 \cong 0.99$).

3.3.2.6 Sample preparation for determination of functional properties

Deamidated samples were prepared in citrate-phosphate-borate buffer (pH 7.0) containing 60 mg/mL SPI and incubated at various time periods (15 min, 2 h, and 12 h). A control sample of soy protein isolate was treated under the same conditions without PG for 2 h. Five hundred milliliters of SPI in mixed buffer solution with PG (E/S of 40 U/g protein) were incubated in 1000 mL reagent bottles with caps. The temperature was controlled in a water bath at 44 ºC. The enzymatic activity was stopped by increasing temperature to 80 °C for 10 min. The resultant solution and precipitate were dialyzed in 0.1 M acetic acid overnight and then freeze dried. The dried samples were stored at 4 °C for the entire study.

3.3.2.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method described by Laemmli (1970). Precast polyacrylamide gel electrophoresis (4-20% Mini-PROTEAN® TGX™; catalog number 456-1093, Bio-Rad Laboratories Inc., Hercules, CA) was used in the study. Each protein sample (~ 2 mg/mL) was diluted (1:1 ratio, volume) with sample loading buffer (950 µL Laemmli sample buffer, Bio-Rad® catalog number 161-0737; and 50 µL 2-mercaptoethanol for electrophoresis, > 98% from Sigma®), then vortexed. Protein samples were boiled for 5 min and then briefly spun (6000 rpm) in a minicentrifuge (Fisher Scientific, Pittsburgh, PA). The samples (30 µL, equivalent to 30 µg of protein) and 5 µL Precision Plus Protein™ Dual Color standard (161-0374, Bio-Rad®) were loaded into the wells of precast gel, run at 200 V for 30 min (PowerPac 300, Bio-Rad®) in Tris/glycine SDS buffer. The gel was placed in fixing buffer (40% methanol and 10% acetic acid) for 15 min then stained overnight with Coomasie blue. The stained gel was de-stained using by 10% acetic acid for 20 min, and then washed with deionized water. The photo of the gel pattern was taken by Kodak Image Station 440CF (Eastman Kodak Co., New Haven, CT). Band intensities of protein samples were used for molecular mass calculation.

3.3.2.8 Circular dichroism (CD)

Soy protein dispersions were prepared at a concentration of 10 µM in phosphate buffer (pH 7.0) at 20 ºC. The CD spectra in the far UV region (190-250 nm) of each sample was determined using JASCO spectropolarimeter (Model J-715, Tokyo, Japan)

equipped with a temperature controller and a water bath (Neslab RTE 111; Thermo Neslab, Newington, NH). The samples were analyzed in a 1-cm path length square quartz cuvette with a Teflon cap with a speed of 50 nm/min, resolution 1 nm, sensitivity 50 mdeg, response 0.5 s; 50 scans were averaged. The molar ellipticity values were calculated using the formula given by Kelly et al. (2005) as:

$$[\theta]_{molar,\lambda}(\text{deg cm}^2\ \text{dmol}^{-1}) = 100 \times \theta_\lambda / m \times d \qquad (3.2)$$

where θ_λ is the observed ellipticity (degrees) at wavelength λ, m is molar concentration of a solute, and d is the pathlength (cm). Prediction of the percent of protein secondary structure from CD spectra was obtained using software from webserver: http://perry.freeshell.org/raussens.html, which uses the method of Raussens et al. (2003).

3.3.2.9 Determination of solubility

Solubility was determined in triplicate according to Puppo et al. (2004) and Yong et al. (2006) with some modifications. The freeze dried samples (1 mg) were dispersed in acetate-phosphate buffers of various pH values (3.0, 5.0, and 7.0) in 1.5 mL microcentrifuge tubes. All samples were kept at 25 °C overnight, then vortexed. The samples were then centrifuged at 3000 rpm (1000 × g) at 10°C by Eppendorf centrifuge model 5417R (Brinkmann Instruments, Westbury, NY) for 10 min. The supernatant (soluble fraction) were collected and total soluble protein was determined. The solubility was calculated as:

$$\text{solubility}\,(\%) = \frac{\text{protein in supernatant (mg/mL)}}{\text{initial protein (mg/mL)}} \times 100 \qquad (3.3)$$

3.3.2.10 Determination of emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of protein samples were determined in triplicate following Pearce and Kinsella (1978) and L'Hocine et al. (2006) with some changes. Emulsions of protein dispersions were prepared by mixing 10 mL of corn oil (Crisco®) with 30 mL of 0.5% (w/v) protein dispersion in 0.1 M acetate-phosphate buffer at pH 7.0. The mixtures were emulsified using a homogenizer (Ultra Turrax® T18, IKA® Works Inc., Wilmington, NC) at 22,000 rpm for 1 min. An aliquot of emulsion was immediately diluted 200 times with 0.1% (w/v) sodium dodecyl sulfate (SDS) solution and held for 15 min after homogenization. The absorbance of diluted emulsion was measured at 500 nm with UV-Visible spectrophotometer (DU®-64, Beckman Coulter, USA). EAI and ESI were calculated by the following equation:

$$\text{EAI}\,(\text{m}^2/\text{g}) = \frac{2\text{T} \times \text{A}_0 \times \text{dilution factor}}{\text{c} \times \Phi \times 10000} \tag{3.4}$$

$$\text{ESI}\,(\text{min}) = \left(\frac{\text{A}_0}{\text{A}_0 - \text{A}_{15}}\right) \times \text{t} \tag{3.5}$$

where T is turbidity (2.303), A_0 and A_{15} are absorbance at time 0 and 15 min, dilution factor is 200, c is the weight of protein per unit volume (g/mL) of protein aqueous phase before forming emulsion, Φ is oil volume fraction of the emulsion (0.23, based on preliminary experiments), and t is time interval (15 min).

3.3.2.11 Determination of foaming properties

Evaluation of foaming capacity (FC) and foaming stability (FS) were performed in triplicate according to the method described by Kanu et al. (2009) with some changes.

Protein dispersions (0.5%, w/v) were prepared in 0.1 M phosphate buffer at pH 7.0 then 50 mL of each sample were poured into a 100 mL graduated cylinder. The aqueous sample was mixed using a homogenizer (Ultra Turrax® T18, IKA® Works Inc., Wilmington, NC) at 18,000 rpm for 1 min inside the cylinder. FC was calculated as the percentage of increasing volume upon mixing. FS was expressed as the percentage of remaining foam after 5, 10, 20, 40, and 60 min without disturbing.

3.3.2.12 Statistical Analysis

Analysis of variance (ANOVA) and lease significant difference (LSD) were used in order to determine the differences among treatments ($p<0.05$) by SAS (SAS Institute Inc., Cary, NC).

3.4 RESULTS AND DISCUSSION

3.4.1 Optimization of Deamidation of SPI by PG

Experimental data from the central composite design were obtained using 17 combinations of three independent variables: enzyme:substrate ratio (E/S), temperature and pH (**Table 3.1**). Two models were fitted with second-order polynomial equations to explain DD and DH using actual values as shown in **Equations 3.6** and **3.7**:

$$DD\ (\%) = -10.218-4.132x_1-1.555x_2+20.188x_3-0.013x_1^2-2.366x_3^2+0.100x_1x_2 +0.670x_1x_3+0.236\ x_2x_3-0.013\ x_1x_2x_3 \quad (3.6)$$

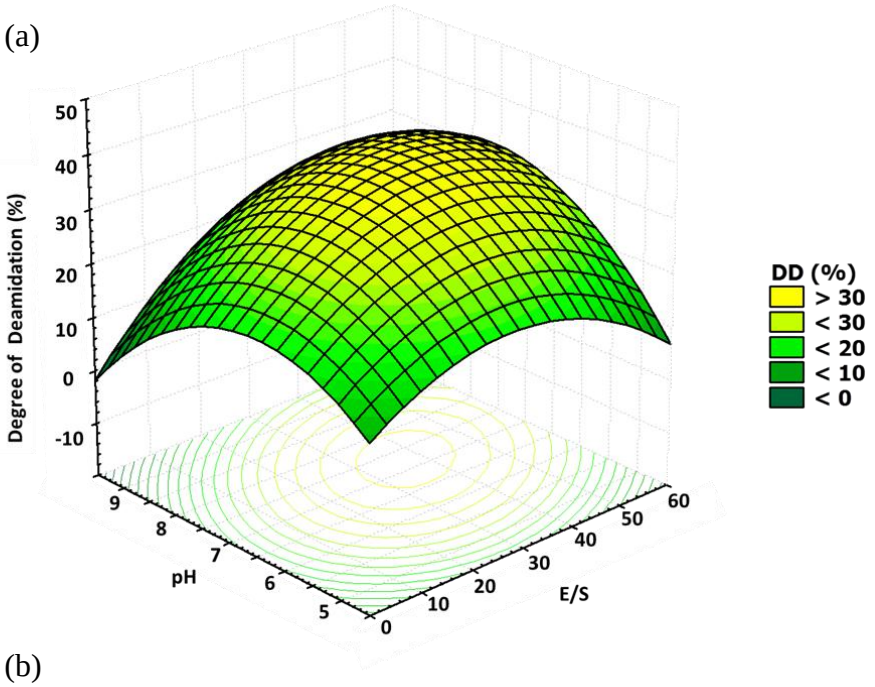
$$DH\ (\%) = 12.365-1.620x_1-0.189x_2+0.001x_1^2-0.005x_2^2-0.350x_3^2+0.029x_1x_2 +0.211x_1x_3+0.090x_2x_3-0.004x_1x_2x_3 \quad (3.7)$$

where DD is degree of deamidation (%), DH is degree of hydrolysis (%), and x_1, x_2 and x_3 represent E/S, temperature (°C) and pH, respectively. Both models (**Equations 3.6** and **3.7**) were examined by ANOVA without considering non-significant term(s). The coefficients of determination (r^2) of the models (DD and DH) were 0.867 and 0.855, respectively. This means that the models explain 86.7% and 85.5% of the total variation for DD and DH, respectively.

Response surface plots (a) and contour plots (b) generated by the models for DD and DH are shown in **Figures 3.1** and 3.**2**, respectively. The plots show the interaction between two independent variables (E/S and pH), while the third variable with the least significance in the fitted model (temperature) was maintained at 44 °C. This was the lowest temperature that gave response values (i.e., DD and DH from the model) that were closest to the experiment data.

Quadratic trends were observed between both independent and dependent variables (**Figures 3.1** and **3.2**). DD increased as a function of E/S until around 36-38 U/g protein, after which it declined with further increases in E/S (**Figure 3.1**). In addition, greater DD was obtained at higher pH values, with the highest DD occurring at around pH 7.1-7.4, after which DD declined at the higher pH values. The decreased activity at higher pH values could be explained by a loss of enzyme stability (Whitaker, 1994a). Furthermore, Yamaguchi et al. (2001) showed that PG was most active at a pH range of 5.0-7.0 and then declined slightly at higher pH values. As shown in **Figure 3.2,** DH was lowest at either the combination of high E/S and low pH or at low E/S and high pH. The lower deamidation activity at low pH (even at high E/S) was expected since soy protein exhibits a low electrostatic repulsion as it draws closer to its isoelectric point (pI ~ 4.5),

(a)



(b)

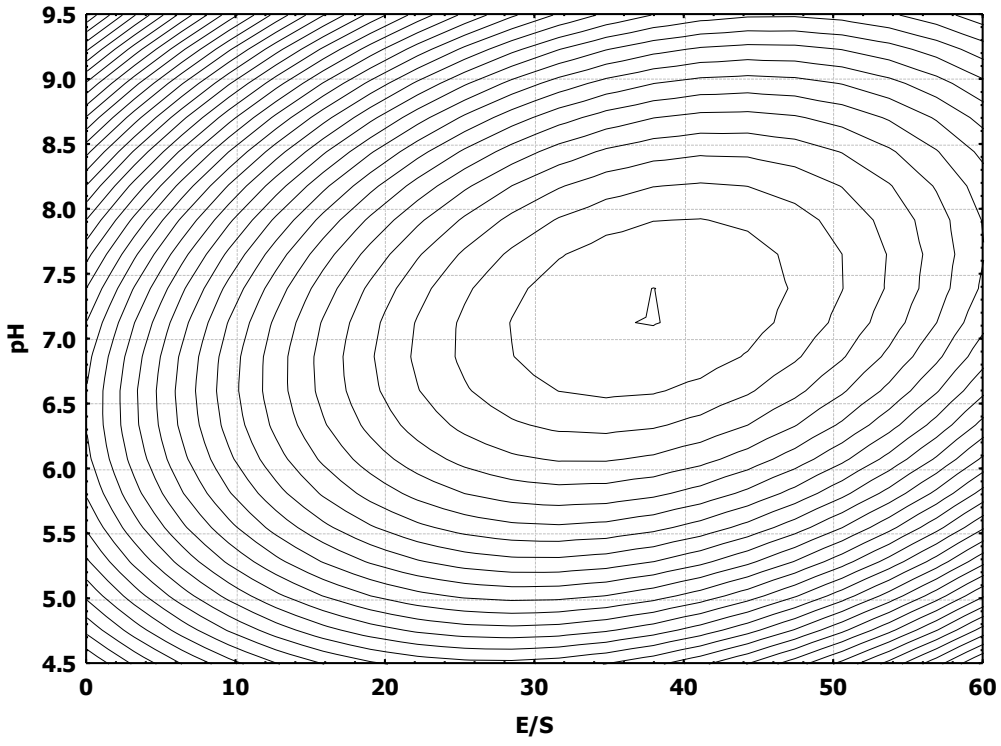


Figure 3.1 Response surface (a) and contour (b) plots of the effect of enzyme:substrate ratio (E/S) and pH on the degree of deamidation (DD, %) of soy protein isolate (SPI) by protein-glutaminase at 44 °C (from **Equation 3.1**).

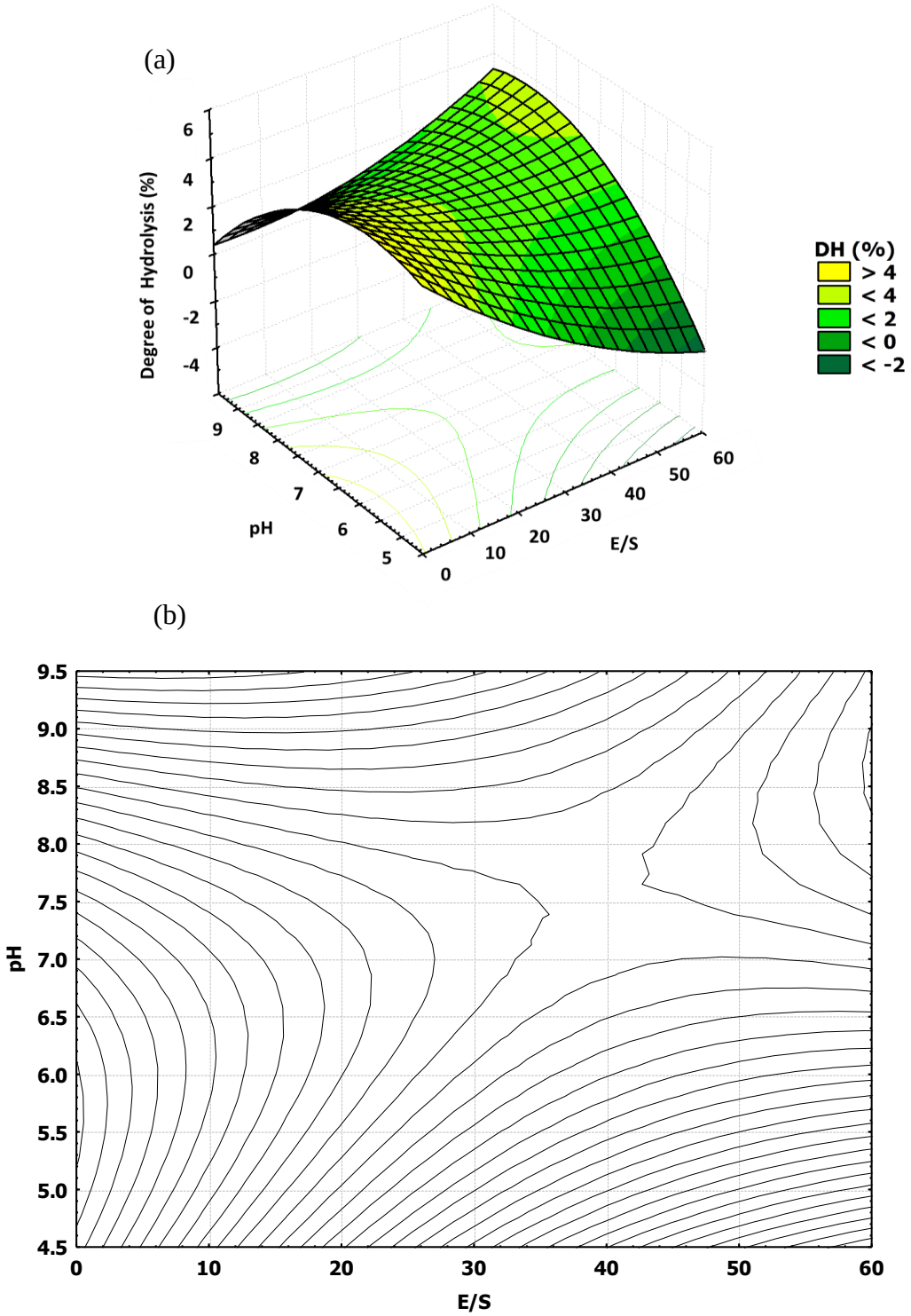


Figure 3.2 Response surface (a) and contour (b) plots of the effect of enzyme:substrate ratio (E/S) and pH on the degree of hydrolysis (DH, %) of soy protein isolate (SPI) by protein-glutaminase at 44 °C (from **Equation 3.2**).

thus reducing the number of sites at which the enzyme can react. In addition, the deamidation activity of PG is lower at higher pH since the pI of this enzyme is around 10.0 (Yamaguchi et al., 2001). In contrast, highest DH was observed at both the combination of low E/S and low pH and at high E/S and high pH (**Figure 3.2**).

In order to optimize the PG deamidation of SPI using RSM, a high DD and low DH were considered as being the most desirable outcome. **Figure 3.3** shows superimposed contour plots of DD and DH. At the highest value of DD (discussed above), the DH was less than 3% which was deemed acceptable.

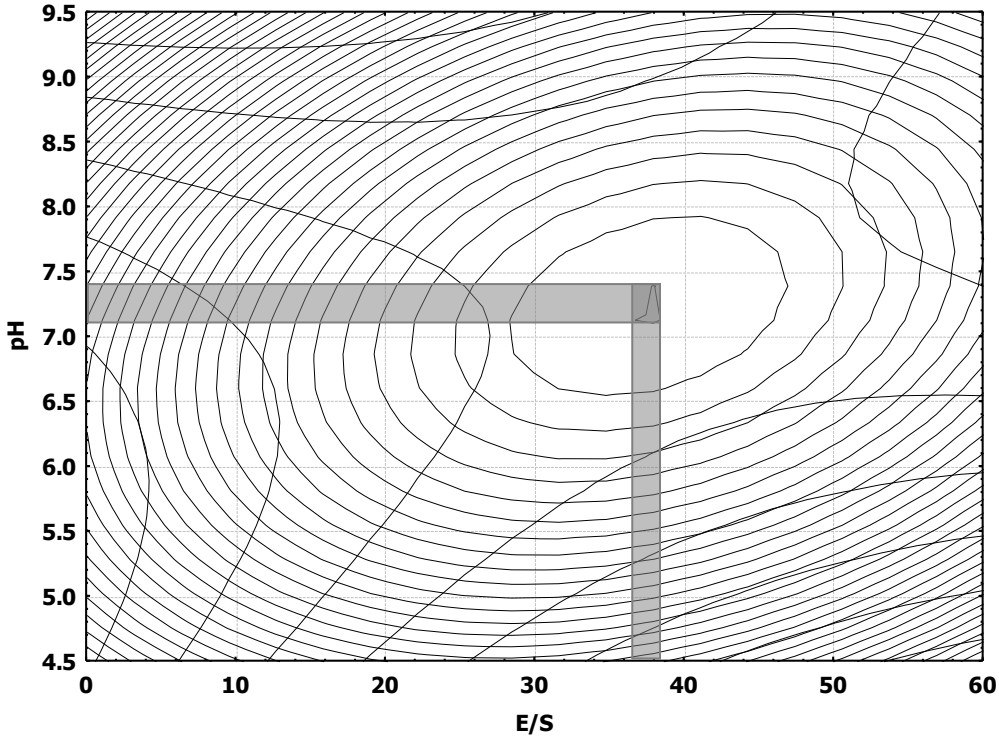


Figure 3.3 Superimposed contour plots for the response variables: degree of deamidation (DD, %) and degree of hydrolysis (DH, %) showing the optimal region for the deamidation of soy protein isolate (SPI) by protein glutaminase at 44 °C.

The adequacy of the DD model was confirmed by conducting an additional RSM experiment using same central composite design discussed previously, with 17 combinations of three independent variables (data not shown). The model was also fitted in the second-order polynomial equation as shown in **Equation 3.8**:

$$DD\ (\%) = -39.656+0.548x_1-0.153x_2+19.437x_3-0.009x_1^2-0.007x_2^2-1.829x_3^2+0.008x_1x_2+0.032x_1x_3+0.114\,x_2x_3-0.001x_1x_2x_3 \quad (3.8)$$

The coefficient of determination (r^2) of the model was 0.933, which means the model could explain 93.3% of total variation. Furthermore, the response surface and contour plots generated for the model from **Equation 3.8** with temperature maintained at 44 °C (data not shown) were similar to those shown previously in **Figure 3.1**. Based on this model, the optimum conditions for the PG deamidation of SPI for the three variables were (approximately) at a temperature of 44 °C, an E/S of 40 U/g protein and a pH of 7.0. These conditions were used for all additional studies.

Changes in DD and DH as a function of reaction time are shown in **Figure 3.4**. DD rapidly increased to about 40% within the first 2 h, and then gradually reached a level of about 53% after 24 h. In addition, the reaction might continue after 24 h since it had not yet reached a plateau at 24 h. The appearance of the deamidated SPI solution was more turbid, had lower apparent precipitate and viscosity compared to the control SPI solution (without PG). The rate of PG deamidation of SPI determined in the present study was faster than what was reported for native state α-lactalbumin and α-zein (Gu et al., 2001; Yong et al., 2004). This agrees with results of Yamaguchi et al. (2001), who reported that the specific activity of the PG on SPI (1.170 μmol/min·mg) was higher than for both α-lactalbumin (0.836 μmol/min·mg) and zein (0.655 μmol/min·mg). DH was

comparatively lower than DD at all time points, but both showed a parallel pattern. DH increased rapidly within first 2 h to approximately 4% then gradually increased to nearly 9% at 24 h.

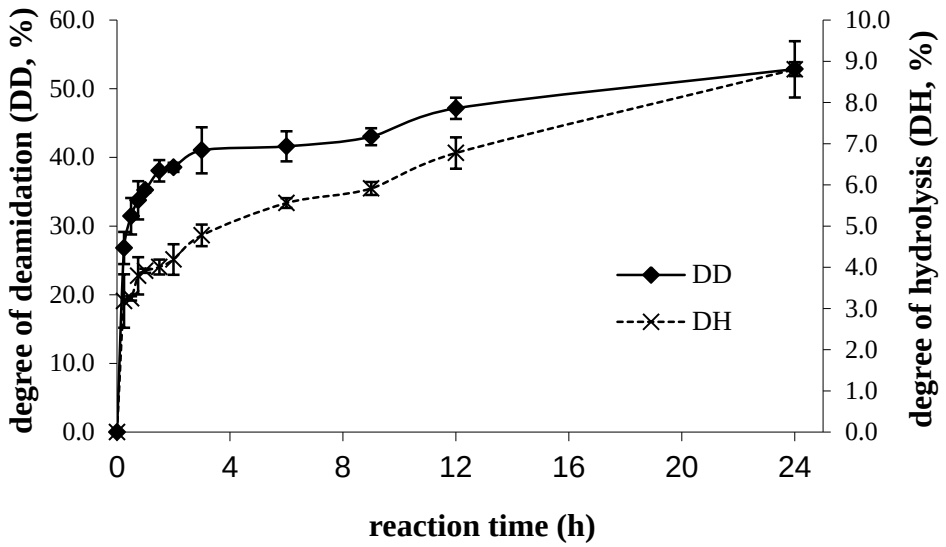


Figure 3.4 Change in degree of deamidation (DD, %) and degree of hydrolysis (DH, %) as a function of reaction time.

Previous reports indicated that PG can catalyze the deamidation of protein without proteolysis (Yamaguchi et al., 2001; Yong et al., 2006); however, the enzyme used in the present study is a commercial product and might contain some residual protease activity. In addition, it is also possible that some of the increase in DH can be attributed to the effects of deamidation. During deamidation amide groups are converted to carboxyl groups (Hamada and Marshall, 1989; Yamaguchi et al., 2001). This increases the number of negative charges and causes an increase in the electrostatic repulsion within the protein molecule. As a consequence protein unfolding might occur

leading to release of small peptide fragments, thus contributing to the increase in the measured DH.

Reaction times of 15 min, 2 h and 12 h were used to prepare larger samples under optimal conditions for use in the determination of functional properties. The DD levels for the resulting 15 min, 2 h, and 12 h deamidated protein samples were 32.9, 43.7, and 52.3%, respectively. Meanwhile, the DH levels for these same samples were 3.45, 4.81, and 10.7%, respectively. DD and DH of the large scale samples were higher than those observed for the aforementioned time dependent study, which might be due to the higher substrate concentration used in the large scale reactions (60 mg/mL) (Whitaker, 1994b). In addition, the large scale reactors were stirred throughout the deamidation process, thus providing better mass transfer between the PG and SPI compared with the time dependent experiments.

3.4.2 Molecular Mass Distribution of Deamidated SPI

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of untreated SPI, control SPI and SPI deamidated for 15 min, 2 h and 12 h are shown in **Figure 3.5**. The average molecular masses of untreated SPI, control SPI and SPI deamidated for 15 min, 2 h and 12 h (lanes 1 - 5, respectively) were approximately 40.2, 42.6, 31.2, 24.5 and 17.4 kDa, respectively. The gel pattern (**Figure 3.5**) shows that the deamidated samples (lanes 3-5, respectively) differed from the untreated SPI and control SPI (lanes 1 and 2, respectively). Band patterns for deamidated proteins indicated a downward shift to lower molecular mass units, which is supported by the observed increase in DH as a function of reaction time. However, Yong et al. (2004, 2006)

reported that no apparent protein hydrolysis occurred for the PG deamidation of other proteins (α-zein and wheat gluten) The untreated SPI and control had similar SDS-PAGE patterns and were in agreement with those previously reported in the literature (Liu et al., 2007). The 20 kDa band had the highest intensity in the 15 min and 2 h deamidated SPI samples (lanes 3 and 4, respectively), while the SPI deamidated for 12 h produced a high intensity band around 15 kDa. Based on these data, among the various protein fractions in SPI, subunit B of glycinin may be the most resistant to hydrolysis by PG, especially for the shorter reaction times of 15 min and 2 h.

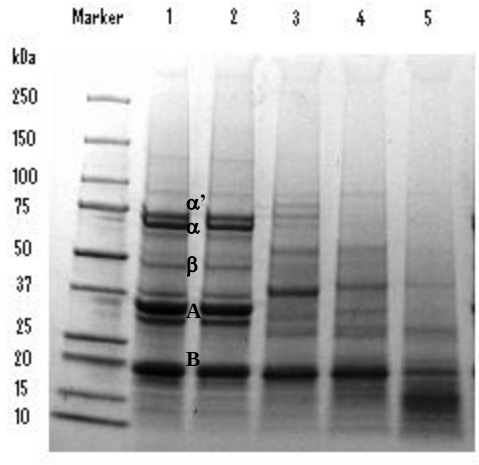


Figure 3.5 SDS-PAGE patterns for non-deamidated and enzyme deamidated soy protein isolate (SPI): (1) untreated SPI; (2) control SPI (SPI treated without PG); (3) SPI deamidated for 15 min (32.9% DD, 3.45% DH); (4) SPI deamidated for 2 h (43.7% DD, 4.81% DH); (5) SPI deamidated for 12 h (52.3% DD, 10.7% DH). α', α, and β indicate subunits β-conglycinin (7S); A, B indicate acidic and basic subunits of glycinin (11S), respectively.

3.4.3 Circular Dichroism (CD)

Secondary structure changes caused by deamidation were evaluated by measuring the far-UV CD spectra (190-250 nm) of the above samples. **Figure 3.6** shows CD spectra of the soluble fractions of the control and deamidated SPI samples. The CD spectra of the untreated and control SPI samples had similar patterns to those previously reported for α + β proteins (Venyaminov and Yang, 1996), which showed a positive band near 190-195 nm and negative board band at around 210-220 nm. The CD spectra of the deamidated SPI samples indicated that the increase in reaction time resulted in a reduction of the α-helix structure. The α-helix content of untreated SPI and control SPI samples were around 21.3 and 18.0%, respectively, and then subsequently decreased to 10.7, 10.2 and 10.0% as a result of deamidation for 15 min, 2 h and 12 h, respectively (**Table 3.2**). The 15 min deamidated SPI sample showed the greatest increase in β-sheet structure, which then decreased as a function of the further reaction time. The increase in β-sheet formation after deamidation agrees with the results of Yong et al. (2004), who reported that β-sheet structure in deamidated α-zein increased from 24% to 32%. In addition, β-turn structure remained constant, while random coil did not differ among samples. These observations are supported by the SDS-PAGE results (**Figure 3.5)**, where α' and α fraction band intensities dramatically decreased, indicating that they might have changed their structure to β-sheet form.

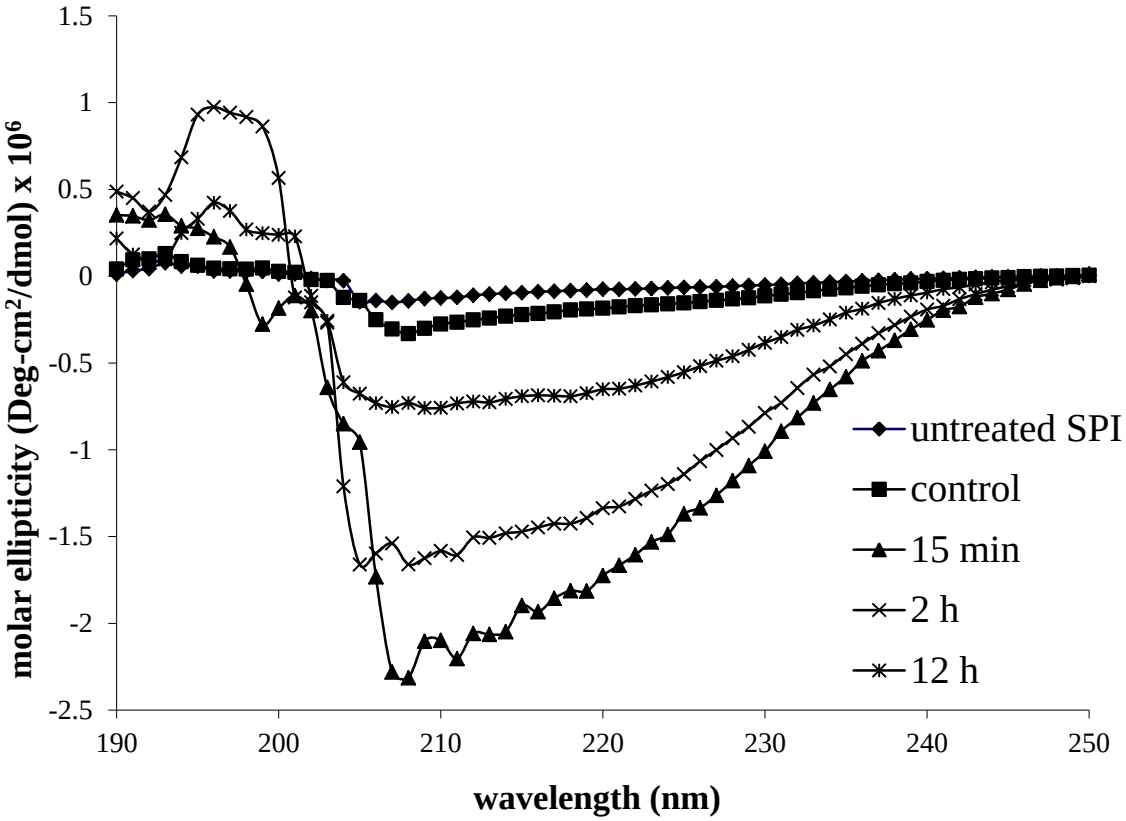


Figure 3.6 Far UV-CD spectra of non-deamidated and enzyme deamidated soy protein isolate (SPI): untreated SPI; control SPI (SPI treated without PG); SPI deamidated for 15 min (32.9% DD, 3.45% DH); SPI deamidated for 2 h (43.7% DD, 4.81% DH); SPI deamidated for 12 h (52.3% DD, 10.7% DH).

Table 3.2 Secondary structures* of non-deamidated and deamidated soy protein isolate

	untreated SPI	control	deamidation reaction time		
			15 min	2h	12h
α-helix (%)	21.3	18.0	10.7	10.2	10.0
β-sheet (%)	22.2	24.9	28.9	25.2	23.4
β-turn (%)	12.5	12.5	12.5	12.5	12.5
Random (%)	37.2	37.5	38.5	37.9	38.3
Sum (%)	93.2	92.9	90.5	85.8	84.5

*Data were derived by analysis of CD spectra.

3.4.4 Effect of Deamidation on Protein Solubility

The effect of deamidation on the solubility of SPI was evaluated for at different pH values (3.0, 5.0, and 7.0) (**Figure 3.7**). The solubility of all samples was highest at neutral pH (pH 7.0) and lowest at low pH values (pH 3.0 and 5.0). At pH 3.0, the solubility of the 12 h deamidated SPI sample was the highest (~32%), while the solubilities of other samples were similarly low (~18-20%). The solubility data for pH 5.0 was similar to that of pH 3.0 except that the control SPI sample showed the lowest solubility. The lower solubility of the control SPI compared to untreated SPI can be explained by its partial protein denaturation caused by the heating step (80 °C for 10 min). This in turn could have resulted in an increase in the hydrophobicity of protein at the surface after rearrangement, thus reducing its water solubility (Damodaran, 1996). Also, after the rearrangement, the pI of control SPI might be increased to nearly 5.0 as a result of the increase in positively charged amine groups at the surface of protein molecule in solution. Thus, the solubility of the control SPI was lowest at pH 5.0. The shift in pI of soy protein from 4.5 to 5.0 was also reported when hydrothermally cooked (154 °C) for 19 s or longer (Wang and Johnson, 2001). At pH 7.0, the solubility of the deamidated samples were much higher (>65%) than those of the untreated SPI and control SPI (~25%). Therefore PG deamidation for even a short period of time (15 min) can lead to an increase in solubility of SPI at neutral pH. These results are in agreement with those of Yong et al. (2006), who demonstrated that partial deamidation of wheat gluten (1 h, 22% DD) remarkably increased its solubility in a neutral buffer (pH 7.0).

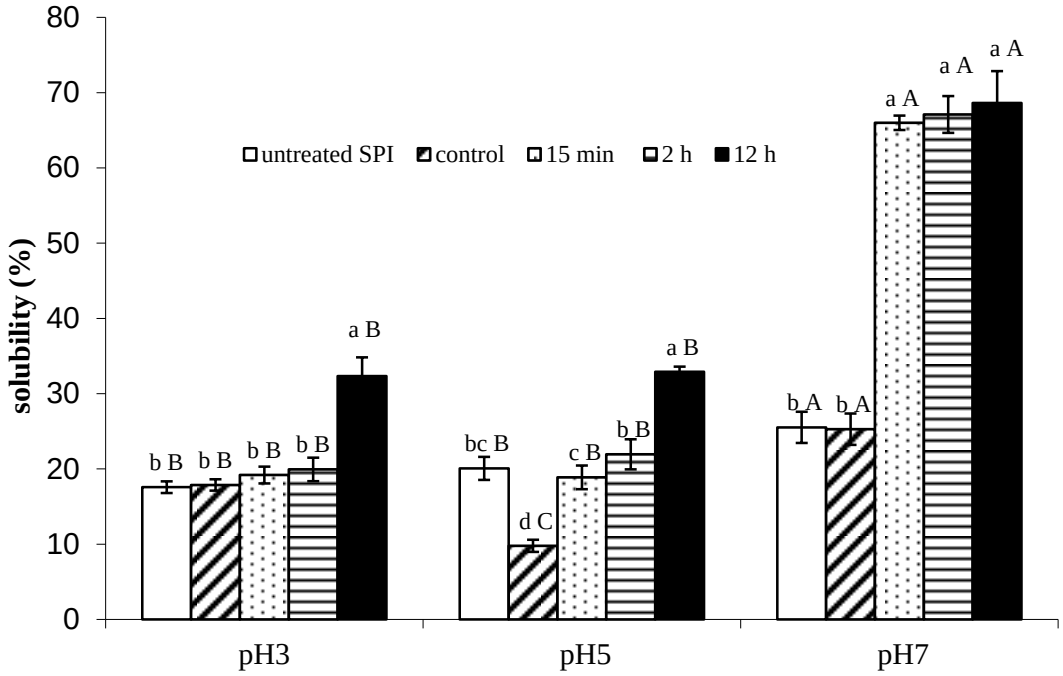


Figure 3.7 Solubility of non-deamidated and enzyme deamidated soy protein isolate (SPI) under various pH conditions: untreated SPI; control SPI (SPI treated without PG); SPI deamidated for 15 min (32.9% DD, 3.45% DH); SPI deamidated for 2 h (43.7% DD, 4.81% DH); SPI deamidated for 12 h (52.3% DD, 10.7% DH). Same lower case letters at same pH are not significantly different ($p>0.05$, $n=3$); same upper case letters within the same sample across different pH values are not significantly different ($p>0.05$, $n=3$).

3.4.5 Effect of Deamidation on Emulsifying Properties

Based on the preliminary experiments (data not shown), emulsifying properties could not be reliably measured at pH 3.0 and 5.0 because of the poor solubility of the protein. Yong et al. (2004, 2006) found that deamidated α-zein and wheat gluten displayed excellent emulsifying properties at pH 7.0. Thus, the emulsifying activity index (EAI) and emulsion stability index (ESI) were determined at only pH 7.0 in the present study (**Table 3.3**). EAI of all deamidated SPI samples were higher than untreated SPI and

control SPI ($p<0.05$). The 15 min deamidated SPI had the highest EAI, which was slightly higher than the EAI values for the 2 and 12 h deamidated SPI samples. These results indicated that deamidation could improve emulsifying properties by increasing the solubility of SPI, thus enhancing the protein's ability to form a layer around fat globules allowing them to better associate with the aqueous phase of the emulsion (Ponnampalam et al., 1990). Mirmoghtadaie et al. (2009) also stated that the improvement in the emulsifying activity of deamidated oat protein isolate was caused by an increase in solubility and surface hydrophobicity, which resulted in a better balance of the hydrophobic and hydrophilic ratio necessary for emulsification. The slightly lower EAI values for the 2 and 12 h deamidation SPI samples compared to the 15 min deamidation SPI sample can be explained by the higher solubility caused by the increase in deamidation. This could increase the net charge of the protein, which could affect protein-protein interactions of the protein film around the fat droplets (Mirmoghtadaie et al., 2009). The ESI values for all deamidated SPI samples were higher than for the untreated SPI and control SPI samples, which also increased as a function of reaction time. The 2 h and 12h deamidated SPI samples had the highest ESI values (~31 min).

Table 3.3 Emulsifying and foaming properties of non-deamidated and deamidated soy protein isolate [a,b,c]

Sample	EAI[d] (m^2/g)	ESI[e] (min)	FC[f] (%)	Foaming stability (%)				
				5 min	10 min	20 min	40 min	60 min
untreated SPI	17.5±0.2 c	20.1±0.3 c	26.0±3.5 c	21.1±3.0 c A	15.3±2.1 d B	11.9±1.6 c BC	9.8±2.2 d C	8.5±1.1 d C
control	16.8±0.7 c	20.0±0.3 c	38.7±2.3 b	29.6±1.1 b A	27.9±0.7 b A	25.0±1.3 a B	21.6±1.8 a C	21.6±1.8 a C
15 min	45.0±1.6 a	26.6±0.4 b	54.7±2.3 a	36.6±3.5 a A	30.6±1.3 ab B	14.9±0.5 c C	11.4±0.2 cd D	10.8±0.7 cd D
2 h	40.4±1.0 b	31.1±0.9 a	50.0±6.0 a	37.3±2.5 a A	24.0±1.7 c B	18.2±1.4 b C	13.8±0.4 bc D	12.5±0.9 c D
12 h	41.2±0.8 b	29.2±0.9 ab	53.3±6.4 a	39.2±1.7 a A	31.8±2.8 a B	26.1±2.9 a C	17.5±3.8 b D	15.7±3.2 b D

[a] Within columns, values with same lower case letters are not significantly different at $p>0.05$. [b] Within rows, values with same upper case letters are not significantly different at $p>0.05$. [c] Average ± standard deviation (n=3). [d] Emulsifying activity index. [e] Emulsifying stability index. [f] Foaming capacity.

3.4.6 Effect of Deamidation on Foaming Properties

Foaming capacity (FC) and foaming stability (FS) were measured in pH 7.0 phosphate buffer (**Table 3.3**). FC of the deamidated SPI samples did not differ from one another and were higher than those of the untreated SPI and control SPI samples. This might be due to the increase in solubility caused by deamidation, since foaming is enhanced by soluble proteins (Panyam and Kilara, 1996). In addition, the FC of the control SPI sample was higher than of that of the untreated SPI sample. Although the increase in %DD enhanced FC, the FS decreased. The FS of the deamidated SPI samples decreased as a function of resting time. This might be due to the reduction of protein-protein interaction which is affected by the excessive increase in protein charge which interferes with the formation of a cohesive protein film at the air-liquid interface (Phillips et al., 1994). These results agree with those of Chan and Ma (1999) who studied the effect of acid deamidation on the functional properties of okara (soymilk residue) protein isolate and found that higher DD leads to greater foaming ability. In that study, the FS decreased over resting time, and also decreased with higher DD. Kanu et al. (2009) also reported a similar result, in that the FC of hydrolyzed defatted sesame flour protein was higher than non-hydrolyzed sample. Furthermore, the FS also decreased over resting time.

3.5 CONCLUSIONS

Optimization of the enzymatic deamidation of SPI by PG was successfully carried out using RSM. The optimum conditions to obtain a deamidated SPI with high DD and acceptably low DH was a temperature of 44 °C, an E/S of 40 U/g protein and a pH of 7.0.

The deamidated SPI had enhanced solubility in both acidic and neutral conditions. The higher DD (longer deamidation time) showed better emulsification properties, including both EAI and ESI. Furthermore, the deamidated SPI had higher FC, but decreased FS over resting time. Deamidation has great potential to produce SPI with modified functional properties that can be used for various purposes in the food industry, especially for use in acidic soy-based beverages. However, studies on the conformational changes and other functional properties, such as impact on the flavor profile and flavor binding properties are still needed. Therefore, the effect of PG deamidation on flavor binding property of soy protein was further evaluated and discussed in the next chapter.

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CHAPTER 4

EFFECT OF ENZYMATIC DEAMIDATION OF SOY PROTEIN BY PROTEIN-GLUTAMINASE ON THE FLAVOR BINDING PROPERTIES OF THE PROTEIN UNDER AQUEOUS CONDITIONS

4.1 ABSTRACT

The effect of the enzymatic deamidation by protein-glutaminase (PG) on flavor binding properties of soy protein isolate (SPI) under aqueous conditions was evaluated by a modified equilibrium dialysis technique. Binding parameters, such as number of binding sites (n) and binding constants (K), were derived from Klotz plots. The partial deamidation of SPI by PG (43.7% degree of deamidation) decreased overall flavor binding affinity (n·K) at 25 °C for both vanillin and maltol by approximately 9 and 4-fold, respectively. The thermodynamic parameters of binding indicated that the flavor-protein interactions were spontaneous (negative $\Delta G°$) and that the driving force of the interactions shifted from entropy to enthalpy driven as a result of deamidation. Deamidation of soy protein caused a change in the mechanism of binding from hydrophobic interactions or covalent bonding (Shiff-base formation) to weaker van der Waals forces or hydrogen bonding.

4.2 INTRODUCTION

Flavor is a major determinant of the consumer acceptance of a food product (Liu, 2005; Cadwallader and Chang, 2010). The availability of a flavor compound for sensory perception is greatly influenced by its interaction with non-volatile food constituents including fats, proteins, and carbohydrates (Hatchwell, 1996; Guichard, 2002; 2006). The binding of flavor compounds to soy protein can lead to a decline in product quality since it can cause flavor fade (loss of flavor or lowering of flavor intensity) and/or a flavor imbalance due to selective binding of certain flavor compounds over others. This makes it difficult to determine the exact flavoring composition and dose for use in a food formulation (Chobpattana et al., 2002).

The type of flavor-soy protein interaction can be reversible (non-covalent) or irreversible (covalent) depending on the nature of protein and flavor compounds (Kühn et al., 2006; Preininger, 2006). While most of the interactions are hydrophobic and reversible (Kühn, 2008), irreversible binding can occur for certain flavor compounds, especially carbonyl containing flavor compounds. These carbonyl groups can form covalent bonds (Schiff-bases) with the amide side chains (e.g., glutamine, asparagine, etc.) of proteins (Hansen and Heinis, 1991; Damodaran, 1996).

Flavor-protein binding interactions can be altered by protein modification. Deamidation is a protein hydrolysis method which can alter primary, secondary, and tertiary structures of protein by removing amide groups. Deamidation can change the functional properties of a food protein such as solubility, foaming capacity and emulsification properties (Hamada, 1994; Suppavorasatit et al., 2011) and also can decrease flavor-protein binding, as demonstrated for a chemically deamidated soy protein

isolate (Lozano, 2009). Enzymatic deamidation is generally more desirable than chemical methods because it is substrate specific, can be conducted under mild reaction conditions, and is perceived as natural and safe (Hamada, 1991; Shih, 1996). Protein-glutaminase (PG), first isolated in 2000, catalyzes the deamidation of protein (Yamaguchi and Yokoe, 2000). PG differs from other enzymes with deamidation activity because it does not produce any side reactions, such as cross-linking (TGase), peptide hydrolysis (protease), and is not limited to the deamidation of glutamine residues in only short peptide chains as with peptidoglutaminase.

In our previous study, we developed a procedure for the deamidation of soy protein isolate by using PG which led to the production of a deamidated protein with modified functional properties (Suppavorasatit et al., 2011). However, that study did not assess the impact of deamidation on the flavor binding properties of the protein. The present study was aimed at testing the hypothesis that the reduction in the glutamine (amide) side chains capable of covalently bonding with carbonyl-containing flavor compound will reduce the overall flavor binding affinity of the protein. Therefore, the objective of the present study was to investigate the effect of deamidation on the binding of selected carbonyl containing flavor compounds to soy protein in an aqueous system using an equilibrium dialysis method.

4.3 MATERIALS AND METHODS

4.3.1 Materials

4.3.1.1 Reagents

Analytical grade (≥ 98% purity) vanillin (4-hydroxy-3-methoxybenzaldehyde), maltol (3-hydroxy-2-methoxyl-*4H*-pyran-4-one), and ethyl maltol (2-ethyl-3-hydroxy-*4H*-pyran-4-one) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Deuterium labeled vanillin (vanillin-d_3; 4-hydroxy-3-(methoxy-d_3)-benzaldehyde) was synthesized following the procedure described by Schneider and Rolando (1992).

Sodium phosphate monobasic (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4) were purchased from Sigma-Aldrich Co. and used for phosphate buffer preparation. Ethyl ether (99.9% purity, < 10.0 ppm BHT) was obtained by Fisher Scientific Inc.

4.3.1.2 Soy proteins

Soy protein isolate (SPI; Profam 974) was purchased from Archer Daniels Midland Company (Decatur, IL) and was vacuum packaged immediately upon receipt. The deamidated SPI (DSPI; 43.7% degree of deamidation and 4.81% degree of hydrolysis) was prepared by PG-deamidation for 2 h following the procedure described by Suppavorasatit et al. (2011). The DSPI sample was kept in a 125-mL amber glass jar and sealed with Teflon lined cap. Both SPI and DSPI were stored at 5 ± 1 ºC.

4.3.1.3 Enzyme

Protein-glutaminase "Amano" 500 (500 U/g) was obtained from Amano Enzyme, Inc. (Elgin, IL).

4.3.2 Methods

4.3.2.1 Preparation of flavor compound solutions

Vanillin (9,960 µg/mL) and maltol (10,500 µg/mL) stock solutions were prepared in odorless distilled water (prepared by boiling glass-distilled water in an open flask until its volume was reduced by one-third of the original volume). Vanillin-d_3 (1,130 µg/mL) and ethyl maltol (1,210 µg/mL) solutions were prepared in methanol and used as internal standards. All solutions were kept in 2-mL amber glass vials sealed with Teflon lined caps and stored at -70 ºC.

4.3.2.2 Analysis of free (unbound) flavor compounds

4.3.2.2.1 Isolation of free flavor compounds

A three mL aliquot of the reaction mixture was transferred to an Amicon® Ultra-4 centrifugal filter tube with 3K molecular weight cutoff (Millipore Corporation; Billerica, MA) and centrifuged at 5,000 × g for 30 min using a refrigerated centrifuge (Sorvall®, Du Pont Co.; Wilmington, DE) controlled at the same temperature used for incubation (5, 15, or 25 ºC). The permeate was spiked with 20 µL of the vanillin-d_3 (or ethyl maltol) internal standard solution and then thoroughly mixed. One mL of the permeate was transferred to a 2-mL glass vial and extracted with 0.5 mL of diethyl ether. The ether

fraction was subjected to GC-MS analysis. A flow diagram illustrating the procedure is shown in **Appendix A**.

4.3.2.2.2 Gas chromatography-mass spectrometry (GC-MS)

A Series II 5890 GC/5970 mass selective detector (MSD) system (Agilent Technologies, Inc. Palo Alto, CA) was used to quantify vanillin. Two μL of each sample was injected in the hot splitless mode (250 °C; 30 sec valve-delay). Separations were performed using an Innowax® column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; J&W Scientific; Santa Clara, CA). The oven was programmed from 150 to 220 °C at a rate of 10 °C/min with initial and final holding times of 2 and 20 min, respectively. Helium was used as carrier gas at a constant rate of 1.0 mL/min. The MSD conditions were as follows: transfer line temperature, 250 °C; ionization voltage, 70 eV; mass range (scan mode), 35 to 400 amu; scan rate, 2 scans/s.

A 6890 GC/5973 mass selective detector (MSD) system (Agilent Technologies, Inc.) was used to quantify maltol. Two μL of each sample was injected in the cold splitless mode (initial temperature, -50 °C; initial time, 0.1 min, ramp rate, 12 °C/s; final temperature, 260 °C; valve-delay time, 1 min). Separations were performed using a Stabilwax® column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Restek; Bellefonte, PA). The oven was programmed from 40 to 225 °C at a rate of 10 °C/min with initial and final hold times of 5 and 20 min, respectively. Helium was used as carrier gas at a constant rate of 1.0 mL/min. MSD conditions were as follows: transfer line temperature, 280 °C, ionization voltage, 70 eV; mass range (scan mode), 35 to 350 amu; scan rate, 5 scans/s.

4.3.2.2.3 Quantification

Quantitative analysis was conducted by using MS response factors (f_i) for vanillin and maltol compared against the internal standards (i.s.; vanillin-d_3 or ethyl maltol, respectively). The f_i of each compound is defined as the inverse of the slope of a plot (standard curve) of peak area ratio (flavor compound/i.s.) versus mass ratio (flavor compound/i.s.) for an ascending series of mass ratios. The f_i of vanillin vs vanillin-d_3 [hot splitless injection mode; using mass chromatography peak areas of ion 151 (vanillin) and ion 154 (vanillin-d_3)] was 1.32. For f_i for maltol vs ethyl maltol [cold splitless injection mode; using total ion chromatogram peak areas of maltol and ethyl maltol] was 1.16. The mass of each flavor compound was calculated as follows:

$$\text{mass of flavor compound} = \text{mass of i.s.} \times f_i \times \frac{\text{peak area of flavor compound}}{\text{peak area of i.s.}} \qquad (4.1)$$

4.3.2.3 Determination of flavor binding equilibration times

Prior to use, all glassware was silanized using 10% (v/v) dimethyl dichlorosilane (Sigma-Aldrich Co.) in toluene (Fisher Scientific Inc.; Pittsburgh, PA) as described by Tsutsumi and others (2003), then thoroughly rinsed with methanol (Fisher Scientifi Inc.), washed and then baked at 190°C. Protein solutions of SPI or DSPI [3% (w/v)] were prepared in aqueous 0.05 M phosphate buffer (pH 7.0), then stored at 4 ºC overnight to allow for complete hydration. Each protein suspension was placed into a 50-mL test tube equipped with a Teflon coated magnetic bars. Vanillin (or maltol) was spiked into each suspension to achieve an approximate concentration of 50 µg/mL. The test tubes were sealed with Teflon lined caps and were incubated with stirring [at speed level-6 using a

VWR magnetic stirrer model 310 (VWR International, LLC.; Arlington Heights, IL)] at a constant temperature (5, 15, or 25 ºC) maintained by using a 1-L low form jacketed beaker water bath (Chemglass, Inc.; Vineland, NJ). At specific time intervals, aliquots of each flavor-protein suspension were withdrawn and the concentration of the free (unbound) flavor compound were determined. Equilibration times were determined from plots of concentration of free flavor compound versus time at constant temperature.

4.3.2.4 Determination of binding properties by equilibrium dialysis technique

The determination of binding properties was performed according to the methods described by Chobpattana et al. (2002) and Li et al. (2000) with some modifications. Protein solutions of SPI or DSPI [3% (w/v)] were prepared in 0.05 M phosphate buffer (pH 7.0), then stored at 4 ºC overnight to allow for complete hydration of the protein. For binding studies, five mL aliquots of each protein suspension were placed into 20-mL glass scintillation vials containing Teflon coated magnetic stir bars and spiked with vanillin (or maltol) to achieve concentrations of 10, 20, 40, 60, 80, or 100 µg/mL and then sealed with Teflon-lined caps. Solutions of 20 µg/mL vanillin (or maltol) in 0.05 M phosphate buffer (pH 7.0) were used as controls. Each set of vials, consisting of a complete concentration range for each flavor plus the control, were incubated with stirring at three different temperatures (5, 15, or 25 ºC) until equilibrium was reached or exceeded (48 h for 5 ºC, 36 h for 15 ºC, and 24 h for 25 ºC), at which point the concentrations of the free (unbound) flavor compounds were determined.

Number of binding sites (n) and binding constants (binding affinity; K) were obtained by generating the double reciprocal plots (Klotz plots) from the Klotz equation

(**Equation 4.2**). This is one of the most commonly used methods for the analysis of protein-ligand binding data, as previously described (Klotz et al., 1946; Suppavorasatit and Cadwallader, 2010):

$$\frac{1}{\upsilon} = \frac{1}{n} + \frac{1}{\mathrm{K}n[L]} \tag{4.2}$$

where ν is the number of moles of ligand (flavor compound) bound per mole of total protein and [L] is the concentration of free ligand (free flavor compound). Based on **Equation 4.2,** the double reciprocal plot of 1/ν vs 1/[L] gives a slope equal to 1/Kn and a y-intercept equal to 1/n.

4.3.2.5 Determination of thermodynamic parameters

Thermodynamic parameters were calculated by using the binding constant (K), derived from the Klotz equation. The Gibb's free energy of binding (ΔG°) for each temperature was calculated from the following equation:

$$\Delta G° = -RT\ln K \tag{4.3}$$

where R is the gas constant (1.9859 cal $K^{-1}mol^{-1}$) and T is the absolute temperature in degrees Kelvin. The enthalpy of binding (ΔH°) was determined from the Van't Hoff equation:

$$\Delta H° = \frac{-\mathrm{R} \cdot d \ln \mathrm{K}}{d(1/\mathrm{T})} \tag{4.4}$$

where K_1 and K_2 are the binding constants at 5 and 25 °C, T_1 and T_2 are the absolute temperatures in degrees Kelvin, and R was the gas constant. The entropy of binding (ΔS°) was determined using the following equation:

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (4.5)$$

4.3.2.6 Statistical analysis

Analysis of variance (ANOVA) and least significant difference (LSD) were used to test for differences among treatments ($p<0.05$) (SAS Institute Inc., Cary, NC).

4.4 RESULTS AND DISCUSSION

A prerequisite to the use of an equilibrium dialysis technique for flavor binding studies is the knowledge of the minimum incubation time necessary for the system to reach equilibrium under the various experimental conditions to be evaluated. Equilibration times were determined by plotting the concentration of free (unbound) flavor compound as a function of incubation time at 5, 15, and 25 °C for both soy protein isolate (SPI) and deamidated soy protein isolate (DSPI). Typical equilibration curves for binding interaction of SPI or DSPI with vanillin and maltol at 25 °C are shown in **Figures 4.1** and **4.2**, respectively.

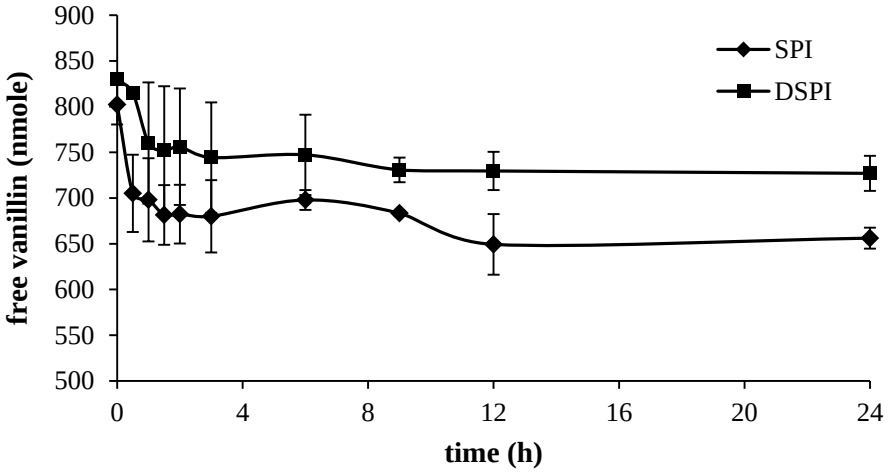


Figure 4.1 Equilibration curves for binding of vanillin with soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) at 25 °C.

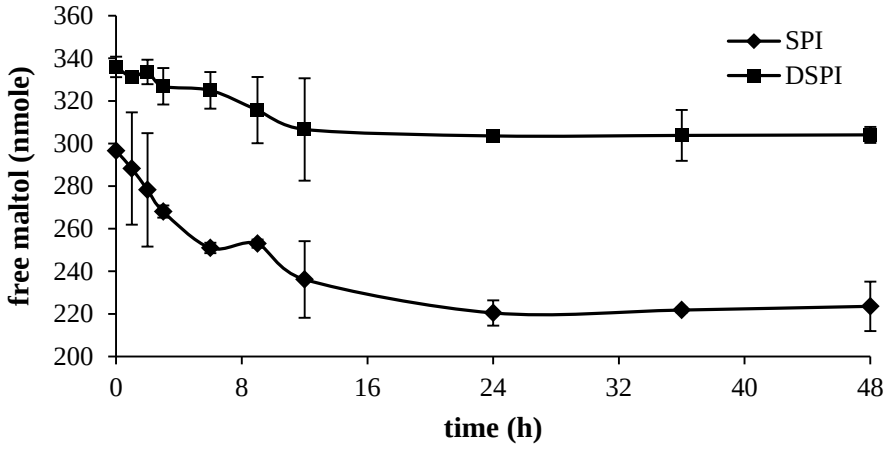


Figure 4.2 Equilibration curves for binding of maltol with soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) at 25 °C.

The equilibration time was considered to be the minimum time necessary for the free flavor compound to reach a stable (lowest) concentration. Equilibration times for binding of vanillin and maltol to SPI and DSPI at the three experimental temperatures used in this study are given in **Table 4.1**.

Table 4.1 Equilibration time for the binding of vanillin and maltol to soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) at different temperatures

soy protein type	Temperature (°C)	minimum time to reach equilibrium (h)	
		vanillin	maltol
SPI	5	48	48
	15	36	36
	25	12	24
DSPI	5	48	48
	15	24	36
	25	9	12

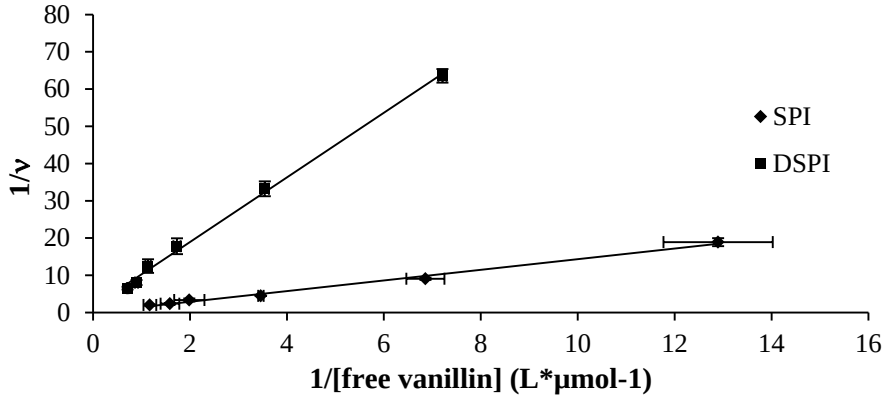
4.4.1 Binding Affinity of Vanillin and Maltol to SPI and DSPI

Figures 4.3a, 4.3b and **4.3c** show double reciprocal plots (Klotz plots) for the binding of vanillin to SPI and DSPI at 5, 15, and 25 °C, respectively. The plots are linear, which demonstrates that vanillin binds independently (non-cooperative interaction) to both SPI and DSPI for the temperature range studied. These results agree with those of Li and others (2000) who reported non-cooperative interaction of vanillin with soy and dairy proteins in an aqueous model system at 4 and 12 °C.

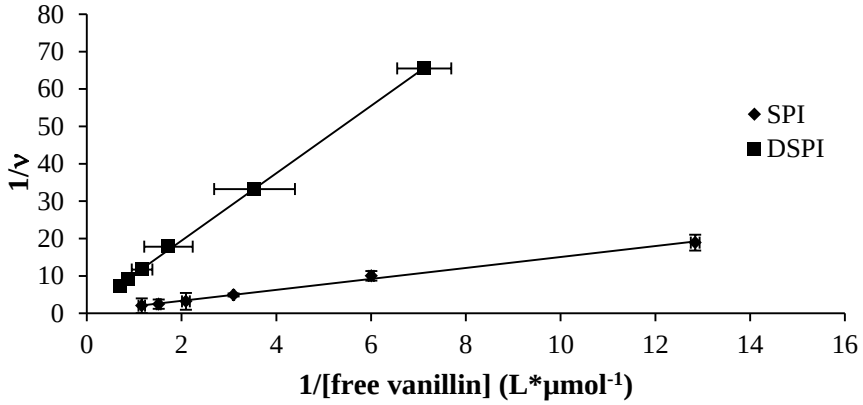
Linear regression equations from the Klotz plots for the binding of vanillin to soy proteins of two replications are presented in **Table 4.2**. The coefficients of determination (r^2) of all equations were greater than 0.97, which means that equations can explain more than 97% of the total variation for the plots.

As mentioned previously, **Equation 4.2** was used to determine the number of binding sites (n) and binding constants (binding affinity; K), which were calculated from

(a) 5 °C



(b) 15 °C



(c) 25 °C

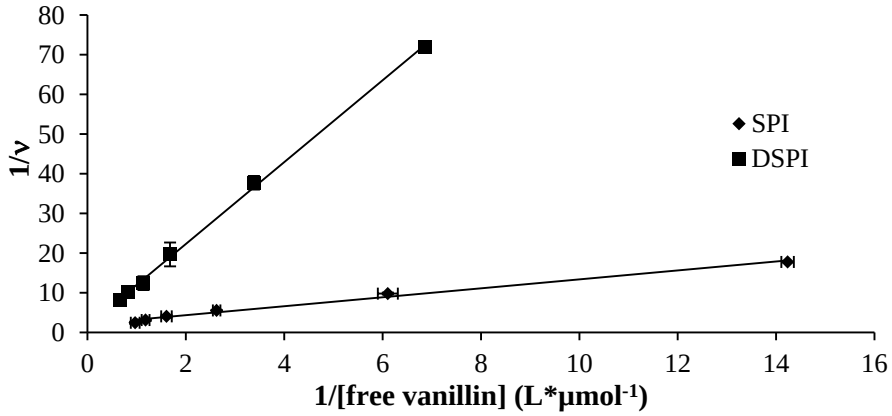


Figure 4.3 Klotz plots for binding of vanillin to soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) at 5 °C (a), 15 °C (b) and 25 °C (c) [Plots represent an average of two complete replications].

the y-intercepts (1/n) and slopes of the plots (1/Kn), respectively. The calculated n and K values for the binding of vanillin to soy proteins are shown in **Table 4.3**. The values for n for the interaction of vanillin with SPI at 5 °C, 15 °C, and 25 °C were 13.6, 2.31 and 0.48, respectively. The n values at 5 and 15 °C are in good agreement to those previously reported by Li et al. (2000) for the binding of vanillin to SPI at 4 and 12 °C (10.92 and 3.81, respectively). The K values for the binding of vanillin to SPI increased ($p \leq 0.05$) with increasing temperature (**Table 4.3**), which agrees with the results of Li and others (2000) who reported that the K values for the binding of vanillin to SPI increased from 468 M^{-1} at 4 °C to 683 M^{-1} at 12 °C.

Table 4.2 Linear equations from Klotz plots for the binding of vanillin and maltol to soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) obtained from two replications

flavor compound	soy protein	T (°C)	replication 1		replication 2	
			equation	r^2	equation	r^2
vanillin	SPI	5	y = 1.3026x + 0.0715	0.9981	y = 1.5616x + 0.0755	0.9840
		15	y = 1.3553x + 0.4217	0.9973	y = 1.5693x + 0.4466	0.9763
		25	y = 1.1376x + 1.9083	0.9833	y = 1.1138x + 2.2768	0.9937
	DSPI	5	y = 8.3793x + 1.5106	0.9940	y = 8.9729x + 1.6973	0.9944
		15	y = 8.7235x + 1.4958	0.9945	y = 9.2997x + 1.3234	0.9965
		25	y = 10.156x + 1.5559	0.9986	y = 10.4793x + 1.7326	0.9921
maltol	SPI	5	y = 0.3107x + 0.0942	0.9984	y = 0.3000x + 0.0920	0.9950
		15	y = 0.3210x + 0.1156	0.9985	y = 0.3103x + 0.1229	0.9960
		25	y = 0.3073x + 0.2985	0.9953	y = 0.3564x + 0.3131	0.9806
	DSPI	5	y = 0.4938x + 0.2554	0.9972	y = 0.4714x + 0.2433	0.9985
		15	y = 0.4665x + 0.2673	0.9993	y = 0.4976x + 0.2213	0.9989
		25	y = 1.1245x + 0.0335	0.9997	y = 1.4234x + 0.0342	0.9995

However, the magnitudes of the K values calculated in the present study are not close to the values reported by Li et al. (2000). This could be due to the fact that the present study was conducted on a different protein source and by different methodologies

which has been reported to cause systematic differences in K values (Kühn et al., 2006; Suppavorasatit and Cadwallader, 2010).

Table 4.3 Binding and thermodynamic parameters[a,b,c] for the binding of vanillin to soy protein isolate (SPI) and deamidated soy protein isolate (DSPI)

parameter	T (ºC)	SPI		DSPI	
n	5	13.6 ± 0.52	a A	0.63 ± 0.05	ns B
	15	2.31 ± 0.09	b A	0.71 ± 0.06	ns B
	25	0.48 ± 0.06	c NS	0.61 ± 0.05	ns NS
K ($\times 10^4$) (M^{-1})	5	5.16 ± 0.46	b B	18.5 ± 0.63	ns A
	15	29.8 ± 1.88	b A	15.7 ± 2.06	ns B
	25	186 ± 25.9	a A	15.9 ± 0.86	ns B
n·K ($\times 10^4$) (M^{-1})	5	70.4 ± 9.00	ns A	11.5 ± 0.56	a B
	15	68.7 ± 7.11	ns A	11.1 ± 0.50	ab B
	25	88.8 ± 1.33	ns A	9.69 ± 0.21	b B
ΔG° (kcal.mol^{-1})	5	-5.99 ± 0.05	a A	-6.69 ± 0.02	a B
	15	-7.21 ± 0.04	b B	-6.84 ± 0.07	a A
	25	-8.54 ± 0.08	c B	-7.09 ± 0.03	b A
ΔH° (kcal.mol^{-1})	5-25	29.5 ± 1.89	A	-1.22 ± 0.16	B
ΔS° (cal.K^{-1}.mol^{-1})	5	106 ± 6.79	ns A	-4.38 ± 0.59	ns B
	15	102 ± 6.55	ns A	-4.22 ± 0.57	ns B
	25	98.9 ± 6.34	ns A	-4.08 ± 0.55	ns B

[a] Within columns, values with the same lower case letters are not significantly different at $p > 0.05$. [b] Within rows, values with same upper case letters are not significantly different at $p > 0.05$. [c] Average ± standard deviation (n = 2).

The n values for the binding of vanillin to SPI decreased with increasing temperature, while the K values increased with increasing temperature (**Table 4.3**). However, the n and K values for the binding of vanillin with DSPI were not significantly

affected by temperature (**Table 4.3**). Values of n were lower for DSPI than SPI at 5 and 15 °C, but did not differ at 25 °C. Furthermore, with respect to vanillin the K values were lower for DSPI than SPI at 15 and 25 °C, but were higher for DSPI at 5 °C.

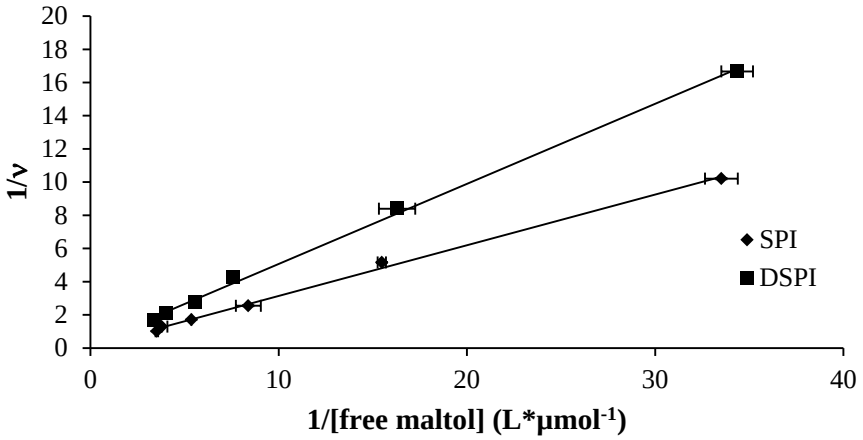
The Klotz plots of the binding of maltol to SPI and DSPI at 5, 15 and 25 °C are shown in **Figures 4.4a**, **4.4b** and **4.4c**, respectively. Linear equations from the Klotz plots for the binding of maltol to soy proteins of two replications are also presented in **Table 4.2**. The equations ($r^2 = 0.98$) explain more than 98% of the total variation of the plots. These results demonstrate that maltol also was bound non-cooperatively to both SPI and DSPI as same as vanillin as explained above.

The n and K values for the binding of maltol to soy proteins are shown in **Table 4.4**. The n values for the binding of maltol to SPI decreased with increasing temperature, while the K values increased with increasing temperature (**Table 4.4**). This is in agreement with the trends in n and K values observed for the binding interaction of vanillin to SPI (**Table 4.3**).

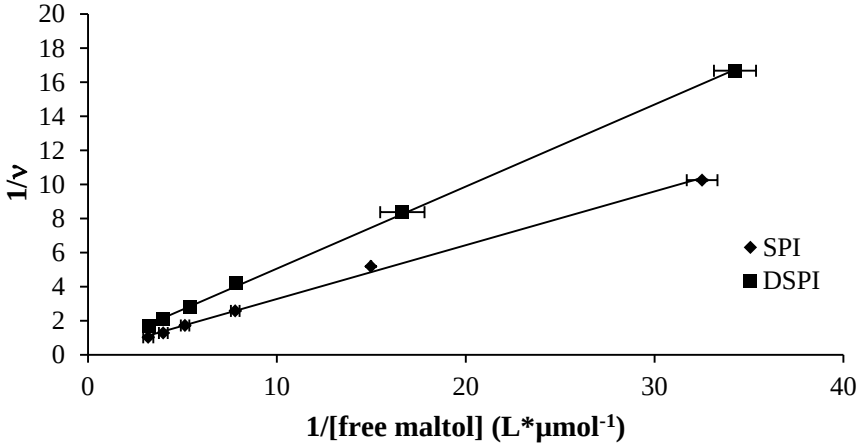
An opposite trend was observed for the binding of maltol to DSPI at 25 °C, where the n values increased and K values decreased with increasing temperature. This differs from the results for binding of vanillin to DSPI (**Table 4.3**), where temperature had no effect on the values of n and K.

The decrease in n with increasing temperature observed for the binding interaction of vanillin and maltol to SPI can be explained by the increase in protein unfolding as a result of the decrease in temperature (Pastore et al., 2007). In addition, lower temperatures may cause a rearrangement of protein subunits due to the weakening

(a) 5 °C



(b) 15 °C



(c) 25 °C

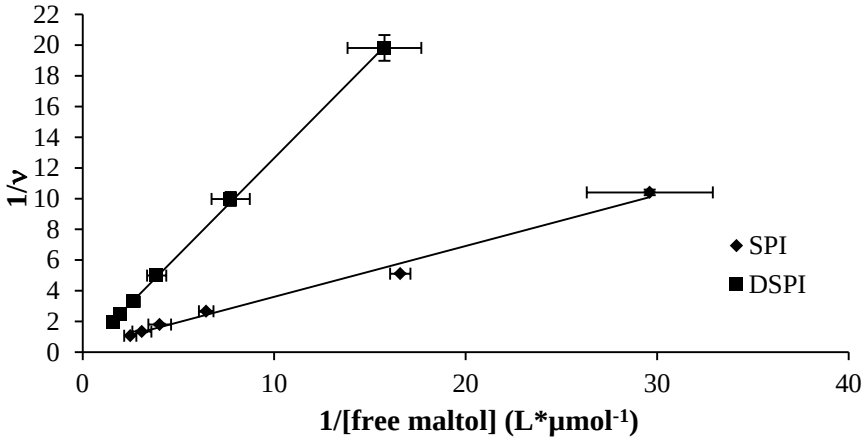


Figure 4.4 Klotz plots for binding of maltol to soy protein isolate (SPI) and deamidated soy protein isolate (DSPI) at 5 °C (a), 15 °C (b) and 25 °C (c) [Plots represent an average of two complete replications].

Table 4.4. Binding and thermodynamic parameters [a,b,c] ± standard deviation for the binding of maltol to soy protein isolate (SPI) and deamidated soy protein isolate (DSPI)

parameter	T (ºC)	SPI		DSPI	
n	5	10.7 ± 0.18	a A	4.01 ± 0.14	b B
	15	8.38 ± 0.35	b A	4.13 ± 0.55	b B
	25	3.27 ± 0.11	c B	29.5 ± 0.43	a A
K ($\times 10^4$) (M^{-1})	5	30.5 ± 0.25	b B	51.7 ± 0.08	a A
	15	37.9 ± 2.47	b NS	50.9 ± 9.07	a NS
	25	92.5 ± 6.57	a A	2.69 ± 0.41	b B
n·K ($\times 10^4$) (M^{-1})	5	328 ± 8.12	ns A	207 ± 6.80	a B
	15	317 ± 7.60	ns A	208 ± 9.48	a B
	25	303 ± 31.7	ns A	79.6 ± 13.2	b B
ΔG° (kcal.mol^{-1})	5	-6.97 ± 0.01	a A	-7.26 ± 0.00	b B
	15	-7.35 ± 0.04	b NS	-7.51 ± 0.10	b NS
	25	-8.13 ± 0.04	c B	-6.03 ± 0.09	a A
ΔH° (kcal.mol^{-1})	5-25	9.12 ± 0.65	A	-24.3 ± 1.24	B
ΔS° (cal.K^{-1}.mol^{-1})	5	32.8 ± 2.34	ns A	-87.6 ± 4.45	ns B
	15	31.7 ± 2.26	ns A	-84.5 ± 4.30	ns B
	25	30.6 ± 2.18	ns A	-81.7 ± 4.16	ns B

[a] Within columns, values with the same lower case letters are not significantly different at $p>0.05$. [b] Within rows, values with same upper case letters are not significantly different at $p>0.05$. [c] Average ± standard deviation (n=2).

of hydrophobic interactions (Damodaran and Kinsella, 1981), which could expose a higher number of binding sites at the outer surface of the protein. Therefore, more binding sites for vanillin and maltol should be available at 5 °C than at 25 °C.

The above trends, i.e., decrease in n and increase in K with increasing temperature, observed for vanillin and maltol are opposite to what has been reported for

some other flavor compounds. Damodaran and Kinsella (1981) reported that the n value for the binding of 2-nonanone with whole soy protein increased with increasing temperature from 5 °C to 25 °C, while the K decreased with increasing temperature. The different behavior of these flavor compounds could be due to the differences in their hydrophobicities as indicated by the difference in their log P values [log P of vanillin; 1.19 (Kikuchi et al., 2008), log P of maltol; 1.40 (Enyedy, et al., 2011), log P of 2-nonanone; 2.90 (Seuvre et al., 2002)], the difference in the functional groups of 2-nonanone (ketone) versus vanillin (aldehyde, phenol, and ether) and maltol (pyranone and hydroxyl), and also the difference in type of soy protein (whole soy protein vs SPI) used in the experiments.

As previously pointed out by Zhou and Cadwallader (2006), use of n or K alone might not be the best way to represent the overall binding affinity of a flavor compound to a protein. Instead, the value of n·K; which is derived from Klotz equation (**Equation 4.2**), can more accurately measure overall binding affinity (Zhou and Cadwallader, 2006; Kühn et al., 2008). The n·K values (at 5, 15 and 25 °C) demonstrate that the overall binding affinity of vanillin (68.7×10^4 to 88.8×10^4 M^{-1}) or maltol (303×10^4 to 328×10^4 M^{-1}) to SPI was greater than to DSPI (9.69×10^4 to 11.5×10^4 M^{-1} for vanillin and 79.6×10^4 to 208×10^4 M^{-1} for maltol) (**Tables 4.3** and **4.4**). These results indicate that deamidation by PG had a significant effect on the binding of vanillin since the n·K for DSPI was around 7-9 times lower than for SPI, while n·K value for the binding of maltol to DSPI was around 1.5-3 times lower than for SPI. It is hypothesized that the overall binding affinities of vanillin and maltol to DSPI decreased, at least in part, as a result of the loss of reactive amide side groups, in particular the loss of glutamine resides, on the

protein. As a result, DSPI would have less ability to bind to vanillin and maltol via Schiff base formation between glutamine resides and the carbonyl groups of the flavor compounds. Since the deamidation caused most of glutamine residues to be replaced by acidic groups, binding of vanillin and maltol to DSPI might also occur via hydrogen bonding and non-specific interactions (Lozano, 2009).

4.4.2 Thermodynamics of Binding of Vanillin and Maltol to SPI and DSPI

The thermodynamic parameters for the binding of vanillin and maltol to SPI and DSPI are shown in **Tables 4.3** and **4.4**, respectively. These data indicate a negative free energy of binding (ΔG°) for both flavor compounds to both SPI and DSPI. This means the binding of vanillin and maltol to both proteins was thermodynamically favorable and, thus, was spontaneous. The ΔG° values indicate that the binding affinities of vanillin to SPI and DSPI were higher at 25 °C than at 5 and 15 °C. Meanwhile, for maltol, the binding affinity was higher for SPI, but lower for DSPI at 25 °C. The ΔG° values determined in the present study for binding of vanillin agree with those of Li and others (2000), who reported ΔG° values of -3.38 and -3.70 kcal·mol^{-1} at 4 and 12 °C, respectively. Taking into consideration of structure of maltol, which contains a ketone group, the ΔG° value for this compound at 25 °C (-8.13 kcal·mol^{-1}) is not much different from the value (-4.1 kcal·mol^{-1}) reported by Arora and Damodaran (2010) for the binding of 2-nonanone to soy protein in an aqueous system.

In the present study, the enthalpy of binding (ΔH°) of vanillin to SPI was highly positive (29.5 ± 1.89 kcal·mol^{-1}) (**Table 4.3**), which indicates that the interaction between vanillin and SPI is not favorable because it is an endothermic reaction. However, the

entropy values (ΔS°) were also high (98.9 ± 6.3 to 106 ± 6.8 cal·K^{-1}·mol^{-1}), which resulted in negative ΔG° values, which indicates that the binding interaction is spontaneous (Li et al., 2000). Similar trends were observed for maltol (**Table 4.4**).

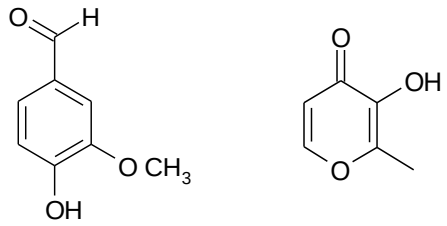
Therefore, it can be concluded that the interaction between vanillin or maltol and SPI is an entropy driven process, which indicates a greater disorder of the system, i.e., protein unfolding (Aspelund and Wilson, 1983; O'Keefe et al., 1991; Li et al., 2000; Price et al., 2001). The greater disorder of SPI results in the exposure of new binding sites for the vanillin (Damodaran and Kinsella, 1981). In addition, if we consider that vanillin has both a carbonyl and an alcohol group in its structure, our results are in agreement with the findings of Aspelund and Wilson (1983). They found that the interactions of SPI with hexanal and 2-hexanone (carbonyl compounds) and 1-hexanol (alcohol) were also entropy driven.

In contrast to the results for SPI, the values of ΔH° for the binding of vanillin and maltol to DSPI were negative (-1.22 ± 0.16 kcal·mol^{-1} and -24.3 ± 1.24 kcal·mol^{-1}, respectively). This means that the binding interaction between DSPI and vanillin or maltol was favorable (exothermic). The ΔS° values for the interaction of both flavor compounds were also a negative; however, these were not of high enough magnitude to cause the value of ΔG° to become positive. Hence, the interactions of vanillin and maltol with DSPI are enthalpy driven processes.

Previous studies have indicated that the binding of carbonyl-containing flavor compounds to various types of the proteins may be caused by Schiff base formation (Hansen and Heinis, 1991; Guichard, 2002; Suppavorasatit and Cadwallader, 2010). Besides covalent bonding, non-covalent interactions might occur at the same time due to

hydrogen bonding, van de Waals forces, and hydrophobic interactions. The ΔH° and ΔS° of a reaction can help to identify these binding modes (Wang and Li, 2011). The values for ΔH° and ΔS° for binding of vanillin and maltol to SPI were positive (**Table 4.3**), which means that hydrophobic interactions would be involved in the interaction of vanillin and maltol to SPI. With respect to binding interactions with DSPI, the values for ΔH° and ΔS° for vanillin and maltol were negative. Therefore, van der Waals force or hydrogen bonding would be involved in interaction of vanillin and maltol with DSPI (Ross and Rekharsky, 1996; Wang and Li, 2011).

Vanillin and maltol appear to undergo similar binding interactions with SPI and DSPI. This might be because both compounds contain a carbonyl and a hydroxyl functional group (**Figure 4.5**). Between the two compounds, maltol showed the greatest overall binding affinity to both SPI and DSPI. This might be due to differences in the orientation of the functional groups on the compounds, thus resulting in different accessibilities to binding sites.



(a) vanillin (b) maltol

Figure 4.5 Chemical structures of vanillin (a) and maltol (b).

4.5 CONCLUSIONS

Partial enzymatic deamidation of SPI affected flavor binding properties of the protein. Vanillin and maltol undergo non-cooperative to both SPI and DSPI. The binding of vanillin and maltol onto SPI is entropy driven, while the binding of the two compounds to DSPI is enthalpy driven. In addition, both vanillin and maltol showed a decrease in binding affinity towards DSPI. The thermodynamic data indicate that vanillin and maltol undergo stronger binding interactions with SPI than with DSPI. It is possible that these differences are due to a shift in the binding mechanisms from predominantly hydrophobic interactions and/or covalent bonding (Schiff-base formation) for SPI to mainly van der Waals force or hydrogen bonding for DSPI. These findings may helpful to soy protein and soy-food manufacturers aiming to reduce the flavor fade problem in aqueous protein-containing foods. However, studies on flavor binding by using only analytical approach cannot demonstrate the actual impact on consumer perception. Thus, further studies using sensory evaluation techniques are still needed.

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CHAPTER 5

EFFECT OF ENZYMATIC DEAMIDATION BY PROTEIN-GLUTAMINASE ON PROTEIN SOLUBILITY AND FLAVOR BINDING PROPERTIES OF SOYMILK

5.1 ABSTRACT

The effect of enzymatic deamidation by protein-glutaminase (PG) on protein solubility and flavor binding potential of soymilk was studied. Treatment of soymilk with PG for 2 h under optimal conditions (temperature of 44 °C and enzyme:substrate ratio (E/S) of 40 U/g protein) resulted in a high degree of protein deamidation (66.4% DD) and a relatively low degree of protein hydrolysis (4.25% DH). Deamidated soymilk (DSM) and control (treated without enzyme) soymilk (CSM) did not differ the with respect to aroma, but differed in taste characteristics. Protein solubility in DSM was enhanced at weakly acidic conditions (pH 5.0), but not differ from non-deamidated soymilk at pH values of 3.0 and 7.0. Odor detection thresholds for the flavor compounds vanillin and maltol were approximately 5 and 3 fold lower, respectively, in DSM than in CSM. The sigmoid curves from Fechner's law plots demonstrated that DSM had lower flavor binding potential than CSM. The *n* exponents from Stevens's power law indicated that vanilla and cotton candy intensities increased, as a function of vanillin or maltol concentration, respectively, at a higher rate in DSM than in CSM. PG deamidation has the potential to reduce flavor binding problems encountered in high protein-containing foods and beverages.

5.2 INTRODUCTION

Soy-based foods and beverages have become popular among consumers because of their potential health benefits. In the United States, the soy-based food and beverage market in 2008 ($1.4 billion in sales) showed a 15% increase over the year 2003. In addition, soymilk sales grew at a rate of 61% from years 2003 to 2008. Soymilk comprises one of the two largest segments of the soy-based foods market along with soy-based snack bars (Heyl-Rushmer, 2009). However, the consumption of soy foods is still limited due to the presence of undesirable off-flavors (Wilson, 1985). Furthermore, flavor compounds added to a food product may interact (binding) with the soy protein and result in a reduction in flavor, so called flavor fade (Kühn and others 2006; Zhou and Cadwallader 2008; Lozano 2009; Suppavorasatit and Cadwallader, 2010).

Flavor-protein binding interactions can be altered by changing the conformation of proteins by physical, chemical, and enzymatic modification (Dufour and Haertlé,1990; O'Keefe and others, 1991; Mikheeva and others, 1998; Liu and others, 2005; Kühn and others, 2008; Lozano, 2009; Suppavorasatit, 2012). Deamidation is one method used for protein modification. It has been used to improve solubility and functional properties of proteins, by converting amide groups into acid residues (carboxyl groups) with a release of ammonia. The conversion of amide groups to carboxyl groups leads to a decrease in isoelectric point (pI) of the protein due to the increase in number of negatively charged carboxyl groups. Thus, the deamidated proteins will be more soluble under weakly acidic conditions (Hamada and Marshall, 1989; Suppavorasatit and others, 2011). Recently, Miwa and others (2010) demonstrated that deamidation of skim milk by protein-

glutaminase (PG) increased protein solubility, relative viscosity, and emulsification capacity as a function of increasing degree of deamidation (DD).

In addition to improvements in solubility and functional properties, deamidation has excellent potential for altering the flavor binding properties of a protein (Lozano, 2009; Suppavorasatit, 2012). Previously, our research group reported that the partial deamidation of SPI by PG improved protein solubility and decreased overall flavor binding affinity to selected carbonyl containing flavor compounds (vanillin and maltol) in an aqueous model system (Suppavorasatit and others, 2011; Suppavorasatit, 2012). However, the impact of PG deamidation on protein solubility and flavor binding potential in an actual aqueous soy food system (e.g. soymilk) has not been studied. Therefore, the objective of the present study was to evaluate by sensory evaluation techniques the potential of the deamidation of soymilk by PG to produce a flavored soymilk with improved protein solubility and decreased flavor fade problems.

5.3 MATERIALS AND METHODS

5.3.1 Materials

5.3.1.1 Soybean seeds

Soybean seeds (IA 3027) were supplied by Clarkson Grain Company, Inc. (Cerro Gordo, IL).

5.3.1.2 Enzyme

Protein-glutaminase “Amano” 500 (500 U/g) was obtained from Amano Enzyme, Inc. (Elgin, IL).

5.3.1.3 Flavor compounds

Food grade (> 98% purity) vanillin (4-hydroxy-3-methoxybenzaldehyde) and maltol (3-hydroxy-2-methoxyl-*4H*-pyran-4-one) were obtained from FONA International Inc. (Geneva, IL). Vanillin and maltol solutions were prepared separately in odorless distilled water (prepared by boiling glass distilled water in an open flask until its volume was reduced by one-third of the original volume).

5.3.2 Methods

5.3.2.1 Soymilk preparation

The soybean variety selected for this experiment was IA 3027 because of its high protein content and higher yield compared with other high protein varieties commonly grown, such as Vinton variety (Winsor, 2009). The soymilk as prepared according to a previously published method (Lozano and others, 2007; Sun and others, 2010). Beans were soaked with odorless water in the ratio of 1:5 w/w (soybeans to water) and placed at 4 ± 1 °C overnight. The soaked soybeans were washed with cold distilled water, then drained. Soybeans were hot-ground (1:7 w/w; beans to odorless distilled water) and processed using a bean disintegrator BMI 300 (Beam Machines Inc., San Francisco, CA, U.S.A.) equipped with a no. 40 stainless steel mesh screen. The filtrate was heated and the temperature kept at 85 ± 5 °C for 15 min for pasteurization. The obtained slurry was filtered (mesh screen no. 200) to separate the soymilk from the okara. The finished soymilk (FSM) was hot filled into polyethylene bottles, cooled down in an ice-water bath, capped and then stored at 4 ± 1 °C.

5.3.2.2 Enzymatic deamidation

Deamidation of soymilk (FSM) was performed for 2 h under the following optimal conditions as described by Suppavorasatit and others (2011): reaction temperature of 44 °C and enzyme:substrate (E/S) ratio of 40 U/g protein. A control soymilk (CSM) sample consisted of FSM treated under the same conditions without the addition of PG for 2 h. Degree of deamidation (DD) and degree of hydrolysis (DH) were measured for deamidated soymilk (DSM) and CSM.

5.3.2.3 Determination of degree of deamidation (DD)

DD was determined according to the methods of Yong and others (2006) and Cabra and others (2007) with some changes. An ammonia assay kit (Sigma-Aldrich, Inc., St. Louis, MO) was used to determine amount of ammonia released from the deamidated glutamine residues. The DD was expressed as the ratio (in percentage) of amount of ammonia released by PG deamidation and the total glutamine residues of protein in soymilk, which was measured by the released ammonia when the soymilk was treated with 2 N sulfuric acid at 100 °C for 4 h.

5.3.2.4 Determination of degree of hydrolysis (DH)

DH was performed according to the method described by Cabra and others (2007) with some modifications. The DH was calculated as the percentage of the dissolved protein in the deamidated soymilk sample after precipitation with 0.2 N trichloroacetic

acid (TCA) and the total dissolved protein, which was measured after complete hydrolysis with 2 N sulfuric acid at 100 °C for 4 h.

5.3.2.5 Determination of total soluble protein (TSP)

TSP was determined by using the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA) as described by Dia and others (2009).

5.3.2.6 Determination of protein solubility

Protein solubility was determined in triplicate using the method described by Puppo and others (2004) and Yong and others (2006) with some changes. Deamidated soymilk and control samples (100 µL) were placed in 1 mL acetate-phosphate buffers of various pH values (3.0, 5.0 and 7.0) in 1.5 mL microcentrifuge tubes. The samples were kept at 25 °C overnight, vortexed, then centrifuged at 3000 rpm (1000xg) at 10 °C using an Eppendorf centrifuge model 5417R (Brinkmann Instruments, Westbury, NY) for 10 min. The supernatants (soluble fraction) were collected to determine total soluble protein. The solubility was calculated as follows:

$$\text{solubility}\ (\%) = \frac{\text{protein in supernatant (mg/mL)}}{\text{initial protein (mg/mL)}} \times 100 \qquad (5.1)$$

5.3.2.7 Sensory evaluation

The sensory protocol conducted in this study was approved by the Institutional Review Board of the University of Illinois at Urbana-Champaign (IRB Protocol Number 12038).

5.3.2.7.1 Difference in aroma and taste of DSM and CSM

For aroma difference testing, soymilk samples (15 mL of DSM or CSM) were transferred to sniff bottles (125-mL FEP [Teflon] squeeze bottles; Nalge Nunc International Corporation, Rochester, NY) as previously described by Zhou and others (2006). Each bottle was covered with aluminum foil and labeled with a three-digit random number. For testing of taste differences, the test samples (20 mL of DSM or CSM) were placed into 1 oz translucent plastic cups with lids (Solo Cup Co., Urbana, IL). The difference in perceived aroma (or taste) of soymilks was determined using the two-alternative forced choice (2-AFC) with warm-up method developed by Thieme and O'Mahony (1990).

The "warm-up" procedure for the 2-AFC test was conducted using the procedure described by Zhou and others (2006). Each panelist was instructed to sniff (or taste) back and forth between a pair of samples labeled "A" and "B" (one was DSM, and the other was CSM) until they could detect a difference in aroma (or taste) between the pair and the nature, as well as the direction, of the difference between the two samples (e.g. "A" is sweeter than "B") was recorded. The sensory attributes described by each panelist (during the warm-up session) were then used in the instructions for the subsequent sample testing. Panelists were instructed to sniff odorless distilled water between aroma evaluations or instructed to cleanse their palates with plain crackers then spring water between taste evaluations. Evaluations were conducted in triplicate by each panelist.

A total of 17 panelists (4 males and 13 females; 23-48 years old) participated in this study. They consisted of staff and students from the Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign. Data from the 2-AFC tests

were analyzed by beta-binomial statistics using the IFPrograms™ software (version 7.3; The Institute for Perception, Richmond, VA) with a null probability of 0.5.

5.3.2.7.2 Determination of odor detection threshold

The odor detection threshold was determined orthonasally using ASTM E697-04 protocol (ASTM, 2004) with some modifications. The test samples (flavored soymilk) at six different concentrations were prepared by adding the same volume (1 mL) of various flavor compound solutions (six different ascending concentrations at 3-fold increments) into 15 mL of either DSM or CSM contained in sniff bottles (Nalge Nunc International Corporation) as previously described by Guadagni and Buttery (1978) and Watcharananun and others (2009). The final concentration of each sample is shown in **Table 5.1**. The test samples were kept in a refrigerator (4 ± 1 °C) overnight (24 h) to allow for equilibration of flavor-matrix interactions before testing.

Table 5.1 Final concentration of flavor compounds in test samples for threshold evaluation

flavor compound	soymilk	concentration (μg/mL)					
		set 1	set 2	set 3	set 4	set 5	set 6
		x/27	x/9	x/3	x[a]	3x	9x
vanillin	control (CSM)	1.667	5.000	15.00	45.00	135.0	405.0
	deamidated (DSM)	0.556	1.667	5.000	15.00	45.00	135.0
maltol	control (CSM)	1.297	3.890	11.67	35.00	105.0	315.0
	deamidated (DSM)	1.297	3.890	11.67	35.00	105.0	315.0

[a] Concentrations were based on the literature and preliminary testing.

Panelists consisted of staff and students from the Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign. Twenty-one panelists (22-

48 years of age; 5 male, 16 female) participated in the determination of the vanillin threshold, while 27 panelists (19-40 years of age; 5 male, 22 female) were involved in the threshold determination of maltol. The day before each sensory study, panelists were given standardized training. They practiced in two sessions using six sets of three-alternative forced choice (3-AFC) tests to identify the sample with the strongest odor (flavored soymilk) in a set of three samples, where two of them were plain soymilks (no flavor added). They were informed that the 3-AFC tests were arranged in ascending concentration, and needed to guess if they were uncertain of the identity of the odd sample.

The test samples were removed from refrigeration approximately 1 h prior to evaluation. To avoid visual bias, all bottles were covered with aluminum foil and labeled with three-digit random codes. Panelists were presented with six sets of three samples, with each set consisting of two blanks and a flavored sample. The samples were randomized within each set and were served in ascending flavor concentration. The panelists were instructed to sniff one set of samples at a time in the order presented and select the odd sample (3-AFC). If uncertain, the panelists were asked make a best guess. The group best estimate threshold (BET) was calculated as the geometric mean of the individual BETs (ASTM, 2004).

5.3.2.7.3 Aroma intensity scaling of flavored soymilks

The aroma intensities of flavored soymilks were determined by rating on a universal (15-point) scale. Twelve panelists between 22-45 years of age (2 males and 10 females; recruited from staff and students of the Department of Food Science and Human

Nutrition, University of Illinois at Urbana-Champaign) participated in this study. Each panelist had previous sensory evaluation experience with food products, especially with aroma intensity evaluation. They received an additional 5 h of training (five 1-h sessions) focused on identifying and rating specific aroma attributes of the flavored soymilks (Mailgaard and others, 2007). During training, panelists evaluated and discussed an array of commercial and experimental flavored soymilks. Specific aroma attributes (vanilla and cotton candy) and references (50 μg/mL vanillin and 250 μg/mL maltol in odorless distilled water) were developed and were in consensus agreement with respect to terminology and aroma intensity among panelists.

The test samples (flavored soymilks) with five different concentrations of added flavor were prepared by adding the same volume (1 mL) of flavor compound solutions (five different concentrations at 5-fold increments) into 14 mL of either DMS or CSM contained in sniff bottles. All samples were kept in a refrigerator (4 ± 1 °C) at least 24 h before testing to allow for equilibration of flavor-matrix interaction. On the testing day, the samples were removed from the refrigeration and condition at room temperature (25 °C) for approximately 1 h prior to evaluation. The sniff bottles were covered with aluminum foil and labeled with three-digit random codes. Panelists were presented five samples in each set, with each set consisting of flavored samples at five different flavor concentrations. Panelists were asked to rate vanilla (or cotton candy) intensity of all samples against the reference using a 15-point scale.

The above dose-response results were plotted based on Fechner's and Steven's power laws. In the case of Fechner's law at plot of the log of concentration versus

perceived intensity should have a sigmoidal relationship (**Figure 5.1**) (Lindinger et al., 2008).

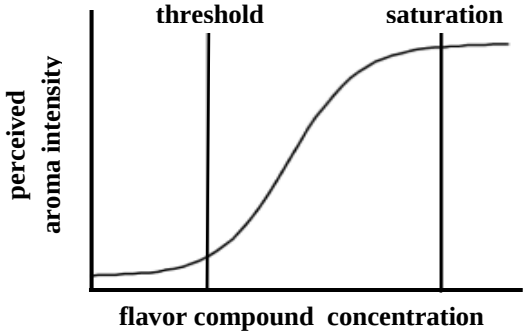

Figure 5.1 Typical sigmoidal relationship between perceived aroma intensity and log concentration of aroma compound (adapted from Lindinger et al., 2008).

The Stevens's power law also explains the relationship between perceived intensity and concentration of sensory stimuli (flavor compound) (Stevens, 1971; Meilgaard and others, 2007). This law can be mathematically expressed as:

$$R = k \cdot C^n \tag{5.2}$$

where R is the perceived aroma intensity, C is flavor compound concentration, k is a constant that depends on the units in which R and C are measured, and n is an exponent of the power function, which indicates how perceived magnitude is affected when the concentration of the stimulus (flavor compound) is raised. The n value is obtained as the slope of a plot of the log of concentration of the flavor compound versus the logarithm of the perceived aroma intensity.

5.3.2.8 Statistical analysis

Analysis of variance (ANOVA) and lease significant difference (LSD) were performed to determine significant differences among treatments ($p > 0.05$) by Statistical Analysis Software (SAS, version 9.2, SAS Institute Inc., Cary, NC).

5.4 RESULTS AND DISCUSSION

5.4.1 Deamidation of Soymilk by Protein-Glutaminase (PG)

The conditions for the deamidation of soymilk by PG were selected based on previous published optimal conditions for production of deamidated soy protein isolated (SPI) (Suppavorasatit and others, 2011) to achieve high degree of deamidation (DD) and low degree of hydrolysis (DH). The DD and DH of deamidated soy milk (DSM) are shown in **Table 5.2**. The DD of DSM (66.4%) was higher than that previously reported for deamidated SPI (DSPI; 43.7%) produced under the same experimental conditions (a temperature of 44 °C and an E/S of 40 U/g protein) for 2 h (Suppavorasatit and others 2011). This difference is most likely due the different substrates used in the two experiments. For example, proteins in soymilk are more soluble than in a SPI suspension. Furthermore, protein unfolding during the heating process of soymilk production could enable the enzyme (PG) to have better access to the substrate (glutamine residues) (Malaypally and Ismail, 2010), thus resulting in the more efficient conversion of glutamine residues to glutamic acid residues (Yamaguchi et al., 2001).

Table 5.2 Degree of deamidation (DD) and degree of hydrolysis (DH) of soymilk after deamidation[a] by protein-glutaminase

	average[b] ± standard deviation
DD (%)	66.4 ± 2.6
DH (%)	4.25 ± 0.42

[a] deamidation under optimal conditions: reaction temperature of 44 °C, enzyme:substrate (E/S) ratio of 40 U/g protein for 2h. [b] n=3.

The DH of DSM was 4.25% (**Table 5.2**), which was similar to what was previous reported for DSPI (4.81%) (Suppavorasatit and others, 2011). Previous studies have reported that PG catalyzes deamination without proteolysis (Yamaguchi and others, 2001; Yong and others, 2006). Hydrolysis might have occurred because the enzyme used in the present study is a commercial product and might contain some residual protease activity. In addition, the release of small peptide fragments during the deamidation process (during protein unfolding) may have also occurred, thus increasing the measured DH (Suppavorasatit and others, 2011).

5.4.2 Effect of Deamidation on Protein Solubility in Soymilk

Protein solubility in FSM, CSM, and DSM was determined at three different pH values (3.0, 5.0, and 7.0) (**Figure 5.2**). There was no difference in protein solubility among soymilks at pH 7.0 and at this pH protein solubility was highest (nearly 100%) for all soymilks. This might be because pH of FSM (6.74) and CSM (6.44) were already in the neutral pH range (Liu and Chang, 2004).

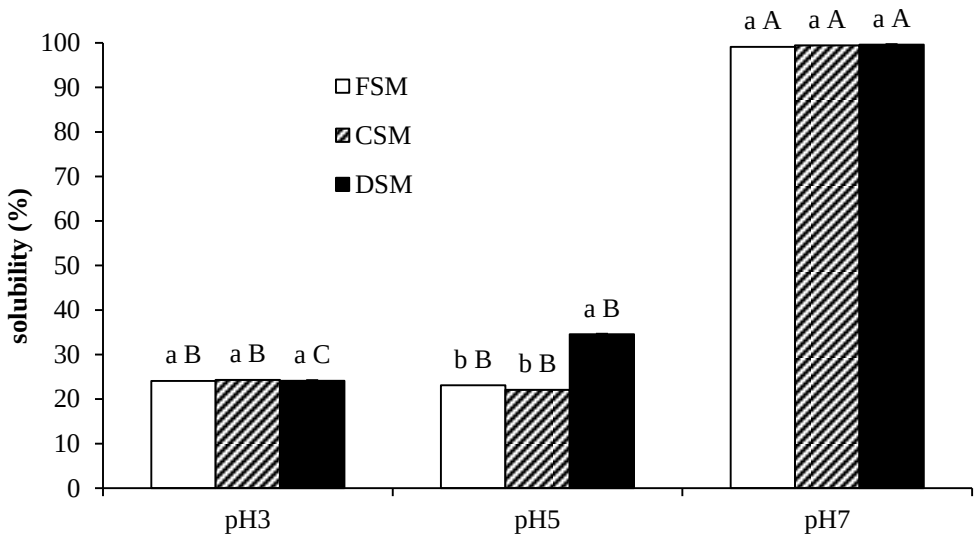

Figure 5.2 Solubility of untreated (FSM), control (FSM treated without PG; CSM) and deamidated (DSM) soymilks under various pH conditions. Values with same lower case letters at same pH are not significantly different ($p > 0.05$, n=3); same upper case letters within the same sample across different pH values indicate a significant difference ($p > 0.05$, n=3).

At low pH (pH 3.0 and 5.0), the protein solubility was significantly lower ($p \leq 0.05$) than at neutral pH for all soymilks. At pH 5.0, the solubility of DSM was the highest (~35%), while the solubilities of FSM and CSM were similarly low (~22-23%). This could be a result of PG deamidation which converts glutamine residues in protein and peptide chains to glutamic acids. The increase in the number of negatively changed carboxyl groups causes a decrease in the isoelectric point (pI) of the protein (Hamada and Marshall 1989). This means that the pI of the soy protein in DSM should be lower than the pI of soy protein (approximately 4.5) in CSM. For this reason the protein solubility of DSM at pH 5.0 was higher ($p \leq 0.05$) than FSM or CSM. This result is in agreement with

the previous results on DSPI (Suppavorasatit and others, 2011). These researchers also found that the solubility of DSPI (52.3% DD) at pH 5.0 was higher than non-deamidated SPI. At pH 3.0, there was no difference ($p > 0.05$) in solubility (~24%) among all soymilks.

5.4.3 Effect of Deamidation on Soymilk Flavor and Flavor Binding

As discussed above, deamidation can cause some protein hydrolysis, which could affect aroma and/or taste of soymilk since there might be a change in the volatile profile or an increase in peptides or amino acids cleaved from the protein chains (Chobert, 2003). For this reason, sensory difference tests were conducted to demonstrate the effect of PG deamidation on aroma and taste of soymilk.

5.4.3.1 Sensory evaluation of aroma and taste differences between CSM and DSM

The 2-Alternative forced-choice (2-AFC) with a warm-up method (Thieme and O'Mahony, 1990) was used to detect perceived differences in aroma or tastes between CSM and DSM. During the warm-up session panelists identified and recorded the main aroma and taste differences between two test samples. Terms that panelists used to identify the differences between the two products are shown in **Table 5.3**.

The results, from β-binomial analysis, demonstated that CSM and DSM did not differ in aroma ($p < 0.4443$; estimated probability of the data, 0.4902; and power of the test, 3.7%). In addition, there was no significant difference in the responses among panelists (no significant over dispersion; estimated gamma value, 0.0000). This result indicated that deamidation did not affect the overall aroma of the soymilk. However,

there was a significant taste difference detected between CSM and DSM ($p < 0.0116$; estimated probability of the data, 0.7059; and power of the test, 75.4%). In addition, there were significantly different responses among subjects (estimated gamma value, 0.3389). Therefore, it can be concluded that deamidation by PG affected the overall perceived taste of soymilk.

Table 5.3 Terms generated by panelists to differentiate aroma and taste of soymilks

attribute	terms generated	attribute	terms generated
aroma	beany	taste	bitter
	burnt sugar		salty
	caramel		sweet
	chickeny		
	creamy		
	vanilla		

The apparent tastes difference between CSM and DSM might be caused by factors other than taste per se (e.g. viscosity, mouthfeel, and aroma by mouth) that panelists perceived during the evaluation. Enzymatic deamidation was shown to increase the viscosity of skim milk (Miwa and others, 2010). Therefore, it is possible that the viscosities of CSM and DSM differed and, thus, mouthfeel of the soymilks may also have differed (Gallardo-Escamilla and others, 2007). This could be one reason why the panelists were able to distinguish the two soymilks by taste.

5.4.3.2 Effect of deamidation on odor detection threshold of vanillin and maltol in soymilk

Protein-flavor binding studies which made use of instrumental techniques provide valuable information but cannot measure the impact that flavor binding has on flavor

perception. For this purpose it is necessary to use sensory evaluation. In the present study, odor detection thresholds were measured and used to demonstrate how deamidation affects protein-flavor binding properties in soymilk.

The group best estimate thresholds (BETs) for vanillin in DSM and CSM are shown in **Table 5.4**. The group BET of vanillin in CSM (9.61 ppm) was close to the literature BET value of vanillin in skim milk (7.41 ppm) reported by Karagül-Yüceer and others (2004). The BET values of soymilk and skim milk may be similar because both fluids contain about the same amount of protein (~3% protein).

Table 5.4 Group best estimate thresholds (BET) of vanillin and maltol in control (treated without enzyme; CSM) and deamidated soymilk (DSM)

flavor compound	BET (μg/mL) ± standard deviation	
	CSM	DSM
vanillin	9.61 ± 1.39	1.80 ± 2.44
maltol	23.8 ± 2.61	7.01 ± 3.71

In addition, the group BET of vanillin in DSM was about 1.80 ppm, which was substantially lower (~5-fold) than the group BET in CSM (9.61 ppm). This indicates that the overall binding of vanillin in CSM was higher than in DSM. The lower overall binding of vanillin in DSM was expected since deamidation by PG reduces the number of available amide groups by converting most of glutamine residues in soy protein to glutamic acids. Therefore, the chance to form covalent bonds (Schiff bases) with the carbonyl group of vanillin was decreased (Suppavorasatit, 2012).

Furthermore, as a result of the deamidation the interaction of vanillin to soy proteins might change from covalent bonding or hydrophobic interactions to weaker interactions such as hydrogen bonding or van der Waals force (Lozano, 2009; Suppavorasatit, 2012). This reduction in the binding of vanillin to soy protein as affected by deamidation was previously reported in **Chapter 4**. It was shown that the overall binding (numbers of binding sites × binding constants; n·K) at 25 °C for vanillin to SPI (88.8×10^4 M^{-1}) was 9-times greater ($p > 0.05$) than to DSPI (9.69×10^4 M^{-1}).

The group BETs of maltol in DSM and CSM are shown in **Table 5.4**. The BET for maltol in CSM was 23.8 ppm, which is similar to the published BET for maltol in skim milk (16.6 ppm) reported by Karagül-Yüceer and others (2004). The BET of maltol in DSM was 7.02 ppm, which was ~3 times lower than the BET for maltol in CSM. The lower BET in DSM could be explained as described above for vanillin. The lower binding affinity of DSM for maltol is also in agreement with the instrumental results of Suppavorasatit (2012). They reported that n·K of maltol to SPI was 303×10^4 M^{-1}, which was about 4 times higher than the n·K value for the binding of maltol to deamidated SPI (79.6×10^4 M^{-1}) at 25 °C.

5.4.3.3 Effect of deamidation on dose-response curves of vanillin and maltol in soymilk

Panelists determined (consensus opinion) that “vanilla” and “cotton candy” were the best attributes to describe aroma perceived from flavored soymilks prepared from vanillin and maltol, respectively. Attribute intensity ratings were determined for five different concentrations of each flavor compound in CSM and DSM (1000, 200, 40, 8,

and 1.6 µg/mL of vanillin or maltol in CSM; and 500, 100, 20, 4, and 0.8 µg/mL of vanillin or maltol in DSM). The flavor compound concentrations were chosen to include levels below the detection thresholds to levels just above aroma saturation (determined during training sessions). Analysis of variance (ANOVA) showed that vanilla and cotton candy intensities increased as a function of concentration of vanillin and maltol, respectively ($p < 0.0001$). The dose-response plots to relate the concentration of vanillin and maltol to perceived aroma intensity as a function of Fechner's and Stevens's power laws are shown in **Figures 5.3** and **5.4**, respectively.

As expected, the plots shown in **Figures 5.3a and 5.4a** demonstrate a sigmoidal relationship between the logarithm of the flavor concentration and the perceived aroma intensity, and thus follow Fechner's law, which states that the intensity of a sensation increases as a function of the logarithm of the stimulus (Lindinger and others, 2008). The sigmoidal relationship between chemical concentration and perceived intensity were also reported in previous studies (Audouin and others, 2001; van Ruth, 2004). The relationship observed for the plots of the logarithm of concentration of the flavor compound versus logarithm of perceived aroma intensity (**Figures 5.3b** and **5.4b**) were linear, and thus followed the Stevens's power law (Kamadia and others, 2006; Pham and others, 2008).

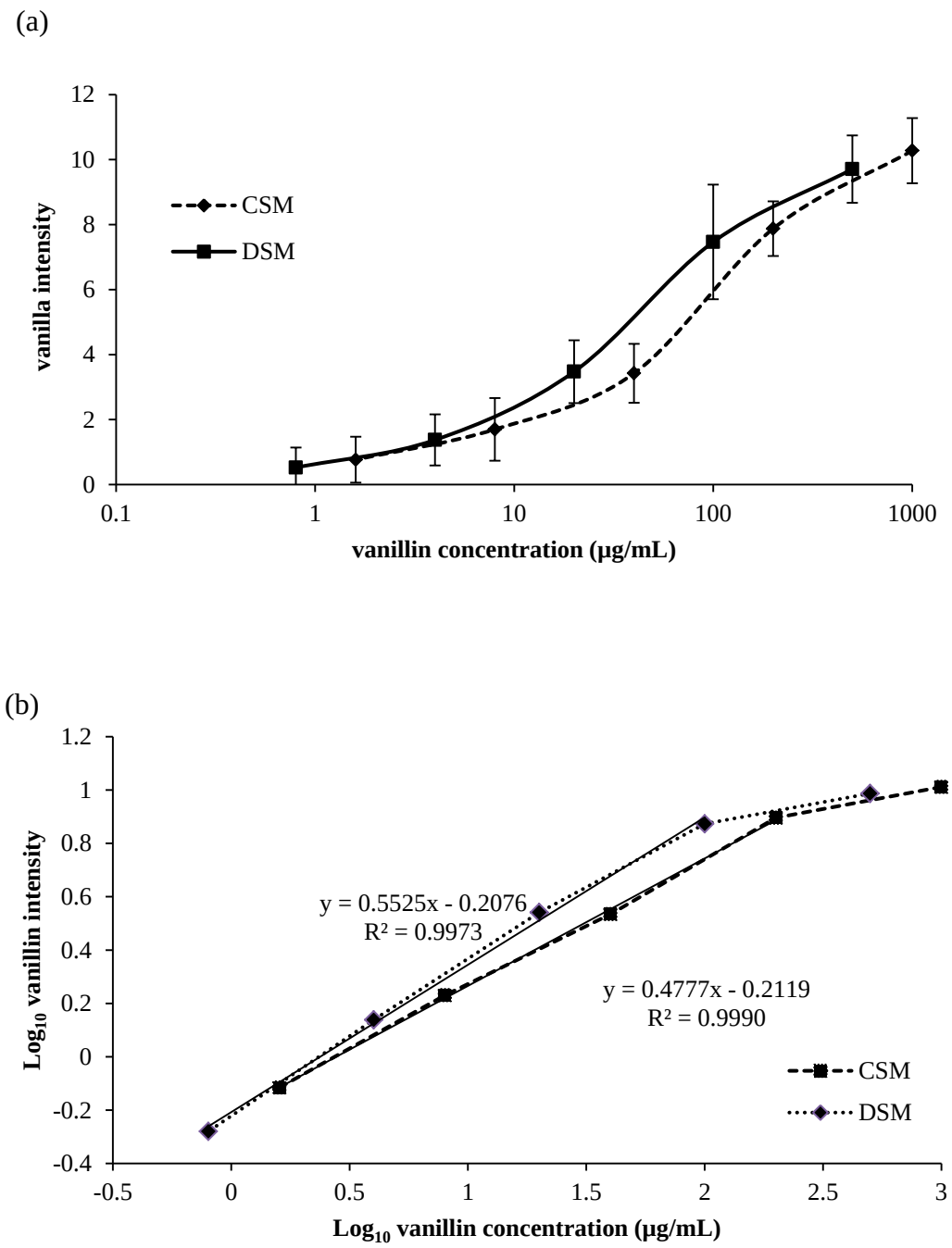

Figure 5.3 Dose-response curves relating vanillin concentration to perceived vanilla intensity of control soymilk (treated without enzyme; CSM) and deamidated soymilk (DSM) as a function of Fechner's law (a) and Stevens's power law (b). The solid lines in (b) represent the linear section of the plots.

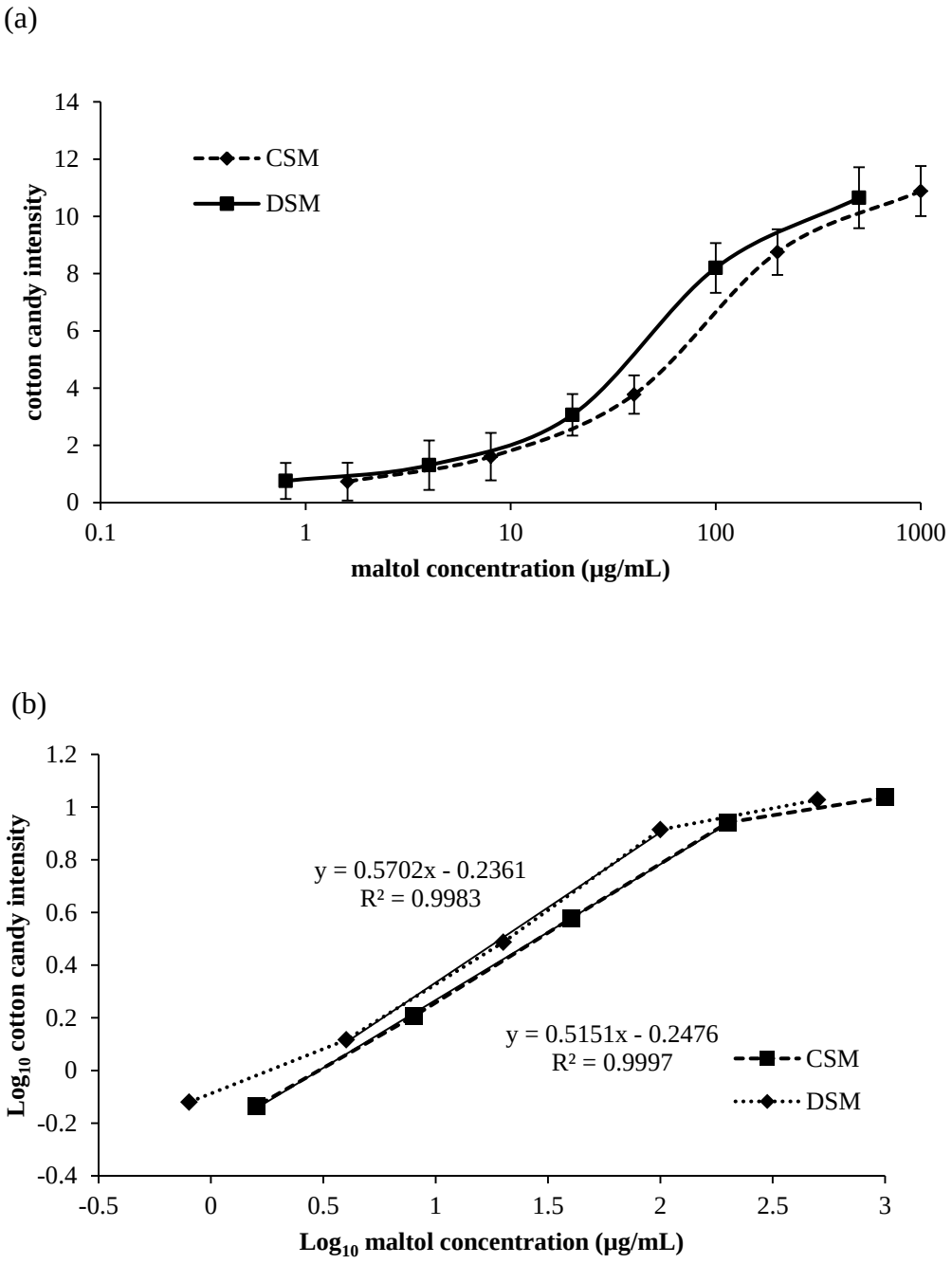

Figure 5.4 Dose-response curves relating maltol concentration and perceived cotton candy intensity of control soymilk (treated without enzyme; CSM) and deamidated soymilk (DSM) as a function of Fechner's law (a) and Stevens's power law (b). The solid lines in (b) represent the linear section of the plots.

The results from sigmoid curves (**Figures 5.3a** and **5.4a**) showed that the deamidation by PG affected how panelists perceived the aroma intensity. For example, vanilla intensity in DSM containing 50 µg/mL vanillin received an intensity rating of 4.4, while the intensity in control sample was rated at 3.0 (**Figure 5.3a**). This is in agreement with the results for odor detection thresholds. It could be explained in terms of flavor-protein interactions in these two soymilks in that the deamidation by PG decreased the interactions between proteins in soymilk and carbonyl groups of the flavor compounds (Lozano, 2009; Suppavorasatit, 2012).

From Stevens's power law, the *n* exponent value (**Table 5.5**) (from a slope of the linear section of the plot) indicated the relationship between the perceived magnitude (aroma intensity) and the concentration of a stimulus (flavor compound concentration) (Stevens, 1971). From **Table 5.5**, all *n* exponents of vanillin and maltol in both CSM and DSM were less than one, which means the aroma intensity increased only marginally as a function of increasing concentration (Pham and others, 2008). The results showed that the *n* exponents for both vanillin and maltol were higher in DSM than in CSM. This demonstrated that vanilla (or cotton candy) intensities increased at a higher rate in DSM than in CSM as a function of vanillin (or maltol) concentration.

Table 5.5 Stevens's power law exponent (*n*) and coefficients of determination (r^2) from the plots of log of flavor compound concentration versus log of perceived aroma intensity of control soymilk (CSM) and deamidated soymilk (DSM)

protein	flavor compound			
	vanillin		maltol	
	n	r^2	*n*	r^2
CSM	0.4777	0.99	0.5151	0.99
DSM	0.5525	0.99	0.5702	0.99

5.5 CONCLUSIONS

Partial deamidation of soymilk by PG under optimal conditions (a temperature of 44 °C and E/S of 40) for 2 h produced a deamidated soymilk (DSM) with 66.4% DD and 4.25% DH. The DSM had enhanced protein solubility under acidic conditions (pH 5.0). The perceived aromas of DSM and control soymilk (CSM) were not different while the taste of the two materials differed. Furthermore, deamidation by PG affected flavor binding property of protein in soymilk by decreasing odor detection thresholds of both vanillin and maltol in DSM compared to CSM. These findings could benefit soy protein and soy-food manufacturers who intend to reduce the flavor fade problem in aqueous food products containing soy proteins, especially for carbonyl containing flavor compounds. In addition, DSM could be used in weakly acidic protein fortified beverages. However, studies on the application of PG in actual weakly acidic beverages or the effect of PG deamidation on low moisture food systems are still needed.

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CHAPTER 6

SUMMARY, CONCLUSIONS, IMPLICATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

The availability of a flavor compound for sensory perception is largely influenced by its interaction with non-volatile food constituents, including fats, carbohydrates and proteins. The binding of flavor compounds to soy protein can result in flavor loss or a decrease in flavor intensity of added flavorings (flavor fade) and hence a decline in product quality. This makes it difficult to determine the exact amount of flavoring that should be used during food formulation. The degree of these flavor-protein binding interactions depends not only on environmental conditions (e.g., pH and temperature) in which the interactions taken place, but also the conformations of both flavor compounds and proteins. The formation of covalent bonds (Schiff-base formation) between carbonyl containing flavor compounds and amide side chains of proteins are problematic since the flavor compounds are not readily released during food consumption. Chemical deamidation, a type of protein modification which results in the selective removal amide groups from protein molecules, has been reported to decrease the overall binding affinity of soy protein to carbonyl containing volatile compounds. In addition, enzymatic deamidation is more desirable than chemical modification because it is substrate specific, can be accomplished under mild conditions and is considered natural and safe.

In the present study, the optimal condition of enzymatic deamidation of soy protein isolate (SPI) by protein-glutaminase (PG) was successfully carried out using response surface methodology. This process has great potential for production of

deamidated SPI (DSPI) with modified functional properties, including greater solubility in both acidic and neutral conditions, improved emulsification properties and increased foaming capacity.

The present study also evaluated the effects of PG deamidation on flavor binding properties of SPI under aqueous conditions. An equilibrium dialysis technique based on use of ultrafiltration was developed to assess binding interactions of individual carbonyl containing flavor compound (vanillin or maltol) to soy proteins (DSPI and untreated SPI). Partial deamidation of SPI by PG decreased the overall flavor binding of the protein to both flavor compounds and changed the nature of this interaction from entropy- to enthalpy-driven. The observed changes in the binding parameters suggest that deamidation by PG could reduce the chance of covalent bonding (Schiff-base formation) and/or reduce hydrophobic interactions. Meanwhile, the interaction of deamidated SPI with the flavor compounds appears to be due to weaker van der Waals force or hydrogen bonding.

The final experiment of this project was conducted to demonstrate the practical application of deamidation of PG on an actual food system. This involved the enzymatic deamidation by PG of soymilk under the optimal condition reported in **Chapter 3**. As expected from our previous study with SPI (**Chapter 3**), the solubility of protein in the deamidated soymilk (DSM) increased at weakly acidic condition (pH 5.0) compared to non-deamidated soymilks. Sensory evaluation results demonstrated that aroma of DSM did not differ from control (treated without enzyme) soymilk (CSM), while the taste of the two products was different. Additionally, odor detection thresholds of vanillin and maltol in the two different matrices (DSM and CSM) were evaluated in order to

demonstrate the potential for PG deamidation to reduce the flavor binding capacity of soymilk. It was shown that odor detection thresholds for the flavor compounds (vanillin and maltol) were lower in DSM than in CSM; therefore, demonstrating that DSM had lower flavor binding potential than CSM. The sigmoidal relationship of dose-response curves relating concentration of flavor compounds to aroma intensity, also demonstrated that deamidation by PG decreased the binding of both vanillin and maltol in soymilks. The *n* exponents from Stevens's power law indicated that vanilla and cotton candy intensities increased, as a function of vanillin or maltol concentration, at a higher rate in DSM than in CSM. The findings can lead to the development of technology to produce proteins with improved functional properties and potentially decreased problems associated with flavor-protein interactions, especially with carbonyl containing flavor compounds. In addition, the use of enzymatic deamidation is potentially safer than chemical methods and is considered as a natural process. The information about binding mechanisms caused by modification of binding sites in protein by PG will allow the protein manufactures and protein food industry to produce protein ingredients, not only from soybean, with desired functional properties and potentially decreased flavor fade problem in food products, especially in acidic protein-fortified beverages.

Further studies about the effect of PG deamination on competitive binding are recommended. The study of binding interactions by using some other techniques, such as NMR spectroscopy, will definitely help to confirm the mechanisms involved. Furthermore, the effect of PG deamidation on flavor binding to other protein sources, such as dairy proteins, would be beneficial to the food industry. In addition, since PG

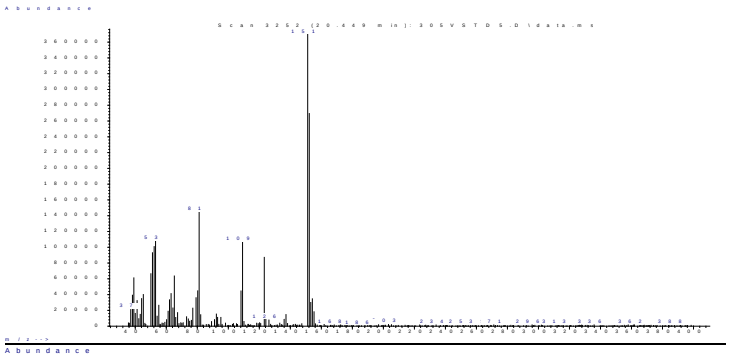
causes some hydrolysis of the protein, a study on the allergenicity of hydrolyzed protein might be beneficial for people who have allergic reactions to certain types of proteins.

APPENDIX A

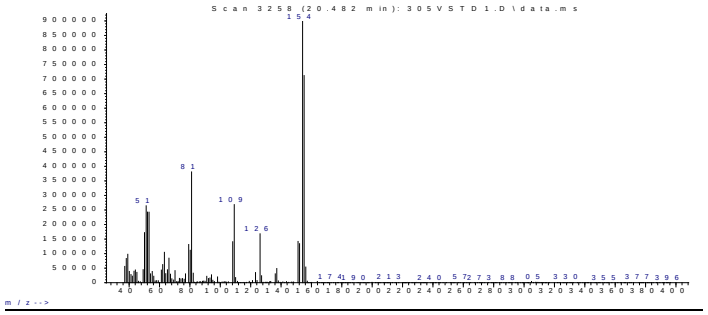
RESPONSE FACTOR OF VANILLIN VS VANILLIN-D_3

	vanillin	vanillin-d_3
Standard:	4-hydroxy-3-methoxybenzaldehyde	4-hydroxy-3-methoxy-d_3-benzaldehyde
CAS:	NA	121-33-5
Mfg/Ref:	NA	Sigma-Aldrich Co. (St. Louis, MO)
% Purity (by GC-FID)	99.9%	99.4%

Spectra

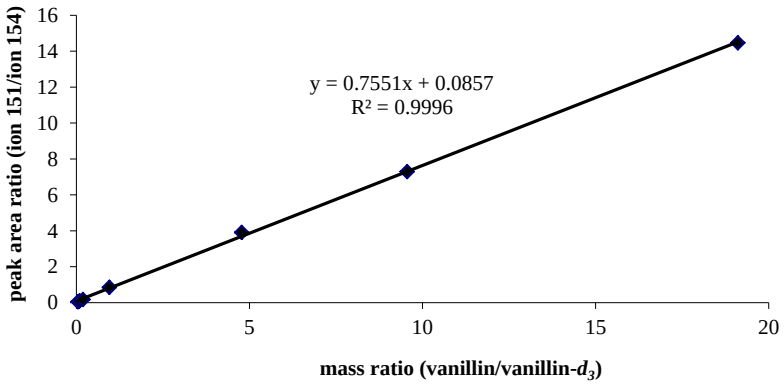


vanillin



vanillin-d_3

Standard curve for vanillin vs vanillin-d_3

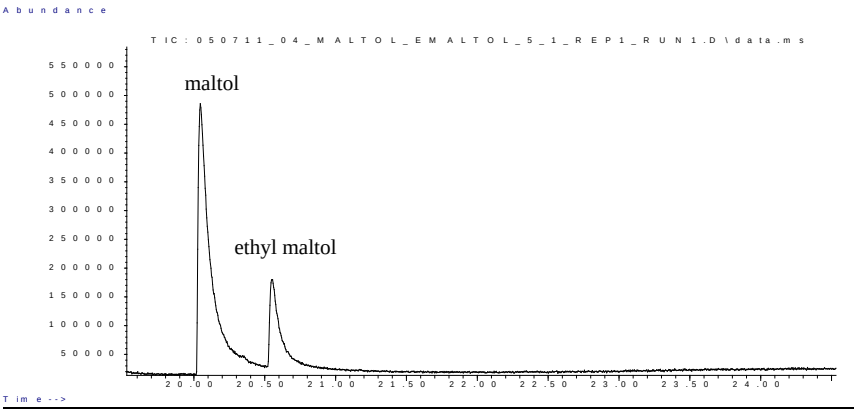


Slope = 0.7551

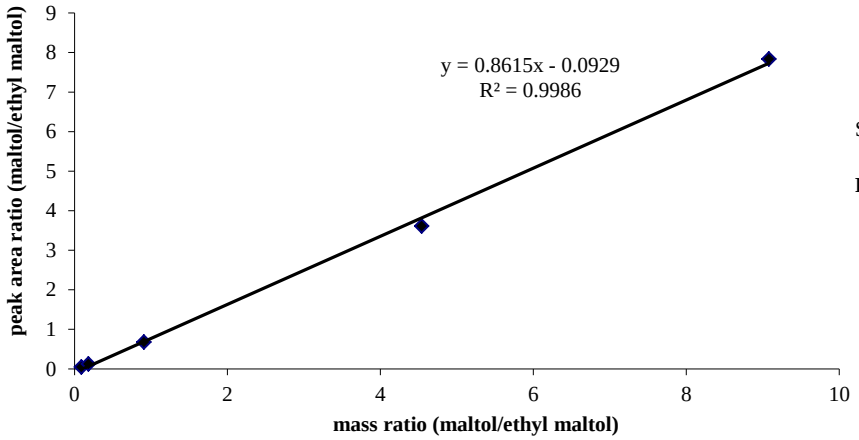
Response factor = 1.32

RESPONSE FACTOR OF MALTOL VS ETHYL MALTOL

	Maltol	ethyl maltol
Standard:	3-hydroxy-2-methoxyl-*4H*-pyran-4-one	2-ethyl-3-hydroxy-*4H*-pyran-4-one
CAS:	118-71-8	4910-11-8
Mfg/Ref:	Sigma-Aldrich Co. (St. Louis, MO)	Sigma-Aldrich Co. (St. Louis, MO)
% Purity (by GC-FID)	100%	99.3%



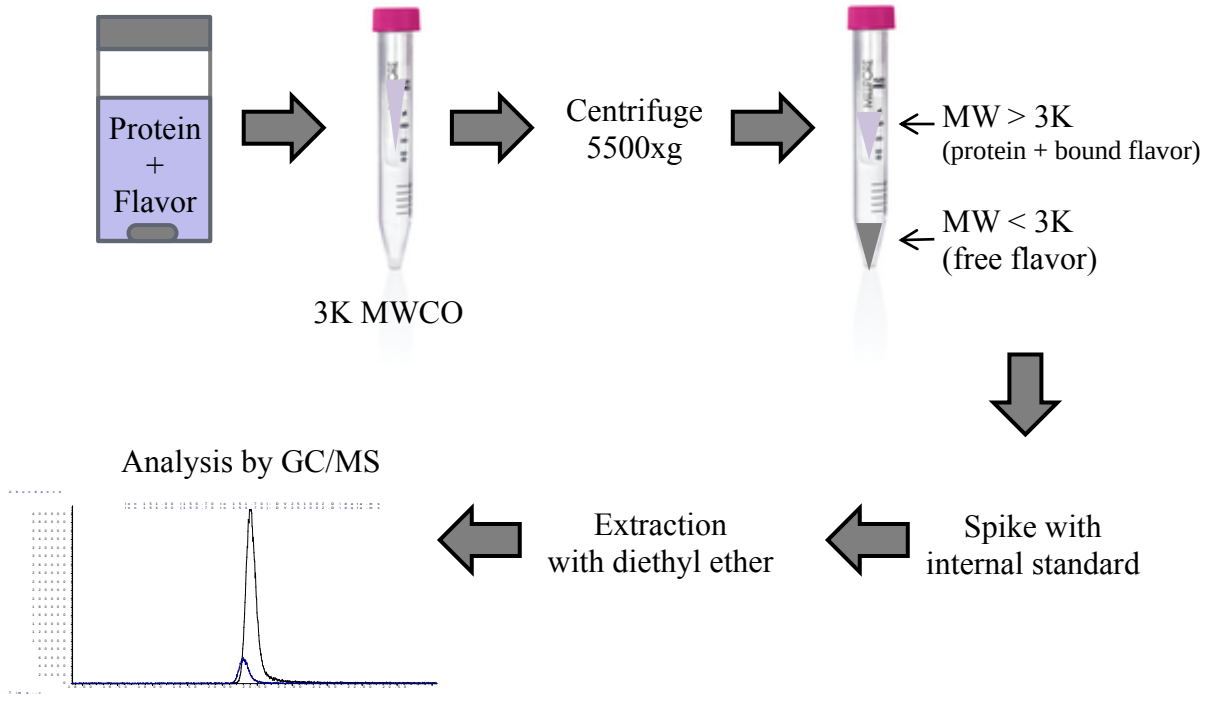
Standard curve for maltol vs ethyl maltol



Slope = 0.8615

Response factor = 1.16

FLOW DIAGRAM FOR ISOLATION AND QUANTIFICATION OF FREE FLAVOR COMPOUNDS



APPENDIX B

WARM-UP SCORECARD (AROMA OF SOYMILK)

Instructions:

First sniff the pair of samples labeled "A" and "B". If you can tell the difference between the two, describe in your own words in the response area below. Your response should indicate the **nature** and the **direction of the difference**. For example: if you are comparing two cookies, a response would look like "cookie A is **sweeter** than cookie B". You can go back and forth between samples "A" and "B" until you can tell the difference.

Response area: What is the difference between sample A and B?

When you finish, go to the next page.

SOYMILK EVALUATION SCORECARD

Instructions:

You have decided that two samples differ in _____. For this pairs of samples presented to you, sniff both samples, and mark "X" in the box of the one that is stronger in_____.

Set 1	813	☐	449	☐
Set 2	307	☐	951	☐
Set 3	103	☐	344	☐

WARM-UP SCORECARD (TASTE OF SOYMILK)

Instructions:

Before tasting, cleanse your palate with plain crackers and spring water before starting the first sample, and between the samples. First taste the pair of samples labeled "A" and "B". If you can tell the difference between the two, describe in your own words in the response area below. Your response should indicate the **nature** and the **direction of the difference**. For example: if you are comparing two cookies, a response would look like "cookie A is **sweeter** than cookie B". You can go back and forth between samples "A" and "B" until you can tell the difference.

Response area: What is the difference between sample A and B?

When you finish, go to the next page.

SOYMILK EVALUATION SCORECARD

Instructions:

You have decided that two samples differ in _____. For this pairs of samples presented to you, taste both samples, and mark "X" in the box of the one that is stronger in_____. Please cleanse your palate with plain crackers and then spring water before starting the first sample, and between the samples.

Set 1	333	☐	451	☐
Set 2	787	☐	116	☐
Set 3	404	☐	181	☐

SCORECARD FOR ODOR DETECTION THRESHOLD EVALUATION

DESCRIPTION OF THE TEST

In this test, you are asked to compare the odor of three test samples. You will be asked to select one sample that has a **strongest** odor from other two samples. After completion of the entire set, you may go back and re-evaluate samples and change your answer if you wish.

Please read the instructions below and make sure that they are completely clear to you. If you have any questions please ask the experimenter.

INSTRUCTIONS

1. You will perform 6 sets of 3-AFC test. Start from the first set.
2. In each set, you have received 3 samples which are labeled with 3-digit numbers.
3. Sniff the sample by gently squeezing the bottle, and sniffing (bunny sniff) the expressed air.
4. Evaluate the sample from the left to right as shown by **serving order** below.
5. Choose one sample in each set that has a **strongest vanilla** odor, and mark and X in the space next to the code of that sample.
6. If samples appear the same, please make a "best guess."

Set 1	___608	___133	___847
Set 2	___823	___548	___605
Set 3	___109	___251	___628
Set 4	___671	___570	___793
Set 5	___512	___372	___747
Set 6	___657	___776	___414

Comments_____

_____

_____

SCORECARD FOR DESCRIPTIVE ANALYSIS BY SCALING

DESCRIPTION OF THE TEST

In this test, you are asked to rate aroma intensity of flavored soy milk. You will be asked to rate aroma intensity of each sample compare to reference. After completion of the entire set, you may go back and re-evaluate samples and change your answer if you wish.

Please read the instructions below and make sure that they are completely clear to you. If you have any questions please ask the experimenter.

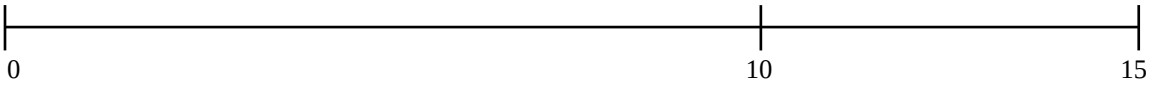
INSTRUCTIONS

1. You will perform 5 samples
2. Each sample is labeled with 3-digit number.
3. Sniff the sample by gently squeezing the bottle, and sniffing (bunny sniff) the expressed air.
4. Rate aroma intensity of each sample compare to the reference by placing a mark on each line.

Vanilla aroma intensity

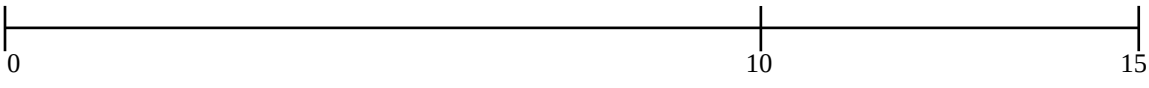
Set 1: Serving order 226 901 813 360 621

none *reference* very strong



Set 2: Serving order 814 966 320 671 254

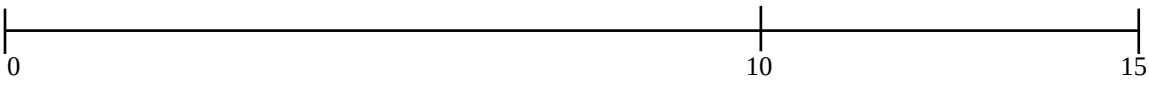
none *reference* very strong



Cotton candy aroma intensity

Set 1: Serving order 950 289 419 802 429

none *reference* very strong



Set 2: Serving order 278 948 650 842 898

none *reference* very strong