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Investigation of the Effect of Sulfitolysis on the Functional Properties and Extrusion Performance of Whey Protein Concentrate

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INVESTIGATION OF THE EFFECT OF SULFITOLYSIS ON THE FUNCTIONAL PROPERTIES AND EXTRUSION PERFORMANCE OF WHEY PROTEIN CONCENTRATE

by

David Taylor

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

2004
Whey proteins have restricted use in many food applications because of limited functional properties. Whey proteins' relatively high content of disulfide bonds may be responsible for their lack of functionality, especially in extrusion applications.

To determine the effect of disulfide bond content on functional properties and extrudate performance, whey protein concentrate was treated with sodium sulfite to achieve four levels of disulfide bond sulfonation (0, 31, 54, and 71%). Sulfonated whey protein functional properties, extrusion-expanded snack properties (32% total protein), and extrusion-textured fibrous product properties (48% protein) were determined. Correlation analysis was performed to determine relationships between functional properties and extrudate performance.
Sulfonation of whey protein concentrate (80% protein) increased foaming and emulsion properties and decreased melt temperatures. These changes were largely attributed to increased protein unfolding and flexibility. Sulfonation decreased gel strength and increased resolubilization after heat treatment. These changes were likely the result of increased electric charge on the proteins, limiting protein-protein interactions during heating.

Snack products extruded from the 31 and 71% sulfonated samples were less expanded and released less protein and carbohydrate during extrudate solubilization. Sulfonation may have promoted protein unfolding, thereby exposing interaction sites and increasing the formation of insoluble protein-starch aggregates. In support of this suggestion, negative correlations were found between extrusion performance and protein functional properties related to flexibility, including emulsification activity index, foam stability, and melt onset temperature. The anomalous behavior of the 54% sulfonated sample may be the result of significant structural and functional changes of α-Lb that are predicted to occur at approximately 50% sulfonation.

Although the textured extrudate produced from all levels of sulfonation (including the control) did not possess typical fibrous texture, sulfonation at 31% and higher decreased stability after hydration. Decreased stability and fibrous texture may have resulted from
decreased protein-protein interactions caused by the repulsion of electric charges contributed by sulfite groups.

In conclusion, sulfonated whey protein functional and extrudate properties were influenced by disulfide bond content. Changes in these properties were attributed primarily to increased protein unfolding and flexibility. Increased electric charge on proteins also played a role where protein-protein interactions were important.
ACKNOWLEDGMENTS

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David P. Taylor
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<tr>
<td>α-Lb</td>
<td>alpha lactalbumin</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>β-Lg</td>
<td>beta lactoglobulin</td>
</tr>
<tr>
<td>β-ME</td>
<td>beta mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>CFR</td>
<td>Code of Federal Regulations</td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
<tr>
<td>EAI</td>
<td>emulsification activity index</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>HSD</td>
<td>honest significant difference</td>
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<td>L</td>
<td>liter</td>
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<td>ln</td>
<td>natural log</td>
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<td>M</td>
<td>molar</td>
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<tr>
<td>Symbol</td>
<td>Standardized Property</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>ml</td>
<td>milliliter</td>
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<td>mM</td>
<td>millimeter</td>
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<td>min</td>
<td>minute</td>
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<tr>
<td>n</td>
<td>number of observations</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>°</td>
<td>emulsified oil volume fraction</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>$T_{1/2}$</td>
<td>foam drainage time</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>protein melt peak temperature</td>
</tr>
<tr>
<td>$T_o$</td>
<td>protein melt onset temperature</td>
</tr>
<tr>
<td>TVP</td>
<td>textured vegetable protein</td>
</tr>
<tr>
<td>TWP</td>
<td>textured whey protein</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>vol</td>
<td>volume</td>
</tr>
<tr>
<td>x g</td>
<td>times gravity</td>
</tr>
<tr>
<td>WAI</td>
<td>water adsorption index</td>
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<tr>
<td>WPC</td>
<td>whey protein concentrate</td>
</tr>
<tr>
<td>WSI</td>
<td>water solubility index</td>
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<td>Wt</td>
<td>weight</td>
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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Extrusion cooking is described as a process whereby moistened starch and protein materials are cooked and worked into viscous, plastic-like dough (1). Extrusion processes are used to produce such foods as ready to eat cereals, snack foods, and meat analogs and substitutes. Although extrusion is a common food process, it is more of an art than a science. While references exist on extrusion process control and factors that affect how well various foods extrude, data is largely empirical. As a result, there is a general lack of understanding regarding many aspects of food extrusion.

Protein functional properties important to food applications include solubility, viscosity, gelation, foam formation and stability, emulsification, and water and fat holding capacity (2). In general, the more functional a protein, the more valuable it is as a food ingredient. Function of native proteins may be enhanced through a number of chemical or enzymatic processes such as alkalization, glycosylation, and enzymatic hydrolysis (2).

The most highly functional proteins have the ability to interact with other proteins, solvents, interfaces, and compounds. The nature and extent of these interactions determine protein functional properties. Whey protein is generally considered a
functional protein and is used in many applications. Whey proteins can replace egg proteins in sponge cakes and other baking applications, and have been used to enhance gelation and eating properties of cheese-like products and meat-like gels (3).

In some applications, however, compact molecular structure and relatively low solubility limit whey protein functionality. Compact proteins may show limited interaction with other proteins and solvents. Opening and unfolding compact proteins may increase solubility and their ability to interact with other compounds, resulting in improved gelation, foaming, solubility, and emulsification properties.

Although often difficult, whey proteins can be extruded at high levels into food products having different properties and applications. Two of such products currently being researched at Utah State University are a fibrous textured whey product, suitable for use as a meat extender or substitute (4,5), and a high protein, expanded snack food.

Development of these products has shown that the ease at which different whey protein concentrates extrude varies with the commercial source of the protein. While some commercial whey proteins work very well in extrusion, others do not. The differences in the proteins’ extrusion performance are likely related to how the functionality of the proteins are altered during their isolation as well as to the content of other components (i.e. lipids, lactose, and minerals). It is not well understood how the functional properties of whey are related to their extrusion performance.
To determine how whey functionality is related to extrusion performance, it is requisite that the functional characteristics be different while differences in minor components of whey (such as lipids, lactose, and minerals) are minimized. One possible way to do this is to sulfonate a single source of whey proteins to various degrees and compare their derivatives with regard to functional properties and extrusion performance.

Sulfitolysis is a chemical reaction where disulfide bonds in a protein are broken by sodium sulfite, producing a free thiol and a sulfonated cysteine derivative. Because disulfide bonds stabilize the compact structure of whey proteins, the removal of these bonds promotes protein opening and unfolding during solvation. This unfolding, along with the addition of negatively charged sulfite groups, alters the functional properties of whey but not in an entirely predictable manner. Whey from a single source can be sulfonated to different levels in order to produce proteins with different structural and chemical characteristics. By sulfonating whey from a single source and treating all of the samples in a similar manner, confounding effects from the minor components are avoided.

Sodium sulfite is a common food additive generally recognized as safe when used in accordance with good manufacturing practice (21 CFR 182.3798) (6), although sulfite must be declared on the label if it was added to impart a functional effect or it is present at greater than the detectable level of 10 ppm (21 CFR 130.9) (6). Sulfonated whey
proteins are precipitated at a pH of < 5.0, leading to significant savings in the energy
and time required for drying. Precipitation also aids in the removal of lactose and excess
sodium sulfite. After precipitation, proteins can be lyophilized and the functionality and
extrusion performance of the residual powder can be measured.

RESEARCH HYPOTHESIS

Functional properties of sulfonated whey proteins are related to their extrusion
performance.

RESEARCH OBJECTIVES

Objective 1. To determine the effect of extent of sulfonation has on traditional
measures of whey protein functionality, including high temperature solubility, gel
strength, emulsification, foaming, and thermal denaturation. This information is of
general interest to food scientists.

Objective 2. To identify correlations between sulfonated whey protein functional
properties and extrusion performance. These correlations could be used to predict and
select raw materials for extrusion.

LITERATURE REVIEW

Extrusion of Whey Proteins in Expanded Snacks. In general, incorporating large
amounts of whey and other proteins into starch-based expanded snacks leads to
detrimental effects on physical properties. However, incorporating small amounts of whey protein into starch based extruded products has been successfully accomplished using traditional extrusion technology. Matthey and Hanna (7) determined the presence of an interaction between amylose and whey proteins. Whey protein concentrate (WPC 80) from 3 sources, containing between 80 and 86% protein, were incorporated at levels up to 30% of the product weight. They concluded that physiochemical interactions developed between amylose and whey proteins. Amylose bound to the whey proteins, preventing extrudate expansion and the degradation of the amylose. Increasing the amount of whey protein from 0 to 30% resulted in extrudates with decreased expansion and a lower apparent amylose content. It was concluded that both whey protein and amylose content should be low to obtain desirable expansion.

Onwulata et al. (8-10) incorporated WPC 34 (containing 34% protein) into extrudates made from corn, rice, and potato flours to determine its effect on extrudate properties. Incorporating 25% or more of the WPC 34 into the extrudates significantly increased water-holding capacity and decreased expansion. As a result of decreased expansion, breaking strength was increased. Increased expansion could, however, be introduced in products containing 25% WPC 80 or whey protein isolates by changing extrusion parameters and through the incorporation of wheat fiber (11). Kim and Maga (12) reported that rice flour extruded with 10 to 20% WPC 34 were preferred by consumers as
compared to extrudates of only rice flour. Consumers preferred the added crunchiness and flavor from the incorporation of whey.

Whey proteins have also been extruded using supercritical carbon dioxide, rather than heat and pressure, as a means to increase expansion in the extrudate (13,14). Whey was incorporated into corn and potato starch extrudates (up to 10% WPC 34) and then extruded with the supercritical carbon dioxide. After extrusion, extrudates are then dried at 70-100 °C to complete expansion. A well-expanded product with uniform air cell distribution could be produced, particularly when dried between 70 and 80 °C. The resulting expansion and cell structure was similar to extrudates expanded using steam.

Until recently, the incorporation of whey proteins into extruded carbohydrate systems has been limited to less than 30% by weight and only in snack-type products. Limitations arise from decreased extrudate expansion, increased extrudate moisture, and an increase in maillard browning from the abundant lactose in the whey. Researchers at Utah State University have developed extrusion processes using WPC 80 to incorporate 30 to 60% whey proteins in extruded products. Through the modification of extrusion parameters and by using WPC 80, product expansion can be controlled and the deleterious effects from lactose are minimized. A puffed snack product containing 16-40% protein has been produced, as well as a textured product containing 48-60% protein.
Extrusion of High Protein Expanded Snacks. A high protein puffed product has been researched at Utah State University. The expanded product is produced using an extruder screw configured to generate high shear and pressure, resulting in product expansion upon exiting the die. This screw configuration is quite different from that used to produce fibrous textured whey, where the screw configuration must give high conveyance at lower pressures. The die used in the expanded snack promotes extrudate expansion, whereas the die used to produce the fibrous textured product allows cooling that limit product expansion (15).

The functional role of whey proteins in the expanded product is different from that in the fibrous textured product. In the expanded product, the structure of the extrudate is primarily developed after the product leaves the die. Upon exiting the die, the product expands rapidly before it cools. This expansion is dependent upon the carbohydrate phase of the extrudate and the amount of WPC incorporated into the product.

Extrusion of Textured Whey Protein. Textured vegetable proteins (TVP), particularly those from soy bean, have been used for many years in the U.S. as a nutritious and inexpensive meat replacer and substitute. Unlike extruded puffed snacks, TVP is composed primarily of protein and the character of the extrudate is due to the protein component of the product. TVP is widely used and had sales of $325 million in the US during the year 1998 (16). TVP has high utilization because of its stability in
various cooking applications and its fibrous meat like texture. It can replace 30% of
meat in products such as patties, meatballs, taco meat, pizza toppings, and chili (17-19).
Although TVP is highly functional, consumer acceptance is generally less than that of
entirely meat products, because of characteristic “beany” and “grassy” off flavors
associated with soy. The extent of TVP included in products can be much greater if the
problem with off flavors could be avoided.

Textured whey protein (TWP) is researched at Utah State University and has shown
considerable promise as an acceptable extender in ground beef patties (4) or as the main
ingredient of non-meat patties (5). The physical configurations of extruder and die
necessary to extrude a textured whey product were similar to those described by Cheftel
et al. (20) and include sections for mixing, the development of necessary temperature and
pressure for protein melting, and for the laminar flow that aligns the whey proteins.
When melted whey proteins align, they layer with an immiscible polysaccharide phase
within the barrel and die of an extruder (21-25). This layering effect is dependent upon
the degree to which whey proteins are unfolded and melted into random structure in the
extruder. Layering is critical to the final structure of the extrudate, as the structure is
formed before exiting the die. A long cooling die reduces expansion of extrudates as they
leave the extruder barrel. The die configuration is critical in the determination of the
final texture of the extruded product (22).
The TWP contains 48-60% protein (wt/wt) and possesses a more complete amino acid profile than TVP. Additionally, TWP does not have the beany off flavor of TVP. Sensory testing indicated that ground beef patties containing up to 30% (w/w) of hydrated TWP were as acceptable as all beef patties and were more acceptable than beef patties containing equivalent amounts of TVP (25). TWP has also been incorporated in products such as taco meat and coarse ground sausage.

**Protein Reactions During Extrusion.** During texturization, the melted proteins experience laminar flow within the extruder barrel, which aligns the proteins in directional flow and exposes covalent bonding sites (22). The high pressures and temperatures found within the extruder barrel aid in the formation of covalent bonds that cross-link protein at these newly formed bonding sites (26). The result is a fibrous, meat-like structure. When carbohydrates are used in conjunction with proteins to form a textured extrudate, layering between the immiscible protein and carbohydrate phases also contributes to texturization.

The covalent cross-linking has a major role in the formation of textured proteins. Cross-linking can be one of two major types: disulfide bonding and nondisulfide bonding. Of the two types of covalent bonding, it appears that the nondisulfide type is more significant in extrusion performance. Rhee et al. (27) showed that extruding soy flour with sodium sulfite, which breaks disulfide bonds, increased extrudate strength.
Extruding with an oxidizing agent (KIO₃), which promotes disulfide bond formation, decreased extrudate strength. These results led to the conclusion that disulfide bonds formed during extrusion are not significant to increased extrusion performance. Types of nondisulfide cross-linking which may be important in extrusion include isopeptide formation and maillard reactions (26).

Less is known about protein reactions in starch based puffed products. When extruding puffed snacks containing whey protein, the proteins bind to amylose to create protein-starch bipolymer s (7). This binding lead to decreased extrudate expansion and increased moisture in the final product. Maillard browning reactions also occur, particularly if the extrudate contains large amounts of a reducing sugar (such as lactose and degraded starch).

**Enhancing Extrudability of Whey.** Whey proteins were modified through esterification, acetylation, and pH adjustment to determine the effects on puffed extrudate expansion and breaking strength (28). At high protein levels (up to 50% by weight), the acetylated whey proteins gave the highest breaking strength and aided in fiber formation, which may have been from a collapse in structure as the extrudate left the extruder. At these high levels of protein, chemical treatment did not affect expansion. All extrudates had similar expansion. At low protein levels, acetylation resulted in lower breaking strength whereas pH modified (alkaline) whey gave higher levels of breaking strength.
Chemical modifications that affect protein solubility may also have an effect on extrusion and extrudates. Recent research indicated that the high temperature solubility (which is related to an open, random structure) of proteins heated at extrusion conditions may largely be dependent on the protein’s content of disulfide bonds (29). As proteins like soy, gelatin, and gluten are heated from 100 °C to 145 °C in a high moisture setting (such as in extrusion) protein solubility initially decreased, then increased as the temperature climbed above 125 °C. Solubility of these proteins at 145 °C approached that of the unheated proteins (generally 80-90% soluble). In the case of whey protein, solubility decreased as the temperature was heated from 100 to 145 °C (to only about 20% soluble). Proteins like soy and gelatin are relatively low in disulfide bonds, whereas whey has a higher content of disulfide bonds.

The effects of breaking disulfide bonds on the extrusion performance of whey proteins have not been directly investigated. Altered extrusion performance by other chemical modifications of whey proteins (e.g. acidification, alkalization, esterification, and acylation) may be as a result of changes in disulfide bonds (28). Breaking disulfide bonds may create a more extrusion-soluble protein with better extrusion properties.

In addition to evaluating the effect breaking disulfide bonds has on extrusion and extrudate quality, it is also of interest to know what effect breaking disulfide bonds has on other measures of protein functionality (i.e. gel strength, foaming, and solubility). By
knowing the effects on both extrusion and protein functionality, conclusions can be drawn on how functionality and extrusion are related.

**Sulfitolysis.** The disulfide bonds in whey proteins are broken by sulfitolysis (sulfonation in the presence of sodium sulfite \((\text{Na}_2\text{SO}_3)\) \((30-34)\). Sulfitolysis of proteins has been studied for many years. The reaction in proteins is as follows:

\[
\text{RS - SR + SO}_3^{2-} \leftrightarrow \text{RS - SO}_3^- + \text{RSH}
\]

\[
\uparrow \quad \text{Oxidizing Agent}
\]

Sulfite cleaves disulfide bonds, forming one sulfonate derivative and one free thiol for each disulfide. An advantage of this form of disulfide reduction is that the sulfonated cysteine residue can no longer undergo oxidation to reform a disulfide, as would be the case when reducing disulfides with 2-mercaptoethanol or dithiothreitol. However, two free thiols formed during the reaction can be reoxidized to form a new disulfide bond by the addition of an oxidizing agent, such as cupric sulfate, as a catalyst. These newly formed disulfide bonds undergo sulfitolysis and the cycle repeats until the majority of the disulfide bonds have been cleaved and converted to the sulfonated derivative.

Some of the early work with sulfitolysis involved studying the effects that different catalysts and denaturants on sulfonation reactions rate \((35-37)\). Sulfitolysis was generally performed in 8 M urea and Tris buffer (pH 7-7.5) with cupric sulfate or iodosobenzoate.
as a catalyst. Kella and Kinsella (38) discovered that sulfitolysis could be accomplished in the absence of a denaturant but still required the addition of cupric sulfate as a catalyst.

Gonzalez and Damodaran (30) investigated the use of a solid state copper carbonate catalyst rather than a soluble copper catalyst to eliminate problems with removing copper after the reaction is complete. Copper carbonate was impregnated on silica beads and was added to the protein reaction solution. It was determined that the use of this solid state catalyst increased the rate of sulfitolysis several fold and the activation energy of the reaction was much lower than with soluble catalyst. When the reaction was incubated at 35 °C, 50% sulfitolysis of a 1% BSA solution was obtained at approximately 10 minutes when using the solid state catalyst.

**Sulfitolysis of Soy Proteins.** Kella et al. (32) used the soy protein glycinin to study the effects of sulfitolysis on solubility and other chemical properties. The sulfitolysis reaction mixture contained 50 mg of glycinin dissolved in 50 ml 0.1M phosphate buffer (pH 7.0) containing 4 M urea and 0.1 M sodium sulfite. Ammoniacal cupric sulfate (pH 9.0) was added at the 400 µM level and molecular oxygen was bubbled through the solution as it incubated at 40 °C. Results from this experiment showed that complete sulfitolysis was obtained and no free thiol groups were left. Sulfitolysis improved the
solubility of glycinin, particularly at pH 2-3 and at pH 7-8. Although the isoelectric point of the protein shifted towards the acidic side, the change was not statistically significant.

Petruccelli and Añón (34) studied the reaction kinetics for partial sulfitolysis on soy protein isolate. Differing levels of ammoniacal cupric sulfate were used to study how the level of catalyst affected the rate of sulfitolysis. Levels which were used were 0, 1.33, and 4.0 mM. Solutions were incubated at 42 °C and were incubated in a laboratory shaker (120 oscillations per minute). Solutions contained 10 mg/ml of soy protein isolate in a 0.1 M phosphate solution pH (7.0) containing 0.1M sodium sulfite. To this solution cupric sulfate was added. Sulfitolysis proceeded when no catalyst was added, although the rate increased with the addition of catalyst (both 1.33 mM and 4.0 mM levels of catalyst had similar reaction rates). All of the reactions reached approximately 75% sulfitolysis after 240 min.

**Sulfitolysis on Whey Protein.** Solid state sulfitolysis increased recovery of whey proteins from raw sweet whey (31). Raw whey, containing 0.1M sodium sulfite, was continuously pumped over the solid state catalyst beads until the desired level of sulfonation was reached. Over 90% of the sulfonated whey protein were recovered by isoelectric precipitation (between pH 4.0 and 5.0) providing an economical way to recover whey proteins from raw sweet whey. Some copper was leached from the catalyst
and was complexed with the protein, however this copper was effectively removed from the protein later by washing with EDTA, pH 4.5.

Klamaszewski and Kinsella (33) studied the effect of sulfitolysis on emulsion properties of whey protein. Sulfitolysis was performed as described by Kella et al. (32), except that the protein level was increased to 10 mg/ml. The reaction was stopped at 50%, 75%, and 100% sulfonation by adding EDTA to chelate the cupric sulfate. Emulsification properties were improved in the samples that were 75% sulfonated. The improvement of emulsification was attributed to changes in unfolding and alignment of the proteins at the air-water interface. It is also believed that the sulfite groups (SO$_3^-$) changed the net charge of the protein enough to cause protein-protein repulsion thereby preventing coalescence of neighboring droplets and stabilizing the emulsion.

Stabilization of oil-in-water emulsions using sulfonated $\beta$-lactoglobulin ($\beta$-Lg) was studied by Lasso and Nakai (39). $\beta$–Lg underwent complete sulfonation. Samples were also conjugated to polyethylene glycol (PEG). Protein samples were emulsified with corn oil and their stability was measured over 7 days. Sulfonated $\beta$–Lg-PEG conjugates had the highest emulsion stability, followed by sulfonated $\beta$–Lg, while unmodified $\beta$–Lg and $\beta$–Lg-PEG conjugates generally were much less stable. The increased stability of the $\beta$–Lg -SO$_3^-$ emulsions was attributed to an increased negative charge on the protein as
well as increased flexibility of the protein. The β−Lg-SO₃-PEG emulsion had the
added protection of the PEG, causing a thick adsorbed layer that prevented coalescence.

Kella et al. (40) analyzed the effects of sulfitolysis on interfacial and foaming
properties of whey protein isolate. Whey protein isolate was sulfonated in the presence
of 4 M urea, 0.1 M sodium sulfite, and 0.8 mM cupric sulfate at 25 °C to achieve
sulfonation levels of 0, 25, 50, 75, and 100%. The sulfonated whey samples had
minimum solubility between pH of 4.0 and 5.0. Surface hydrophobicity increased with
sulfonation up to 50% sulfitolysis, but decreased with 75 and 100% sulfonation. The
initial increase was believed to be from exposure of nonpolar amino acid residues to the
surface, while the decrease at 75% sulfonation was from the increase of the charged
sulfite derivatives on the surface. When looking at surface adsorption rates, data varied
among the different levels of sulfonation and could not be explained by current theories.

Foaming capacity (volume of foam) increased with increasing levels of sulfitolysis,
but only as time was increased from 10 to 20 minutes of whipping. Foam stability
increased in the samples as sulfonation increased to the 75% level, but decreased in the
100% level. It was concluded that as sulfonation increased, molecular flexibility and net
charge increased. This resulted in increased protein-protein interactions and water
retention. Above 75%, the increase in net charge may have been too great, resulting in
the repulsion of proteins in the film and a decrease in stability.
Sulfitolysis Catalysts. Soy isolate has been reported to be able to undergo sulfitolysis without the addition of any catalyst (34). However, research has shown that BSA undergoes very little sulfitolysis (less than 10% in 8 hours time) in the absence of a catalyst, despite having molecular oxygen bubbled though (38). Our preliminary research with fresh whey has indicated that the sulfitolysis reaction does not proceed significantly without a catalyst (less than 10% sulfitolysis could be obtained over several hours of reaction time).

Although a solid state catalyst may help to eliminate the problems with the removal of copper, it is anticipated that sulfitolysis of whey protein concentrate would be very difficult to control. Gonzalez and Damodaran (30) reported that in using a solid state catalyst system, bovine serum albumin could be 50% sulfonated in less than 10 min. Whey protein concentrate may reach that level much quicker since the relative number of disulfides in whey is much less than BSA. It would be very difficult to accurately obtain low levels of sulfitolysis (i.e. 10-30% sulfitolysis). Since using soluble copper as the catalyst is much slower, it may be easier measure and stop the reaction at desired low levels of sulfitolysis.

Protein Solubility. Solubility of a protein is of primary importance because most other functional properties, such as emulsification, foaming, and gelation develop from soluble protein. A protein's solubility is dependent upon its surface active properties,
which result from amino acid composition, amino acid distribution, molecular flexibility, size, and shape of the protein (2). The number and placement of hydrophobic and hydrophilic regions on the surface of the protein are extremely important to solubility. At equilibrium, hydrophobic and hydrophilic interactions of a protein can be expressed as the following equilibrium:

\[
\text{Protein-Solvent} \leftrightarrow \text{Protein-Protein} + \text{Solvent-Solvent}
\]

which suggests that a decrease in protein-protein or solvent-solvent interactions results in more protein-solvent interaction (41).

Protein solubility can often be improved by increasing the number of hydrophilic groups on the surface of the protein. This can be accomplished through a number of chemical means, including deamidation, covalent attachment of hydrophilic amino acid residues, introduction of glyco groups, and the addition of phosphates (42). Addition of other charged groups to the surface of the protein may also improve solubility.

The solubility of a protein is affected by environmental factors including pH, ionic strength, temperature, solvent composition, and other chemicals present in solution (2). The pH of the environment has a large effect on solubility. At the protein’s isoelectric point, the net charge on the protein is zero and protein-protein interactions increase because electrostatic forces that normally keep proteins from interacting with one another
are not present. Also the minimally charged proteins are less likely to interact with water molecules. The result is protein aggregation and possible protein precipitation.

At a pH above or below the isoelectric point, the protein takes on a net charge, which increases the protein’s interaction with water and decreases protein-protein interactions. Extremely high or low pH, however, can cause a protein to unfold and denature. This may expose hydrophobic regions that normally would be buried within the structure of the protein leading to aggregation and precipitation.

Ionic strength of the protein solution affects the solubility of proteins, especially globulins and some albumins (2). Salt ions interact with oppositely charged groups on a protein, decreasing electrostatic interactions between protein molecules. This also increases the protein’s interaction with water and solvation. Neutral salts in the range of 0.1-1.0 M may increase the solubility of a protein, depending upon the protein, salt, pH, and temperature. This phenomenon is known as “salting in.”

At salt concentrations greater than 1 M, hydrophobic interactions increase and proteins begin to aggregate and precipitate. This decrease in solubility is known as “salting out.” At these high salt concentrations, salts compete with the protein for solvation by water. Protein-protein interactions increase until they overcome protein-solvent interactions and precipitation results.
Protein solubility is generally increased as temperature is increased from 0 °C to 40-50 °C. Temperature often affects the influence of hydrophobicity on the protein’s physical properties (43). When a protein is exposed to higher heat or heat for an extended period of time, proteins unfold and denature. This unfolding exposes hydrophobic regions, leading to aggregation, coagulation, and precipitation. The pH and ionic strength of the protein solution affect the actual temperature where proteins denature.

The solvent used to dissolve the protein is important to the overall solubility of the protein (2). In general, water-miscible organic solvents decrease solubility of proteins. These solvents denature and unfold proteins. The presence of other chemicals can also affect the solubility of proteins. Chemicals such as picarate, tungstate, and trichloroacetate precipitate proteins. Lipids can interfere with protein-water interactions and may decrease solubility, as does polyethylene glycol. Surface active agents and reducing agents can also effect solubility.

Through sulfitolysis, the number of net charges on the protein will be increased. The number of negative charges will be increased as the $\text{SO}_3^-$ groups are added to the cysteine residues. The increase of negative charges will be proportional to the degree of sulfonation. This addition of negative charges should help to increase the solubility of the protein. However, the elimination of disulfide bonds may facilitate the unfolding of
the protein and expose hydrophobic regions, resulting in more protein-protein interactions and a decrease in solubility.

**Protein Gel Formation.** A protein gel is defined as a protein aggregation phenomenon in which attractive and repulsive forces are balanced, resulting in a well-ordered tertiary network, capable of holding water (44). When attractive forces predominate, the coagulum is formed and water is expelled from the gel matrix. If repulsive forces predominate, the tertiary network will not be formed. Gelation occurs in two steps. The first step involves the partial denaturation of protein molecules, whereas the second is the gradual association of these proteins (2).

Fibrous proteins form gels through the connection of long linear molecules (45). Networks are formed from polypeptide chains of random or helical structures. The gelation of globular proteins is somewhat different. Globular protein gels are often formed by end-to-end aggregation where the individual proteins retain their globular structure. Two main types of globular protein gels exist (46). The first is described as a “string of beads,” while the second is a network formed by random aggregation. Strings of bead gels are typically transparent or translucent in appearance. Examples of these gels are serum albumin, insulin, lysozyme, and soybean glycinin (47,48). Gels made by random aggregation have a more turbid appearance. Examples of this type of gel include whey proteins and myosin (49,50).
Several chemical and physical properties affect protein gels. The amount of hydrophobic amino acid residues has been correlated to properties of protein gels. Heating protein solutions enhances hydrophobic interactions of non-polar segments of adjacent polypeptides (2). The location and amount of the hydrophobic segments also effects the type of gel formed. Shimada and Matsushita (51) showed that proteins containing less than 31.5% mole percent of non-polar amino acid residues formed translucent gels, where proteins containing 31.5% mole percent or more of non-polar residues produced coagulation-type gels.

Electrostatic repulsion of highly charged proteins can inhibit gelation of proteins (44). In contrast, gels can quickly form at the isoelectric point for a protein as well as at other pH levels if salts are added to shield the charged amino acid residues from each other (2). The addition of salts often leads to the formation of turbid gels consisting of large aggregates.

Hydrogen bonds contribute to the formation of protein gels, although their effect is difficult to demonstrate in whey protein gels (52). With other gels, the involvement of hydrogen bonds can be demonstrated by melting gels at elevated temperatures (2). Intermolecular covalent disulfide bonds are not believed to be initial gel network formers, but they are believed to enhance gel strength (53).
The effect of sulfitolysis on a protein’s ability to form a gel is not well understood. While there may be increased exposure of hydrophobic regions as a few disulfide bonds are broken, the addition of the SO$_3^-$ groups at higher levels of sulfonation may result in repulsion of proteins and inhibition of gelation. The addition of salt may eliminate some electrical charges on the protein and a strong gel may still be obtainable with proteins having undergone extensive sulfonation.

**Protein Emulsification.** An emulsion is defined as a dispersion or suspension of two immiscible liquids (54). The most common type of emulsion is the oil-in-water emulsion, where small droplets of oil are suspended in an aqueous phase. Because of van der Waals and other forces, suspended oil droplets are attracted to one another and will aggregate together (flocculation), combine to form larger droplets (coalescence) or rise to the top of the aqueous solution (creaming) (55). In order to prevent this from happening, repulsive forces, such as electrostatic or steric forces must balance the attractive forces.

Proteins often have the ability to stabilize the dispersed oil droplets, preventing them from flocculation, coalescence, and creaming (55). Protein molecules, often having both hydrophobic and hydrophilic regions, align themselves at the interface between the oil droplet and the aqueous interface. Portions of the protein protrude both into the oil droplet and out into the aqueous phase. These “loops” and “tails” which protrude out into...
aqueous phase create steric forces, which prevent other oil droplets from approaching the droplet.

In addition to having both hydrophobic and hydrophilic regions, a protein must also be highly soluble and have a flexible structure in order to be an effective emulsifier (2). Heat denaturation and the breakage of disulfide bonds often aids in improving solubility and flexibility and may enhance emulsification properties. Sulfitolysis, which breaks disulfide bonds, creates a more flexible protein, and increases solubility is an effective means to improve emulsification (33).

**Protein Foaming.** The ability of a protein to form foam is an important functional property, particularly in products such as meringues, mousses, and whipped products. In protein foam formation, proteins adsorb at the interface between the liquid and gas phase. Proteins interact with one another by the formation of electrostatic, hydrophobic, hydrogen, and covalent bonds to form a stiff, viscoelastic adsorbed layer (2). This viscoelastic layer stabilizes the foam, preventing the coalescence of air bubbles.

Two distinct phases of foaming exist: gas encapsulation (foamability) and the lifetime of the foam (foam stability) (2). Foamability is usually dependent upon the rate at which a protein is transported to the gas/fluid interface and adsorbed. Foam stability is dependent upon how well the adsorbed protein creates an intact structure that can resist collapse. Some proteins have excellent foamability properties, while others often will
excellent foam stability properties. Not all proteins have both of these important properties.

Protein properties that affect foam stability include the flexibility and structure of a protein, surface hydrophobicity, and size of the protein. The net charge of the protein also has a large effect on protein foam stability, as highly charged proteins tend to repel one another and will not adsorb well to the interface. For this reason, ionic strength of the foam also will affect a protein’s ability to form foam.

A protein’s foamability can be altered through a number of methods. Decreasing the size of the protein through enzymatic digestion increases the rate of diffusion to the interface and improves foamability (56). Alkylation increases the hydrophobicity and surface activity of the protein, making adsorption more favorable (57). Chemically changing the isoelectric point also affects foamability, shifting ideal foaming conditions to a different pH. Protein glycosylation can also improve protein flexibility and rate of adsorption (58, 59).

Properties that affect foam stability include the number of charged amino acid residues on a protein, the ability of a protein to form disulfide bonds with other neighboring interfacial proteins, and glycosylation (60-62). Charged amino acids prevent proteins on two interfaces from coming together, which reduces drainage and increases foam life. Bonds formed between adjacent proteins increases interfacial surface
viscosity, which also prevents liquid drainage. Glycoproteins with large oligosaccharides protruding into the interlamellar fluid cause liquid flow more slowly, minimizing drainage. Foaming stability of proteins can be altered through the addition of more charges on a protein or through chemical glycosylation.

As reported by Kella et al. (40) sulfitolysis has an affect on both foamability and foam stability. Excessive sulfonation increases the number of negative charges on the protein, which may cause proteins to repel from one another at the air-water interface and result in a decrease of foamability. However, the breaking of the disulfide bonds will help to open the protein, which would increase protein flexibility and improve the rate of adsorption to the interface. The increase of negative charges will help improve foam stability by decreasing the rate at which proteins at the interface come together and cause collapse of the foam structure.

**Thermal Analysis of Whey Protein Using Differential Scanning Calorimetry.**

Differential scanning calorimetry (DSC) is a method of thermal analysis where the instrument scans the temperature of both experimental and reference samples (63) as they are heated. Differences in the heat input to the sample compared to that of the reference is recorded as a peak on a thermogram. When extra energy is required to heat the sample in comparison to the reference, then an endothermic peak is recorded on the thermogram.
In contrast, an exothermic peak is recorded when energy is liberated from the sample and less energy input is required.

DSC is widely used in the food research industry to measure thermal properties of foods, particularly in protein research. The DSC is used to induce structural changes in proteins, from a native or ordered state to one of denaturation and disorder (63). By measuring the temperatures at which these changes occur, it can be determined thermal stability and denaturation temperatures of these proteins.

Whey proteins, particularly β−Lg and α-lactalbumin (α−Lb) have been well studied using DSC techniques. Bernal and Jelen (64) demonstrated that whey protein denaturation temperature is dependent on many factors. These factors included the pH of the whey sample, the presence of milk sugars, and the presence of fatty acids. β−Lg was most heat stable at a pH of 3.5 while α−Lb was most stable between pH 4.5 to 6.5. The presence of fatty acids stabilized bovine serum albumin (BSA) such that it denatures at a higher temperature. Thermal analysis on fresh whey showed that the thermal behavior was dominated by the β−Lg fraction in the sample.

Paulsson and Dejmek (65) investigated the effect casein had on thermal denaturation of pure individual whey proteins. It was discovered that β-casein had no effect on enthalpy, while α-casein lowered denaturation temperatures of all the whey proteins by 2 to 3 °C. Κ-Casein also lowered the denaturation temperature of β−Lg by 3 °C. In a
review of previous literature, Paulsson and Dejmek reported that in 10-20% protein concentrations in 0.1M phosphate (pH 7.0) and heated at a 5 °C per minute rate, β-Lg had a $T_{\text{max}}$ of 79.0 °C. α-Lb had a $T_{\text{max}}$ of 64.8 and BSA had a $T_{\text{max}}$ of 64 °C.

Anema (66) reported that the denaturation temperature of β-Lg was dependent upon the protein and milk solid concentrations. At higher solids concentrations, thermal denaturation temperature was increased beyond the characteristic 75 °C. It was hypothesized that the free thiol on β-Lg is involved in aggregation with other whey proteins and casein via disulfide aggregation, resulting in thermal stabilization.

Boye et al. (67) reported that α-Lb has two reversible thermal transitions. The transitions, attributed to two different isoforms of the protein, occur at 39.6 °C and 64.8 °C. When the pH was changed to pH 3, the first transition was only partially reversible while the second is completely reversible. At pH 9, both transitions were completely reversible. When α-Lb was heated at pH 9, a translucent gel was formed. At pH 3, aggregation occurred but no gelling was observed.

Particular attention has been given to studying the effects that disulfide bonds have on the thermal denaturation of these whey proteins. Disulfide crosslinks (two of which are present in β-Lg and 4 in α-Lb) act as physical restraints for the freedom of motion after thermal denaturation (68). The presence of these bonds plays an important role in the determination of denaturation temperature.
de Wit and Klarenbeek (69) studied the denaturation of β–Lg up to 160 °C. The typical denaturation endotherm was present at 80 °C. However, an additional endothermic event occurred between 130 and 150 °C. This peak disappeared when the 2-mercaptoethanol was added to the sample, supporting the hypothesis that the endothermic event is due to the melting of residual protein conformations and perhaps the breakdown of disulfide bonds. Partial stabilization occurs at 80 °C (from disulfide exchange), followed by destabilization of the residual structure at 140 °C.

Above 40 °C, β–Lg dimers begin to dissociate into monomers. These monomers are the most significant species of β–Lg during heat denaturation (70). The free thiol on β–Lg is capable of forming new disulfide bonds with other thiols, especially at pH 6.8 or above.

de Wit and Swinkels (70) reported that above 70 °C the denaturation behavior of β–Lg was changed, possibly because of aggregation. Above 70 °C, denaturation becomes irreversible. Below 70 °C, the changes that occur to the structure of β–Lg are reversible upon cooling.

LITERATURE CITED


CHAPTER II

THE INFLUENCE OF DISULFIDE BOND CONTENT ON THE FUNCTIONAL PROPERTIES OF WHEY PROTEIN CONCENTRATE

ABSTRACT

Whey protein concentrate (WPC 80) was treated with sodium sulfite to achieve 4 levels of disulfide bond sulfonation (0, 31, 54, and 71%). The effects on protein gel strength, foam overrun and stability, emulsified oil fraction (φ) and activity index (EAI), melt temperature, and protein resolubilization after heating were evaluated. Sulfonation increased foam overrun, foam stability, and EAI and decreased gel strength. These changes were attributed to increased protein flexibility and unfolding as disulfide bonds were cleaved, although at the higher sulfonation levels increased electrostatic charges from sulfite groups may have predominated to decrease gel strength. Protein melt temperature decreased at a sulfonation level of 71%. A complex three-way interaction between level of sulfonation, heating liquid (water or NaOH), and dissolving solvent (1% SDS or SDS/β-ME) affected protein resolubilization. Heating in NaOH decreased resolubilization, perhaps by enhancing covalent crosslinking in the control sample. Sulfonation increased resolubilization likely because electrostatic repulsion of sulfonated samples precluded protein-protein interaction during heating. Functional properties
related to protein unfolding and solubilization (including protein resolubilization) were positively correlated to level of sulfonation, whereas protein melt temperatures and gel strength were negatively correlated, supporting the conclusions that sulfonation improved protein flexibility and resolubilization and decreased protein stability during heating and gel strength. Related functional properties (foam overrun and stability, onset and peak melt temperatures, and protein resolubilization) were correlated to each other.

**INTRODUCTION**

In many food applications whey proteins can only be used in low concentrations because of limited functionality, which may be a result of their high disulfide bond content. Whey protein concentrate consists of many different proteins, primarily β-Lg (52-65% of total protein by weight) and α-Lb (15-21%) (1) and has a high disulfide bond content compared to many other common food proteins (2). β-Lg and α-Lb have two and four disulfide bonds per protein molecule, respectively. The relatively high concentration of disulfide bonds in whey protein contributes to compact molecular structures and limit their functional performance in food applications. Under physiological conditions, compact proteins often show restricted interaction with other proteins and solvents and thus have may have poor functional characteristics where protein-protein interactions are required.
Proteins are often evaluated for their usefulness in food systems by in vitro measurement of their functional properties including gel formation, foam volume and stability, and emulsion formation. Functional proteins need some or all of these properties to be useful food ingredients. In general, open unfolded proteins tend to have better gel, foam and emulsion properties (3, 4). Whey proteins, with their high disulfide bond content and compact structure, have limited gel, foam, and emulsion properties.

Less common functional measures such as protein melt temperature and protein resolubilization after heating may be used to evaluate a protein’s performance in specific processes like extrusion. These measurements evaluate protein’s ability to unfold and interact with other proteins and components during heating. While whey proteins have been incorporated into extruded puffed snacks (5-8), textured meat extenders (9) and textured meat patties (10), the amount that can be used has been limited by poor product texture and expansion. Supercritical carbon dioxide extrusion has also been used to increase product expansion of whey containing cereals at low temperatures (11-13), but the use levels were still less than 10%. It has also been hypothesized that unfolded protein is important for texturization in high protein extruded foods (14). This may be one reason why whey proteins have limited use in extrusion and may necessitate modifying whey proteins to increase their extrudability.
Sulfitolysis is a common method for reducing disulfide bond concentration in proteins (15). As protein is treated with sodium sulfite, disulfide bonds react to form one sulfonated cysteine and a free thiol. Two free thiols can then be oxidized to form a new disulfide, which can then be cleaved by additional sulfite. The reaction continues until the majority of the cysteine residues in the protein have been sulfonated.

Sulfonation alters the solubility of soy proteins (16) as well as emulsion and foaming properties of whey protein isolates (17, 18). In these studies, both increases and decreases in the solubility of soy protein fractions were attributed to protein unfolding and increased electrical charge. Improvements in the emulsification and foaming properties were attributed to the breakage of disulfide bonds that allowed increased protein unfolding and the exposure of hydrophobic residues.

We hypothesize that functional properties of whey protein concentrate are limited by disulfide bond concentration and that reducing the concentration of disulfide bonds through sulfonation will enhance general functional and extrusion properties. Thus, the objective of this research is to compare functional measures of whey protein concentrates (80% protein) sulfonated to different levels. Functional measures of general importance to food systems were evaluated, including protein gel strength, foam overrun and stability, and emulsification activity index. To model possible effects on extrusion performance, the protein melt temperature and protein resolubilization after high
temperature heating were evaluated. Additionally, correlation analysis was performed to determine correlation among functional properties and level of sulfonation.

MATERIALS AND METHODS

Materials. Whey protein concentrate (WPC 8000) was obtained from Proliant Inc., Ames, IA. BCA and Modified Lowry protein assay kits were purchased from Pierce, Rockford, IL and 5,5'-Dithiobis (2-nitro-benzoic acid) was obtained from Sigma Chemical Co, St. Louis, MO.

Sulfitolysis of Whey Protein Concentrates. Sulfitolysis was performed according to Petruccelli and Añón (19) in large single flask batches with the following modifications: 1500 ml of WPC solution (50 mg/ml) was prepared in 0.1 M Tris buffer, pH 8.0, containing 0.1 M Na₂SO₃ in a 2 L Erlenmeyer flask. The solution was incubated with shaking until the solution temperature reached 42 °C. Ammoniacal CuSO₄ (0.1 M), pH 10.0, was added to obtain a 4.0 mM Cu²⁺ concentration in the reaction solution. This catalyzes the reaction and was replaced by distilled water to prevent sulfonation of the control sample. The solution was shaken at 150 oscillations/min while being held at 42 °C in a laboratory shaker. Sulfonation was monitored at 5 minute intervals according to Petruccelli and Añón as described below (19). Disulfide content of the control sample (0% sulfonation) was measured at 5-min intervals and compared to that of a 50-mg/ml solution of WPC dissolved in distilled water to ensure that sulfitolysis did not occur.
When the desired level of sulfonation was reached, the reaction was stopped through the addition of 180 ml of 0.2 M EDTA, pH 7.0, to chelate the catalytic copper in the reaction.

Target sulfonation levels were 25, 50, and 75%. To achieve 25, 50, and 75% sulfonation levels, samples were allowed to react for 6.5, 20, and 120 minutes, respectively.

**Recovery of Sulfonated Whey Proteins.** After the addition of EDTA to stop the sulfonation reaction, whey proteins were precipitated by slowly adding concentrated HCl to pH 4.0. The solution was centrifuged at 2800 x g and the protein pellet was washed in 3 volumes of 0.1 M EDTA (pH 4.0) to remove residual copper from the pellet. The suspension was centrifuged at 2800 x g and the pellet was suspended in an equal amount of 0.1 M phosphate buffer (pH 7.0) and the pH was adjusted to 7.0 with NaOH. The suspension was then frozen, lyophilized, and then stored at -20 °C. The lyophilized protein from 20 individual flasks of the same sulfonation level was pooled together before analysis to ensure a homogenous protein concentrate at each level. Samples were analyzed for composition by SDS-PAGE analysis (20).

**Determination of Disulfide and Protein Content.** Protein solutions (10 mg/ml) were prepared from the dried sulfonated and control WPCs. Synthesis of 2-nitro-5-thiosulfobenzoate (NTSB) using 5,5'-Dithiobis (2-nitro-benzoic acid) and the
determination of disulfide content were performed according to Petruccelli and Añón (19). Protein contents of the WPC samples were determined by the BCA method using bovine albumin as standard.

**Gel Strength.** A solution (12 g) containing approximately 15% of the control and sulfonated WPC (12% total whey protein), 2% NaCl, and 83% distilled water (wt/wt) was placed in 6 cm petri dishes. The pH of the solution was adjusted to 7.0 with NaOH, if necessary. The solutions were heated to 70 °C in a water bath for 60 minutes and then cooled overnight at 4 °C. After allowing to warm to room temperature, gel strength (G*) was measured using the HAAKE Rheostress 75 rheometer (Paramus, NJ) with a 35 mM probe. G* was measured using a stress sweep test (10 Pa increments from 10 to 100 Pa) and a frequency of 0.464 Hz. Maximum G* observed over the measurement period was recorded.

**Foam Overrun and Stability.** Foam generation was performed based on procedures outlined by Luck et al. (21). Distilled water solutions containing approximately 9% WPC (7.2% actual whey protein) and 6% sucrose (wt/wt) were allowed to equilibrate overnight. The pH of the solutions was adjusted to 7.0 with NaOH, if necessary. Solutions (100 ml) were added to a 4.3 L stainless steel bowl of a Kitchen Aid mixer and mixed for 10 minutes with a wire whisk on a speed setting of 9 (1950 rpm). Foam overrun and stability were measured after 10 minutes of whipping.
according to Phillips et al. (22) with minor modifications. To determine foam stability, approximately 40 g of foam was quickly transferred to 250 ml plastic beaker and weighed. The beaker was positioned on its side over a graduated cylinder on a tarred balance. The time for 50% of the original foam weight to drain ($T_{1/2}$) was recorded.

**Emulsification Activity Index (EAI).** Emulsions were made based on methods by Lasso and Nakai (23). Approximately 0.5 g of control and sulfonated WPC (0.4 g of actual whey protein) was added to 79.5 g of 0.05 M phosphate buffer (pH 7.0). Soybean oil (20 g) was added and the solution was homogenized using a polytron (Ultra-Turrax T25, Janke & Kunkel, Germany) on maximum speed for 5 minutes. The oil fraction volume ($\phi$) was measured according to Pearce and Kinsella (24) and EAI was calculated as described by Cameron et al. (25).

**Thermal Denaturation and Melt Temperatures.** Differential scanning calorimetry (DSC) analysis was employed to determine thermal denaturation and melt temperature of the control and sulfonated WPC. Analysis was done according to Paulsson and Dejmek (26). Control and sulfonated WPC (100 mg) was suspended in 1.0 ml of 0.1 M phosphate buffer (pH 7.0) and the suspension (20 µl) was placed in hermetically sealed aluminum pans and heated from 20 to 100 °C using a TA Instruments 2910 DSC (Wilmington, DE). Samples were heated to 145 °C at 5 °C per minute in a DSC cell pressurized to 689 kPa using nitrogen gas. The DSC was calibrated using thermograms
for ice and indium. Denaturation onset temperature ($T_o$) and denaturation peak temperature ($T_{max}$) were measured using TA Instruments Universal Analysis software.

**Protein Resolubilization after High Temperature Heating.** Control or sulfonated WPC (100 mg) was blended with 67 mg of commercially available cornstarch. The blend was placed in 3-ml glass vials and 170 µl of a heating liquid (distilled water or 0.2 M NaOH) was added to the vial and vortexed. This mixture of WPC, starch, and heating liquid is typical of that extruded into meat alternatives (9, 10). Vials were sealed and heated in an aluminum heating block at 145 °C for 10 minutes to simulate possible extrusion conditions. The heated slurries were transferred to 15 ml centrifuge tubes containing 10 ml of a dissolving solvent, 1% sodium dodecyl sulfate (SDS) or 1% SDS and 1% beta-mercaptoethanol (β-ME). The tubes were placed overnight on a laboratory rocker (Rocking Platform 200, VWR Scientific, Bristol, CT) on a rock setting of 4. Soluble protein analysis was performed according to Mohammed et al. (27) using the Modified Lowry protein assay.

**Statistical Analysis.** Statistical analysis was performed using the MANCOVA procedure of Statistica (Statsoft, Tulsa, OK). Significance was determined at $p < 0.05$ and post-hoc mean comparisons were performed using the Tukey’s HSD (Honest Significant Difference) test. Total protein and protein solubility measurements were made using $n = 9$ measurements. All other functional properties were measured using
n = 5. The samples for the measurements were randomly selected from a bag containing the WPC pooled from 20 different sulfonation batches. Functional property means were correlated using Statistica with significance determined at p < 0.05. ANOVA tables for the statistical analyses are contained in Appendix A.

RESULTS AND DISCUSSION

**Protein and Disulfide Content of the Samples.** The sulfonation levels (the percentage of disulfide bonds cleaved by sodium sulfite) of the pooled samples were 0, 31, 54, and 71%. Differences between the actual sulfonation level and the target levels were likely the result the inability to more accurately control the sulfonation reaction. The pooled samples contained between 72 and 83% protein as determined from the BCA assay (Table I). Variability in the final protein content may have been the result of differences in precipitation efficiency during the recovery of sulfonated proteins. The proportion of the individual whey protein components in the samples was shown to be consistent by SDS-PAGE (see Appendix B). The amount of sample used in each functional test was varied to account for the differences in protein content and ensure that the same amount of total WPC protein was used in each test.

**Gel Strength, Foaming, and Emulsification.** Sulfonation generally decreased gel strength (G*) and increased foaming and emulsification properties. Increased sulfonation decreased protein gel strength from 370 Pa in the control to 50 Pa in the 31% sulfonated
Table 1. Mean Protein Content for Control and Sulfonated Samples

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sulfonation Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>78 ± 2 ab</td>
</tr>
</tbody>
</table>

abc Values with common letters were not different (p > 0.05). Mean ± SD (n = 9). SD: Standard deviation.

sample (Table 2). As sulfonation level increased to 54 and 71%, samples did not gel during the heating or subsequent cooling. Sulfonation at all levels increased foam overrun compared to the control (Table 2) and the greatest overrun was observed at the 31% level (350% overrun compared to 60% overrun in the unsulfonated control). Foam stability increased at all levels of sulfonation compared to the control (Table 2).

Sulfonation at the 31% level yielded the most stable foam (T_{1/2} of 2200 seconds compared to 5 in the control).

Sulfonation increased emulsification properties of the WPC. Although sulfonation increased the volume fraction of oil (φ) emulsified by a protein, there were no significant differences between the three sulfonation levels (Table 2). Sulfonation at the 71% level increased the EAI compared to the control (Table 2). Sulfonation at high levels leads to more oil being emulsified into smaller oil droplets.

Protein unfolding was most important in describing the effect of sulfonation on foam overrun, foam stability, and EAI. This increased unfolding was evident in the decreased melt temperatures of the DSC analysis (see below). At the air-water interface of foams,
<table>
<thead>
<tr>
<th>Functional Measurement</th>
<th>0%</th>
<th>31%</th>
<th>54%</th>
<th>71%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G* (Pa)</td>
<td>370 ± 18 a</td>
<td>50 ± 15 b</td>
<td>0.14 ± 0.11 c</td>
<td>0.38 ± 0.22 d</td>
<td>0.0001</td>
</tr>
<tr>
<td>Foam Overrun (%)</td>
<td>60 ± 15 a</td>
<td>350 ± 35 b</td>
<td>240 ± 22 c</td>
<td>150 ± 21 d</td>
<td>0.0000</td>
</tr>
<tr>
<td>T1/2 Foam Drainage Time (s)</td>
<td>5 ± 2 a</td>
<td>2200 ± 220 b</td>
<td>430 ± 51 c</td>
<td>900 ± 120 d</td>
<td>0.0000</td>
</tr>
<tr>
<td>Oil Volume Fraction (φ)</td>
<td>0.22 ± 0.01 a</td>
<td>0.24 ± 0.01 b</td>
<td>0.25 ± 0.01 b</td>
<td>0.24 ± 0.01 b</td>
<td>0.0016</td>
</tr>
<tr>
<td>EAI</td>
<td>78 ± 9 a</td>
<td>94 ± 7 ab</td>
<td>80 ± 13 a</td>
<td>110 ± 10 b</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

abcd Values with common letters across rows are not different (p > 0.05). Mean ± SD (n = 5).

Flexible proteins interact with one another to form a viscoelastic film (3) resulting in increased foam formation and stability. Increased protein flexibility also increases emulsification capacity and stability by allowing for more protein to properly align at the interface (17).

Electrical charge may mask the benefits of unfolding and explain the decreased gel strength and why foam overrun, foam stability, and φ were highest at the 31% level. As charge increased, so did electrostatic repulsion between proteins. Protein-protein interactions, required for gel formation (28), were decreased. The addition of 2% NaCl, used to shield electrostatic charges, was not sufficient for gelation in the highly sulfonated samples. In foaming and emulsification, the increased repulsion from electrostatic charges may have reduced the protein-protein interactions that are
responsible for the formation of the viscoelastic film in the foams and emulsions.

Thus, the effect of increased protein flexibility and disulfide cleavage is diminished by increased electrostatic repulsion. EAI was, however, highest at the 71% sulfonation level.

The increase in foam stability at the 71% sulfonated level compared to the 54% level may also have been from both increased unfolding and electrical charge. Water binding is based on physiochemical properties of the protein (amino acid composition, pI) as well as the openness of the protein’s structure (29). Increased unfolding at the 71% level, coupled with the increased electrostatic charge from the sulfonated cysteine residues, helped bind water and prevented drainage from the lamellae. As water is prevented from draining from the lamellae, the foam remains stable for longer periods of time (3).

**Protein Melt Temperature and Resolubilization after High Temperature**

**Heating.** At the highest level of sulfonation (71%), the melt temperature of the samples decreased (Table 3). $T_m$ decreased from 128 °C in the unsulfonated control to 119 °C at the 71% level. $T_{max}$ also decreased from 133 °C in the control to 122 °C in the 71% sulfonated sample.

The endothermic event of whey protein observed at 130°C is attributed to the melting of β-Lg and is largely dependent on the breakdown of disulfide bonds (30). Thus, it was
Table 3. Mean Protein Melt Onset ($T_o$) and Maximum Peak ($T_{max}$) Temperatures of WPC at Each Level of Sulfonation

<table>
<thead>
<tr>
<th>Melt Temperature Measurement</th>
<th>Sulfonation Level</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
<td>31%</td>
<td>54%</td>
<td>71%</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>$T_o$ ($°C$)</td>
<td>128 ± 2 a</td>
<td>127 ± 4 a</td>
<td>126 ± 3 a</td>
<td>119 ± 3 b</td>
<td>0.0154</td>
<td></td>
</tr>
<tr>
<td>$T_{max}$ ($°C$)</td>
<td>133 ± 3 a</td>
<td>132 ± 3 ab</td>
<td>127 ± 4 ab</td>
<td>122 ± 2 b</td>
<td>0.0002</td>
<td></td>
</tr>
</tbody>
</table>

a b Values with common letters across rows were not different (p > 0.05). Mean ± SD (n = 5).

It is not surprising that increased sulfonation resulted in decreased melt temperatures. Proteins with lower concentrations of disulfide bonds melt and unfold more easily than those with higher concentrations. This is perhaps why soy (with a relatively low disulfide bond concentration) has enhanced extruder functionality compared to whey proteins.

The analysis of protein resolubilization showed a complex three-way statistical interaction (p < 0.006) between the level of sulfonation, the heating liquid, and dissolving solvent (Table 4). Heating in NaOH decreased resolubilization of the control in SDS/β-ME, suggesting that increased intermolecular bond formation was promoted during heating with NaOH. As the samples were heated to 145 °C in the presence of starch, the increased pH associated with the use of NaOH may have increased the formation of isopeptide and maillard crosslinks, thereby decreasing resolubilization (27). The choice of heating solvent did not effect protein resolubilization in the sulfonated samples,
Table 4. Mean Percent Soluble Protein After Heating Protein-Starch Mixtures to 145°C Using Distilled Water or 0.2 M NaOH as the Heating Liquid and 1% SDS or 1% SDS/β-ME as the Dissolving Solvent

<table>
<thead>
<tr>
<th>Heating Liquid</th>
<th>Dissolving Solvent</th>
<th>Sulfonation Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Water</td>
<td>SDS</td>
<td>26 ± 4 a</td>
</tr>
<tr>
<td>Water</td>
<td>SDS/β-ME</td>
<td>96 ± 8 ef</td>
</tr>
<tr>
<td>NaOH</td>
<td>SDS</td>
<td>21 ± 3 a</td>
</tr>
<tr>
<td>NaOH</td>
<td>SDS/β-ME</td>
<td>83 ± 13 bcd</td>
</tr>
</tbody>
</table>

abcdef Values with common letters were not different (p > 0.05). Mean ± SD (n = 9).

suggesting that sulfonation inhibited the formation of those bonds otherwise promoted by heating in NaOH. The increased resolubilization in SDS/β-ME, which breaks disulfide bonds, suggests that disulfide bonds were the primary cause of insolubility in the control sample dissolved in SDS.

Sulfonation lead to increased resolubilization in SDS and resolubilization was similar when either SDS or SDS/β-ME were used. Together these observations suggest that sulfonation inhibited intermolecular disulfide bonding during heating. Although sulfonation would allow for easier unfolding and exposure of possible covalent bonding sites, the negatively charged sulfite groups may have blocked disulfide bonding and limited protein-protein interactions via electrostatic repulsion.
We expect that the effect of sulfonation on the extrudability of whey proteins will be twofold. First, sulfonation may enhance the extrusion performance though decreased melt temperature and decreased disulfide bonding. Unfolded, soluble protein is important to proper layering of proteins and carbohydrate during laminar flow in the extruder (14, 31). However, decreased protein-protein interactions and covalent bonding as sulfonated samples are heating may prevent proper protein crosslinking during texturization (14), yielding a product that may be less stable.

Sulfonation improved foam and emulsion properties, suggesting that these properties are greatly influenced by disulfide bond content and to some extent electrostatic charge. While native whey proteins, with a high concentration of disulfides, do not unfold as easily when subjected to mechanical work, sulfonated proteins are more likely to unfold and be flexible. This is a possible explanation of why whey proteins are not as functional as other proteins in foams and emulsions. Decreased gel strength with increasing sulfonation, as well as decreased foam overrun and stability at high levels of sulfonation (greater than 54%), suggest that increased electrostatic charges are also important to a protein’s functional characteristics. While the decreased melt temperature and increased protein resolubilization in SDS at suggest that sulfonated WPC may have enhanced extrudability, decreased protein-protein interactions may limit proper texturization and stability of the extrudate.
Correlation among WPC Functional Properties. Correlation analysis was performed to determine the correlation among functional properties and to the level of sulfonation. Percent sulfonation was correlated to all functional properties except EAI, $T_{1/2}$, and foam overrun and every individual functional property was correlated to at least one other property (Table 5). In general, sulfonation was positively correlated to properties related to protein unfolding and protein-solvent interactions ($\phi$ and protein resolubilization) and negatively correlated to properties related to resistance to unfolding ($T_o$ and $T_{max}$) and protein-protein interactions ($G^*$). These correlations support the previous drawn conclusions that sulfonation increased functional properties related to flexibility and decreased those related to protein-protein interactions.

Functional properties negatively correlated included EAI to $T_o$, $G^*$ to $\phi$ and resolublized WPC protein, and $T_{max}$ to resolublized WPC protein after heating in water. Generally positive correlations were present between $T_{1/2}$ to foam overrun, $T_o$ to $T_{max}$, and all of the measures of resolublized WPC to one another.

EAI and $T_o$ both are related to protein unfolding. In general, proteins with high EAI values are flexible and have open structure (2) while proteins with a high $T_o$ values resist unfolding during heating. Since these properties are inversely related to one another, the negative correlation between the two was expected. $G^*$ measures the strength of a gel
Table 5. Coefficients of Correlation Between Sulfonated WPC Functional Properties. Significant (p < 0.05) Coefficients are Displayed with Bold Text

<table>
<thead>
<tr>
<th>Property</th>
<th>Sulfonation</th>
<th>EAI</th>
<th>$T_o$</th>
<th>$G^*$</th>
<th>$\phi$</th>
<th>$T_{max}$</th>
<th>Water/SDS</th>
<th>Water/βME</th>
<th>Base/SDS</th>
<th>Base/βME</th>
<th>$T_{\mu}$</th>
<th>Overrun</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAI</td>
<td>0.678</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_o$</td>
<td>-0.824</td>
<td>-0.870</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G^*$</td>
<td>-0.902</td>
<td>-0.561</td>
<td>0.554</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\phi$</td>
<td>0.802</td>
<td>0.259</td>
<td>-0.324</td>
<td>-0.944</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{max}$</td>
<td>-0.929</td>
<td>-0.688</td>
<td>0.935</td>
<td>0.679</td>
<td>-0.549</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water/SDS</td>
<td>0.948</td>
<td>0.610</td>
<td>-0.643</td>
<td>-0.993</td>
<td>0.921</td>
<td>-0.763</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water/βME</td>
<td>-0.909</td>
<td>-0.322</td>
<td>0.624</td>
<td>0.796</td>
<td>-0.828</td>
<td>0.856</td>
<td>-0.841</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base/SDS</td>
<td>0.906</td>
<td>0.624</td>
<td>-0.593</td>
<td>-0.997</td>
<td>0.914</td>
<td>-0.690</td>
<td>0.992</td>
<td>-0.767</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base/βME</td>
<td>0.873</td>
<td>0.425</td>
<td>-0.461</td>
<td>-0.987</td>
<td>0.984</td>
<td>-0.638</td>
<td>0.974</td>
<td>-0.832</td>
<td>0.971</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{\mu}$</td>
<td>0.199</td>
<td>0.489</td>
<td>-0.048</td>
<td>-0.511</td>
<td>0.363</td>
<td>0.085</td>
<td>0.437</td>
<td>0.114</td>
<td>0.548</td>
<td>0.433</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Overrun</td>
<td>0.285</td>
<td>0.159</td>
<td>0.145</td>
<td>-0.668</td>
<td>0.683</td>
<td>0.085</td>
<td>0.574</td>
<td>-0.162</td>
<td>0.663</td>
<td>0.672</td>
<td>0.852</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Sulfonation: % sulfonation  
EAI: Emulsification activity index  
$T_o$: Protein melt onset temperature (°C)  
$G^*$: Gel strength (Pa)  
$\phi$: Emulsified oil volume fraction  
$T_{max}$: Protein melt maximum temperature (°C)  
Water/SDS: Resolubilized WPC protein heated in water and dissolved in 1% SDS  
Water/βME: Resolubilized WPC protein heated in water and dissolved in 1% SDS/β-ME  
Base/SDS: Resolubilized WPC protein heated in 0.2 M NaOH  
Base/βME: Resolubilized WPC protein heated in 0.2 M NaOH and dissolved in 1% SDS/β-ME  
$T_{\mu}$: Foam stability (s)  
Overrun: Foam overrun (%)
(and protein-protein interactions) whereas $\phi$ is often reflective of protein-solvent interactions. Hence, the negative correlation between the two is intuitive. $G^*$ was also generally negatively correlated to protein resolubilization while $\phi$ was generally positively correlated.

Some of the measurements of protein functionality evaluate very similar properties and thus were correlated to each other. $T_{1/2}$ and foam overrun both are related to protein unfolding at the aqueous-air interface and their ability to form a foam, $T_o$ and $T_{max}$ both measure protein unfolding during heating, and the measures of resolublized WPC protein each measure resolublized protein in given conditions. Thus it was not surprising that these sets of measurements were each correlated to one another.

In summary, limited sulfonation (up to 31%) generally enhanced functional performance because of unfolding of the otherwise compact protein. Sulfonation to greater degrees may decrease their functional performance in general food systems because of increased electrostatic charge that limits interaction of the proteins with themselves and other components. Correlation between the functional properties and level of sulfonation supported these conclusions.

SAFETY

All experiments were conducted using standard laboratory safety procedures.
LITERATURE CITED


CHAPTER III

INFLUENCE OF SULFONATION ON THE PROPERTIES OF EXPANDED EXTRUDATES CONTAINING 32% WHEY PROTEIN

ABSTRACT

Whey protein concentrate (WPC) was treated with sodium sulfite to achieve 4 levels of disulfide bond sulfonation (0, 31, 54, and 71%). Sulfonated WPC was blended with cornstarch to a 32% final protein concentration and extruded into an expanded snack product. Extrudates were collected at two temperatures (160 and 170 °C) and analyzed for physical (air cell diameter, expansion ratio, breaking strength, and density) and chemical (water adsorption index (WAI), water solubility index (WSI), moisture content, resolublized protein and soluble carbohydrates) properties. The control and 54% sulfonated samples had larger expansion ratios and air cell diameters and smaller densities and breaking strengths than the 31 and 71% sulfonated samples. Expansion properties increased at the higher collection temperature in the sulfonated samples, but not in the control. WAI was influenced by the interaction of sulfonation and temperature while the other chemical properties (except moisture content) were influenced only by sulfonation level. Differences within WAI and WSI were minor, as these characteristics are primarily influenced by the starch properties. Resolublized protein and soluble carbohydrate were highest in the control and 54% sulfonated samples. The anomalous
behavior of the 54% sulfonation level may have been the result of significant structural and functional changes of α-Lb that are predicted to occur at approximately 50% sulfonation. Correlation analysis was performed using the means of functional properties measured in Chapter II (i.e. emulsification activity index, foam stability, and protein melt temperatures) and the means of the extrudate properties. The same increased protein unfolding that altered functional properties as a result of sulfonation may have promoted both smaller air cells and protein-starch interactions. Both may have contributed to the limited expansion in the 31 and 71% sulfonated extrudates.

INTRODUCTION

Extrusion cooking is described as a process whereby moistened starch and protein materials are cooked and worked into viscous, plastic-like dough (1). Extrusion processes are used to produce such foods as ready to eat cereals, snack foods, and meat extenders. Although extrusion is a common food process, it is more of an art than a science. While references exist on extrusion process control and factors that affect the extrusion of various foods, data is largely empirical. As a result, there is a general lack of understanding regarding many aspects of food extrusion.

In general, the incorporation of whey proteins into starch-based expanded snacks leads to detrimental effects on physical properties. Matthey and Hanna (2) extruded starch based extrudates containing 30% WPC 80 (80% protein) and concluded that both whey
protein and amylose content should be low to obtain desirable product expansion.

Onwulata et al. (3,4) incorporated WPC 34 (34% protein) into extrudates made from corn, rice, and potato flours and concluded that using 25% or more of the WPC 34 led to increased water holding capacity, decreased expansion, and increased breaking strength (all undesirable qualities). However, using 25% WPC 80 or whey protein isolates in conjunction with wheat fiber greatly increased extrudate expansion (5). Whey protein expanded snacks have also been extruded using supercritical carbon dioxide, rather than heat and pressure, as a means to increase expansion in the extrudate (6-8). Whey protein use levels, however, were limited to 10% protein by weight.

It is not well understood why whey proteins must be used at lower concentrations than other proteins (i.e. soy) in extruded snack products. Typical soy based snacks contain 25% or more total protein (9). One possible explanation is the relatively high concentration of disulfide bonds in whey proteins compared to other food proteins (10). Disulfide bonds stabilize tertiary protein structure and may limit unfolding and denaturation during extrusion. Extruding other proteins with chemical reducing agents, which cleave disulfide bonds, has been shown to improve the flow and melt characteristics of proteins while in the extruder barrel (11). Reducing the disulfide bond content in whey proteins may improve the extrudate properties and allow for increased amounts of protein to be incorporated into snack products.
Sulfitolysis is a chemical reaction where disulfide bonds in a protein are broken by sodium sulfite, producing a free thiol and a sulfonated cysteine derivative (12). Free thiols can be reoxidized in the presence of catalyst and oxygen to disulfides and the sulfonation process repeats until the majority of the disulfides are cleaved or the reaction is chemically stopped. Through the sulfonation of disulfide bonds prior to extrusion, the effects of disulfide bond concentration on extrusion performance can be determined.

Sulfonation of whey proteins significantly changes their functional properties, including gel strength ($G^*$), foam overrun, foam stability, emulsified oil volume fraction ($\phi$), emulsification activity index (EAI), denaturation temperatures ($T_o$ and $T_{max}$), and resolublized protein after heating. Similarly, we hypothesize that the physical and chemical properties of an expanded whey product are related to the concentration of disulfide bonds. Thus, the main objective of this research was to extrude expanded snacks using sulfonated whey protein concentrates (of varying disulfide contents) and measure their chemical characteristics (water absorption index, water solubility index, resoluble protein, soluble carbohydrate, and moisture content) and physical properties (air cell diameter, expansion ratio, breaking strength, and density). We also explored whether extrudate characteristics are correlated with the sulfonated whey proteins’ functional properties measured in Chapter II.
MATERIALS AND METHODS

**Materials.** Sulfonated whey protein concentrate used in extrusion is described in Chapter II. Degree of sulfonation and protein content analysis are also described there.

**Experimental Design and Statistical Analysis.** WPC from each level of sulfonation was extruded twice (2 replicates) and during each replicate extrusion the extrudates were collected at 2 exit temperatures (160 and 170 °C) (see Appendix C for the experimental design diagram). Extrudates from each combination of sulfonation level, replicate, and collection temperature were measured and analyzed using the MANCOVA procedure of Statistica (Statsoft, Tulsa, OK). The interaction effects from the variable “Replicate” were pooled with the error to control for variability between replicates. Significance was determined at p < 0.05 and post-hoc mean comparisons were performed using the Tukey’s HSD test. Air cell diameter analysis of extrudates from each combination of sulfonation level, replicate, and temperature was made with 20 measurements. Extrudate density, breaking strength, and expansion ratio were made with 10 measurements (the natural log of breaking strength was used in the statistical analysis to normalize the data for unequal variance between samples). All physical properties were measured using 3 measurements, except soluble carbohydrate (six measurements) and moisture (two measurements). ANOVA tables for the statistical analyses are contained in Appendix D.
Correlation analysis was performed on Statistica using a spreadsheet containing mean values for extrudate characteristics and functional properties at each level of sulfonation. Extrudate characteristics of the expanded snack included moisture content, WAI, WSI, resolublized protein, soluble carbohydrate, air cell diameter, expansion ratio, breaking strength, and density. Functional properties of the control and sulfonated WPCs included gel strength (G*), foam overrun, foam drainage time (T½), emulsified oil volume fraction (φ), emulsification activity index (EAI), protein melt temperatures, and resolublized protein after heating (see Tables 2, 3, and 4). Significance of the correlation coefficients was determined at p < 0.05.

**Extrusion of Expanded Snacks.** The expanded snacks were extruded from a dry mix containing 32% total whey protein (approximately 40% WPC (80% protein) and 60% cornstarch (purchased locally)). The content of WPC and cornstarch were individually determined for each sulfonation level to account for variability in the actual protein content of the WPCs at each level. Extrudates were produced using an APV Baker MPF 19 twin screw extruder (Grand Rapids, MI). The final protein content of the dry mix was 32%. Approximately 1200 g of dry mix was loaded into the feed hopper. Extruder screws were configured to promote increased shear and pressure inside the barrel (see Appendix E). Sodium hydroxide (0.1 M) was pumped into the barrel at a rate of 4 g/min while the dry mix was fed into the barrel at a rate of 23 g/min. Extrudates were collected
at exit temperatures of 160 and 170 °C. Extrudates were allowed to dry overnight at room temperatures before storing in airtight plastic bags.

**Average Air Cell Diameter.** Approximately five randomly selected extrudates from each combination of block, sulfonation level, and collection temperature were embedded in melted household wax (Parowax, Roswell, GA) and allowed to cool overnight. Embedded extrudates were cut lengthwise with a razor to expose the longitudinal cross section. Images of the cross section were taken using a digital camera (Nikon Coolpix 5700) mounted to a stand. The camera lens was positioned approximately 6.5 cm above the extrudates. Camera settings included a focal length of 15.7 mm and a picture setting of Fine (2560 x 1920 pixel resolution).

Twenty air cells from the images of the cross sections were randomly selected and analyzed using Adobe PhotoShop (Adobe Systems Inc., San Jose, CA). Air cell surface area was estimated using the Magnetic Lasso tool to determine the air cell circumference and a total pixel count within that circumference. The diameters were calculated from the pixel count using a standard curve made from paper cut to known areas and photographed in the same manner of the extrudates.

**Extrudate Density.** Ten extrudate “sticks” were randomly selected from each combination of block, sulfonation level, and collection temperature and trimmed at one end to give an approximate weight of 1.1 g. Samples were weighed and placed into 100-
ml graduated cylinder before the both were tarred. Ottawa sand (60 mesh, average density of 1.68 g/cm$^3$) was added to the cylinder and the full cylinder was reweighed. The density of the extrudate was determined as follows:

\[
\text{Weight of the extrudate} \times (\text{Weight of } 100 \text{ cm}^3 \text{ of sand-weight of sand to fill the tarred cylinder})/(1.68 \text{ g/cm}^3)
\]

**Breaking Strength and Expansion Ratio.** A Salter 235 shear device with a Warner-Bratzler shear cell (GR Electric Manufacturing, Manhattan, KS) was used to determine breaking strength of the extrudates. Ten extrudates sticks were randomly selected from each combination of block, sulfonation level, and collection temperature and sheared at approximately 3, 6, and 9 cm from one end. The diameter of the break was measured to determine the expansion ratio. Breaking strength force was calculated using the following equation:

\[
\text{Force (Pa)} = \frac{(9.7865 \text{ N/kg}) \times \text{(breaking strength (kg))}}{\pi \times \text{(extrudate diameter (m))/2}}^2
\]

The values for both expansion ratio and breaking strength from the three breaks on each stick were averaged to obtain one mean value for each extrudate, and these values were used in further statistical analysis.

**Moisture Determination.** Two small sections of dried extrudate (0.5 g) were randomly selected and placed in pre-weighed aluminum dishes and dried overnight at 70
°C in a drying oven. Pans were allowed to cool and were weighed to determine the moisture content.

**Water Adsorption Index (WAI) and Water Solubility Index (WSI).** The water adsorption index (WAI) and water solubility index (WSI) were performed according to Jin et al. (13) with minor modifications. Ground sample from each combination of block, sulfonation level, and collection temperature (0.5 g), that passed through a 60-mesh screen, was combined with 5.0 ml of distilled water in a tarred centrifuge tube. The mixture was sealed, vortex, and allowed to hydrate for 10 min. The sealed tube was inverted three times at both 5 and 10 min to ensure proper mixing. After the 10 minutes, samples were centrifuged for 15 min at 1000 x g using a Sorvall RC-5B fixed angle rotor (DuPont Instruments, Wilmington, DE) and the supernatant was decanted into a pre-weighed aluminum dish. The tube was inverted for 5 min over the dish to catch residual moisture. The dish was allowed to dry overnight in a drying oven (70 °C) and the centrifuge tube was reweighed to determine the weight of the sediment. WAI was calculated by dividing the sediment weight by the dry sample weight while WSI was calculated by dividing the dried supernatant weight by the dry sample weight (13).

**Resolublized Protein and Soluble Carbohydrate.** Extrudates were ground until they passed through a 16-mesh screen and were retained in a 60-mesh screen. Ground sample (200 mg) was placed into a 15-ml centrifuge tube and a solution (10-ml) of 1-%
sodium dodecyl sulfate (SDS) and 1-% beta-mercaptoethanol (β-ME) was added. The tubes were sealed and were rocked overnight on a laboratory rocker (Rocking Platform 200, VWR Scientific, Bristol, CT) on a rock setting of 4. The samples were centrifuged, filtered, and analyzed for soluble protein according to Mohammed et al. (14) using a modified Lowry protein assay (Pierce, Rockford, IL) with bovine serum albumin as standard. The filtered supernatant (200 µl) was diluted 1:4 and soluble carbohydrate analysis was performed using the Dubois Assay (15) with glucose used as standard.

RESULTS AND DISCUSSION

Air Cell Diameter, Expansion Ration, Breaking Strength, and Density. While only sulfonation level influenced air cell diameter (p = 0.0002), density was influenced by sulfonation level (p = 0.0070) and temperature (p = 0.0132). Breaking strength was influenced by a sulfonation level-temperature interaction (p = 0.0124) while expansion ratio was influenced by both sulfonation level (p = 0.0104) and temperature (p = 0.0397). In general, physical measurements of the control extrudates were not affected by temperature. However, temperature had a significant influence on these measurements at the other three sulfonation levels. In each of the other levels, extrudate density and breaking strength generally decreased as collection temperature was raised to 170 °C, while expansion ratio increased. Increased temperature may have increased both flashing
off of moisture from the extrudate upon exiting the die and the content of gelatinized starch, both of which are correlated to increased extrudate expansion (11).

The control and 54% sulfonated samples had greater air cell diameters and expansion ratio and smaller densities and breaking strengths than the 31 and 71% samples (Figure 1 and Table 6). Protein-carbohydrate interactions may have been responsible for limiting product expansion, particularly at the 31% and 71% sulfonation levels. Insoluble protein-starch aggregates, which are known to limit expansion (2), may have formed. Increased protein unfolding, resulting from disulfide bond cleavage and sulfonation, may have exposed potential protein-carbohydrate interaction sites.

Since extrudate expansion is important to the overall acceptance of extruded snack foods (16), it may be important to limit protein-carbohydrate interactions. Limiting these interactions may be accomplished through the use of a protein with a compact molecular structure (like the control sample) and by reducing the amount of carbohydrate (such as lactose and degraded starch) available for interaction with proteins. In addition to limiting protein-carbohydrate interaction, higher collection temperatures may also promote expansion.

**Moisture, WAI, WSI, Resolublized Protein, and Soluble Carbohydrate.** Neither sulfonation nor collection temperature had an effect on the final moisture levels
Figure 1. Longitudinal cross sections of the control and sulfonated extrudates collected at 170 °C. A: Control (0% sulfonated), B: 31% sulfonated; C: 54% sulfonated; D: 71% sulfonated. Bar: 5 mm.

Table 6. Mean Values for Physical Tests of Control and Sulfonated Extrudates Collected at 160 and 170 °C

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Collection Temperature</th>
<th>N</th>
<th>0%</th>
<th>31%</th>
<th>54%</th>
<th>71%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air Cell Diameter (mm)</td>
<td>160</td>
<td>40</td>
<td>8.0 ± 1.2 a</td>
<td>6.1 ± 1.0 b</td>
<td>7.7 ± 0.8 a</td>
<td>6.4 ± 1.6 b</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>40</td>
<td>8.1 ± 1.2 b</td>
<td>6.4 ± 1.5 b</td>
<td>8.2 ± 1.4 a</td>
<td>6.2 ± 1.4 b</td>
</tr>
<tr>
<td>Density (g/cm³)</td>
<td>160</td>
<td>20</td>
<td>0.13±0.04 ab</td>
<td>0.31±0.08 c</td>
<td>0.18±0.04 ab</td>
<td>0.28±0.09 c</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>20</td>
<td>0.13±0.03 ab</td>
<td>0.20±0.12 b</td>
<td>0.10±0.01 a</td>
<td>0.19±0.05 b</td>
</tr>
<tr>
<td>Break Strength (ln Pa)</td>
<td>160</td>
<td>20</td>
<td>3.8±0.5 b</td>
<td>5.6±0.5 e</td>
<td>3.9±0.5 b</td>
<td>5.8±0.5 e</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>20</td>
<td>3.7±0.4 b</td>
<td>5.0±0.3 d</td>
<td>3.3±0.4 a</td>
<td>4.0±0.6 c</td>
</tr>
<tr>
<td>Expansion Ratio</td>
<td>160</td>
<td>20</td>
<td>3.6±0.5 c</td>
<td>2.8±0.6 b</td>
<td>3.4±0.3 c</td>
<td>2.3±0.3 a</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>20</td>
<td>3.6±0.5 c</td>
<td>3.5±0.7 c</td>
<td>4.1±0.3 d</td>
<td>2.8±0.3 b</td>
</tr>
</tbody>
</table>

abcde Values with common letters for each measurement were not different (p > 0.05). Mean±SD.
of the extrudates \( (p = 0.3346 \text{ and } 0.6924, \text{ receptively}) \) (Table 7). Moisture levels were approximately 6.6-7.2\%. Sulfonation level and extrudate collection temperature interacted to affect WAI but only at the 31\% sulfonation level \( (p = 0.0043) \). At the 31\% sulfonation level, WAI was lowest at 160 °C and was highest in samples collected at 170 °C. WAI at all other levels of sulfonation and collection temperatures were not significantly different. Level of sulfonation influenced WSI \( (p = 0.0389) \), resolublized protein \( (p = 0.0084) \), and soluble carbohydrate \( (0.0124) \).

While WSI was generally lower in the control and higher in the other sulfonated samples, resolublized protein was highest in the control and 54\% sulfonated samples and was generally lower at the 31 and 71\% levels (Table 7). Soluble carbohydrate levels were similar in the samples sulfonated up to 54\%. The 71\% sulfonated sample had less soluble carbohydrate than the control or 54\% sulfonated samples.

WAI is primarily dependent on the concentration of unbroken polymer chains, such as the cornstarch in the extrudates (13). In general, unbroken starch molecules bind water better than broken ones and result in higher WAI values. A high WAI level is correlated to poor consumer acceptability, especially in breakfast cereal applications, because broken starch rapidly absorbs water and reduces product crispiness and crunchiness (6). Since WAI is primarily dependent upon the starch and not the protein, it is not surprising that samples had similar WAI values. Differences in the 31\% sulfonated sample
Table 7. Mean Values for Chemical Tests on Control and Sulfonated Extrudates Collected at 160 and 170 °C

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Collection Temperature</th>
<th>N</th>
<th>0%</th>
<th>31%</th>
<th>54%</th>
<th>71%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>160</td>
<td>4</td>
<td>6.6±0.1 a</td>
<td>6.6±1.1 a</td>
<td>6.6±0.3 a</td>
<td>7.2±0.2 a</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>4</td>
<td>6.6±0.1 a</td>
<td>6.9±0.1 a</td>
<td>6.6±0.1 a</td>
<td>6.7±0.3 a</td>
</tr>
<tr>
<td>WAI</td>
<td>160</td>
<td>6</td>
<td>3.0±0.2 a</td>
<td>2.7±0.3 a</td>
<td>3.3±0.2 a</td>
<td>3.3±0.1 a</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>6</td>
<td>3.0±0.2 a</td>
<td>3.8±0.3 b</td>
<td>3.1±0.2 a</td>
<td>3.2±0.1 a</td>
</tr>
<tr>
<td>WSI</td>
<td>160</td>
<td>6</td>
<td>38±5 ab</td>
<td>43±3 abcd</td>
<td>44±2 cd</td>
<td>43±4 bcd</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>6</td>
<td>38±5 ab</td>
<td>40±3 abc</td>
<td>46±1 d</td>
<td>45±1 cd</td>
</tr>
<tr>
<td>Resolubilized Protein (%)</td>
<td>160</td>
<td>9</td>
<td>78±7 ab</td>
<td>68±7 ab</td>
<td>84±6 a</td>
<td>65±10 b</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>9</td>
<td>78±7 ab</td>
<td>64±7 b</td>
<td>82±13 a</td>
<td>64±15 b</td>
</tr>
<tr>
<td>Soluble Carbohydrate (%)</td>
<td>160</td>
<td>12</td>
<td>40±4 ab</td>
<td>38±6 abc</td>
<td>40±6 ab</td>
<td>33±5 d</td>
</tr>
<tr>
<td></td>
<td>170</td>
<td>12</td>
<td>42±2 a</td>
<td>39±6 abc</td>
<td>41±5 ab</td>
<td>35±4 cd</td>
</tr>
</tbody>
</table>

abcd Values with common letters within each chemical test were not different (p > 0.05)
Mean ± Standard deviation (SD).
collected at 170 °C may have resulted from protein-starch interactions. Whey proteins
have been shown to interact with starch and prevent its degradation (2).

WSI was lowest in the control sample and increased in the sulfonated samples (Table
6). Although differences were significant, they were relatively small. As with WAI,
increased WSI is correlated to poor consumer acceptability (6). WSI is a measure of the
extent the extrudate is solubilized during hydration, thus it depends upon starch
degradation (more degradation is correlated to higher WSI values) and the concentration
of other soluble molecules, such as minerals and lactose (13). Because WSI is primarily
dependent upon carbohydrates and other small molecules, and extrudates had similar
starch concentrations, it was not unexpected that the difference observed between the
samples was small.

The differences in the levels of resolublized protein and soluble carbohydrate are
likely the result of protein-carbohydrate interactions. Decreases in both resolublized
protein and soluble carbohydrate at the 71% sulfonation level suggest that carbohydrate-
protein interactions (possibly mallard type reactions) increased. Similar reactions were
likely present in the 31% sulfonated samples but was less prevalent in the control or 54%
sulfonated samples. As disulfide bonds were cleaved by sulfonation, the proteins likely
had increased ability to unfold in the extruder barrel. This unfolding may have allowed
more amino acid residues to be exposed for potential protein-carbohydrate interaction, promoting the formation of insoluble protein-starch aggregates.

It is not understood why the 54% sulfonated sample retained high levels of resolublized protein and soluble carbohydrate and had similar physical properties to the control sample. One possible hypothesis is that this anomalous behavior may have been the result of the significant changes in the \( \alpha\)-Lb fraction of the WPC. In a WPC containing 52-65\% \( \beta\)-Lg, 15-21\% \( \alpha\)-Lb, and 3-5\% BSA (17), the percentage of disulfides in the WPC from these three fractions would be approximately 40, 30, and 30\%, respectively. While the rate and order at which the different whey protein fractions undergo sulfonation is not well known, it is believed that the majority of the first bonds cleaved are in the \( \beta\)-Lg and \( \alpha\)-Lb fractions. Disulfide bonds in BSA, which were shown to be difficult to sulfonate (18), presumably are cleaved last. At the 31\% sulfonation level, the majority of the bonds cleaved were likely those of \( \beta\)-Lg. As the sulfonation reaction proceeded to the 54\% sulfonation level, approximately half of those in \( \alpha\)-Lb were additionally cleaved. Cleavage of these bonds may have significantly changed the physical structure of the protein, inducing a structure similar to the molten globule conformation (19). This structure has markedly different functional properties than the unaltered protein and may be significantly changing the overall performance of the WPC. This significant change may have been responsible for the 54\% sulfonated sample's
unusual behavior compared to the other sulfonated samples. As the sulfonation level increased to 71%, the remaining disulfide bonds in α-Lb are likely cleaved, further changing its properties and the overall characteristics of the WPC.

**Correlation among Extrudate Properties.** Most extrudate physical properties (air cell diameter, density, breaking strength, and expansion ratio) were correlated to the amount of resolublized protein and soluble carbohydrate of the extrudates (Table 8). Additionally, air cell diameter, density, breaking strength, and expansion ratio were all correlated to one another. Moisture content was negatively correlated to soluble carbohydrate. No correlations existed between WAI and WSI with the other extrudate characteristics.

The amount of protein and carbohydrate in the extrudate that can be resolublized in 1% SDS/β-ME may be important factors in determining extrudate air cell diameter, density, breaking strength, and expansion ratio. As proteins and carbohydrates interact and form insoluble aggregates, air cell diameter and expansion decreased while density and breaking strength increased. Thus, it may be important to maintain high levels of soluble carbohydrate and protein to promote extrudate expansion. Possible reactions that lead to protein and carbohydrate insolubility include maillard and isopeptide reactions (3).
Table 8. Coefficients for Correlation Between Measured Expanded Extrudate Characteristics. Significant (p < 0.05) Coefficients are Displayed with Bold Text

<table>
<thead>
<tr>
<th></th>
<th>Cell</th>
<th>Density</th>
<th>Break</th>
<th>Expansion</th>
<th>Protein</th>
<th>Carbs</th>
<th>WAI</th>
<th>WSI</th>
<th>Moist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Density</td>
<td>-0.885</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Break</td>
<td>-0.805</td>
<td>0.966</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expansion</td>
<td>0.795</td>
<td>-0.849</td>
<td>-0.784</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>0.917</td>
<td>-0.754</td>
<td>-0.731</td>
<td>0.710</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbs</td>
<td>0.776</td>
<td>-0.732</td>
<td>-0.663</td>
<td>0.902</td>
<td>0.755</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WAI</td>
<td>-0.197</td>
<td>0.165</td>
<td>0.103</td>
<td>0.056</td>
<td>-0.291</td>
<td>-0.160</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSI</td>
<td>-0.248</td>
<td>0.132</td>
<td>0.001</td>
<td>-0.188</td>
<td>-0.021</td>
<td>-0.369</td>
<td>-0.025</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Moist</td>
<td>-0.527</td>
<td>0.655</td>
<td>0.687</td>
<td>-0.622</td>
<td>-0.638</td>
<td>0.744</td>
<td>0.541</td>
<td>0.037</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Cell: Air cell diameter (mm)
Density: Extrudate density (g/cm³)
Shear: Breaking strength (In Pa)
Expansion: Extrudate expansion ratio
Protein: Resolubilized protein in 1% SDS/B-ME
Carbohydrate: Resolubilized carbohydrate in 1% SDS/B-ME
WAI: Water adsorption index
WSI: Water solubility index
Moist: % moisture
The negative correlation between soluble carbohydrate and moisture level may be related to starch degradation because intact starch (which is less soluble) binds and holds more water (13). WAI and WSI, which are largely dependent upon the amount of starch degradation and gelatinization (4), were not correlated to other characteristics.

**Correlation Between WPC Functional Properties and Expanded Extrudate Characteristics.** All WPC functional properties were correlated to at least one extrudate property, with the exception of foam overrun (Table 9). EAI, perhaps the most significant functional property in relating extrudate and WPC properties, was positively correlated to extrudate density and negatively correlated to resolublized protein, soluble carbohydrate, cell diameter, and extrudate expansion. $T_{1/2}$ was positively correlated to extrudate density and breaking strength and negatively correlated to cell diameter. $T_0$ was positively correlated to soluble carbohydrate and expansion ratio.

EAI, $T_{1/2}$, and $T_0$ are reflective of a sulfonated whey protein's molecular flexibility. While EAI and $T_{1/2}$ are specifically related to a protein's ability to be flexible at the interface between polar and non-polar phases, $T_0$ is related to protein flexibility and unfolding during heating. The correlation between these properties and extrudate expansion suggest that protein flexibility may be related to air cells size and expansion ratio.
Table 9. Coefficients of Correlation Between Sulfonated WPC and Expanded Extrudate Characteristics. Significant (p < 0.05)

Coefficients are Displayed with Bold Text

<table>
<thead>
<tr>
<th></th>
<th>WAI</th>
<th>WSI</th>
<th>Protein</th>
<th>Carbs</th>
<th>Moist</th>
<th>Cell</th>
<th>Density</th>
<th>Break</th>
<th>Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAI</td>
<td>0.238</td>
<td>0.376</td>
<td>-0.879</td>
<td>-0.949</td>
<td>0.700</td>
<td>-0.874</td>
<td>0.733</td>
<td>0.650</td>
<td>-0.847</td>
</tr>
<tr>
<td>T_{1g}</td>
<td>0.246</td>
<td>0.161</td>
<td>-0.700</td>
<td>-0.336</td>
<td>0.301</td>
<td>-0.814</td>
<td>0.751</td>
<td>0.732</td>
<td>-0.379</td>
</tr>
<tr>
<td>T_{o}</td>
<td>-0.189</td>
<td>-0.538</td>
<td>0.553</td>
<td>0.899</td>
<td>-0.622</td>
<td>0.548</td>
<td>-0.428</td>
<td>-0.317</td>
<td>0.723</td>
</tr>
<tr>
<td>T_{max}</td>
<td>-0.200</td>
<td>-0.734</td>
<td>0.275</td>
<td>0.744</td>
<td>-0.487</td>
<td>0.339</td>
<td>-0.252</td>
<td>-0.118</td>
<td>0.527</td>
</tr>
<tr>
<td>G*</td>
<td>-0.330</td>
<td>-0.872</td>
<td>0.317</td>
<td>0.521</td>
<td>-0.356</td>
<td>0.541</td>
<td>-0.482</td>
<td>-0.349</td>
<td>0.355</td>
</tr>
<tr>
<td>Overrun</td>
<td>0.288</td>
<td>0.883</td>
<td>-0.004</td>
<td>-0.240</td>
<td>0.140</td>
<td>-0.265</td>
<td>0.251</td>
<td>0.127</td>
<td>-0.079</td>
</tr>
<tr>
<td>Water/SDS</td>
<td>0.324</td>
<td>0.889</td>
<td>-0.327</td>
<td>-0.584</td>
<td>0.395</td>
<td>-0.535</td>
<td>0.468</td>
<td>0.329</td>
<td>-0.402</td>
</tr>
<tr>
<td>Water/βME</td>
<td>-0.209</td>
<td>-0.894</td>
<td>-0.110</td>
<td>0.384</td>
<td>-0.213</td>
<td>0.065</td>
<td>-0.038</td>
<td>0.103</td>
<td>0.161</td>
</tr>
<tr>
<td>Base/SDS</td>
<td>0.336</td>
<td>0.851</td>
<td>-0.392</td>
<td>-0.578</td>
<td>0.401</td>
<td>-0.604</td>
<td>0.535</td>
<td>0.403</td>
<td>-0.416</td>
</tr>
<tr>
<td>Base/βME</td>
<td>0.313</td>
<td>0.895</td>
<td>-0.165</td>
<td>-0.397</td>
<td>0.259</td>
<td>-0.409</td>
<td>0.371</td>
<td>0.239</td>
<td>-0.229</td>
</tr>
</tbody>
</table>

EAI: Emulsification activity index  
T_{1g}: Foam stability (s)  
T_{o}: Protein melt onset temperature (°C)  
T_{max}: Protein melt maximum temperature (°C)  
G*: Gel strength (Pa)  
ϕ: Emulsified oil volume fraction  
Overrun: Foam overrun (%)  
Water/SDS: Resolubilized WPC protein heated in water and dissolved in 1% SDS  
Water/βME: Resolubilized WPC protein heated in water and dissolved in 1% SDS/β-ME  
Base/SDS: Resolubilized WPC protein heated in 0.2 M NaOH and dissolved in 1% SDS  
Base/βME: Resolubilized WPC protein heated in 0.2 M NaOH and dissolved in 1% SDS/β-ME
In addition to promoting increased protein-starch interactions (as discussed above), flexible proteins may also directly influence air cell size. Proteins with increased flexibility often have increased ability to interact with the different phases in both emulsions and foams, creating smaller air or oil droplets that are more stable to coalescence (20). Extrusion may be similar in that flexible proteins may promote the formation of smaller air cells, resulting in decreased expansion as the extrudate leaves the barrel. This decreased expansion then translates into higher extrudate density and breaking strength. Similar EAI values for both the control and 54% sulfonated WPC help to explain why the 54% sample had extrudate properties similar to the control compared to the other sulfonated extrudates.

Although WAI was not correlated to any functional property of sulfonated WPC, WSI was correlated to $T_{\text{max}}$, $G^*$, $\phi$, and resolublized WPC protein. Both $T_{\text{max}}$ (high $T_{\text{max}}$ indicates resistance to unfolding) and $G^*$ (a measure of protein-protein interactions as proteins are heated) were negatively correlated to WSI. Decreased protein unfolding and increased protein-protein interactions may limit a protein’s ability to interact with and dissolve in water. Thus, the negative correlation between WSI is expected. $\phi$ and resolublized WPC, both of which are related to unfolding and protein-water interactions, were positively correlated with WSI.
As previously mentioned, extrudate expansion is related to the content of protein and carbohydrate that remains soluble during extrusion. It is thus not surprising that the some of the functional properties correlated to expansion were also correlated to soluble protein and carbohydrate. Resolublized protein was negatively correlated to EAI while soluble carbohydrate was negatively correlated to EAI and positively correlated to $T_o$ and $T_{max}$. Less protein flexibility (corresponding to low EAI and high $T_o$ and $T_{max}$) may limit both protein-protein and protein-carbohydrate interactions. As these interactions were limited, so would the formation of insoluble protein and starch aggregates. The net result would be higher amounts of soluble protein and carbohydrate.

In conclusion, disulfide bond content and sulfonation did have significant effects on the chemical and physical characteristics of an extrusion-expanded product. Generally, disulfide cleavage through sulfonation had either no beneficial or deleterious effects on extrudate characteristics. The decrease in expansion and air cell diameter and increase in density and breaking strength at the 31 and 71% sulfonated samples may have been the result of increased protein-carbohydrate reactions (as evidenced by the lower amounts of resolublized protein and soluble carbohydrate). These interactions should be limited to increase extrudate expansion. The anomalous behavior of the 54% sulfonated sample may have been the result of significant changes that are believed to occur in the $\alpha$-Lb fraction at approximately 50% sulfonation.
The correlation between functional properties reflective of protein flexibility (i.e. EAI, $T_{1/2}$, and $T_o$) also support the conclusion that protein-carbohydrate interactions were responsible for decreased expansion at the 31 and 71% sulfonation levels. Unfolded and flexible proteins (like those with increased EAI and $T_{1/2}$ values) are likely to have increased exposure of interaction sites, promoting increased protein-carbohydrate interaction. In addition to promoting starch-protein interactions, flexible proteins may have stabilized smaller air cell size, resulting in decreased air cell size and expansion and increased breaking strength and density.

LITERATURE CITED


ABSTRACT

Whey protein concentrate (WPC 80) was treated with sodium sulfite to achieve 4 levels of disulfide bond sulfonation (0, 31, 54, and 71%). This protein was blended with cornstarch to a 48% final protein concentration and extruded under conditions to produce a fibrous product. No extrudates, including the control (0%), developed the fibrous, meat-like texture typical of textured whey protein. Extrudates were hydrated with water (3.85% wt/vol) and incubated for 90 minutes. The control (0% sulfonated) sample had the lowest percentage of extrudate solids lost and the highest soluble protein in the supernatant. Conversely, the 54% sulfonated sample had the highest extrudate loss and lowest soluble protein. Protein-protein interactions may have decreased as a result of increased electrostatic charges on the proteins, suggesting that these interactions are critical for texturization and stability. Increased charge may have prevented the necessary protein crosslinking that enhances extrudate stability and aids in the production of a fibrous extruded product. No significant correlations were found between extrudate stability and sulfonated WPC functional properties.
INTRODUCTION

Textured vegetable proteins (TVP), typically made of extruded soy protein, is a common and inexpensive meat replacer. Recent reports put TVP sales at over $325 million in the U.S. (1). TVP can successfully replace up to 30% of meat in products such as patties, meatballs, taco meat, pizza toppings, and chili (2-4) but the consumer acceptance is generally less than that of all meat products. The characteristic "beany" and "grassy" off flavors associated with soy are often present in the final product. The extent that TVP can be included in products may be greater if the problem with off flavors could be avoided.

Textured whey protein (TWP) was patented by Utah State University and has shown considerable promise as an acceptable extender in ground beef patties (5) or as the main ingredient of non-meat patties (6). During whey protein extrusion, the proteins melt and layer with an immiscible polysaccharide phase within the barrel and die of the extruder (7-11). It is believed that the layering effect is largely dependent upon the degree to which the proteins unfold and melt into random structures. In addition to the layering effect, the unfolded proteins chemically bond with one another, creating a protein network that adds to the stability of the final product (8,12). A cooling die at the end of the extruder limits expansion and maintains the final texture of the extruded product (8).
The extent to which proteins can successfully be incorporated into textured foods may be dependent upon their disulfide bond content (13). Some proteins, like soy, have relatively low disulfide bond contents and have excellent extrusion performance. Whey proteins have a relatively high disulfide content (14) leading to a compact structure that may limit their performance. Through the elimination of disulfide bonds, it may be possible to improve the extrusion of whey proteins, allowing them to be extruded at higher concentrations or lower temperatures than is currently used. TWP made at higher protein concentrations and lower temperatures may improve consumer acceptance (through higher protein content and improved extrudate color and flavor) and lower energy costs.

Sulfitolysis is a chemical reaction where disulfide bonds in a protein are broken by sodium sulfite, producing a free thiol and a sulfonated cysteine derivative (15). This form of disulfide reduction is advantageous compared to reducing by dithiothreitol or beta-mercaptoethanol, because the sulfonated cysteines are not able to oxidize back to the disulfide. The elimination of disulfide bonds may increase protein unfolding in the extruder, exposing potential covalent bonding sites essential for texturization and increasing the layering effect with the polysaccharide phase.

In addition to determining the effects of disulfide bond concentration on the characteristics of textured whey protein, it is of interest to know if textured extrudate
properties are related to the functional properties of the sulfonated WPC. Significant changes occurred in the functional properties of WPC as they were sulfonated (Chapter II) and many of these properties were related to the extrusion of expanded puffed snacks (Chapter III). Significant correlation between functional properties and textured whey properties allow prediction of an extrudates characteristics prior to extrusion.

The hypothesis of this research is that the reduction of disulfide bonds through sulfonation will improve the extrusion performance of whey protein concentrates and that extrusion performance is correlated to the functional properties of sulfonated WPC. The approach was to sulfonate whey protein concentrate to varying levels of disulfide bond content and to extrude them according to patented procedures (16), examine the stability (total solids lost and soluble protein lost) of extrudates, and correlate the stability of the extrudates to the functional properties of the sulfonated proteins reported in Chapter II.

MATERIALS AND METHODS

**Materials.** Preparation of sulfonated whey protein concentrates. Whey protein concentrates (WPCs) used are described in Chapter II.

**Experimental Design and Statistical Analysis.** Each level of sulfonation was extruded in two replicates. Three samples of extrudates from these replicate extrusions were used in the analysis for solids lost and soluble protein in the supernatant. Statistical analysis was performed using the MANCOVA procedure of Statistica (Statsoft, Tulsa,
OK). The effects from the interaction between level of sulfonation and replicate were pooled into the error during the analysis. Significance was determined at $p < 0.05$ and post-hoc mean comparisons were performed using the Tukey's HSD test. ANOVA tables for the statistical analysis are contained in Appendix F.

Correlation analysis was performed using Statistica. Mean values for extrudate stability and functional properties at each level of sulfonation (Chapter II) were used in the analysis. Functional properties of the control and sulfonated WPCs included gel strength ($G^*$), foam overrun, foam drainage time ($T_{1/2}$), emulsified oil volume fraction ($\phi$), emulsification activity index (EAI), protein melt temperatures, and resolublized protein after heating (see Tables 2, 3, and 4). Significance of the correlations was determined at $p < 0.05$.

**Extrusion of Textured Whey Protein.** Extrusion of textured whey protein was performed based on procedures outlined by Hale et al. (5) using a similar screw configuration (see Appendix C). The extrudate was extruded on an APV Baker MPF 19 twin screw extruder (Grand Rapids, MI) from a dry mix containing approximately 60% control or sulfonated WPC and 40% cornstarch (purchased locally). Protein content of the dry mix was 48% (by weight). Sodium hydroxide (0.2 M) was pumped into the barrel at a rate of 8 g/min while the dry mix was fed into the barrel at a rate of 9 g/min. Extrudates exited the extruder barrel at 160 °C and passed through a cooling die before
being collected. Extrudates were dried overnight at room temperatures before storing in airtight plastic bags.

**Soluble Protein in Supernatant and Extrudate Stability.** Soluble protein in the supernatant and extrudate stability (retention) were measured according to Hale et al. (5). Extrudate samples were broken in a blender and passed through sieves. Particles less than 4 mesh but larger than 8 mesh were retained for analysis. Particles (0.385 g) were combined with 10 ml of distilled water and sealed in a 15 ml centrifuge tube. Tubes were placed on a laboratory rocker (Rocking Platform 200, VWR Scientific, Bristol, CT) on a rock setting of 2 and allowed to hydrate for 90 minutes. After 90 minutes, the samples were centrifuged at 3000 x g for 15 minutes. Protein content in the supernatant was determined with the BCA method (Pierce, Rockford, IL) using bovine serum albumin as standard. Soluble protein lost was calculated as a percentage of total protein. The pelleted hydrated sample was placed on an 8-mesh screen and briefly shaken. The hydrated particles retained on the screen were placed in a preweighed aluminum dish and dried overnight at 70 °C. The dried samples were reweighed and the percentage of sample lost and was determined on a dry weight basis. Additional textured whey was hydrated and inspected for fibrous, meat like texture.
RESULTS AND DISCUSSION

**Water Soluble Protein and Extrudate Stability.** After hydration, all samples were soft, pasty and had very little visual textured structure. The texture was more similar to cooked pasta than to a textured meat extender. While all hydrated samples had limited stability, significant differences were present among samples in the solids lost during hydration (p = 0.0500) and water-soluble protein (p = 0.0148). Loss was lowest in the control sample and highest in the 54 and 71% sulfonated samples (Table 10). Solids lost in the samples likely comprised both protein and carbohydrate. Conversely, soluble protein in the supernatant was highest in the control and lowest in the 54% sulfonated sample (Table 10).

Covalent crosslinking between proteins and the layering of protein and starch phases are important for texturization and product stability (12). The additional electrostatic charges resulting from protein sulfonation may have caused protein-protein repulsion. This repulsion may prevent proteins from layering with the starch phase and also

<table>
<thead>
<tr>
<th>Sulfonation (%)</th>
<th>Extrudate Solids Lost (%)</th>
<th>Soluble Protein Lost (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>38±4 a</td>
<td>11±1 a</td>
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<tr>
<td>31</td>
<td>48±3 b</td>
<td>7±1 b</td>
</tr>
<tr>
<td>54</td>
<td>53±5 c</td>
<td>5±1 c</td>
</tr>
<tr>
<td>71</td>
<td>51±3 bc</td>
<td>10±2 d</td>
</tr>
</tbody>
</table>

abcd Means with common letters within columns are not different. Means ± Standard Deviation.
interacting with each other to form stabilizing crosslinks. It is likely that the proteins and starch were dispersed within a continuous matrix rather than a layered, fibrous network.

Although the control extrudate was more stable than the sulfonated samples (likely the result of fewer electrostatic charges on the proteins), it did not possess the texture or stability of typical textured whey protein. Preparation of the control WPC, which included acid precipitation and treatment with EDTA, may have altered the proteins and mineral content such that texturization was inhibited. The removal of calcium may have prevented the formation of salt bridges and subsequent crosslinking during the later stages of extrusion (5).

Correlation Between WPC Functional Properties and Textured Extrudate Characteristics. No significant correlation (p > 0.05) was found between the WPC functional properties and the textured extrudate characteristics. Although emulsification activity index (EAI) was highly correlated to many properties of the expanded snack (Chapter III), it was not correlated to extrudate stability. While protein-carbohydrate interactions were likely the most important factor in determining the characteristics of the expanded snack, protein-protein interactions are likely more critical to the overall texture and stability of the textured whey product.
In conclusion, all samples (including the control) did not possess fibrous, meat-like texture typical of TWP. This was contrary to what was hypothesized. Although no samples had typical texture, the properties of the sulfonated samples had decreased during hydration compared to the control. Decreased stability may have been the result of decreased protein-protein interactions. Increased protein flexibility, coupled with decreased protein-protein interactions, may have been responsible for the lack of stability in the sulfonated extrudates. Raw materials and processing conditions that may decrease protein-solvent interactions may improve overall product stability of texture whey proteins. No significant correlations were found between extrudate stability and the functional properties of the sulfonated WPC.

LITERATURE CITED


HYPOTHESIS

“Functional properties of sulfonated whey proteins are related to their extrusion performance.”

Whey proteins are limited in their use in food systems, including extrusion, because of poor functional properties. Limited functionality may be related to the high content of disulfide bonds. Sulfonation with sodium sulfite cleaves disulfide bonds and adds a sulfate group that prevents oxidation that would reform another disulfide bond. The controlled sulfonation of disulfide bonds in whey proteins enabled the investigation of how disulfide bond content affects protein functionality. By extruding sulfonated whey proteins into expanded snack and textured products, it is possible to determine disulfide bond content’s effect on extrusion as well as be able to correlate sulfonated whey protein functional properties to extrudate characteristics.

OBJECTIVES

Objective 1. To determine the effect of extent of sulfonation has on traditional measures of whey protein functionality, including high temperature solubility, gel
strength, emulsification, foaming, and thermal denaturation. This information is of
general interest to food scientists.

Disulfide bond content significantly affected the functional properties of whey protein
concentrate. In general, sulfonation increased protein foam overrun and stability,
emulsified oil volume fraction and emulsification activity index, and protein
resolubilization after heating.

Sulfonation led to decreases in protein gel strength and melt temperatures. The
increase in foam overrun, foam stability ($T_{1/2}$), emulsified oil volume fraction ($\phi$), and
emulsification activity index (EAI) and decreases in melt temperatures ($T_{\text{gel}}$ and $T_{\text{melt}}$) were
largely attributed to increased protein flexibility. However, increased electrical charge
may have negated some of the increase in foam overrun and $T_{1/2}$ at the 54 and 71%
sulfonation levels. Increased flexibility enabled more protein surface to interact at the
interfaces between and aqueous and non-polar solvent (either air or oil). Decrease in gel
strength ($G^*$) of the sulfonated samples was most likely the result of increased
electrostatic repulsion resulting from the added sulfite groups on the proteins. Increased
repulsion prevented proteins from interacting and forming a stable gel network. Changes
in protein resolubilization was likely the result of the sulfite groups limiting disulfide
bonding during high temperature heating due to increased electrostatic charge preventing
protein-protein interaction and perhaps increased flexibility allowing for increased
protein-solvent interaction. Correlation analysis among the functional properties support these conclusions in that sulfonation was positively correlated to properties related to protein unfolding and protein-solvent interactions ($\phi$ and protein resolubilization) and negatively correlated to properties related to resistance to unfolding ($T_o$ and $T_{max}$) and protein-protein interactions ($G^*$).

Objective 2. To identify correlations between sulfonated whey protein functional properties and extrusion performance. These correlations could be used to predict and select raw materials for extrusion.

Properties of the expanded snacks were significantly affected by disulfide bond sulfonation. In the expanded snack, sulfonation at the 31 and 71% levels generally lead to decreased extrudate expansion and air cell size and increased density and breaking strength compared to the control and 54% sulfonated sample. Chemical properties of the extrudates such as soluble protein and carbohydrate, were also lower in the 31 and 71% levels, suggesting that the amount of protein and carbohydrate that becomes crosslinked and insoluble may be important to extrudate expansion.

Increased protein flexibility and unfolding may have promoted protein-carbohydrate interactions, resulting in the decreased expansion. This conclusion was supported by correlation between many whey protein functional properties related to protein flexibility ($EAI$ and $T_o$) and extrudate expansion properties. Less flexible proteins, that shield
potential interaction sites on the interior of the protein, may yield extrudates with increased expansion. EAI was correlated to many extrudate properties, suggesting that it may be an important indicator of extrudate characteristics.

The properties of the fibrous, textured product also were affected by sulfonation. None of the samples, including the control, possessed the fibrous, meat like texture typical of textured whey. This was likely the result of increased charge on the proteins from the added sulfite groups that prevented necessary protein-protein crosslinks essential for stability. The removal of minerals from the protein samples during their preparation also likely decreased the extent of texturization at all sulfonation levels.

Sulfonated samples lost more solids during hydration. Soluble protein lost to the supernatant was also affected, although it was lowest at the 54 and 71% sulfonation levels.

CONCLUSIONS

Sulfonation of disulfide bonds altered the functional properties of whey protein concentrate and these changes were correlated to the properties of expanded snacks product. Increased protein flexibility, and the resulting increase in protein-carbohydrate interactions, is likely an important factor in limiting the expansion and physical properties of this product. The flexibility of a protein and its likelihood to participate in protein-carbohydrate interactions may be as important to extrudate properties as both the
protein and reducing sugar contents of the dry mix. Correlation between measures of protein flexibility and extrudate characteristics may allow for prediction of extrudate characteristics based on the properties of the raw materials.

Although increased protein unfolding was hypothesized as being a critical factor for protein texturization, strong protein-protein interactions need to occur. The increased electrostatic charge resulting from sulfonation likely prevented crosslinking necessary to produce a stable, textured product.

**FUTURE RESEARCH**

To evaluate the usefulness of the correlation between WPC functional properties and extrusion performance in the expanded snack product, several sources of WPC should be evaluated for both their functional properties and extrusion performance. Because EAI had the most significant correlations to extrudate properties it may be the single most important property to measure before extrusion. If functional properties of the WPCs are correlated to their extrusion performance, then broad recommendations could be made on predicting extrudate characteristics based on functional properties and it may be possible to select raw materials prior to extrusion.

Additionally, sulfonated whey proteins may be extruded with cation salts or other charge-shielding components to determine if the effects of increased electrostatic charge can be minimized. Extruding with calcium may be one possibility to accomplish this.
The calcium cations may shield the increased electric charge on the whey proteins so that they can more readily engage in the protein-protein interactions essential to proper texturization.
APPENDICES
APPENDIX A

ANOVA TABLES FOR CHAPTER II STATISTICAL ANALYSIS

Table A1. ANOVA Table for mean total protein in sulfonated samples

<table>
<thead>
<tr>
<th>Effect</th>
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<th>MSS</th>
<th>F</th>
<th>p-value</th>
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<td>Error</td>
<td>20</td>
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Table A2. ANOVA Table for mean gel strength (G*)

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<th>p-value</th>
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<tbody>
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Table A3. ANOVA Table for foam overrun (%)

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<th>p-value</th>
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<tbody>
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Table A4. ANOVA Table for $T_{1/2}$ foam drainage time (s)

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Table A5. ANOVA Table for oil volume fraction ($\phi$)

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### Table A6. ANOVA Table for emulsification activity index (EAI)

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<tbody>
<tr>
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<td>94.52</td>
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### Table A7. ANOVA Table for protein melt peak temperature ($T_{\text{max}}$)

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### Table A8. ANOVA Table for protein melt onset temperature ($T_o$)

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### Table A9. ANOVA Table for resolublized protein analysis

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<td>Error</td>
<td>128</td>
<td>69.61</td>
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</table>

1= Sulfonation, 2 = Heating Liquid, 3 = Dissolving Solvent
APPENDIX B

SDS-PAGE ANALYSIS OF SULFONATED WHEY PROTEIN CONCENTRATES

Figure B. SDS-PAGE analysis of sulfonated WPC (80% protein). Lane M, protein molecular weight marker. Lane Pro, Proliant WPC 8000. Lane 0%, control sample. Lane 31%, 31% sulfonated sample. Lane 54%, 54% sulfonated sample. Lane 71%, 71% sulfonated sample. Lane STD, whey protein standards (BSA, 66 kDa; β-Lg, 18.4 kDa; α-Lb, 14 kDa).
# APPENDIX C

**EXPERIMENTAL DESIGN OVERVIEW AND DIAGRAM**

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<td>Replication</td>
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<td>Collection Temperature</td>
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<table>
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<th>Measured Properties</th>
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<td>Air Cell Diameter</td>
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<td>Density</td>
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<td>Breaking Strength</td>
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<td>Expansion Ratio</td>
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<td>Moisture</td>
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<tr>
<td>WAI</td>
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<tr>
<td>WSI</td>
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<tr>
<td>Resolublized Protein</td>
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<table>
<thead>
<tr>
<th></th>
<th>0%</th>
<th>31%</th>
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<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
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<tr>
<td></td>
<td>160</td>
<td>170</td>
<td>160</td>
<td>170</td>
</tr>
</tbody>
</table>

N* Number of measurements (non-independent) on extrudates from each combination of sulfonation, replication, and temperature.
APPENDIX D

ANOVA TABLES FOR CHAPTER III STATISTICAL ANALYSIS

**Table D1.** ANOVA Table for air cell diameter (mm)

<table>
<thead>
<tr>
<th>Effect</th>
<th>df</th>
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<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonation</td>
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<td>79.28</td>
<td>32.47</td>
<td>0.0002</td>
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<td>Temperature</td>
<td>1</td>
<td>2.72</td>
<td>1.11</td>
<td>0.3261</td>
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<tr>
<td>Sulfonation x</td>
<td>3</td>
<td>1.68</td>
<td>0.69</td>
<td>0.5861</td>
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<tr>
<td>Temperature</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Error</td>
<td>7</td>
<td>2.44</td>
<td></td>
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</tr>
</tbody>
</table>

**Table D2.** ANOVA Table for extrudate density (g/cm³)

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<tr>
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<th>p-value</th>
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<tbody>
<tr>
<td>Sulfonation</td>
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<td>0.17</td>
<td>9.62</td>
<td>0.0070</td>
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<td>Temperature</td>
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<td>0.19</td>
<td>10.87</td>
<td>0.0132</td>
</tr>
<tr>
<td>Sulfonation x</td>
<td>3</td>
<td>0.02</td>
<td>1.34</td>
<td>0.3355</td>
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<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>7</td>
<td>0.02</td>
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<td></td>
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</tbody>
</table>

**Table D3.** ANOVA Table for breaking strength (ln Pa)

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<tbody>
<tr>
<td>Sulfonation</td>
<td>3</td>
<td>27.29</td>
<td>38.65</td>
<td>0.0001</td>
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<td>Temperature</td>
<td>1</td>
<td>21.57</td>
<td>30.54</td>
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<tr>
<td>Sulfonation x</td>
<td>3</td>
<td>5.50</td>
<td>7.79</td>
<td>0.0124</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>7</td>
<td>0.70</td>
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</tbody>
</table>
Table D4. ANOVA Table for expansion ratio

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<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonation</td>
<td>3</td>
<td>11.03</td>
<td>8.31</td>
<td>0.0104</td>
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<td>Temperature</td>
<td>1</td>
<td>8.44</td>
<td>6.37</td>
<td>0.0397</td>
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<tr>
<td>Sulfonation x</td>
<td>3</td>
<td>1.14</td>
<td>0.86</td>
<td>0.5047</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>7</td>
<td>1.33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table D5. ANOVA Table for water adsorption index (WAI)

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<tr>
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<th>MSS</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
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<td>0.11</td>
<td>1.32</td>
<td>0.3416</td>
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<td>Temperature</td>
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<td>6.50</td>
<td>0.0382</td>
</tr>
<tr>
<td>Sulfonation x</td>
<td>3</td>
<td>0.98</td>
<td>11.48</td>
<td>0.0043</td>
</tr>
<tr>
<td>Temperature</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>7</td>
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</table>

Table D6. ANOVA Table for water solubility index (WSI)

<table>
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<th>Effect</th>
<th>Df</th>
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<th>p-value</th>
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<td>Sulfonation</td>
<td>3</td>
<td>122.48</td>
<td>4.87</td>
<td>0.0389</td>
</tr>
<tr>
<td>Temperature</td>
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<td>0.75</td>
<td>0.03</td>
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</tr>
<tr>
<td>Sulfonation x</td>
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<td>25.05</td>
<td>0.99</td>
<td>0.4487</td>
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<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>7</td>
<td>25.16</td>
<td></td>
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</tbody>
</table>

Table D7. ANOVA Table for moisture content (%) 

<table>
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<td>Sulfonation</td>
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<td>0.03</td>
<td>0.16</td>
<td>0.6924</td>
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<tr>
<td>Sulfonation x</td>
<td>3</td>
<td>0.19</td>
<td>1.12</td>
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<tr>
<td>Temperature</td>
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</tr>
<tr>
<td>Error</td>
<td>7</td>
<td>0.18</td>
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</tbody>
</table>
### Table D8. ANOVA Table for Resolublized protein (%)

<table>
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<tr>
<td>Sulfonation x Temperature</td>
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<td>5.70</td>
<td>0.05</td>
<td>0.9832</td>
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<tr>
<td>Error</td>
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<td>110.21</td>
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</table>

### Table D9. ANOVA Table for soluble carbohydrate (%)

<table>
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</thead>
<tbody>
<tr>
<td>Sulfonation</td>
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<td>225.61</td>
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<td>0.0124</td>
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<td>Temperature</td>
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<td>57.41</td>
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<td>4.34</td>
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<td>Error</td>
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<td>28.97</td>
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</table>
APPENDIX E

EXTRUDER SCREW CONFIGURATION DIAGRAMS

Figure E1. Extruder screw configuration for producing the expanded snack product.

Figure E2. Extruder screw configuration for producing the fibrous, textured product.
APPENDIX F

ANOVA TABLES FOR CHAPTER IV STATISTICAL ANALYSIS

**Table F1.** ANOVA Table for extrudate solids lost (%)

<table>
<thead>
<tr>
<th>Effect</th>
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<td>Sulfonation</td>
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<td>287.41</td>
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<td>0.0500</td>
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<td>Error</td>
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<td>31.31</td>
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</table>

**Table F2.** ANOVA Table for soluble protein in supernatant (%)

<table>
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<th>F</th>
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<td>Sulfonation</td>
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<tr>
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<td>1.86</td>
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</table>
CURRICULUM VITAE

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900 N. 300 W. #A4
Logan, UT 84321

435-713-7082
dptaylor30@yahoo.com

ACADEMIC PREPARATION:

Ph.D. in Food Science
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- Coursework emphasis: Protein chemistry, protein purification, dairy chemistry and processing
- Dissertation: Investigation of the Effect of Sulfitolysis on the Functional Properties and Extrusion Performance of Whey Protein Concentrate
- Advisors: Dr. Marie K. Walsh and Dr. Charles E. Carpenter
- GPA: 3.9

M.S. in Food Science, December 2000
Department of Food Science and Nutrition, Brigham Young University
- Research emphasis: Continuous carbonation of yogurt
- Coursework emphasis: Food chemistry, electron microscopy
- Thesis: Carbonation of Viscous Fluids
- Advisor: Dr. Lynn V. Ogden
- GPA: 3.8

B.S. in Food Science, December 1998
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Food Analysis Laboratory Assistant, 2002
Utah State University
- Prepared laboratory materials, taught laboratory lessons and supervised students while in the laboratory

Food Commodity Processing Laboratory Teaching Assistant, 1999-2000
Food Engineering Laboratory Teaching Assistant, 1999-2000
Brigham Young University
- Prepared laboratory materials and supervised students in the laboratory
• Responsible for laboratory teaching and grading of assignments
• Substitute instructor for the Food Commodity Processing lecture class

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PROFESSIONAL MEMBERSHIPS:

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• Gandhi Scholarship, Utah State University, 2001-2003
• Graduated Cum Laude, BS if Food Science, Brigham Young University, 1998

ACADEMIC ACTIVITIES:

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• Food Science Club Secretary, 1998-1999, 2001-2003
• USDA/ADSA Dairy Product Evaluation Content participant and assistant coach, 1998-2000
• IFT College Bowl Team participant, 1998-1999