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Modification of egg albumen to improve thermal stability

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by

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LIST OF ACRONYMS AND ABBREVIATIONS

BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CE	Commercial Fresh Egg White Not De-glucosed
CEd	Commercial Fresh Egg White De-glucosed
CEW	Commercial Fresh Egg White
CFEW	Commercial De-glucosed Fresh Egg White
CFR	Code of Federal Regulations
CMC	Carboxymethylcellulose
Cys	Cysteine
DEW	Commercial Dried Egg White
DSC	Differential Scanning Calorimetry
EWP	Egg White Protein or Egg White Powder
FE	Fresh Egg White
FEW	Fresh Egg White or Fresh Hen Egg White
GRAS	Generally Recognized As Safe
H	With Pre-heating
HCMC	Hydrolyzed Carboxymethylcellulose
HPLC	High-performance Liquid Chromatography
LPC	Lysophosphatidylcholine
M_w	Molecular Weight
N	No Pre-heating
NS	Not Significantly Different

OSA	Octenyl Succinic Anhydride
OSACF	Octenyl Succinylated CFEW
OSAE	Octenyl Succinylated EWP
OSAF	Octenyl Succinylated FEW
PCA	Principle Component Analysis
pI	Isoelectric Point
S	Significantly Different
SA	Succinic Anhydride
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SH	Sulfhydryl
Sig	Significance
Son	Sonication or Sonicated
SPI	Soybean Protein Isolate
S-S	Disulfide
T_d	Denaturation Temperature
TNBS	2, 4, 6-Trinitrobenzene Sulfonic Acid Picrylsulfonic Acid
WPC	Whey Protein Concentrate
WPI	Whey Protein Isolate

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ABSTRACT

Eggs are an excellent source of high quality protein for consumers that can be economically produced. This dissertation focuses on improving the thermal stability of egg albumen or egg white protein. As consumers are demanding more protein in their diets, egg white proteins can provide the supply to meet those needs. A major barrier of egg white protein's use as a mainstream protein in beverages, further food processing, or other consumer food is its sensitivity to heat (precipitating out of solution when heated). If this functional property can be resolved, it would not only add value to the egg processing industry, but also would have an expanded use in various other applications. Three specific types of modification to the egg albumen as a whole were studied for this dissertation research. A series of thermal stability evaluations was used to determine the improvement in stability of the protein at 75°C, 95°C, and 121°C.

Since egg albumen contains a mixture of several different proteins, working on the protein dispersion, as a whole is a challenge. The mechanism of protein aggregation is caused by hydrophobic protein-protein interaction after the protein has partially unfolded due to heating. As the proteins aggregate, it can either form a linear soluble aggregate or insoluble coagulum, which can precipitate. Glycation with partially hydrolyzed carboxymethylcellulose (HCMC), succinylation with octenyl succinic anhydride (OSA), and combinations of physical treatment (ultrasound) and the addition of OSA were investigated to prevent these aggregations leading to improved thermal stability of the protein.

The HCMC is an anionic polysaccharide that can increase the electrostatic repulsion between proteins, similar to what OSA can do. However, OSA contains a hydrophobic carbon chain that may further interact with the hydrophobic groups of the unfolded proteins

delaying aggregation. The use of ultrasound and OSA addition provides a more consumer friendly approach that helps to disperse the OSA while hydrolyzing the anhydride simultaneously, creating a system that is more uniform with a dispersion of the charged OSA without chemical modification.

CHAPTER I. GENERAL INTRODUCTION

Rationale

The modification of egg white proteins to make it tolerate high thermal processing temperatures can increase its use by food manufacturers and to meet the consumers demand for a variety of food or drink choices with higher concentrations of protein.[1] By improving the heat stability of egg proteins, processors can also pasteurize egg whites to a higher temperature to ensure safety, quality, and increase shelf life stability. Egg whites already function as good emulsifying, foaming, and gelation agents. A thermal stable egg white protein would have potential applications in the protein beverage industry, competing with other animal and plant protein drinks currently on the market.

Literature Review

Composition of hen albumen [2-4]

A chicken egg can be separated into the shell, albumen, and the yolk components. The albumen or egg white makes up 60-63% of the egg. The amount of total solids can vary, depending on the breed and age of hen, ranging from 11-13% of the egg white portion. The albumen consists of four layers: an outer thin white layer, thick white layer, inner thin white layer, and the chalaza. Each albumen layer is 23.2, 57.3, 16.8, and 2.7%, respectively. Albumen is a non-Newtonian, pseudoplastic fluid that decreases in viscosity with increase shearing. The thin or thick layer has affluent amount of ovomucin protein, with more present in the thick layer. The pH of a fresh or newly laid egg is between 7.6 and 8.5. As the egg is stored, carbonic anhydride escapes as CO_2 through the pores of the eggshell, causing the pH of the albumen to become more alkaline as it ages.

The egg albumen contains approximately 84-88% moisture, between 9.7-10.6% protein, 0.03% lipid, 0.4-0.9% carbohydrate (free, bound or combined with protein), and 0.5-0.6% ash. Egg whites contain sulfur, potassium, sodium, phosphorus, calcium, magnesium, iron and B vitamins. A variety of proteins can be found in egg albumen with its own amino acid sequence, structure, and functionality.

Major proteins found in egg white [2-5]

There are approximately 40 different proteins found in egg whites. Ovalbumin, ovotransferrin (conalbumin), lysozyme, ovomucoid, and ovomucin make up over 80% of the heterogeneous protein composition. Other egg white proteins include; G2, G3 glycoprotein, ovoflavoprotein, cystatin, avidin, ovomacroglobulin, and others detected in trace amounts. A summary of the major protein percentage by weight, isoelectric point, molecular weight, denaturation temperature, free sulfhydryl groups, and disulfide bonds is shown in Table 1.

Ovalbumin

Of all the egg white proteins, ovalbumin comprises 54% of all the proteins. It is a phosphoglycoprotein that is part of the serine protease inhibitor superfamily.[5] As the egg ages or is stored over time, ovalbumin changes to S-ovalbumin. This conformation change has been shown to be more heat resistant, but its mechanism of formation is not understood. S-ovalbumin is more heat stable and can form after a heat treatment at 55°C at a pH of 9.9, as described in a method by Smith and Back.[7] It is the major protein found in egg whites and is responsible for the higher denaturation peak detected by differential scanning calorimetry (DSC) at 75-84°C.[6]

Ovalbumin is separated into 3 main components A1, A2, and A3, which are separated based on the number of phosphate groups. Each component has two, one, and no phosphate groups, respectively.[8,9] The anionic phosphate groups found on the protein may stabilize the structure by providing electrostatic repulsions between the phosphate groups once they are exposed by heating leading to a higher denaturation temperature. Ovalbumin contains one disulfide bond per molecule and four free sulfhydryl-groups.

Table 1. Physiochemical Properties of Egg White Proteins (Modified from Mine and Yang, 2010 and Donovan et al., 1975)

Protein	% (w/w)	pI	M_w (kDa)	T_d (°C)	Cys	SH	S-S
Ovalbumin	54	4.5-4.9	45	75-84	6	4	1
Ovotransferrin (Conalbumin)	12-13	6.0-6.1	77.7	61-65	30	-	15
Ovomucoid	11	4.1	28	77	18	-	9
Ovomucin	1.5-3.5	4.5-5.0	110, 5.5-8.3x10^3, 220.0-270.0x10^3	hs	-	-	-
Lysozyme	3.4-3.5	10.7	14.3-14.6	69-77	6	-	4
G2 ovoglobulin	1.0	4.9-5.5	47-49	-	-	-	-
G3 ovoglobulin	1.0	4.8-5.8	49-50	-	-	-	-
Ovomacroglobulin	0.5	4.5	796	60	-	-	-
Ovoflavoprotein	0.8	4.0	32-35, 80	-	5	-	2
Cystatin	0.05	5.1	12.7	-	-	-	-
Avidin	0.05	10	68.3	95	2	-	1

pI - isoelectric point of protein, M_w - molecular weight, T_d - denaturation temperature, Cys – cysteine, SH – sulfhydryl, S-S – disulfide, hs- heat stable

Ovotransferrin or conalbumin

Ovotransferrin is the second most abundant glycoprotein, as it makes up 12-13% of egg albumen. It is the most heat sensitive protein unless bound to a metallic ion such as iron at pH ≥ 6.[3,6,10] The complexes formed with metallic ions are also resistant to proteolysis. The protein has 15 disulfide bridges and has no phosphorous or any free sulfhydryl groups. Most of the carbohydrates that are found in the proteins C-terminal are oligosaccharide chains. The denaturation peak of ovotransferrin is 61-65°C.

Ovomucoid

Ovomucoid makes up 11% of the egg white proteins, and it has a denaturation temperature of about 77°C. Ovomucoid is another heat-resistant glycoprotein in an acidic solution, but it is altered at an alkaline pH of 9. The protein inhibits trypsin and contains as much as 25% carbohydrates (three oligosaccharides, joined by an asparaginyl residue). At pH 7.6, the protein has low heat stability.

Lysozyme

Egg albumen contains 3.4-3.5% of this antibacterial protein. It is also known as *N*-acetylmuramoyl hydrolase because it can hydrolyze β-1,4-linkages of bacterial cell wall polysaccharide. Lysozyme is a basic protein that has four disulfide bonds. Its denaturation temperature is between 69-77°C.

Ovomucin

Ovomucin is a sulfated glycoprotein (up to 33% carbohydrate content) with a soluble and insoluble fraction. The soluble and insoluble fraction contains a 40:3 and 84:20 ratio of α to β subunits, respectively.[2] Its composition causes the protein to have a huge molecular weight range. The insoluble ovomucin is found in the thick albumen, while the soluble ovomucin is present in the thin albumen fraction that is responsible for the jelly-like texture of the albumen.[5] Ovomucin has shown to precipitate and can be fractionated out when diluted with water (1:3 dilution at pH of 6.5), but is soluble again in basic conditions.[3,11] The protein does not have a

thermal transition temperature because of it native structure existing as a "random coil" and is considered a heat stable protein.[11]

Other protein of importance

Avidin has been shown to be a very heat stable protein when bound. Differential scanning calorimetry (DSC) data showed that the complex was stable at about 131°C compared to 85°C (or 95°C according to Donovan et al.[6]) when it was bound to the B vitamin, biotin.[12] The heat stability of the protein was increased by 46°C.

Industrial processing of egg white proteins [4,13-15]

A typical process for producing the dry albumen powder is to separate it from the yolk portion, de-glucose by fermentation or enzymatic means with glucose oxidase, spray dry, and application of additional heat treatment. The spray dried powder is stored at 54.4°C for 7-10 days to pasteurize the product and improve its functionality meeting Title 9 Code of Federal Regulations (CFR) 590.575.[13] Once it has tested negative for *Salmonella*, the product is ready to be released to the market. The entire process exposes egg white proteins to shearing, air, and heat during the pumping, concentration, filtering, and the drying process, which can affect its structure.[13-15]

Defining thermal stability and protein aggregation

A thermal stable protein is defined as a protein that when heated resists changes in solubility. A precipitation of the protein is caused by unfolding of the native protein structure, increasing surface hydrophobicity, leading to protein-protein interactions by hydrophobic

assoication leading to aggregation and formation of a precipitate.[16] A flow chart by Kitabatake et al.[17] illustrates what happens to protein as it is heated and aggregated (Figure 1).

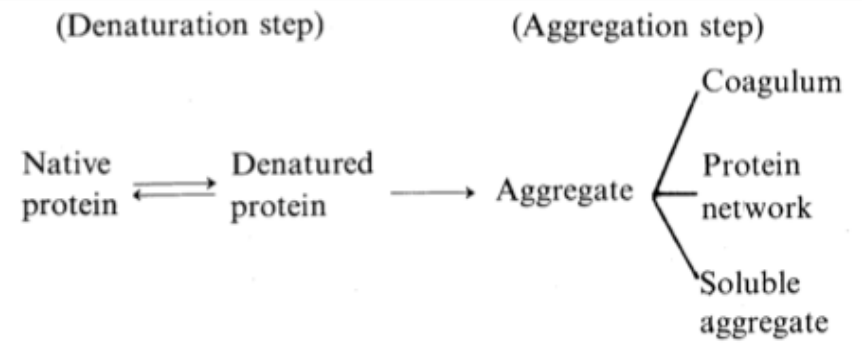

Figure 1. Flow Chart of proteins as it is being heated from denaturation to aggregation (modified from Kitabatake et al., 1987).

When proteins are exposed to heat they undergo a denaturation and aggregation step.[17,18] The molten globule state is established when the protein starts to denature, loses its tertiary structure, and can aggregae, but does not form a gel.[19] After denaturation the protein can aggregate either into soluble aggregates (linear, organized aggregation) or a coagulum (random aggregation) which can eventallually lead to the formation of a gel if a protein network is formed.[17,18] Careful control during processing is important to prevent the formation of a coagulum since it is usually irreversible.

Techniques for thermal stability measurement

The main techniques used to quantify the thermal stability of proteins are through differential scanning calorimetry or turbidimetric methods, and change in protein solubility. DSC gave three different peaks conalbumin, lysozyme (intermediate peak), and the ovalbumin peak

using fresh eggs.[6] These peaks have been suggested to over shadow the other proteins found in egg white. Ibanoglu and Erçelebi[20] using hydrated hen egg albumen powder found only two major denaturation peaks at 68.5 ± 0.4°C and 84.1 ± 0.5°C. The researcher identified the first peak to be both conalbumin (now known as ovotransferrin) and lysozyme, and the second peak to be ovalbumin. However, there are other proteins in egg white besides ovalbumin, conalbumin, and lysozyme. According to Ternes,[5] at higher pH, the denaturation temperature of the egg proteins increases (Table 3). Exposure of the proteins to elevated temperatures has been shown to reduce the DSC peaks, which would make it harder to quantify and compare among different samples.[21] The peaks have been shown to vary by pH, different heating rates, and buffer.[6] Based on this information, DSC may not be the best and most sensitive method to evaluate thermal stability when modifying heterogeneous protein dispersions since the small shift in peak temperature could be due to other factors if modification is not extreme enough or can change due to the food additive used.

Table 3. Denaturation temperature and pH of egg white proteins (Modified from Mine and Yang, 2010; Ternes, 2001)

Protein	Range T_d (°C)	pH			
		4	**5**	**7**	**9**
Ovalbumin	75-84	66	72	81	84
S-Ovalbumin	88.5	75	79	85	92
Ovotransferrin (Conalbumin)	61-65	48	56	62	60
Ovomucoid	77	-	-	90	-
Ovomucin	Heat stable	-	-	-	-
Lysozyme	69-77	78	-	81.5	-

T_d – denaturation temperature

Turbidity measurements depend on concentration of the protein and pH values.[22,23] Low protein concentrations can result in high turbidity values that can be observed at narrow pH

values, i.e., around the isoelectric point (pI) of the protein. While at high protein concentrations, the turbidity of proteins can be analyzed in a wider range of pH. Previous research attributed this to the suppression of electrostatic forces measured by isoionic point measurements by electrometric titration. Shimada and Matsushita[23] showed no turbidity at 4.5% protein concentration after heating at 80°C for 15 minutes when pH was >11. However, at pH values <pH 11 the turbidity values did increase with a plateau of turbidity values around pH 8.[23] To ensure that the changes in turbidity are due to heat treatment, the concentration or change in protein should be measured. Turbidity measurements are a better way to quantify an improvement in protein stability as long as the concentration and pH at evaluation is controlled.

Methods to improve heat stability of proteins

Several methods are available to improve the thermal stability of egg white protein (EWP) with most of the studies investigating its effect on ovalbumin. EWP and other proteins can be modified by enzymatic, physical, or chemical methods. These methods prevent the aggregation of globular proteins by physical or enzymatic means that reduce aggregate, particle size, or molecular weight of proteins enough to prevent aggregation. The other approach is to use chemical modification or additives that increase the degree of electrostatic repulsions between proteins, preventing the hydrophobic protein-to-protein aggregation.

Enzymatic means

Enzymatic modification of proteins with proteases (as papain) has been shown to be a very effective type of modification. Depending on the type of enzyme used, different pH, temperature, reaction time, and proper concentrations of the substrate and enzyme are needed for

modification.[24,25] The higher the degree of hydrolysis with a greater decrease in protein molecular size, the more thermal stable the protein can become. Ryan et al.[25] showed an increase in thermal stability of soy protein hydrolysate compared to soy protein. Enzyme hydrolysis is able to cleave the protein into smaller fractions while increasing its solubility and retaining its nutritional properties.

However, enzyme hydrolysis can be a challenge for a heterogeneous system such as egg albumen, compared to working with an isolated protein. The pH, temperature, time, and deactivation process of enzyme modification may affect the variety of proteins differently. Another disadvantage is the production of bitter compounds, hydrophobic peptides, that can be formed as found when casein and soy are hydrolyzed.[24] Limited proteolysis, careful control, a enzyme cocktail, and other additives to solve these limitations have been studied. Studies dealing with protein hydrolysis along with other further chemical reactions such as succinylation in combination have been investigated.[26]

Physical methods of modification

Ultrasound or sonication physical treatment

Sonication, using low frequency and high energy, is a novel processing technique that causes similar changes to the protein structure as when it is heated with dry heating as an increase in surface hydrophobicity is observed.[27] The use of ultrasound generates heat (by friction) and mixes by cavitation (formation and collapse of air bubbles in solution that generates pressure and increasing temperature indirectly).[28] Arzeni et al.[27] found a reduction in aggregate size of whey protein concentrate (WPC) and soybean protein isolate (SPI) with ultrasound. The heat stability of whey proteins was improved through a two-step heat denaturation and sonication

process as demonstrated by Ashokkumar et al.[29] Sonication treatment is important once the proteins are partially denatured first, at a lower than denaturation temperature. The investigators discussed how sonication disrupted or dispersed the initial hydrophobic or possible disulfide bonds that had formed in the more heat sensitive proteins after it was first heated. When the dispersion was heated again, an improvement occurred since the heat sensitive proteins were dispersed already. Delaying or reducing the amount of further aggregation would reduce the amount of protein precipitation indirectly, thus improving the overall thermal stability of the protein.

Martini and Walsh[30] conducted a sensory test on sonicated whey protein, and they showed no significant difference in sensory between the control and sonicated samples. Ultrasound has been studied and shown to have the potential to sterilize (combination of bactericide and sonication affecting bacterial cell walls), inactivate or activate enzymes (at high intensity enzyme is denatured), help distribute substrate to immobilized enzyme, form a more stable emulsion with phospholipids (good distribution of layers and smaller droplet size), increase gelation time (uniformity of mixture of proteins and exposure of thiol groups), and improve other functionality.[31] The use of sonication in combination with other types of modification method may produce novel techniques for producing thermal stable proteins.

Dry heat treatment

Physical methods such as moisture controlled dry heating (5.7-8.5% moisture, 75-80°C dry heat temperature over time) have shown to improve egg white and ovalbumin protein functionality of dried powder.[21,32-34] Dry heating at different conditions have shown to not only partially unfold the protein structure, but also cause deamidation (increasing electrostatic

repulsions among proteins when heated), hydrolyze some peptide bonds, and cause polymerization.

The amount of deamidation and protein hydrolysis was shown to occur at a pH between 3.0 and 8.0 (no deamidation pH > 9.5) and depending on other factors such as water activity, amino acid composition, protein concentration, and temperature.[33-35] Mine[33] found little deamidation with spray dried and pH 7 egg white after drying for 15 days at 75°C. However, Matsudomi et al.[21] determined a 6% protein deamidation after 1 day compared to 12% after 10 days with freeze dried and pH 7 freeze dried ovalbumin. At a pH of 9.42 and dry heating for 15 days at 75°C, 12.2% of ovalbumin was deamidated with an increasing percent at pH 10.40.[33] Deamidation of glutamine and asparagine would increase negative charges on the protein surface, leading to an increase in thermal stability of the protein.[33]

As proteins are heated, they aggregate and can polymerize with the same or other proteins. Kato[32] determined the average molecular weight of the aggregates of dry heat-treated proteins by heating a 0.15% dispersion of protein at various temperatures for 20 minutes and then analyzing the aggregates with high-performance liquid chromatography (HPLC, TSK gel G3000SW column). The average molecular weight of the polymerized egg white aggregates decreased, along with the degree of polymerization with increasing time dry heating.[32] When the pH of egg whites was increased (7.04, 9.42, to 10.40 pH) and they were dry heated, it took a shorter time to increase the surface hydrophobicity of the protein[33] At pH 10.40, insoluble proteins formed after 3 days of dry heating. Some of the nutritional value of the protein can also be lost through dry heating and partial protein denaturation.

The dry heating process has several advantages since the reaction is mild depending on the temperature, the technology is simple, and the method is recognized as GRAS status

(generally recognized as safe).[36] At the same time, dry heating can also reduce the microbial population of the EWP and is already used in the egg white drying industry.[13] Other reactions can occur at the same time if protein was not the only substrate present when dry heated.

Chemical modification

Chemical modification is an effective method to improve the thermal stability of food proteins (casein, whey, egg, soy), but a disadvantage is consumer acceptance of these products. The process needs to be carefully controlled, since it can reduce the availability of the essential amino acid, such as lysine. Glycation and succinylation are two chemical modification techniques used successfully to modify food proteins and could be further investigated by changing the type of carbohydrate or dicarboxylic acid used to modify the protein

Glycation

Proteins can be glycosylated with carbohydrates through the non-enzymatic Maillard reaction using the dry heating process.[37-40] Zhu et al.[41] showed that the conjugation of whey protein isolate (WPI) can also occur in aqueous solution with simple sugars, such as dextran. The introduction of an anionic group of the carbohydrate moiety would improve the thermal stability of the protein. The negative charges will increase the steric hindrance between the proteins, slowing down protein aggregation and possibly the formation of irreversible disulfide bonds. Dry heat glycation is a condensation reaction with the conjugation of a polysaccharide with a free reducing sugar and a free secondary (ε-amino group) or terminal amino group. de Groot et al.[9] showed that when ovalbumin was de-glycosylated, the heat stable S-ovalbumin could not form

after 72 hour heat treatment at 55°C and a pH of 9.9. In addition, various sugar moieties have been shown to be able to conjugate with ovalbumin and other food proteins.

The amino acids in dried egg white powder were shown to react with glucose, a reducing sugar.[40] Kline and Stewart[40] showed that when amino acids reacted with glucose, it reduced the solubility of the protein by 32% after 85 hours. However when the researchers substituted the glucose with oligosaccharides such as trehalose, raffinose, and sorbitol the solubility did not change over 50 days compared to control, at a pH of 9.5. The other benefit of using oligosaccharides and polysaccharides compared to glucose is that the browning or change in color may not be as prevalent due to the molecular size of the carbohydrate hindering further browning reaction. Kato et al.[38] showed that ovalbumin could be conjugated with dextran (molecular weight 60-90 kDa) when dry heated at 60°C at 65% relative humidity for 3 weeks. However, an increase in browning color developed. Aoki et al.[39] showed that ovalbumin could conjugate with glucuronic acid when dry heated at 50°C at 65% relative humidity. Sun et al.[37] conjugated ovalbumin with D-psicose (rare ketohexose), fructose, and glucose when dry heated at 55°C at 65% relative humidity.

Succinylation

Using the same mechanism, succinylation of proteins can also improve the thermal stability of proteins and other functional properties of egg white, casein, and soy proteins.[42-44] Egg albumen has been succinylated with succinic anhydride (SA), showing a visual improvement in turbidity with an increase in degree of modification, based on free amine (27, 45, to 100% modification).[42] Its thermal stability was increased. SA is an anhydride of dicarboxylic acid that contains two charged acids and its mechanism would be similar to the

conjugation of anionic carbohydrates to proteins. An 2% egg white concentration (with a ratio of 10:1 protein to succinic anhydride at pH 5.5 and 8.0) after heating at 100°C for 3 minutes was still a clear or transparent solution. The pI of the succinylated protein (10:1 ratio) shifted from 4.65 to 3.70 with an increase in surface hydrophobicity and sulfhydryl content. As the degree of succinylated casein increased, a decrease in solubility of proteins in the acidic range also occurred.[43] The succinylation of soy proteins shifted the pI from 4.5 to 4.0 when over 90% of the free amine was modified.[44] Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) also showed evidence of the formation of soluble aggregates with higher molecular weight bands without the addition of 2-mercaptoethanol, but disappeared after its addition.[42] Some disadvantages to this method is the solubility of SA, consumer acceptance, and the amount of salt needed to maintain the pH of the succinylation reaction. Dialysis of the salt after reaction can also be costly.

The use of other food additives

The addition of anionic additives is another method that can improve the thermal stability of proteins.[45] Researchers found that a small concentration of added anionic detergents (as 2-decylcitric acid, sodium dodecyl sulphate (SDS), and lauric acid) were able to elevate the aggregation temperature of ovalbumin when heated at a weak alkaline pH. The addition of 2-decylcitric acid was shown to increase the aggregation temperature in the pH region of 5.5-9.5, form progels at 85-100°C, with no precipitation observed at 100°C at pH of 7.5. The addition of SDS increased the aggregation temperature at higher pH and lowered the isoelectric point of ovalbumin by 0.15 pH units. Hegg and Löfqvist[45] suggested that a thermostable complex between ovalbumin and SDS might have formed through internal stabilization, the hydrocarbon

chain of the detergent strengthening the hydrophobic core of the protein, based on calculated dielectric constants and the net charge of the protein. Lauric acid addition, at 5 mM concentration had similar results with the aggregation temperature increasing at pH 7 and 9, from 58 to 85°C and 61 to 100°C, respectively. The addition of n-dodecanol, a nonionic detergent helped improve the thermal stability of the protein slightly with increasing pH (pH 9 compared to 7).

It has also been shown that adding an amphiphilic phospholipid along with a pre-heating step prior to sonication treatment improved the thermal stability of egg white proteins. Istarova et al.[46] and Oshima and Nagasawa[47] demonstrated that there is an electrostatic interaction between phospholipids and proteins (sodium caseinate, ovalbumin). The interaction is dependent upon the pH, concentration of phospholipid, and hydrophobic interactions between the tail of the phospholipid and the hydrophobic side groups of the protein. The phospholipid would also interfere with aggregation through its hydrophilic tail, improving the dispersion of the protein in an aqueous system.

Mine et al.[48] showed a conformation change when lysophosphatidylcholine (LPC) and linoleic acid were added to ovalbumin. Circular dichroism measurements showed native egg white proteins to have 40.6% helix, 15.8% β-sheet, 15.5% β-turn, and 28.2% unordered secondary structures.[16] As the protein is heated from 60-100°C, an increase in unordered, β-sheet %, and a decrease in alpha helix and β-sheet turn % occured.[16] The LPC changed the conformation of ovalbumin to have an increase in amount of α-helix and a reduction in the amount of β-sheet when sonicated together with the protein. Mine et al.[48] suggested that the β-sheet structure changed to α-helix by the hydrophobic portion of LPC shielding the interior of the

protein. The LPC molecule has a charged group, an alcohol, and a fatty acid suggesting a similar or any amphilphilic molecule may be effective in increasing the heat stability of other proteins.

Other modifications to investigate

Even though there are already several modifications available to increase the heat sensitivity of proteins, more research is needed to find a more effective method that would work for egg white dispersion and is accepted by consumers. Using hydrolyzed carboxymethylcellulose (HCMC) to glycate egg white proteins with dry heating, octenyl succinic anhydride (OSA, food additive used in the starch industry) to succinylate egg white proteins, and combination of OSA and physical treatment to stabilize egg proteins are three methods that are explained in this research. All three methods would improve the thermal stability of egg white proteins by increasing the electrostatic repulsion among proteins when heated at high temperatures.

Dissertation Organization

This dissertation contains a general introduction, a rationale for research, literature review, followed by three research papers corresponding to the required *Journal of Agricultural and Food Chemistry* formats, and a general conclusions section.

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CHAPTER 2. IMPROVING ALBUMEN THERMAL STABILITY BY GLYCATION

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Abstract

Glycation of heat sensitive egg white proteins with partially hydrolyzed carboxymethylcellulose (HCMC) was studied through dry heating to produce a thermal stable protein. The heat stability of the modified protein was evaluated by changes in turbidity at 95°C for 1 hour and protein solubility during heating at 75°C. The 1:1 molar ratio of free amine of egg protein to reducing end of HCMC showed improved reaction with increasing dry heating time at 55°C (65% relative humidity). Such product had an increased thermal stability. The 5:1 molar ratio also showed an improvement in heat stability. However, little change in free amine was observed suggesting that the protein was not glycated as expected. The change in free amine, protein pattern by SDS-PAGE, and soluble protein profile indicate that the 1:1 molar ratio of protein to HCMC did lead to glycation and the optimal dry heating time was between 2 and 7 days. The natural occurrence of glucose in albumen was shown to interfere with the glycation reaction.

Keywords: *carboxymethylcellulose, egg white, glycation, protein, heat stability, sonication*

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Introduction

Hen egg white or albumen consists of a mixture of proteins that are sensitive to heat. Dry-heat glycation of food proteins with anionic carbohydrates through the Maillard reaction may change the net charge and amphiphilic nature of modified proteins. The change in protein structure through glycation can lead to improved protein solubility, emulsification, foaming, gelation, and heat stability of proteins.[1-8] If the heat stability of the egg white protein can be further improved, its use and field of application in the food industry can be further expanded, especially in protein beverages. A thermal stable protein is a protein that does not precipitate or aggregate after heating of the protein dispersion and is tolerant to very high temperature.

Egg white proteins are a complex, heterogeneous mixture of about 40 different proteins with some proteins still not identified.[9] The heat stability of egg white proteins is dependent upon the ability of the system to delay hydrophobic aggregation among the unfolded protein molecules during heating. As protein-protein hydrophobic aggregations occur, the proteins will begin to precipitate and settle out of solution. Previous research on dried egg white or specific egg white proteins such as ovalbumin has shown that dry-heat glycation is an effective method to improve heat stability using oligogalacturonic acids prepared by hydrolysis of pectin and pectic acid.[2] Mastsudomi et al.[10] showed that dry heating ovalbumin at 80°C leads to deamidation, partial denaturation, and the formation of soluble aggregates. Typically, after spray drying, the dried protein powder is required to be stored in a heated, controlled humidity storage area for several days as the pasteurization step to control for *Salmonella.* This storage step is suitable for dry-heat glycation.[11] However, the process of dry heating needs further investigation because it may affect glycation reaction as well as protein structural changes.

An anionic polysaccharide such as carboxymethycellulose (CMC) that is partially hydrolyzed to give more reactive sites could improve the degree of protein glycation. As CMC is not fully hydrolyzed, its high molecular weight (M_w) can limit browning while an increased number of reducing ends is available that can glycate with egg proteins.[2] CMC is also considered a dietary fiber and food additive, so it is attractive and safe to use.

Sonication of fresh egg white proteins has shown to destabilize the quaternary structure of proteins.[12] The partial denaturation of the protein increases the exposure of free terminal and secondary amine groups that serves as binding sites for glycation. This could potentially improve the rate of glycation during the dry-heat glycation process. This physical treatment could also accelerate the rate of reaction by dispersing the fresh egg white more uniformly with the carbohydrate. We hypothesized that a higher degree of glycation with the anionic carbohydrate can increase the net charge of the proteins that would decrease its heat sensitivity.

The glycation reaction of proteins after spray drying, the dry heating time, and the effect of sonication pretreatment on rate of glycation are unknown. In this study, the effects of two different molar ratios of protein free amine group to CMC hydrolysate's reducing end, sonication pretreatment, and time of dry heating on glycation reaction efficiency and heat stability of modified egg white were investigated.

Materials

Grade A, large chicken eggs were purchased from the local market (Ames, IA, U.S.A.) with similar sell-by dates for each replication. Oskaloosa Food Products Corp. (Oskaloosa, IA) provided raw fresh egg whites before and after fermentation, which was to de-glucose the fresh egg white. Carboxymethylcellulose (CMC) of 90 kDa was from Sigma-Aldrich, (St. Louis, MO) and a cellulase enzyme was from Amano Enzyme USA Co., Ltd. (Elgin, IL). The Cellulase DS

is from a strain of *Aspergillus niger* that is a mixture of cellulase and β-glucosidase with an activity of not less than 100,000 u/g. All other materials and chemicals were purchased from Fisher Scientific. The CMC hydrolyzed substrate was prepared in the lab and measured for the concentration of reducing ends before use.

Methods

Egg white glycation process

Controlled enzyme hydrolysis of CMC substrate

Controlled enzymatic hydrolysis of CMC was done using a modified pH-STAT method of Alder-Nissen[13]. A 718 Titrino pH-STAT model (Brinkmann, Switzerland) operated with a jacketed glass container connected to an iso-thermal water bath was used for the CMC hydrolysis reactions. An Erlenmeyer flask and water bath (Fisher Scientific, Pittsburgh, PA) was used to prepare the 3-liter HCMC batch. The detailed flow chart of enzymatic hydrolysis of CMC is shown in Figure 1.

A 4% solution or 40 mg/g of CMC with Milli-Q water (w/w) was mixed overnight. The solution was heated in a water bath to 60°C before the pH was adjusted to 4.5 with a 2 N hydrochloric acid solution. The Cellulase DS enzyme was added at a concentration of 2% based on CMC substrate for 1 hour with mixing. After the reaction was completed the solution was boiled for at least 5 min and up to 1 hour to deactivate the enzyme. The concentration of reducing ends of hydrolyzed CMC (HCMC) was quantified using the Somogyi-Nelson method for reducing sugar quantification.[14,15] The standard curve was established using a 1 mg/mL solution of glucose. Samples were measured at an absorbance of 520 nm and compared to the standard curve to determine the concentration of free reducing ends for the HCMC substrate. The calculated average molecular weight of the CMC after hydrolysis was 8.4 kDa.

Physical treatment of fresh egg white protein

The fresh eggs (Ames, IA) were broken and the white was separated from the yolk. The fresh egg white (FE) was then mixed in a walk-in refrigerator at 4°C. The egg white samples from Oskaloosa Food Products Corp. (de-glucosed and not de-glucosed) had 0.02% of sodium azide (w/w) added before mixing overnight. A portion of the fresh egg white stock solution was diluted with HCMC at ratios of 1:0, 5:1, and 1:1 molar ratios of FE free amino group to HCMC free reducing group (5:1 molar ratio is equivalent to 70% of a 10% protein dispersion mixed with 30% of the 4% HCMC solution; 1:1 molar ratio is equivalent to 30% protein dispersion mixed with 70% of HCMC solution). The mixed FE with HCMC was then sonicated (Son) for 4 min using a Fisher Scientific model 500 Ultrasonic Dismembrator. A 200 mL sample volume was sonicated at a time in an ice bath for 4 min using a 1/2-inch horn at 70% amplitude. This step was repeated until there was enough material of each treatment to make 1-liter for spray drying.

Spray drying and dry heat glycation

Each treatment was spray dried using a conical spinning-disk atomizer feed spray dryer (APV Crepaco Inc, Getzville, NY). The inlet and outlet temperature was between 180-195°C and 88-110°C, respectively. The dried samples are then incubated at 55°C with a relative humidity of 65% (using a saturated KI solution inside a desiccator). The samples were collected after 12 hours, 1, 2, and up to 7 days. The 0-time treatment was the spray dried powder that was not incubated. The treatments we studied were two ratios of reactants, effect of using sonication step, and the four different dry heating times. Table 1 outlines the different variables and acronyms of the experiment.

Quantitative and qualitative analysis of glycated proteins

Degree of glycation determination using a free amine test

The degree of glycation reaction was determined by using the TNBS (2, 4, 6-trinitrobenzene sulfonic acid picrylsulfonic acid) method that quantifies the concentration of free amine of the modified egg white.[16,17] The TNBS kit was purchased from G-Biosciences (St. Louis, MO). Different concentrations of glutamic acid were used to establish the standard curve and the free amine calculated is the equivalent of glutamic acid. The free amine was calculated based on 20 mg of protein. The protein content of the sample was measured for the free amine calculation.

SDS-PAGE analysis of protein molecular size

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli[18] to observe changes in molecular size using a Bio-Rad Mini-PROTEAN® Tetra System. A 4-20% Mini-Protean ® TGX™ precast gel (Bio-Rad Laboratories, Hercules, CA), Laemmli buffer solution, and Precision Plus Protein™ Dual Color Standard were used. The dried modified protein sample was dissolved in Milli-Q water with Laemmli buffer before the addition of β-mercaptoethanol. The mixture was then loaded (40 ug) onto the pre-cast gel well at a constant voltage of 150 V in running buffer (1X Tris/Glycine/SDS buffer) for about 47 min. The gel was stained with coomassie blue dye (50% methanol, 22% coomassie blue solution of 1% concentration, 18% Milli-Q water, and 10% glacial acetic acid) and de-stained using 10% acetic acid, 15% methanol, and 75% distilled water solution. The upward shift of M_w bands would indicate glycation since the proteins are conjugated to the HCMC.

Determination of protein solubility profile

The solubility profile of a sample was measured using a modified protein method of Banach et al.[19] and Rickert et al.[20]. A 1% (w/w) dispersion of the dried egg white was prepared using Milli-Q water and mixed for 1 hour. The dispersion was then separated into separate beakers and the pH was adjusted with 2N NaOH or 2N HCl. The pH was measured at intervals of approximately 15, 30, and 60 min with the pH adjusted if needed. The dispersion was then transferred to 2 mL Eppendorf tubes and centrifuged at 10,000 *g* for 10 min at room temperature. The total dispersion and supernatant after centrifuge was measured for protein content using the biuret method. Bovine serum albumin (BSA) was used as the standard. The % soluble protein at each different pH was calculated as based on the initial protein in the dispersion.

Thermal stability evaluation

Heat stability evaluation at 95°C for 1 hour

A turbidity and protein solubility method was developed to determine the thermal stability of the modified egg white protein at a harsh temperature condition. A 2.5% dispersion (w/w) of the spray dried egg white protein treatments with 0.1 M sodium phosphate buffer (pH 7.0) was well mixed for at least 12 hours. The solution was further diluted to approximately 25 mg/mL after protein quantification. A 1-mL aliquot was transferred to 5 mL of pH 7 buffer, heated in disposable glass tubes at 95°C for 1 hour. The test tubes were then allowed to cool to room temperature in a cooling water bath before measurement of the turbidity and protein solubility. There was a set of diluted samples not heated that were measured for turbidity also before heating. The initial protein content was measured without centrifugation and was used for % protein solubility calculation.

The % transmittance of the treatments was determined at 600 nm using a DU720 spectrophotometer (Beckman Coulter Inc., Brea, CA). The turbidity was calculated by subtracting 100 from the % T value. The turbidity should increase in value, as the protein unfold or becomes denatured via aggregation. A constant or decreased turbidity of the heated samples compared to the unheated samples indicates improved heat stability. The concentration of soluble protein was measured to determine if the decrease in turbidity was due to a decreased soluble protein. Bicinchoninic acid (BCA) protein assay was used to measure the protein content.[21]

Thermal stability evaluation at 75°C over time

The protein solubility in the supernatant of samples heated at 75°C over time was measured to indicate the thermal stability of the modified egg white protein at a less harsh heating condition. A 2.5 to 5.0% dispersion (w/w) of the spray dried egg white protein product in 0.1M sodium phosphate buffer (7.0) was prepared and mixed for at least 12 hours. The dispersion was then transferred to separate 15 mL capped centrifuge tubes, vortexed, and placed in an agitating water bath (Fisher Scientific, Pittsburgh, PA) set at 75°C. The centrifuge tubes were collected at specified time points of 3, 5, 10, 15, and 25 min. Time zero is the test tube that was not heated and that was not placed in the water bath. A 2-mL aliquot was transferred and centrifuged in Eppendorf tubes at 12,000 *g* x 30 min. The supernatant was analyzed for protein content using the BCA method. The percent protein retained was calculated by dividing the concentration of protein of the supernatant to the protein content of the sample (not heated, or centrifuged) multiplied by 100. The degree of decrease in percent protein retained in solution over time reflected the loss of thermal stability of the treatment.

Statistical Analysis

Two replicates of the glycation using fresh egg white and three replicates of the glycation using commercial fresh egg white were conducted. Duplicate analytical measurements were conducted to obtain the average value of each treatment replicate. The statistical analysis was conducted using SAS (Version 9.4, SAS Institute Inc., Cary, NC) with replications being a blocking factor. The data was analyzed using PROC GLM, PROC MIXED, or PROC GLIMMIX with Tukey adjustment on the mean values to determine if there was any significant difference among the means with a p-value of 0.05.

Results and Discussion

Effect of fresh egg white glycation on thermal stability

Effect of hydrolyzed CMC, sonication, and dry heating time on the degree of fresh egg white (FE) glycation

The free amine (mg / 20 mg of protein) and change in free amine concentration can determine the effect of addition of hydrolyzed CMC, sonication, and dry heating time on the degree of glycation of FE (Table 2). Comparing the treatments with one another at each dry heating time, there was no significant difference in concentration of free amine at 0, 12 hours, 1 day, or 2 days. The control treatment showed a % reduction in free amine even though no HCMC was added. There was also significant change in % reduction in free amine between 1:1FE and 5:1FE after 12 hours of dry heating. However, the percent change in free amine of the Son 5:1FE showed an increase in concentration with increasing dry heating time. The 1:1 molar ratio of protein to HCMC, showed a general increase in percent reduction of free amine at 12 hours, but decreased at 1 and 2 days dry heating. After 2 days of dry heating, sonication did not seem to have an effect, as the % reduction was similar without this physical pretreatment. The

modified egg white protein was visually darker in color for all the treatments as the dry heating time increased, indicating that Maillard browning has occurred.

Native chicken egg white contains about 0.4-0.9% carbohydrates that is mainly glucose.[9] The variability of free amine quantified after the reaction may be affected by the glucose inherent in the FE material reacting with the protein and competing with the HCMC for glycation binding sites. This may explain the 7.10% reduction of amine after 2 days of dry heating. It is known that glucose reacts with protein more efficiently than a longer chained polysaccharide during the Maillard reaction.[2] Aoki et al.[2] showed that oligogalacturonic acid had a lower percentage of modification compared to the use of galacturonic acid, 20-25% compared to 31%. Aoki et al.[3] using glucuronic acid was able to conjugate 45% of the free amine after 2 days of incubation. Dry heating proteins has also been shown to partially denature proteins which may have slightly increased the level of free amine being exposed.[10,22] Dry heating egg protein in alkaline pH has also resulted in the deamidation of proteins with longer incubation times.[23] Mine[24] showed that dry heating changed the concentration of free amine slightly at a pH of 8.55, with up to 15 days of drying at 75°C with 8.5% moisture.

Analyses of the changes in molecular weights of the FE glycated protein showed similar changes for all treatments. Figure 2 shows the SDS-PAGE of the FE compared to 1:1 FE glycated. The bands between the treatment and control are very similar from dry heating 0 to 2 days (lane 1 to 4). The bands became more smear-like with certain bands fading when increasing dry heating time, especially the bands near 45 kDa (ovalbumin), and 75 kDa (ovotransferrin). The other major egg white proteins are ovomucoid at 28 kDa and lysozyme at 14 kDa.[9] Mine[23] dry heated de-glucosed egg white at pH 9.42 and showed the disappearance of the ovotransferrin band after dry heating. After 2 days of dry heating a band at the top of the lane appeared

indicating a heavier molecular weight protein was formed for both the control and treatment (lane 4) which could have been caused by the conjugation of the HCMC or some partial hydrolysis of the protein at alkaline pH. Handa and Kuroda[25] found that prior to desurgarization, egg white contained 0.34% (w/v) of glucose. The SDS-PAGE bands of their dry heated un-desugared egg white bands (dry heated at 55°C at 35% relative humidity) were similar to our study. The change in molecular weight of all the products seemed identical at each dry heating time (SDS-PAGE of other treatments not shown). It suggests that the glucose present in the fresh egg white may be glycating with the egg white protein instead of the HCMC.

Effect of FE glycation on protein thermal stability

The thermal stability of the glycated FE heated at 95°C for one hour was measured by turbidity and protein content. The turbidity results are shown in Figure 3. There was no difference among the treatments for the initial and after heating turbidity after 0, 12 hours, and 1 day of dry heating. Even though the calculated % change in turbidity was significantly different comparing control (FE) after 12 hours of dry heating to 1:1FE 12H, the concentration of soluble protein before and after heating were also significantly different (data not shown) making the drawing of conclusion difficult. The % change in turbidity was affected by the difference in soluble protein. The higher concentration of dispersible protein of the 1:1FE 12H treatment may explain its higher turbidity after heating.

After 2 days of dry heating, a significant difference in turbidity and % change among the treatments occurred (Figure 3). The FE 2D and 1:1FE 2D treatment was not different from each other, but they were different from 5:1FE 2D and Son 5:1FE 2D treatments. The difference was not attributed to the change in protein since the concentration of soluble protein was similar for all 2D treatments before (~4.32 mg / mL) and after heating (~3.81 mg / mL). All the treatments

showed an improvement in thermal stability even for the control treatment, which may have been affected by the free glucose present in the fresh egg white. The effect of the quantity of HCMC and sonication is not clear since the control treatment showed similar improvement in thermal stability. The dry heating time did affect the protein. Overall, the graphs show that with heating time all proteins had gradually improved thermal stability. The 1 day heating may be the best because with prolonged heating, protein heat denaturation can be a significant problem too as shown in the 2 day heating "before" samples, which had high initial turbidity values.

Effect of glycation of commercial de-glucosed fresh egg white (CEd) on thermal stability

Effect of hydrolyzed CMC, sonication, and dry heating time on degree of CEd glycation

Commercially de-glucosed and non de-glucosed fresh egg whites were used as two controls for studying the glycation of CEd product. The free amine and % reduction in free amine was significantly different among the treatments dry heated for 12 hours to 7 days (Table 3). When the spray dried powders were not dry heated, the level of free amine among all the samples was not significantly different. All the samples had about 1.00 mg / 20 mg of protein as free amine in general. Commercial fresh egg white that was not de-glucosed (CE) showed considerable reduction in free amine with dry heating time. The free glucose must have reacted with the protein. The concentration of free amine for the CEd sample on the other hand, did not show considerable change with heating time. The free amine of both the 5:1CEd and Son 5:1CEd treatment did not change much with increasing dry heating time. The low concentration of HCMC and its high molecular weight could be contributing to this low reactivity. Aoki et al.[2] found that the reactivity of glycating ovalbumin with glucose and oligomaltosaccharides decreased with increasing chain length. However, the 1:1CEd treatment showed a gradual reduction in free amine with increasing dry heating time. The % reduction in free amine was also

significantly different among the CEd treatment at 12 hours, 1 day, and 2 days but not at 7 days. Aoki et al.[2] found that 31% of the ovalbumin free amine groups was modified with galacturonic acid after 3 days of incubation at 50°C with 65% relative humidity, a value similar to what we observed.

SDS-PAGE (Figure 4) shows no change in molecular weight pattern after spray drying. The heat exposure through the spray drying process was not sufficient to glycate the proteins because the pattern is also similar to raw egg white protein SDS-PAGE (data not shown). As the dry heating time increased a noticeable shift in bands was observed. After 2 days the CE treatment started to fade and was almost unnoticeable after 7 days. The band showed smearing similar to the dry heated bands of the FE control. This may be due to the protein becoming increasingly insoluble with dry heating time as well as the glucose in the sample reacting with the proteins causing the smears. Mine[23] showed dried egg white protein becoming insoluble after 3 days of dry heating at pH 10.42. As the incubation time increased the CEd bands were slightly changing. The bands for 5:1CEd and Son 5:1CEd are similar to one another. The 1:1CEd treatment had a clear upward shift and smear of the protein bands that may relate to an increased degree of glycation reaction with HCMC. The free amine quantification and SDS-PAGE both indicate that sonication did not have an effect, but the concentration of reducing ends and dry heating time had a significant effect on glycation.

A protein solubility profile of the CEd treatment and 1:1CEd treatment after 1 day dry heating was used to further determine if the protein was modified (Figure 5). The isoelectric point of the 1:1CEd shifted to the left and showed a more significant change in protein solubility at lower pH values. The solubility was lowest at a pH of 3 for the modified protein. This glycated protein behaved more like a typical acidic food protein. The isoelectric point of the unmodified

protein was about at pH 5 and it had much higher solubility at the acidic pH range. The solubility profile shows that the egg white protein was modified.

Effect of CEd glycation on thermal stability

Figure 6 shows the turbidity before, after heating at 95°C for 1 hour. The calculated % change in turbidity and the protein content before and after heating is shown in Table 4. At 0 days with no dry heating all the turbidity after heat treatment were greatly increased. The CE treatment % turbidity change in turbidity was significantly higher compared to Son 5:1CEd and 1:1CEd. All the de-glucosed treatments had a significantly lower concentration of soluble protein after undergoing thermal treatment. Since there was no difference in free amine values (Table 3), no improvement in thermal stability was expected and was observed.

As the CE treatment was continually dried its initial turbidity increased but the turbidity after heating greatly decreased. This is explained by the protein insolubilization as shown in Table 4.The SDS PAGE band for CE dry heated for 7 days, was very faint, which is also due to the insolubility of the protein (Figure 4). A similar trend was observed for the de-glucosed CEd. Although, the turbidity was significantly higher after dry heating at 1, 2, and 7 days.

The initial turbidity of the HCMC glycated CEd treatments increased with increasing dry heating time. The % turbidity after the glycated treatments improved after 12 hours of dry heating compared to CEd 12H. The 1:1CEd 12H treatment was significantly lower in turbidity after heating. After 2 and 7 days of dry heating the % turbidity after heating, was similar among all the glycated CEd treatments. Even though the turbidity was not significantly different, the Son 5:1CEd had a significantly higher concentration of soluble protein after heating at 7 days compared to CE, CEd, and the 5:1CEd treatment. With increasing dry heating time, the 1:1CEd treatment showed an increase in soluble protein after heating. Thus, considering the turbidity, the

% change in turbidity, and protein solubility, the glycated treatments showed improved thermal stability compared to the two controls (CE and CEd).

A different mechanism may have caused the improvement in thermal stability for the 5:1CEd and Son 5:1CEd treatments, since the concentration of free amine did not change. Ibanoglu[26] using differential scanning calorimetry (DSC) showed an increase in enthalpy or thermal stability of BSA, lysozyme, and whey protein isolate (WPI) with the addition of hydrocolloids (pectin, guar gum, and ι-carrageenan). The researcher speculated that there might be an ingredient interaction with the hydrocolloids blocking the hydrophobic protein sites, preventing aggregation and improving thermal stability. Using a two-step heating and sonication process, Ashokkumar et al.[27] was able to improve the thermal stability of whey proteins. The initial hydrophobic protein-to-protein interactions were dispersed with sonication, causing a delay in aggregation formation with the second heating step at higher temperatures, leading to an increase in thermal stability.

Effect of CEd glycation on thermal stability at 75°C

The 1-day dry heated proteins were selected for further thermal stability testing to further examine the effect of glycation. Figure 7 shows the soluble protein during heating at 75°C for 25 min. The control CE treatment had a significantly lower concentration of soluble protein compared to all the other treatments at each time point. The CEd treatment showed a high soluble protein concentration during heating for the 5 min, but the solubility significantly reduced during the remaining of the 25 min heating. The other treatments were similar to one another in soluble protein over time. The data suggests the glycation modification process did improve the thermal stability of the protein.

Conclusions

Glycation was successfully used to improve the thermal stability of egg white proteins. This study showed key factors when glycating egg white protein with HCMC. The egg white protein was not glycated through spray drying alone and needed to be dry heated for the reaction to occur. The higher quantity of HCMC improved the thermal stability of egg white proteins. Increasing incubation time also improved the reaction and stability of the protein. Sonication was not important in assisting the substrate dispersion and reaction. Most importantly, the initial sugar content of egg white is the most critical factor in the glycation reaction. In the future, a shorter carbohydrate chain with higher reactivity causing Maillard reaction should be explored to further the glycation study. Spray dried, de-glucosed egg white protein can be glycated with polysaccharide to produce a protein with improved thermal stability.

Acknowledgments

We would like to thank Dr. Hui Wang at the Iowa State University Center for Crops Utilization Research pilot plant for his help in spray drying the samples. Beijing DQY Agricultural Technology Co., Ltd and the Chinese National Egg Engineering Research Center. Beijing, China for providing financial support. Oskaloosa Food Products Corp. for providing the fresh egg white material.

Abbreviations Used

BCA, bicinchoninic acid; BSA, bovine serum albumin; CE, commercial fresh egg white not de-glucosed; CEd, commercial fresh egg white de-glucosed; FE, fresh egg white; HCMC, hydrolyzed carboxymethylcellulose; M_w, molecular weight; NS, not significantly different; S significantly different; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel

electrophoresis; Sig, Significance; Son, sonicated; TNBS, 2, 4, 6-trinitrobenzene sulfonic acid picrylsulfonic; WPI, whey protein isolate.

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Table 1. Treatment acronyms for the evaluation of effect of HCMC glycation on protein thermal stability.

Starting Material	Molar ratio of protein to HCMC	Sonication Step	Dry heating (Time)	Treatment Abbreviation
A. Fresh egg white – (FE)	1:0	No	0	FE
			12 hrs	FE 12H
			1 day	FE 1D
			2 days	FE 2D
	5:1	No	0	5:1FE
			12 hrs	5:1FE 12H
			1 day	5:1FE 1D
			2 days	5:1FE 2D
	5:1	Yes	0	Son 5:1FE
			12 hrs	Son 5:1FE 12H
			1 day	Son 5:1FE 1D
			2 days	Son 5:1FE 2D
	1:1	No	0	1:1FE
			12 hrs	1:1FE 12H
			1 day	1:1FE 1D
			2 days	1:1FE 2D
B. Commercial fresh egg white not de-glucosed (CE)	1:0	No	0	CE
			12 hrs	CE 12H
			1 day	CE 1D
			2 days	CE 2 D
			7 days	CE 7D
C. Commercial fresh egg white de-glucosed (CEd)	1:0	No	0	CEd
			12 hrs	CEd 12H
			1 day	CEd 1D
			2 days	CEd 2D
			7 days	CEd 7D
	5:1	No	0	5:1CEd
			12 hrs	5:1CEd 12H
			1 day	5:1CEd 1D
			2 days	5:1CEd 2D
			7 days	5:1CEd 7D
	5:1	Yes	0	Son 5:1CEd
			12 hrs	Son 5:1CEd 12H
			1 day	Son 5:1CEd 1D
			2 days	Son 5:1CEd 2D
			7 days	Son 5:1CEd 7D
	1:1	No	0	1:1CEd
			12 hrs	1:1CEd 12H
			1 day	1:1CEd 1D
			2 days	1:1CEd 2D
			7 days	1:1CEd 7D

HCMC – hydrolyzed carboxymethylcellulose

Table 2. Effect of molar ratio, sonication, and drying time on free amine (mg / 20 mg protein) and % amine reduction of the glycated fresh egg white (FE)[x]

Treatments	FE	5:1FE	Son 5:1FE	1:1FE	Sig
0 time dry heating					
Free amine (mg/ 20 mg protein)	1.06 ± 0.13	0.77 ± 0.08	1.03 ± 0.34	1.29 ± 0.96	NS
Reduction amine (%)	n/a	n/a	n/a	n/a	n/a
12 hours (12H) dry heating					
Free amine (mg/ 20 mg protein)	0.92 ± 0.03	0.80 ± 0.09	0.94 ± 0.17	0.82 ± 0.55	NS
Reduction amine (%)	13.01 ± 7.88^{a,b}	-3.59 ± 1.33^{b}	6.19 ± 14.53^{a,b}	34.35 ± 6.00^{a}	S
1 day (1D) dry heating					
Free amine (mg/ 20 mg protein)	0.96 ± 0.07	1.01 ± 0.25	0.84 ± 0.19	0.92 ± 0.60	NS
Reduction amine (%)	8.97 ± 4.84	-30.40 ± 18.95	16.79 ± 8.63	25.84 ± 8.43	NS
2 day (2D) dry heating					
Free amine (mg/ 20 mg protein)	0.99 ± 0.14	0.59 ± 0.11	0.60 ± 0.11	1.03 ± 0.36	NS
Reduction amine (%)	7.10 ± 1.92	24.17 ± 6.65	40.02 ± 8.86	4.21 ± 43.41	NS

[x]All values are the means ± standard deviations of three replicates. Different letters in each row represent significant differences ($P<0.05$). Sig – Significance, NS – not significantly different and S for significantly different

Table 3. Effect of protein to HCMC molar ratio, sonication, and drying time on free amine (mg/ 20 mg protein) and % amine reduction of the glycated commercial fresh egg white.[x]

Treatments	CE	CEd	5:1CEd	Son 5:1CEd	1:1CEd	Sig
0 time dry heating						
Free amine (mg/ 20 mg protein)	0.92 ± 0.03	1.01 ± 0.06	0.98 ± 0.04	1.00 ± 0.05	0.89 ± 0.03	NS
Reduction amine (%)	n/a	n/a	n/a	n/a	n/a	n/a
12 hours (12H) dry heating						
Free amine (mg/ 20 mg protein)	$0.87 \pm 0.13^{b,c}$	1.15 ± 0.14^{a}	$1.05 \pm 0.07^{a,b}$	$1.01 \pm 0.15^{a,b,c}$	0.80 ± 0.06^{c}	S
Reduction amine (%)	$5.93 \pm 11.01^{a,b}$	-13.40 ± 12.30^{c}	$-7.49 \pm 7.30^{c,b}$	$-0.48 \pm 13.10^{a,b,c}$	9.80 ± 4.85^{a}	S
1 day (1D) dry heating						
Free amine (mg/ 20 mg protein)	0.24 ± 0.12^{d}	1.17 ± 0.05^{a}	0.97 ± 0.02^{b}	$1.03 \pm 0.05^{a,b}$	0.71 ± 0.01^{c}	S
Reduction amine (%)	73.79 ± 12.22^{a}	-15.62 ± 5.60^{c}	0.83 ± 2.29^{c}	-2.31 ± 0.99^{c}	20.57 ± 1.60^{b}	S
2 days (2D) dry heating						
Free amine (mg/ 20 mg protein)	0.14 ± 0.09^{c}	1.15 ± 0.25^{a}	$0.91 \pm 0.06^{a,b}$	$0.93 \pm 0.06^{a,b}$	0.58 ± 0.05^{b}	S
Reduction amine (%)	85.08 ± 9.03^{a}	-13.50 ± 21.59^{c}	$7.52 \pm 2.19^{b,c}$	$7.22 \pm 4.39^{b,c}$	34.62 ± 3.48^{b}	S
7 days (7D) dry heating						
Free amine (mg/ 20 mg protein)	0.45 ± 0.15^{b}	$0.88 \pm 0.32^{a,b}$	0.93 ± 0.06^{a}	0.99 ± 0.04^{a}	$0.66 \pm 0.02^{a,b}$	S
Reduction amine (%)	51.58 ± 15.54^{a}	$12.61 \pm 34.09^{a,b}$	$4.93 \pm 4.21^{a,b}$	0.81 ± 4.95^{b}	$25.96 \pm 0.26^{a,b}$	S

[x]All values are the means ± standard deviations of three replicates. Different letters in each row in represent significant differences ($P<0.05$). Sig – Significance, NS – not significantly different, S - significantly different, CE – commercial fresh egg white not de-glucosed, and CEd – commercial egg white de-glucosed

Table 4. Effect of molar ratio, sonication, and drying time on thermal stability of glycated protein: change in turbidity and protein solubility before and after 95°C heat temperature evaluation.

Control – 0 day dry heating	CE	CEd	5:1CEd	Son 5:1CEd	1:1CEd	Sig
Change turbidity (%)	85.1 ± 0.8^{a}	77.0 ± 2.0^{a,b}	83.0 ± 1.6^{a,b}	74.9 ± 7.0^{b}	74.9 ± 1.4^{b}	S
Before protein (mg / mL)	4.30 ± 0.10	4.31 ± 0.21	4.18 ± 0.14	4.22 ± 0.06	3.97 ± 0.16	NS
After protein (mg / mL)	3.89 ± 0.14^{a}	3.03 ± 0.05^{b}	3.09 ± 0.09^{b}	3.11 ± 0.09^{b}	3.10 ± 0.26^{b}	S

12 hrs dry heating	CE 12H	CEd 12H	5:1CEd 12H	Son 5:1CEd 12H	1:1CEd 12H	Sig
Change turbidity (%)	6.7 ± 6.2^{c}	68.8 ± 0.6^{a}	31.3 ± 3.0^{b}	31.0 ± 6.8^{b}	11.1 ± 1.1^{c}	S
Before protein (mg / mL)	4.41 ± 0.14^{a,b}	4.48 ± 0.10^{a}	4.43 ± 0.02^{a,b}	4.47 ± 0.05^{a}	4.22 ± 0.04^{b}	S
After protein (mg / mL)	3.63 ± 0.13^{a}	3.28 ± 0.19^{b}	3.62 ± 0.02^{a}	3.68 ± 0.04^{a}	3.48 ± 0.04^{a}	S

2 days dry heating	CE 2D	CEd 2D	5:1CEd 2D	Son 5:1CEd 2D	1:1CEd 2D	Sig
Change turbidity (%)	-38.8 ± 8.7^{c}	40.0 ± 12.5^{a}	1.5 ± 0.6^{b}	3.2 ± 3.7^{b}	-2.8 ± 2.9^{b}	S
Before protein (mg / mL)	1.19 ± 0.21^{b}	4.12 ± 0.48^{a}	4.23 ± 0.05^{a}	4.29 ± 0.08^{a}	4.09 ± 0.06^{a}	S
After protein (mg / mL)	0.96 ± 0.15^{b}	3.27 ± 0.36^{a}	3.59 ± 0.08^{a}	3.63 ± 0.06^{a}	3.55 ± 0.01^{a}	S

7 days dry heating	CE 7D	CEd 7D	5:1CEd 7D	Son 5:1CEd 7D	1:1CEd 7D	Sig
Change turbidity (%)	-55.1 ± 2.4^{b}	-21.7 ± 12.3^{a}	-5.8 ± 0.9^{a}	-5.4 ± 8.3^{a}	-10.8 ± 2.9^{a}	S
Before protein (mg / mL)	0.69 ± 0.11^{c}	2.28 ± 0.49^{b}	3.56 ± 0.08^{a}	4.17 ± 0.11^{a}	4.07 ± 0.02^{a}	S
After protein (mg / mL)	0.46 ± 0.04^{d}	1.70 ± 0.39^{c}	3.15 ± 0.14^{b}	3.67 ± 0.15^{a,b}	3.76 ± 0.01^{a}	S

[x]All values are the means ± standard deviations of two replicates. Different letters in each bar of graph or row in table represent significant differences (P<0.05).
Sig – significance, NS – not significantly different, S – significantly different

Figure captions:

Figure 1. Flow chart of egg white protein preheat glycation treatments.

Figure 2. SDS-PAGE of glycated egg white protein, 40 ug, with β-mercaptoethanol added. The gels are separated out based on treatment FE and 1:1 FE.

Figure 3. Effect of drying time on thermal stability of HCMC glycated FE proteins heated at 95°C for 1 hr. Values are the means ± standard deviations of two replicates. Different letters in each graph represent significant differences ($P<0.05$).

Figure 4. SDS-PAGE of glycated CEd 40 ug loaded. The first five lanes are based on treatments with reducing agent added. The gels are separated out based on dry heating time.

Figure 5. Protein solubility profile of spray dried HCMC glycated commercial fresh egg white (CEd, de-glucosed and 1:1CEd dry heated for 1 day).

Figure 6. Effect of drying time on thermal stability of HCMC glycated CE proteins heated at 95°C for 1 hr. Values are the means ± standard deviations of three replicates. Different letters in each graph represent significant differences ($P<0.05$).

Figure 7. Effect of protein glycation (dry heated 1 day) on thermal stability evaluated at 75°C over 25 minutes.

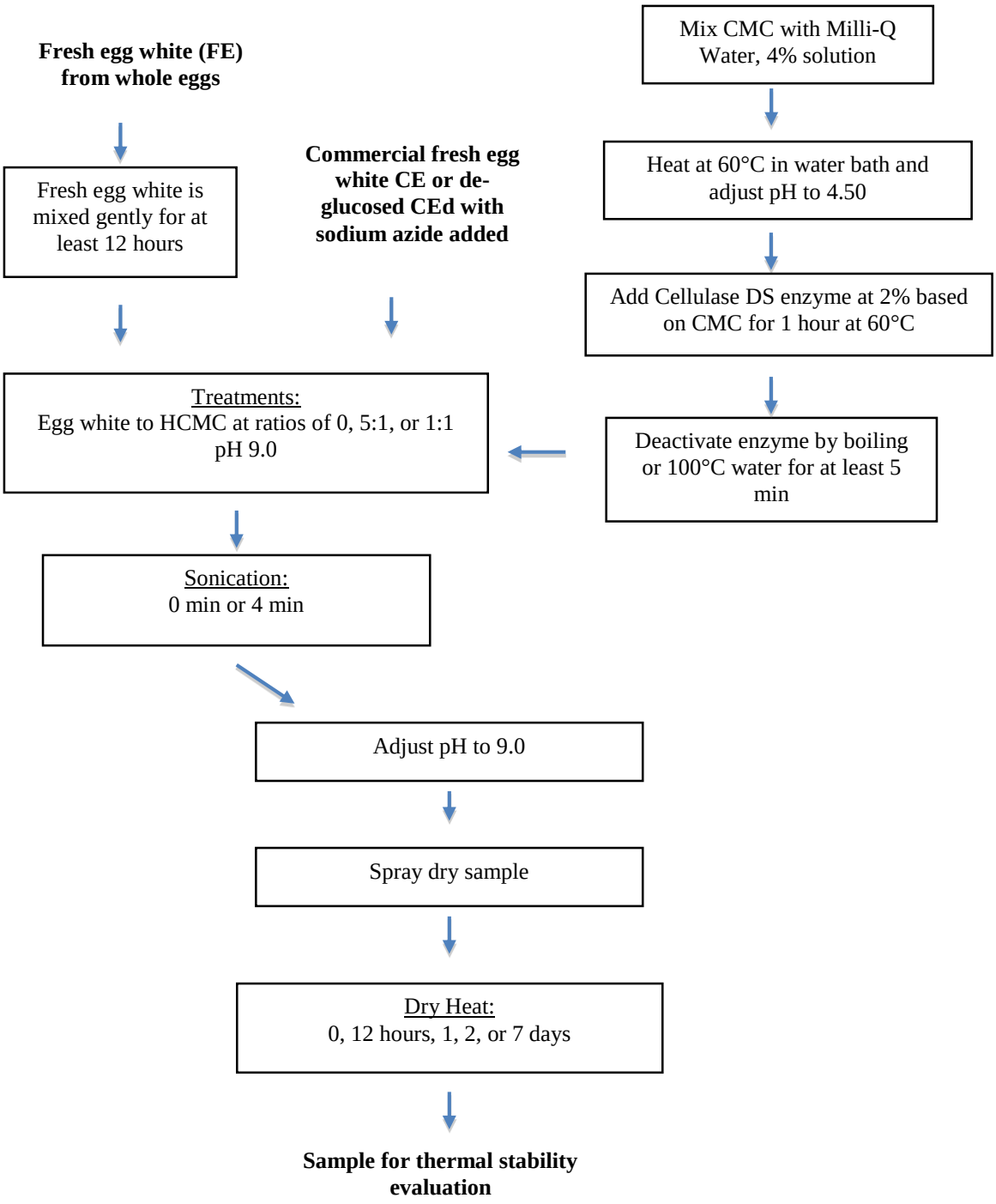


Figure 1. Flow chart of egg white protein preheat glycation treatments.

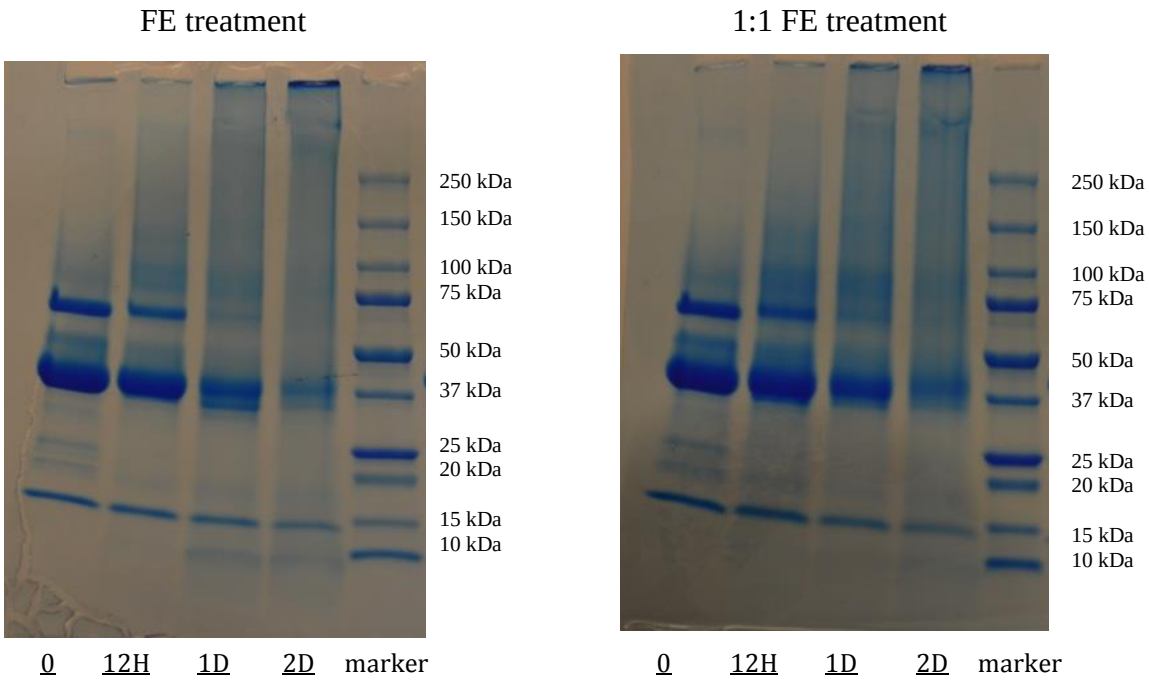


Figure 2. SDS-PAGE of glycated egg white protein, 40 ug, with β-mercaptoethanol added. The gels are separated out based on treatment FE and 1:1 FE.

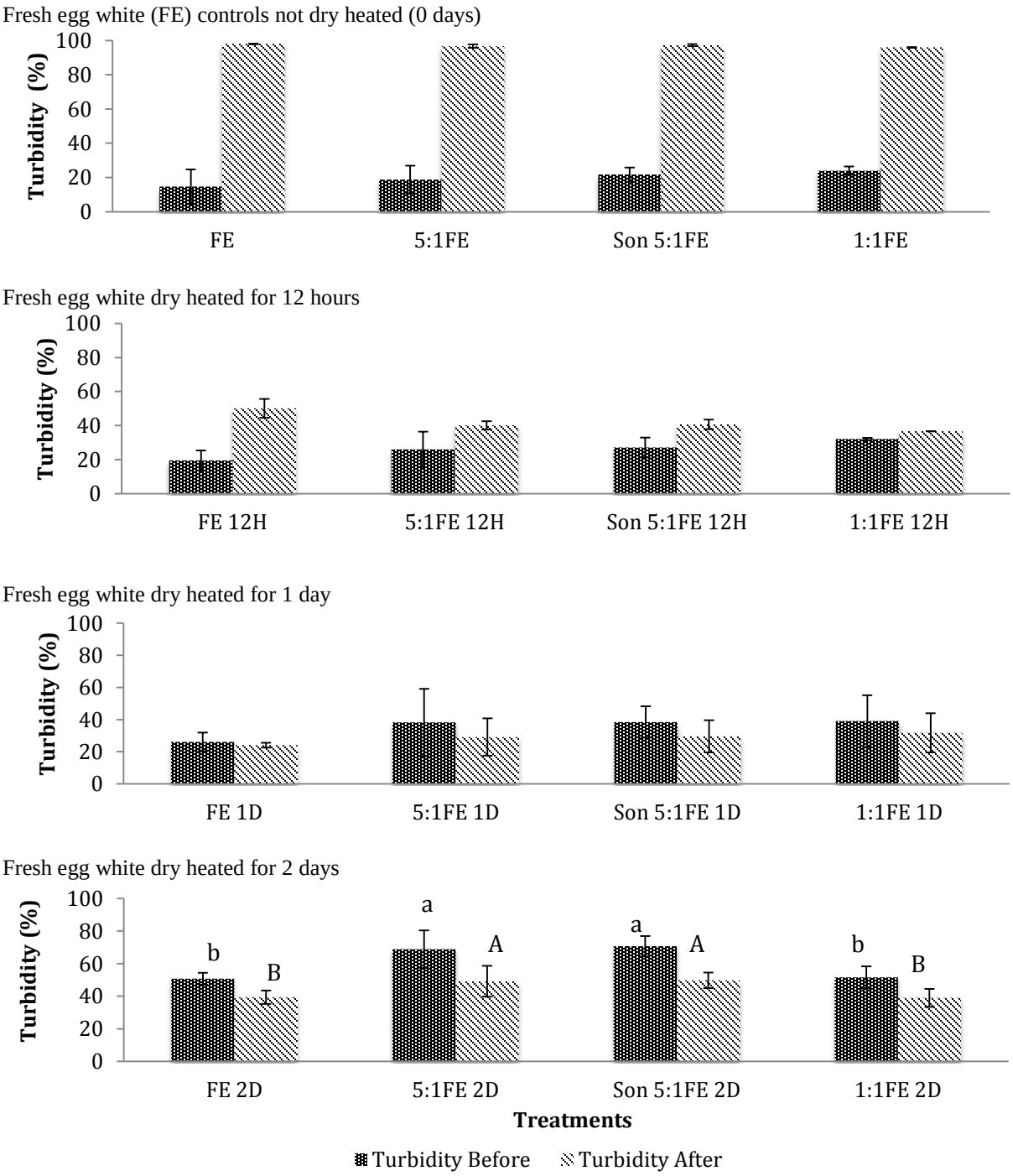
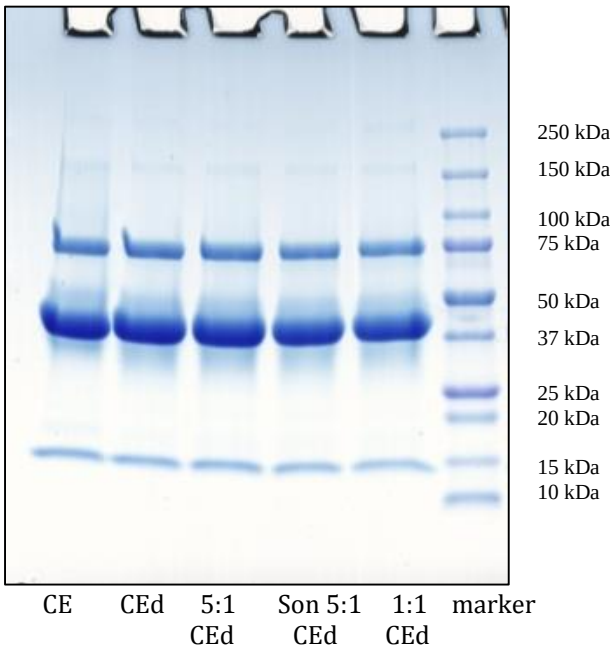
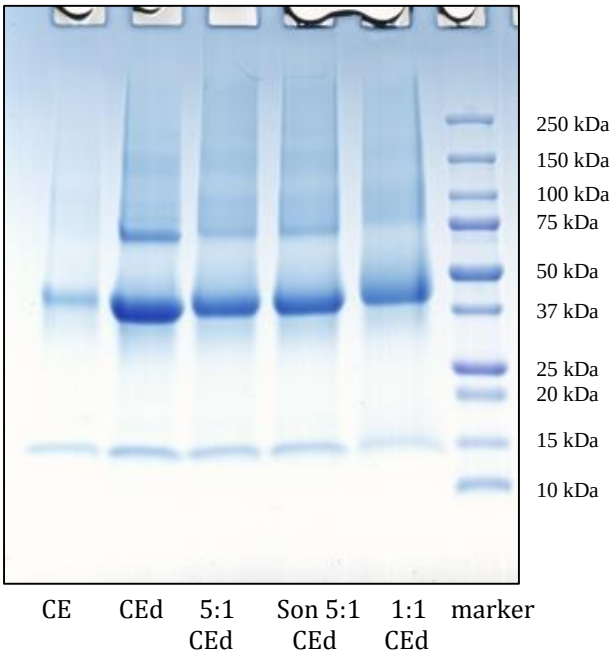


Figure 3. Effect of drying time on thermal stability of HCMC glycated FE proteins heated at 95°C for 1 hr. Values are the means ± standard deviations of two replicates. Different letters in each graph represent significant differences ($P<0.05$).

0 day dry heating time



2 day dry heating time



7 days dry heating time

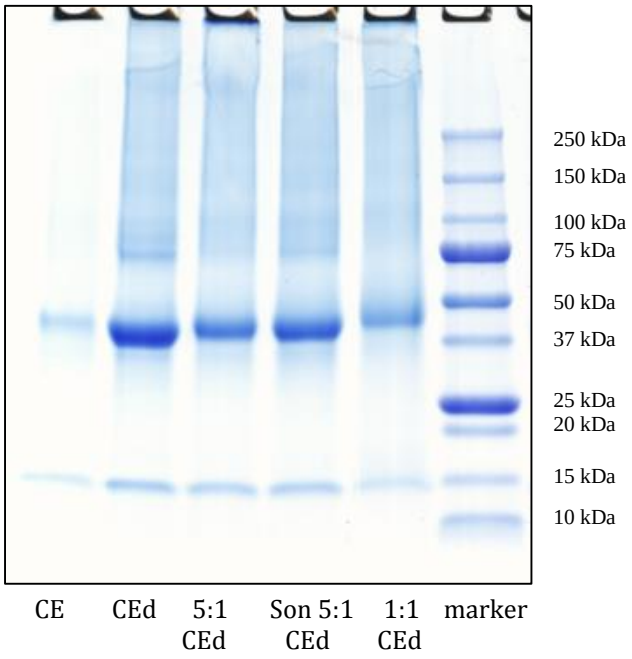


Figure 4. SDS-PAGE of glycated CEd 40 ug loaded. The first five lanes are based on treatments with reducing agent added. The gels are separated out based on dry heating time.

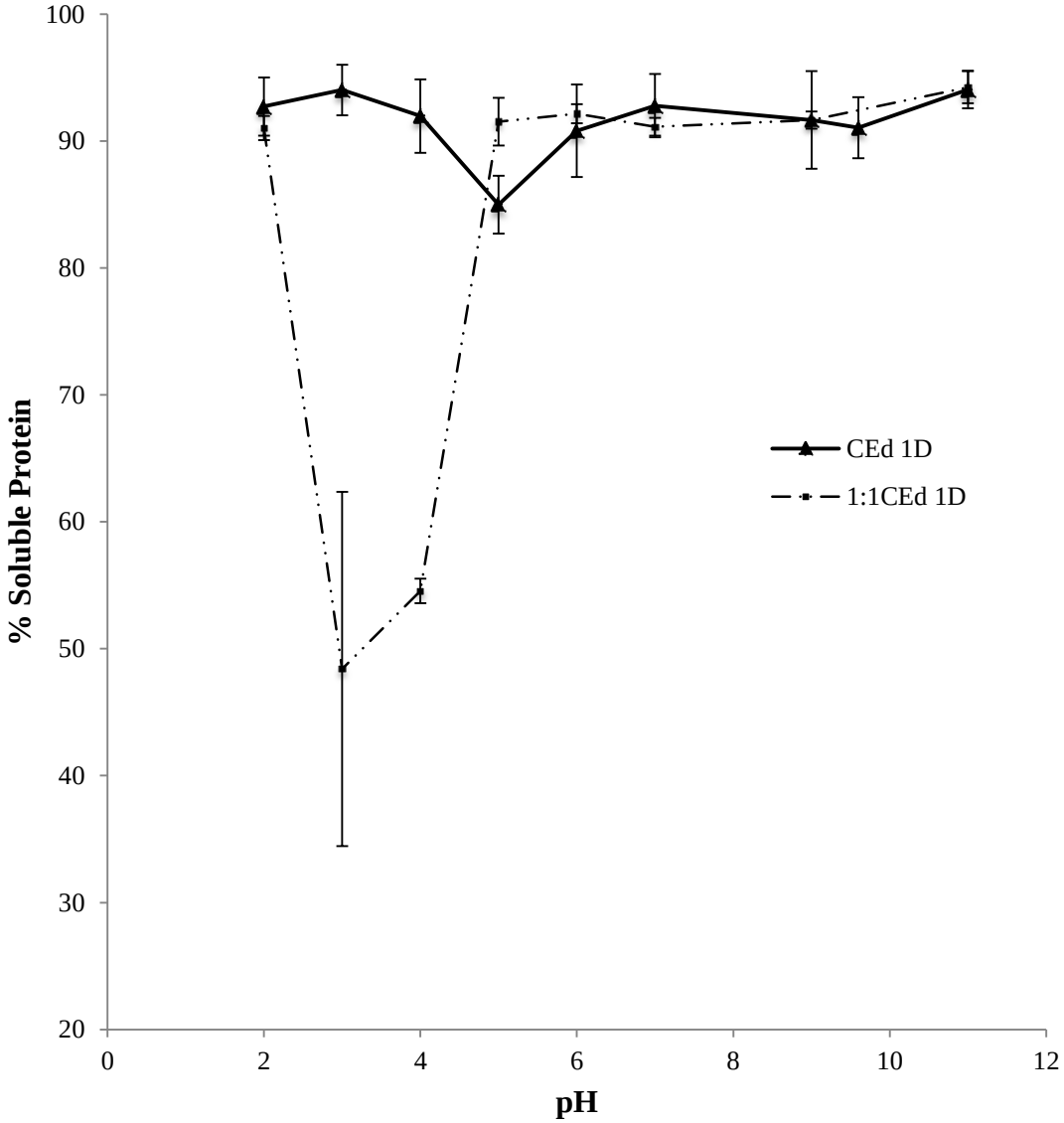


Figure 5. Protein solubility profile of spray dried HCMC glycated commercial fresh egg white (CEd, de-glucosed and 1:1CEd dry heated for 1 day).

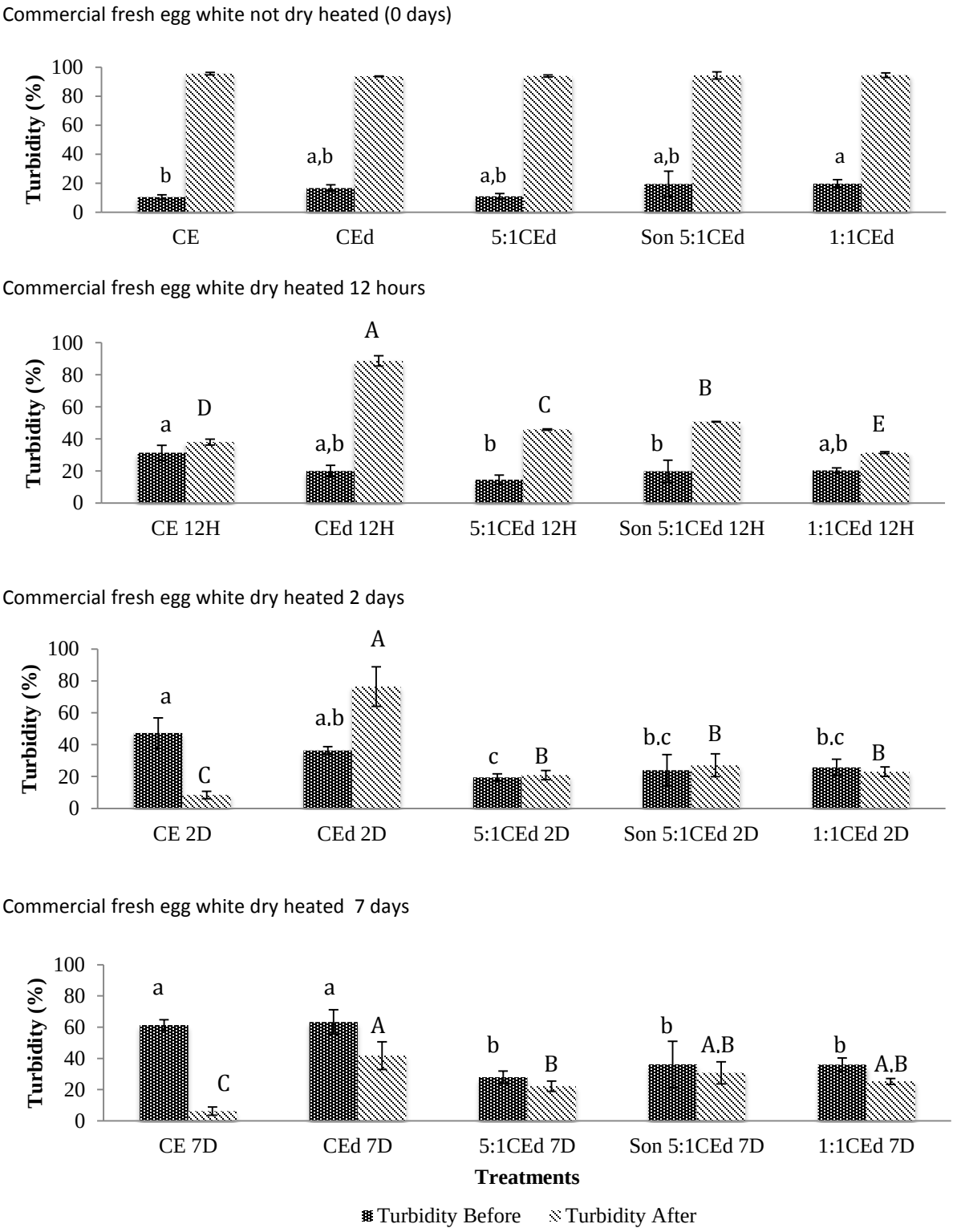


Figure 6. Effect of drying time on thermal stability of HCMC glycated CE proteins heated at 95°C for 1 hr. Values are the means ± standard deviations of three replicates. Different letters in each graph represent significant differences ($P<0.05$).

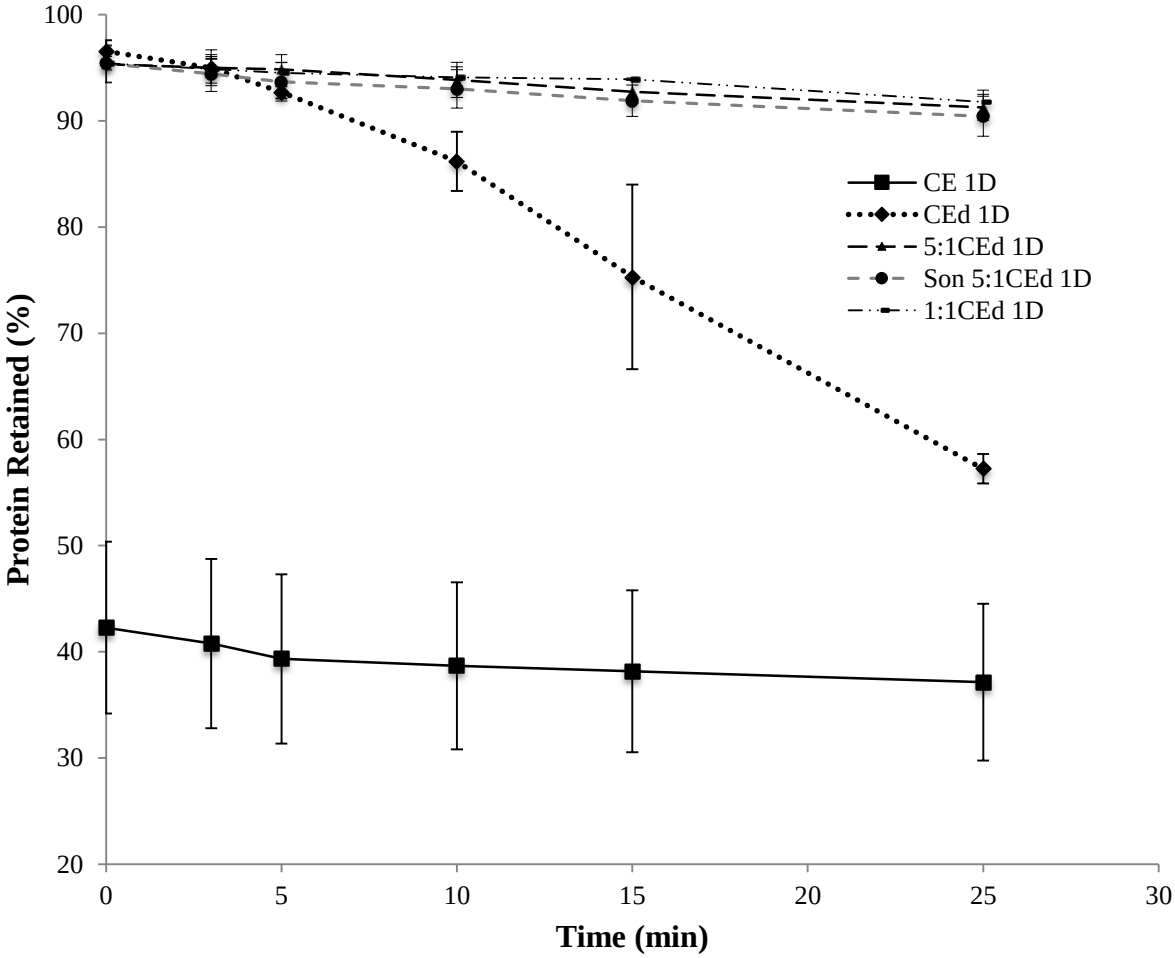


Figure 7. Effect of protein glycation (dry heated 1 day) on thermal stability evaluated at 75°C over 25 minutes.

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CHAPTER 3. ALBUMEN SUCCINYLATION WITH OCTENYL SUCCINIC ANHYDRIDE TO IMPROVE THERMAL STABILITY

A manuscript to be submitted to *Journal of Agricultural and Food Chemistry*

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Abstract

The modification of egg white proteins with octenyl succinic anhydride (OSA) improved thermal stability of the protein. The use of sonication pretreatment with OSA succinylation further improved the efficiency of the reaction while reducing the concentration of OSA used to achieve stability. Three different egg white materials were tested; they were rehydrated egg white powder (EWP), fresh egg white (FEW), and commercial de-glucosed egg white (CFEW). Each egg white material and reaction process had a different effect on the degree of OSA succinylation. At higher temperature of thermal stability evaluation, 95°C compared to 75°C, the sonicated and 10% addition of OSA CFEW showed the greatest level of improvement, but not to the extent of the freeze dried FEW. The sonicated 10% OSA CFEW treatment showed slight changes in molecular weight in SDS-PAGE but significant change in protein solubility profile. A reduction in concentration of added OSA is still possible if the modification process was further optimized.

Keywords: *egg white, albumen, protein, succinylation, thermal stability*

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Introduction

Chemical modification of proteins is effective in improving the functionality of food proteins. Egg white proteins have been chemically modified through acylation or succinylation with oleic acid, succinic anhydride, 3,3-dimethylglutaric anhydride, and other compounds to improve its functionality.[1-4] Succinylated soy, fish myofibrillar, and hen egg proteins have shown to have an improvement in solubility, gelation, emulsification, foam, heat stability, and these modified protein are intended for food uses.[3-6] Palacian et al.[7] suggested that succinylation produced a more stable protein than acylation. As the demand for consumption of protein increases, there is a need to improve functional egg white proteins to expand its application in the food processing industry, especially its heat stability properties.[8]

The functionality improvements from succinylation are attributed to the change in protein structure, because the charges from succinic anhydride for example affects the electrostatic and steric hindrance of the protein.[3,9,10] The succinylation of proteins occurs on the free amine, hydroxyl, and sulfhydryl groups of proteins.[3,7,11,12] The reactivity of each functional group varies and is affected by different reaction conditions. A limited degree of sulfhydryl modification occurs with the hydroxyl containing amino acids serine being more reactive than threonine.[11] Achouri and Zhang[9] found a decrease in esterification of the hydroxyl groups at higher levels of succinylation. However, the highest succinylation reactivity occurs on the secondary amino groups of amino acids such as lysine. Succinic anhydride has been used successfully to succinylate proteins, but the study of octenyl-succinic anhydride (OSA) modified proteins is limited. OSA not only contains a dicarboxylic acid that provides the charges upon reaction, as from succinic anhydride, it also has a hydrophobic group, thus can potentially prevent protein-to-protein hydrophobic aggregation. The proteins' hydrophobic moieties exposed during heating

could interact with OSA's eight-carbon tail instead of with the hydrophobic moiety of other proteins during heating, delaying protein-protein aggregation.

Hen egg white albumen is a heterogeneous mixture that contains over 40 different proteins with ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), and lysozyme (3.4%) representing over 80% of its protein.[13] Sonication can physically alter the structure of proteins, having the potential to increase the efficiency of a chemical modification reaction of a heterogeneous system. The physical treatment uses sound waves at low frequency with high power to mix substrates by cavitation (formation and collapse of air bubbles generating pressure and heat indirectly).[14] The use of ultrasound or sonication could increase the reaction of many chemical modifications such as succinylation. It is difficult to obtain a uniform mixture of fresh egg white with OSA. The use of sonication would improve the uniformity of fresh egg white OSA mixture and may partially denature the proteins and expose reactive sites by unfolding the protein through sound waves.[14] Arzeni et al.[14] demonstrated how sonication could increase protein surface hydrophobicity. This partial unfolding of the thermal unstable proteins, at lower than the denaturation temperatures, may assist the chemical modification of egg white proteins.

We hypothesized that the thermal stability of egg white proteins can be improved by modification with OSA, creating a more thermal stable protein product. OSA has been used to modify food starch in the U.S. and has been widely used in the food industry.[15] There is currently no research on succinylating egg white proteins with OSA, especially with the assistance of sonication. Three different sources of egg white protein were modified to determine their effectiveness, mimicking actual commercial production. The objectives of this study are to

modify egg white proteins with OSA, to study the effects of pretreatment using sonication on reaction efficiency, and to evaluate the changes in thermal stability of the protein.

Materials

Dried egg white powder (EWP) was purchased from Honeyville Grain Inc. (Brigham City, UT). Large grade A chicken eggs were purchased with similar sell-by dates from a local supermarket (Ames, IA) and the fresh egg white (FEW) was manually separated. Oskaloosa Food Products Corp. (Oskaloosa, IA) provided raw fresh egg white (commercial de-glucosed fresh egg white, CFEW) that was already mixed and de-glucosed by fermentation. OSA and other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Methods

Modification of egg white protein

Succinylation of egg white

Succinylation of commercial dried egg white or fresh egg white was accomplished through a similar method of Ball and Winn.[16] The process flow chart is outlined in Figure 1 and the treatment and acronyms are listed in Table 1. The EWP was mixed as 10% protein dispersion for at least 12 hours before centrifugation at 2,000 *g* for 2 minutes to remove any insoluble components. The supernatant was then mixed into a big batch and used for OSA modification. The FEW that was manually separated from the egg yolk was mixed gently for at least 12 hours. The CFEW was already pre-mixed and de-glucosed before further modification. For fresh or commercial de-glucosed fresh egg white, the OSA was added directly to the mixture.

The different egg white dispersions (EWP, FEW, and CFEW) were transferred to the pH-STAT (718 Titrino, Brinkmann, Switzerland) for 1-liter batch reactions to control the pH during

the chemical reaction. The concentration of OSA added ranged from 1-10% (1, 2, 4, 5, and 10%) based on the estimated protein content. The pH was maintained at 8.5 with 2 N NaOH for 3 hours at 35°C with mild mixing. The pH was adjusted to 6.5 using 2N HCl to stop the reaction and the mixture was dialyzed (M_w cutoff 6,000 to 8,000 Daltons) to remove any salts or unreacted OSA for 3 days in a refrigerated room (4°C). The water was changed two times a day. The CFEW treatments were not dialyzed due to the large quantity intended for spray drying.

Succinylation of egg white protein with sonication pretreatment

Fresh egg white was prepared as before, but was sonicated using a Sonicator (Fisher Scientific model 500 Ultrasonic Dismembrator, Pittsburgh, PA). Sonication was done before the reaction with OSA in small batches of between 150-200 mL for 4 minutes using a ½ inch horn at 25-35% amplitude (set at 70%) in an ice bath. Gordon and Pilosof[17] reported a decrease in particle size after sonicating whey protein isolate (WPI) for 2 minutes (using a 13 mm probe threaded to a 3 mm tapered microtip) with no further decrease up to 20 minutes in 10 mL protein batches. For the CFEW modified final product to be spray dried, the reacted mixture was sonicated again for another 4 minutes after reaction to disperse the precipitated particles.

Drying of succinylated egg white protein

After dialysis, the EWP and FEW products were freeze-dried then ground with a mortar and pestle. The CFEW samples were not dialyzed, but underwent filtering using cheesecloth before being spray dried. The spray dried products were produced using a conical spinning-disk atomizer feed spray dryer (APV Crepaco Inc, Getzville, NY). The inlet and outlet temperature was between 180-195°C and 88-110°C, respectively. The flow rate was adjusted to control the outlet temperature. The powder was collected and stored for future analyses.

Characterization of OSA succinylated products

Degree of succinylation using a free amine test

The degree of succinylation reaction was determined by using the TNBS (2, 4, 6-trinitrobenzene sulfonic acid picrylsulfonic acid) method that measures the concentration of free amine according to a method by Habeeb.[18] A TNBS kit was purchased from G-Biosciences (St. Louis, MO). A standard curve established using glutamic acid was used and the free amine calculated is the equivalent of glutamic acid. The free amine was calculated based on 20 mg of protein.

SDS-PAGE analysis of protein molecular size

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli,[19] using a Bio-Rad Mini-PROTEAN® Tetra System to observe changes in molecular size. A 4-20% Mini-Protean ® TGX™ precast gel and Precision Plus Protein™ Dual Color Standards were used. The protein sample was dissolved in Milli-Q water with Laemmli buffer. One set had β-mercaptoethanol added and another did not. The sample (40 ug of protein) was loaded onto the pre-cast gel well at a constant voltage of 120V in running buffer (1X Tris/Glycine/SDS buffer) for 1 hour. The gel was then stained with coomassie blue dye (50% methanol, 22% coomassie blue at 1% concentration, 18% Milli-Q water, and 10% glacial acetic acid) and de-stained with a de-staining solution (10% acetic acid, 15% methanol, and 75% distilled water) before analysis.

Determination of protein solubility profile

The solubility profile of the protein was measured using a modified method of Rickert et al.[20] by Banach et al.[21] A 1% (w/w) dispersion of the dried egg white protein was prepared using

Milli-Q water and mixed for 1 hour. The dispersion was separated into separate beakers and the pH was adjusted with 2N NaOH or 2N HCl to 2 to 11 pH within 0.5 units. The pH was tested at intervals of approximately 15, 30, and 60 min with the pH adjusted if needed. The dispersion was then transferred to 2 mL Eppendorf tubes and centrifuged at 10,000 *g* for 10 min at room temperature. The total dispersion before centrifuge and supernatant after centrifuge were measured for protein content using the biuret method.[22] Bovine serum albumin (BSA) was used as the standard for protein quantification. The percent soluble protein at each different pH was then calculated.

Thermal stability evaluation

Thermal stability evaluation by measuring solubility of protein during 75°C heating

A protein solubility method was used to indicate the thermal stability of the modified egg white protein based on the method of Ball and Winn.[16] A 5% dispersion of the dried egg protein powder (w/w) with pH 7.0 buffer (0.1M sodium phosphate) was prepared and mixed for at least 12 hours. An aliquot, 5 mL, was then transferred to 15 mL Corning centrifuge tubes and placed in a shaking water bath set at 75 ± 2°C. The centrifuge tubes were taken out at specific time points (0, 3, 5, 10, 15, and 25 minutes). Time 0 was the as-is, samples that were not heated. All the samples were allowed to cool before further dilution and measurement for soluble protein content.

Two mL of the modified protein dispersion was transferred to a 2 mL Eppendorf test tube for centrifugation at 12,000 *g* for 30 minutes. The bicinchoninic acid (BCA) protein assay was used to measure the protein content of the supernatant portion.[23] The absorbance was measured at an absorbance of 562 nm using a DU720 spectrophotometer (Beckman Coulter Inc., Brea, CA). BSA standard was used for establishing the standard curve.

Thermal stability evaluation by measuring turbidity and solubility at 95°C

A turbidity and solubility method was also used to evaluate the thermal stability of the modified egg white protein. A 5.0% dispersion (w/w) of the dried modified egg white protein with pH 7.0 buffer (0.1M sodium phosphate) was mixed for at least 12 hours. The dispersion was further diluted to 25 mg protein / mL. The diluted dispersion of 1 mL was then transferred to 5 mL of pH 7 buffer and heated in a disposable glass tube at 95°C for 1 hour. The % transmittance (T) of the samples was measured at 600 nm. The turbidity was calculated as the value after 100 was subtracted from the %T. The dispersion at the same concentration but not heated to 95°C was also determined as the sample's starting turbidity. The concentration of soluble protein of the supernatant was measured using the BCA protein assay to ensure that a decrease in turbidity was not due to a reduction in soluble protein after heating.

Statistical Analysis

Three replicates of each treatment were conducted on EWP and CFEW. Two replicates were performed on FEW treatments. Each sample was measured twice for all analyses. The statistical analysis was carried out using SAS version 9.4 (SAS Institute Inc., Cary, NC). The data was evaluated using PROC GLM, PROC MIXED, or PROC GLIMMIX with Tukey adjustment to determine if there was any significant difference among the means with a p-value of 0.05. Replicates were the blocking factor. Comparisons were made between the control and OSA modified treatments within each egg protein substrate with OSA added, with and without sonication pre-treatment.

Results and Discussion

Effect of OSA concentration on degree of modification

The free amine and calculated % reduction of amine group are shown in Table 2 separated based on type of protein, i.e., EWP, FEW, or CFEW. A larger decrease in free amine group indicates a higher degree of protein modification. The EWP samples had a significant decrease in the concentration of free amine between the Control treatment and different levels of OSA starting at the 2%. The % reduction in free amine was significantly different comparing 1% OSAE (octenyl succinylated EWP, 10.09%) to 2% OSAE (30.64%). However, between 2% OSAE and 4% OSAE (39.05%) the difference in free amine and % reduction was not statistically different. Higher levels of OSA addition may not further improve the functionality of OSA modified EWP.

The modified FEW treatments also showed a reduction in free amine. At 4% OSA addition, the measured free amine was significantly different from Control. The degree of reduction at 10% OSA was significantly different compared to 2% OSA. The reduction in free amine percentage was different between 1% OSAF treatment and 10% OSAF treatment. The higher concentration of OSA needed compared to the modified EWP treatments may be due to the FEW not being de-glucosed. It is a common practice of producing commercial spray dried egg white powder to de-glucose the albumen before drying to prevent Maillard browning reaction.[24] OSA could have reacted with the glucose along with the proteins.[25]

Table 2 also shows free amine results for CFEW. Even with 5% OSA addition the level of free amine was not statistically different from the Control CFEW treatment. However, compared to the freeze-dried FEW, the initial free amine value of CFEW was much lower. Increased CFEW protein unfolding and aggregation could have occurred during the fermentation step with mild elevated temperatures for several hours, or due to the elevated temperatures

during spray drying. These can cause the control treatment to have lower free amine value compared to the freeze-dried FEW. Similar to dry heating or heating proteins in solution, an increase in degree of sulfhydryl, surface hydrophobicity, and deamidation could also occur.

Effect of sonication (Son) on degree of succinylation

Sonication had a significant effect on reaction efficiency with the same concentration of OSA use. However, these increases were lower than those of FEW, but sonication still improved the efficiency of the reaction. Sonication significantly increased free amine reduction particularly at 2 and 4% OSA, with twice as much reduction compared to without sonication. Nonetheless, sonication and higher level addition of OSA at 10% both greatly reduced the free amine content indicating an improved reaction. Succinylation of unpasteurized, fresh egg white with succinic anhydride at 5% was also shown to react with 52% of the total free amine groups.[2] The rates of succinic anhydride modified myofibrillar proteins at 5% and 10% addition were 30% and 48% of the ε-amine groups with a reaction time of 1-2 hours.[26] In comparison, at 4% addition of OSA to FEW, there was 29.37% reduction in free amine value. OSA reaction was not as efficient as using succinic anhydride, which may be due to the hydrophobic carbon tail. However, with sonication the reduction in free amine increased to 60.46%. The use of sonication may have helped increase reaction efficiency by uniformly mixing the egg white protein, making it easier for the OSA to mix into solution, and react with the protein substrate in the dispersion. The difference in reaction conditions such as mixing speed, pH, and time are all factors that may affect the efficiency of the OSA succinylation reaction, and these should be further optimized.

Even though sonication may have improved the reaction efficiency, the type of product drying also indirectly affected the degree of modification. The Control CFEW and the degree of decrease in free amine of modified OSA CFEW treatment were lower compared to the freeze-

dried FEW (Table 2). The extent of OSA succinylation between Son 10% OSAF (octenyl succinylated FEW) and of 10% OSACF (octenyl succinylated CFEW) was different, 85.75% free amine reduction compared to 39.87%, respectively. The lower reduction of free amine observed in the spray dried CFEW compared to the freeze-dried FEW may be due to the proteins' increased exposure to heat and sonication. The sonicated egg white protein was shown to have significantly increased surface hydrophobicity, allowing for more protein aggregation.[14]

The sonication control treatment showed a slight increase in free amine compared to the Control treatment, 0.97 to 0.90 mg / 20 mg of protein or a 7.12% increase in free amine group (Table 2), albeit the increase was not significantly different. All the treatments in the spray dried samples except the Control treatment were sonicated. The starting concentration of free amine among the Control EWP, FEW, and CFEW treatments were different which provides evidence that the process has an effect on the level of free amine available for modification.

Effect of modification on molecular weight profile

SDS-PAGE results indicated that there was no significant change in molecular weight of the protein up to about 5% OSA addition. The major egg white proteins and their molecular weights are ovalbumin (45 kDa), ovotransferrin (77 kDa), ovomucoid (28 kDa), ovomucin (110, 5-8 x 10^3, and 220-270 x 10^3 kDa) and lysozyme (14 kDa).[27] There is no concern that a high degree of modification of the protein will lead to change in molecular weight as shown in Figure 2, lane 3 and lane 8. There was a small upward shift of the bands with the 10% OSAF treatment, especially for Son 10% OSA treatment sample without reducing agent, both the freeze-dried FEW and spray dried CFEW (Figure 2, A lane 5, 10 and B lane 5, and 10, respectively). Since OSA is such a small molecule the shift would be very slight. Succinylated egg albumen with succinic anhydride also showed similar upward shifts bands for a 10:1 or 10% succinic

anhydride modified treatment by SDS PAGE with and without β-mercaptoethanol.[3] Sonication with OSA seemed to indicate a larger shift in molecular weight, which agrees with the higher reduction in free amine or greater degree of OSA succinylation from Table 2.

There were signs of higher molecular weight proteins near the top of the lane, and a decrease in band intensity for the lysozyme protein band in lanes with no reducing agent added (Figure 2, A lane 9, 10 and B lane 9, 10). Comparison of the SDS-PAGE lanes with and without β-mercaptoethanol at the higher levels of OSA indicates that some protein cross-linking may have occurred. This would explain the appearance and disappearance of some of the bands observed. At a lower addition of OSA succinylation the protein molecular weight increase is not noticeable. The SDS-PAGE suggests a certain % of the free amine ends may need to react before any changes in molecular weight can occur.

Effect of OSA modification on solubility profile of protein

Figure 3 shows the solubility profile of two spray dried modified proteins, the Control CFEW and Son 10% OSACF treatment. The Control CFEW was highly soluble from pH 2-11. The isoelectric point or the pH value where the protein solubility seemed to decrease slightly was at the pH range of 5-7. The Control treatment had a similar profile as reported, such that the solubility of the spray dried material at pH 5 and 7 were 96.53, and 97.46%,[28] respectively compared to 91.54 and 91.13% for the Control in the study. The solubility of the OSA succinylated protein was much less than the Control CFEW particularly at the pH range between 2-6. It had the lowest solubility at a pH of 4, with 30.95% soluble protein. A succinylated soy protein also showed a similar change in solubility profile to the OSA succinylated CFEW. An increasing concentration of succinic anhydride added decreased the solubility of the modified

soy protein further near its isoelectric point.[5] This change in isoelectric point of the egg protein provides further evidence that the protein was modified by OSA succinylation.

Thermal stability evaluation of OSA succinylated egg white proteins

Thermal stability of OSA succinylated egg white proteins at 75°C

As the protein is heated, it denatures and precipitates through protein-protein hydrophobic interactions. As the protein becomes insoluble the concentration of soluble protein decreases leading to a reduction of the protein solubility over time. The treatment with the highest concentration of soluble protein maintained over time indicates a thermally stable protein. Figure 4, 5, and 6 show the protein solubility of OSA succinylated EWP, FEW, and CFEW.

Control EWP was lower in protein solubility compared to all the other OSA modified, freeze-dried EWP treatments (Figure 4). Comparing the protein solubility of Control EWP over time, it was significantly reduced after 3 minutes of heating. The 1, 2, and 4% OSAE treatments showed some differences within 10 minutes, however they were all thermally unstable.

Figure 5 shows effect of heating at 75°C on solubility of OSA modified FEW with and without the use of sonication. The sonicated 10% OSA succinylated proteins had a higher percentage of soluble protein at certain time points (5, 10, 15, and 25 min) compared to OSA modified FEW at the same OSA level. Considerable difference in protein solubility was shown for most products except for Son 10% OSAF. The Son 10% OSAF treatment did not have a significant reduction in soluble protein during the 25 minutes of heating, thus indicating an increased thermal stability.

The initial solubility of the 10% OSAF and Son 10% OSAF treatments at time zero were lower and significantly different from the Control and other treatments. The addition of OSA

seemed to decrease the initial solubility of the unheated protein dispersion, which may be due to the hydrophobic portion or carbon tail of OSA preventing a fraction of the protein from being soluble. Therefore, greater concentration of OSA with the assistance of sonication can help form a better complex. Succinic anhydride succinylated egg white heated at 56°C showed that the modified egg white protein was more soluble than the unmodified egg white protein up to 10 minutes, but had a similar decrease in solubility after 15 minutes of heating.[16]

The spray dried products produced from de-glucosed fresh egg white did not show the same improvement as the freeze-dried FEW, especially for the Son 10% OSACF treatment (Figure 6). The protein solubility of the spray dried treatments was slightly higher in general than the other treatments. The combination of sonication for pretreatment and after reaction before spray drying showed an increase in concentration of free amine (Table 2). At the same time, it may have increased the exposure of hydrophobic moieties, which could explain why the functionality of the sample did not improve as much as the FEW treatments. Moreover, the spray drying itself may have further denatured the protein and promoted protein aggregation since the inlet and outlet temperature used in the study was higher compared to temperatures used in other studies. Mine[29] spray dried liquid egg white with an exhaust or outlet temperature of 65-70°C. Ayadi et al.[30] dried egg white at moderate air inlet temperatures of 110-125°C and showed improved functionality. In addition, the CFEW was exposed to a mild temperature for a long period of time during fermentation before it was modified. These factors could have suppressed the improvement of heat stability as observed with the freeze-dried FEW.

Thermal stability of OSA succinylated egg white proteins at 95°C

The turbidity and protein solubility of the modified EWP are shown in Figure 7. The Control EWP was more turbid before and after heating at 95°C compared to other samples. Even

though the initial turbidity of 1% OSAE was significantly different, its turbidity after heating was not different from the Control. Both the 2% OSAE and 4% OSAE treatment, had turbidity significantly different from the Control EWP. The protein content in the dispersions was not significantly different before or after heating indicating the turbidity reduction was not due to protein precipitation. The turbidity indicates that an improvement in thermal stability occurred with 2 and 4% OSA level addition.

The turbidity and protein solubility of the modified FEW are shown in Figure 8. The Control FEW treatment had a much lower turbidity before heating compared to the Control EWP. This may be due to additional heat treatment the EWP had gone through compared to the FEW as it was likely dry heated after being spray dried. However, after heating, the FEW dispersions turbidity was much higher and was similar to the turbidity of the Control EWP. The turbidity of the 4% OSAF after heating were similar to Control. The Son 4% OSAF treatment was significantly different, with a significant reduction in turbidity after heating compared to without sonication. The Son 10% OSAF treatment had the lowest turbidity after heating. The protein solubility of the treatments was not significantly different before or after heating. Figure 8 clearly indicates the higher concentration of OSA used and sonication treatment had produced more thermally stable egg white protein.

The turbidity and protein solubility of the modified CFEW are shown in Figure. 9. The use of sonication or increase in added OSA raised the turbidity of the dispersion before the heating evaluation. After heating, the turbidity reduced with the increasing concentration of OSA and sonication pretreatment. Son 10% OSACF had the lowest turbidity after heating. The protein solubility of the samples seemed to be different before and after heating, but did not perform as well as the freeze-dried OSA modified FEW.

Conclusions

An improvement in thermal stability of the OSA succinylated egg white protein was observed. The degree of improvement in thermal stability depended upon the concentration of OSA and degree of protein modification. The sonicated 10% OSA treatment was the best treatment. The type of initial material i.e., EWP, FEW, or CFEW, and processing conditions after the modification treatment can impact quality and functional properties of the final product significantly. Sonication pretreatment greatly increased the degree of OSA modification. The OSA succinylated egg white proteins could add value to egg white proteins and its use in the food processing industry. The modification process could be further optimized with reduction in sonication time, increase usage of OSA with simultaneous sonication of egg white and OSA before the reaction occurs.

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Abbreviations Used

BCA, bicinchoninic acid; BSA, Bovine serum albumin; CFEW, commercial de-glucosed fresh egg white; EWP, egg white power; FEW, fresh egg white; NS, not significantly different; OSA, octenyl succinic anhydride; OSACF, octenyl succinylated CFEW; OSAE, octenyl succinylated EWP; OSAF, octenyl succinylated FEW; S, significantly different; SDS-PAGE,

sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Son, sonication or sonicated; TNBS, 2, 4, 6-trinitrobenzene sulfonic acid picrylsulfonic; WPI, whey protein isolate.

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Table 1. Treatments and acronyms for the evaluation of the effect of OSA concentration and sonication on protein thermal stability.

Starting Material	OSA Concentration (% relative to protein)	Sonication Step	Treatment Abbreviation
Commercial dried egg white protein (EWP)	0	No	Control EWP
	1	No	1% OSAE
	2	No	2% OSAE
	4	No	4% OSAE
Commercial fresh egg white (FEW)	0	No	Control FEW
	1	No	1% OSAF
	2	No	2% OSAF
	4	No	4% OSAF
	10	No	10% OSAF
	2	Yes	Son 2% OSAF
	4	Yes	Son 4% OSAF
	10	Yes	Son 10% OSAF
Commercial fresh egg white – de-glucosed (CFEW)	0	No	Control CFEW
	0	Yes	Son control
	5	No	5% OSACF
	5	Yes	Son 5% OSACF
	10	Yes	Son 10% OSACF

Table 2. Effect of OSA concentration and sonication on free amine and % reduction of free amine of OSA modified egg white proteins EWP, FEW, and CFEW.[x]

Commercial dried egg white protein (EWP)

Treatments	Control EWP	1% OSAE	2% OSAE	4% OSAE
Free amine group (mg / 20 mg of protein)	2.11 ± 0.77[a]	1.92 ± 0.78[a,b]	1.50 ± 0.64[b]	1.37 ± 0.86[b]
Reduction in free amine (%)	n/a	10.09 ± 6.11[b]	30.64 ± 6.20[a]	39.05 ± 22.45[a]

Fresh egg white (FEW)

Treatments	Control FEW	1% OSAF	2% OSAF	Son 2% OSAF	4% OSAF	Son 4% OSAF	10% OSAF	Son 10% OSAF
Free amine group (mg / 20 mg of protein)	1.61 ± 0.05[a]	1.40 ± 0.10[a,b]	1.41 ± 0.01[a,b]	0.90 ± 0.10[c,d]	1.14 ± 0.01[b,c]	0.64 ± 0.05[d,e]	0.60 ± 0.23[d,e]	0.23 ± 0.06[e]
Reduction in free amine (%)	n/a	13.08 ± 8.84[c]	12.57 ± 2.14[c]	44.12 ± 4.39[c,b]	29.37 ± 1.52[c,b]	60.46 ± 1.95[a,b]	62.79 ± 13.08[a,b]	85.75 ± 3.93[a]

Commercial de-glucosed fresh egg white (CFEW)

Treatments	Control CFEW	Son Control	5% OSACF	Son 5% OSACF	Son 10% OSACF
Free amine group (mg / 20 mg of protein)	0.90 ± 0.03[a]	0.97 ± 0.03[a]	0.95 ± 0.04[a]	0.76 ± 0.05[b]	0.54 ± 0.03[c]
Reduction in free amine (%)	n/a	-7.12 ± 5.18[c]	-5.42 ± 6.68[c]	15.70 ± 7.42[b]	39.87 ± 4.72[a]

[x]All values are the means ± standard deviations of three replicates except FEW with two replicates. Different letters in the same row represent significant differences ($P<0.05$).

Figure captions:

Figure 1. Flow diagram of OSA succinylation of egg white proteins.

Figure 2. Qualitative change in molecular weight of OSA modified A) freeze dried FEW and B) spray dried CFEW by SDS-PAGE with 40 ug loaded in each lane except the standard ladder, which was 10 ul.

Figure 3. Solubility curve of Control and Sonicated 10% OSA CFEW modified protein.

Figure 4. Effect of dose on thermal stability of pH 7 adjusted OSA succinylated EWP during 75°C heating.

Figure 5. Effect of dose and sonication on thermal stability of pH 7 adjusted OSA FEW measured as solubility during 75°C heating out.

Figure 6. Effect of dose and sonication on thermal stability of pH 7 adjusted OSA CFEW measured as solubility during 75°C heating.

Figure 7. Effect of dose on thermal stability of pH 7 adjusted OSA EWP proteins after 1 hour at 95°C. All values are the means ± standard deviations of three replicates. Different letters in each bar of graph or row in table represent significant differences ($P<0.05$), NS – not significantly different, S – significantly different.

Figure 8. Effect of dose and sonication on thermal stability of pH 7 adjusted OSA FEW proteins after 1 hour at 95°C. All values are the means ± standard deviations of two replicates. Different letters in each bar of graph or row in table represent significant differences ($P<0.05$), NS – not significantly different, S – significantly different.

Figure 9. Effect of dose and sonication on thermal stability of pH 7 adjusted OSA CFEW proteins after 1 hour at 95°C. All values are the means ± standard deviations of three replicates. Different letters in each bar of graph or row in table represent significant differences ($P<0.05$), NS – not significantly different, S – significantly different.

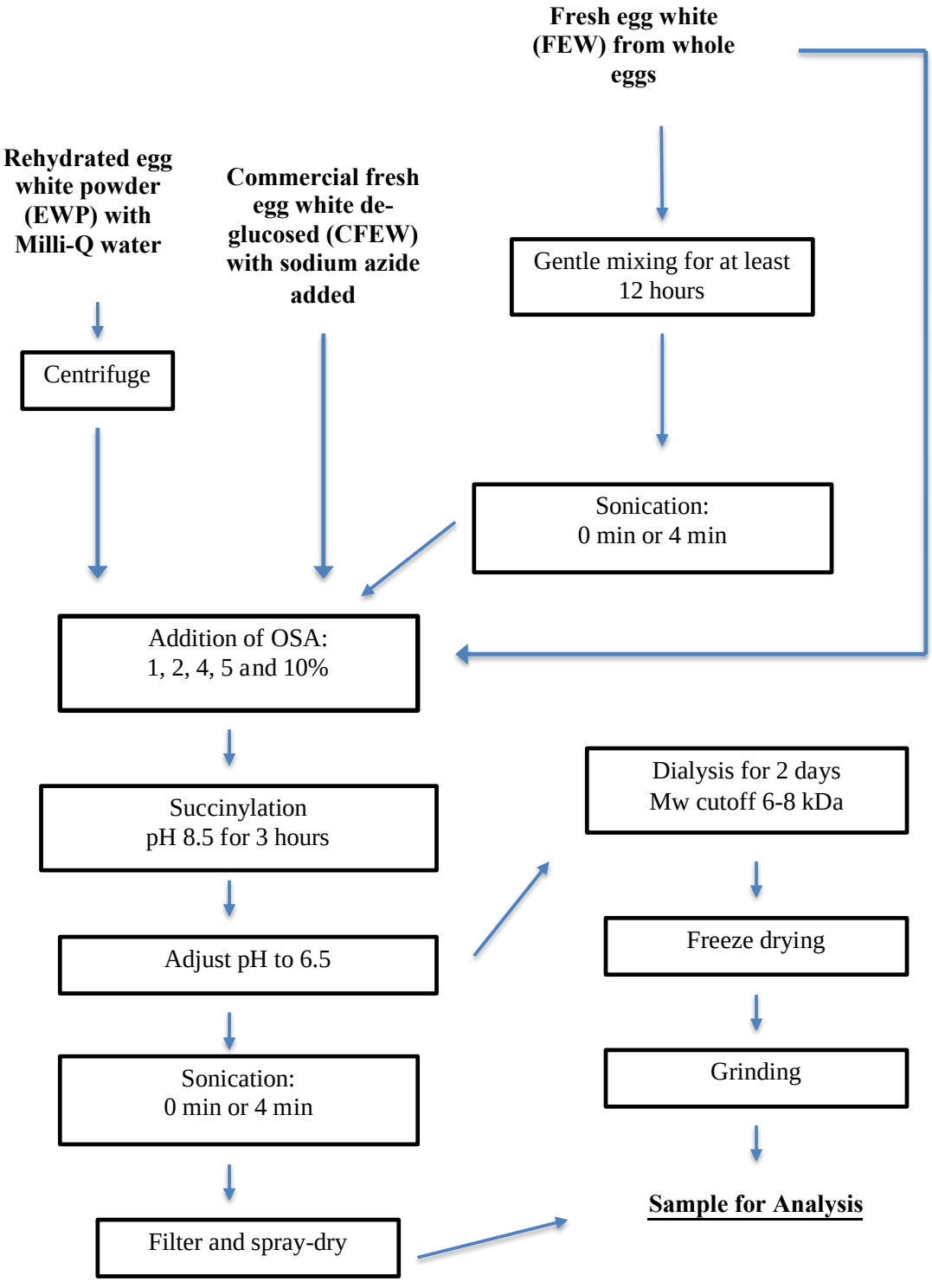
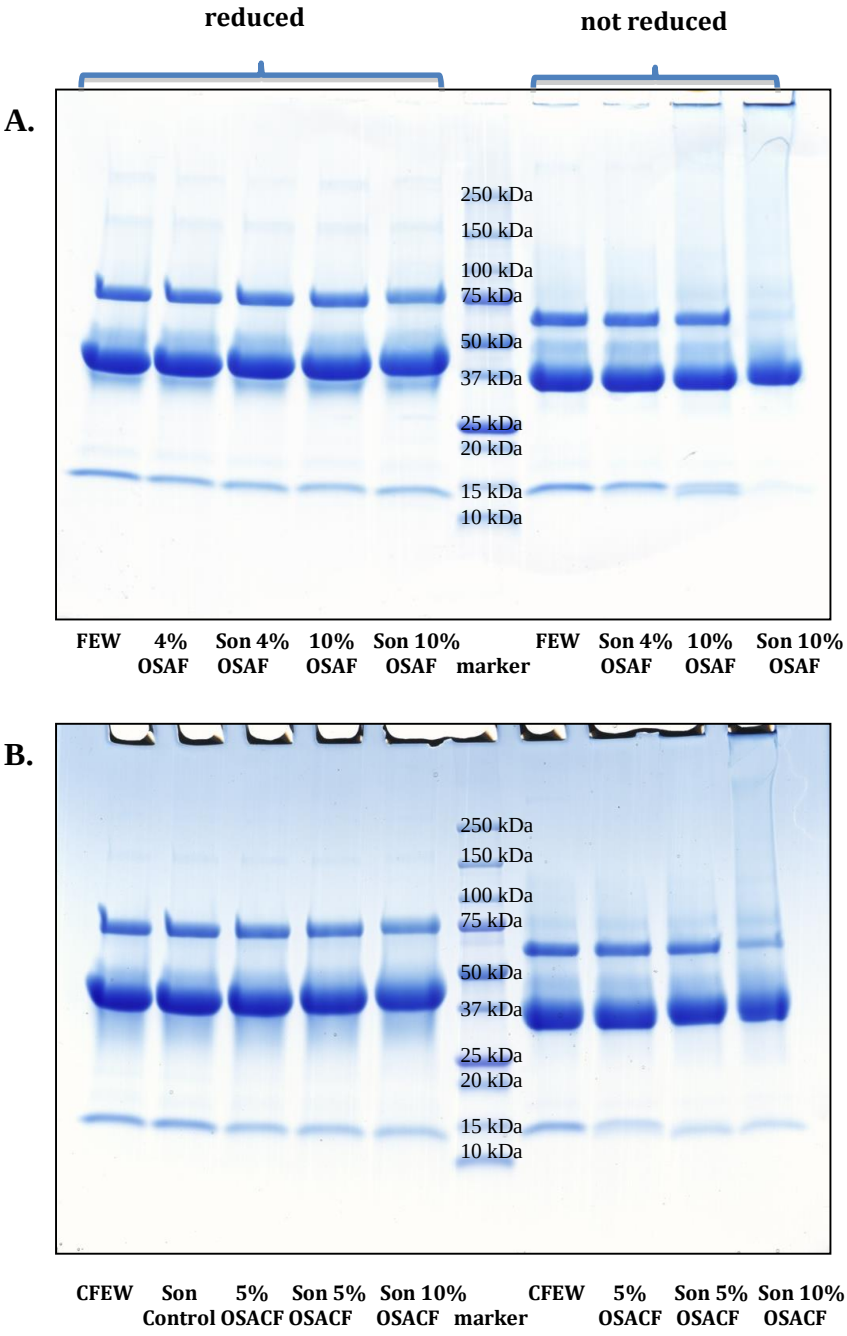


Figure 1. Flow diagram of OSA succinylation of egg white proteins.



The first five lanes have reducing agent (β-mercaptoethanol) added. The lanes after the standard ladder has no reducing agent added.

Figure 2. Qualitative change in molecular weight of OSA modified A) freeze dried FEW and B) spray dried CFEW by SDS-PAGE with 40 ug loaded in each lane except the standard ladder, which was 10 ul.

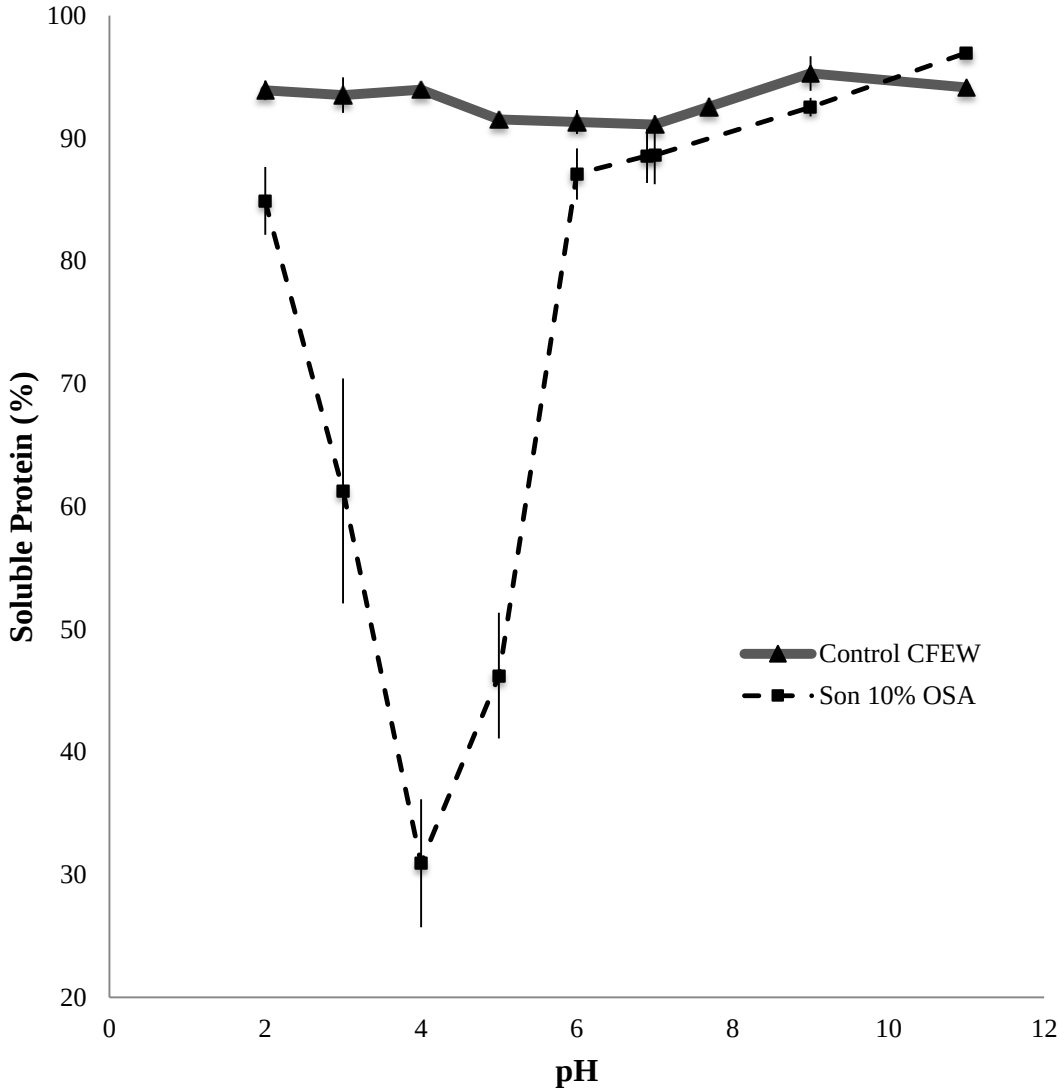


Figure 3. Solubility curve of Control and Sonicated 10% OSA CFEW modified protein.

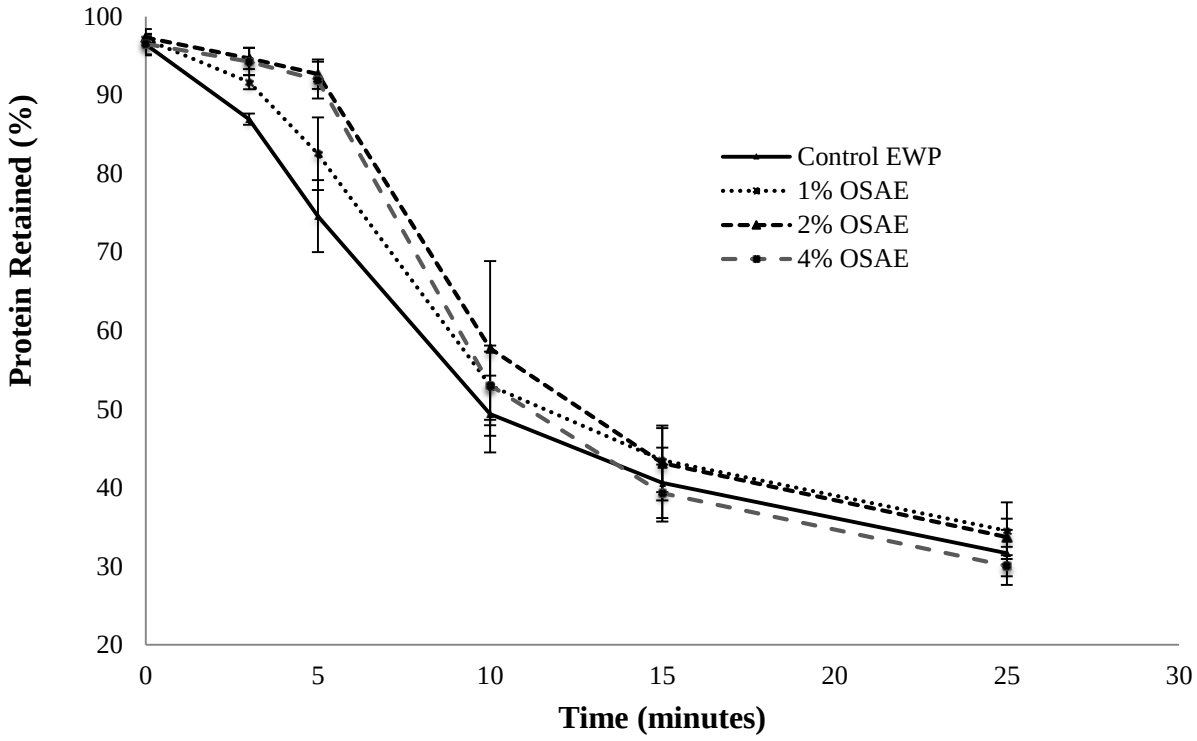
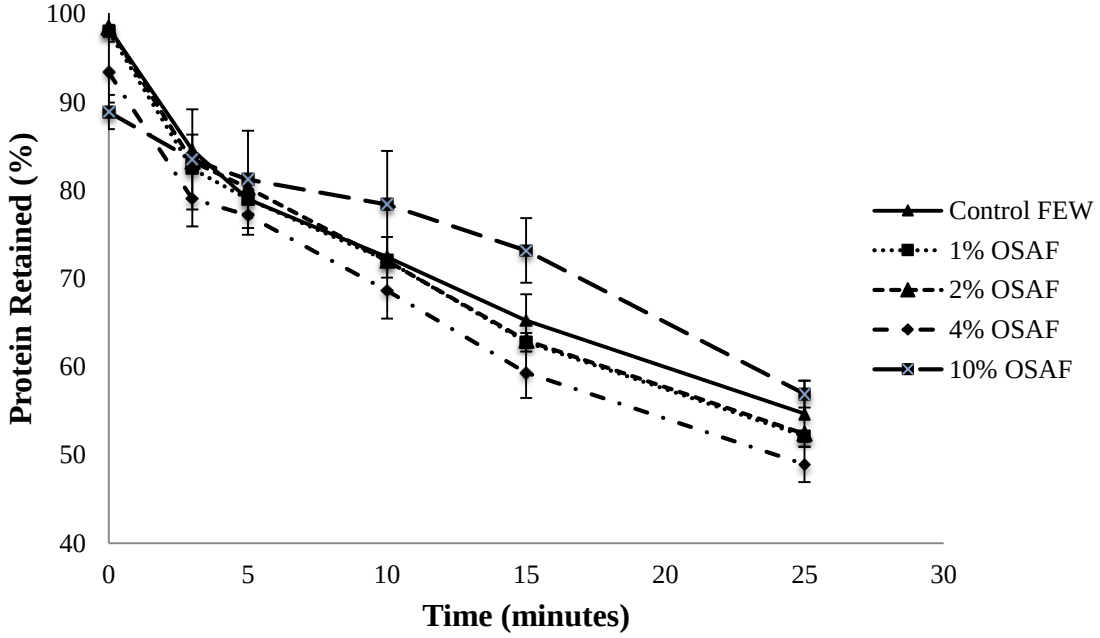


Figure 4. Effect of dose on thermal stability of pH 7 adjusted OSA succinylated EWP during 75°C heating.

OSA succinylated FEW without sonication



OSA succinylated FEW with sonication

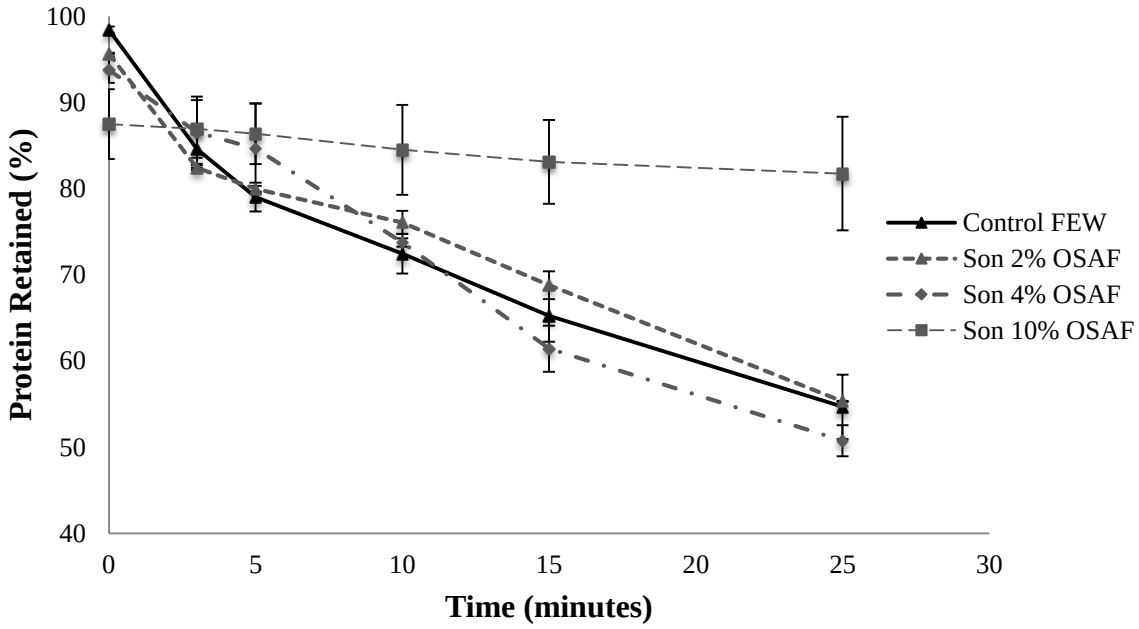


Figure 5. Effect of dose and sonication on thermal stability of pH 7 adjusted OSA FEW measured as solubility during 75°C heating out.

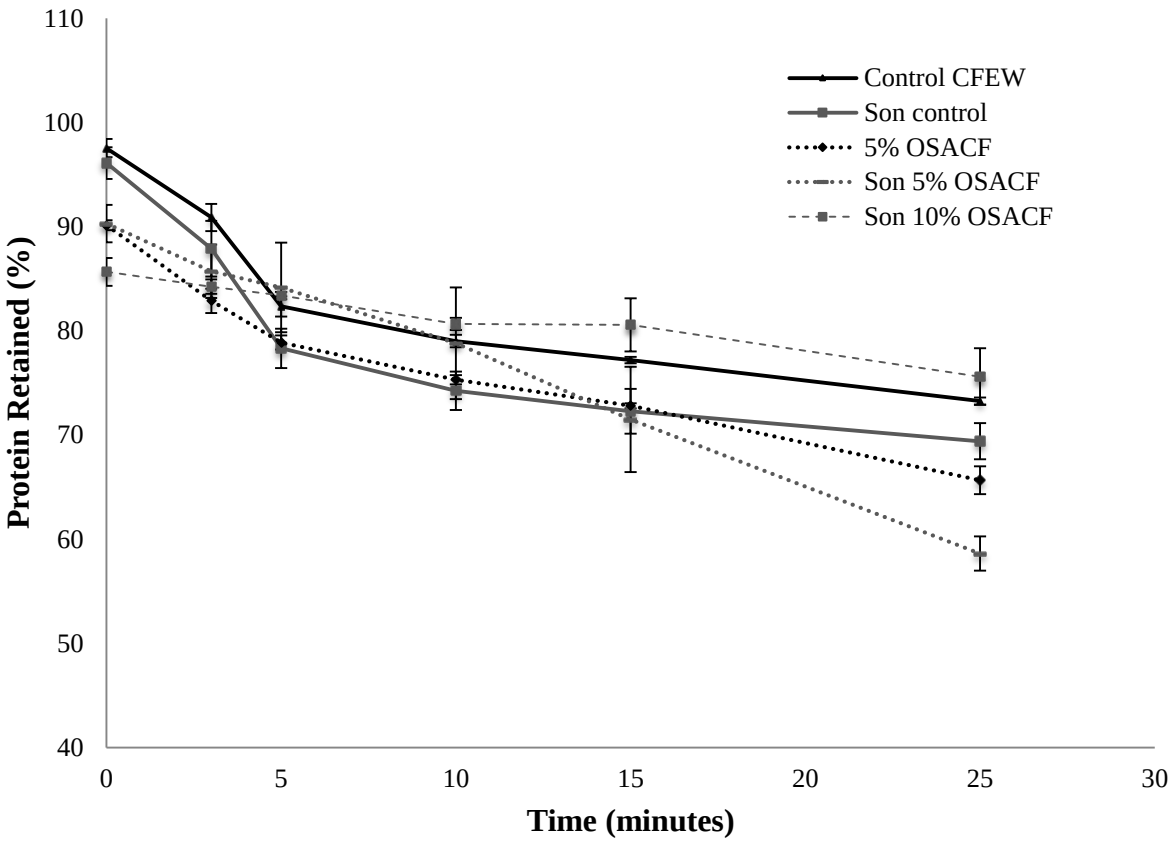
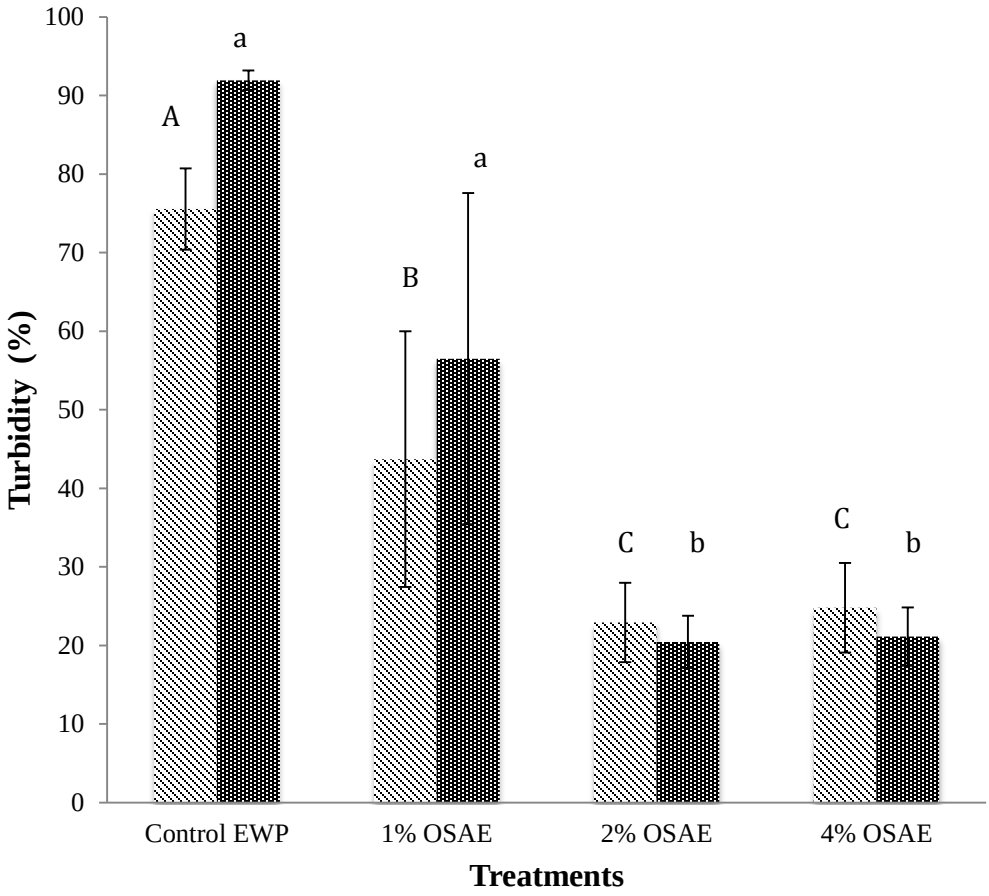
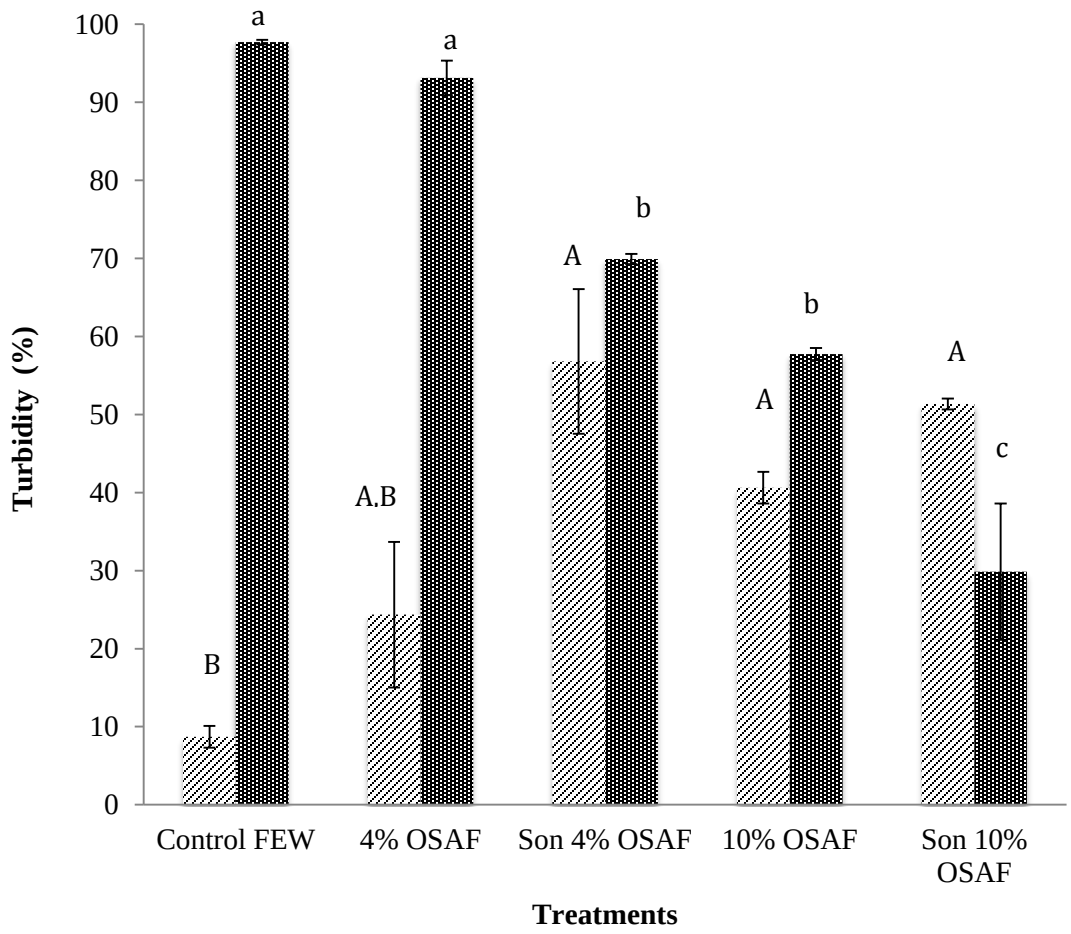


Figure 6. Effect of dose and sonication on thermal stability of pH 7 adjusted OSA CFEW measured as solubility during 75°C heating.



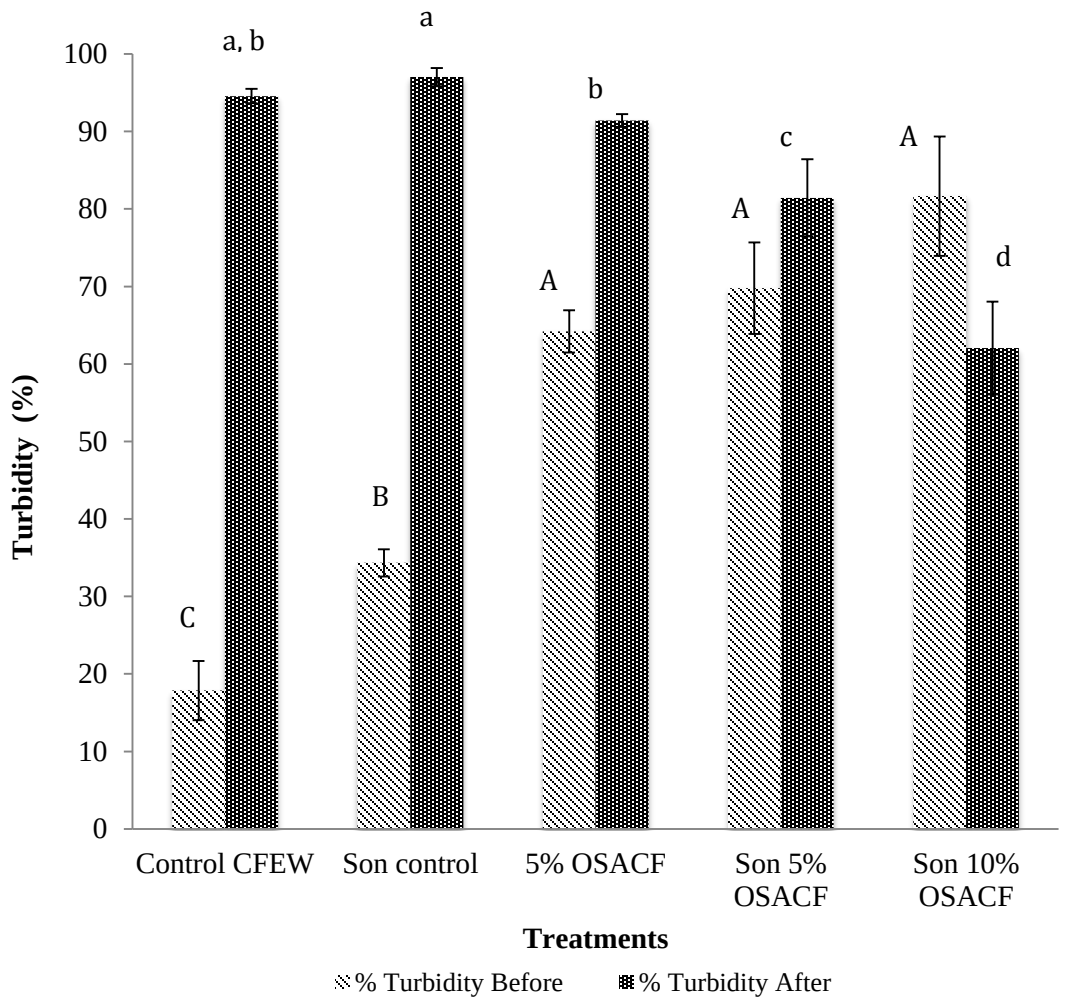
Freeze-dried	Control EWP	1% OSAE	2% OSAE	4% OSAE	Significance
Change in turbidty (%)	16.4 ± 4.7[a]	12.8 ± 5.3[a]	-2.5 ± 2.0[b]	-3.7 ± 3.9[b]	S
Before protein (mg / mL)	7.32 ± 0.41	7.55 ± 0.66	7.68 ± 0.67	7.56 ± 0.78	NS
After protein (mg / mL)	7.09 ± 0.62	7.26 ± 0.82	7.34 ± 0.81	7.28 ± 0.86	NS

Figure 7. Effect of dose on thermal stability of pH 7 adjusted OSA EWP proteins after 1 hour at 95°C. All values are the means ± standard deviations of three replicates. Different letters in each bar of graph or row in table represent significant differences ($P<0.05$), NS – not significantly different, S – significantly different.



Freeze-dried	Control FEW	4% OSAF	Son 4% OSAF	10% OSAF	Son 10% OSAF	Sig-nificance
Change in turbidity (%)	89.0 ± 1.3[a]	68.8 ± 11.6[a]	13.1 ± 8.6[b,c]	17.1 ± 2.8[b]	-21.5 ± 8.0[c]	S
Before protein (mg / mL)	4.61 ± 0.31	4.28 ± 0.01	4.42 ± 0.01	4.05 ± 0.24	4.21 ± 0.24	NS
After protein (mg / mL)	3.46 ± 0.15	3.44 ± 0.11	3.68 ± 0.02	3.35 ± 0.24	3.72 ± 0.03	NS

Figure 8. Effect of dose and sonication on thermal stability of pH 7 adjusted OSA FEW proteins after 1 hour at 95°C. All values are the means ± standard deviations of two replicates. Different letters in each bar of graph or row in table represent significant differences ($P<0.05$), NS – not significantly different, S – significantly different.



Spray dried	Control CFEW	Son control	5% OSACF	Son 5% OSACF	Son 10% OSACF	Sig-nificance
Change in turbidity (%)	76.7 ± 3.0^{a}	62.7 ± 1.9^{b}	27.2 ± 2.0^{c}	11.7 ± 10.9^{d}	-19.6 ± 2.5^{e}	S
Before protein (mg / mL)	4.07 ± 0.13^{a,b}	4.15 ± 0.08^{a}	3.96 ± 0.06^{a,b}	4.08 ± 0.7^{a,b}	3.80 ± 0.22^{b}	S
After protein (mg / mL)	2.88 ± 0.05^{b}	3.17 ± 0.21^{a,b}	3.40 ± 0.04^{a}	3.58 ± 0.07^{a}	3.35 ± 0.20^{a}	S

Figure 9. Effect of dose and sonication on thermal stability of pH 7 adjusted OSA CFEW proteins after 1 hour at 95°C. All values are the means ± standard deviations of three replicates. Different letters in each bar of graph or row in table represent significant differences (P<0.05), NS – not significantly different, S – significantly different.

CHAPTER 4. DIFFERENT COMBINATIONS OF PHYSICAL TREATMENT TO IMPROVE THERMAL STABILITY OF EGG ALBUMEN

A manuscript to be submitted to *Journal of Agricultural and Food Chemistry*

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Abstract

The physical means of modification of fresh hen egg white (FEW) with combined pre-heating, ultrasound, and the addition of octenyl succinic anhydride (OSA) improved the thermal stability of the protein. The pH of the modified FEW dispersion has an effect on the protein's thermal properties. The pre-heating step and higher levels of OSA addition in the process were beneficial when the protein was evaluated at higher temperatures of 95°C or 121°C compared to 75°C. The low level of OSA treatments showed improved thermal stability evaluated by turbidity and protein solubility at 75°C. Thus, the optimal addition level of OSA would depend on the degree of thermal stability improvement needed. The Raman spectroscopy shows evidence of a physical interaction occurring with high OSA concentration and this complex seemed to be more resistant to change by heating.

Keywords: *egg white, physical complex, octenyl succinic anhydride, sonication*

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Introduction

The main limitation of wide utilization of egg white protein in the food industry is its sensitivity to heat. By improving the thermal stability of egg protein, an opportunity exists to expand its use in the food processing industry as demand for high protein food products continue to increase.[1-3] Past research has focused on modifying these major egg albumen proteins through enzymatic, chemical, and physical methods to improve functionality with no significant advance in improving the thermal stability as a whole.[4-6] Consumers may scrutinize certain modification methods such as chemical modification of proteins, as they are becoming more knowledgeable and aware of food safety.

Ultrasound or sonication is a physical processing method that could be used in egg processing to improve its thermal stability if combined with other treatments. The use of ultrasound generates heat and cavitation through the formation and collapse of air bubbles in solution with minimal incorporation of air.[7-10] The physical process has been shown to form more stable emulsions and increase protein gelation properties.[11-13] Sonication (Son) is ideal for mixing egg white proteins since it can minimize foam formation during mixing with other food ingredients. In chemical reactions with succinic anhydride, the solubility or dispersibility of the compound can be increased by using ultrasound.[14] Sonication can also partially expose moieties of proteins with mild heat treatment, denaturing the protein, leading to improved physical interactions with other food additives. However, these have not been investigated thoroughly to our knowledge.

Ashokkumar et al.[7] improved the heat stability of whey protein through a two-step heat denaturation and sonication process. Arzeni et al.[12] found no improvement in heat stability of rehydrated egg white powder with sonication alone. Thus, the use of food ingredients in combination with sonication in the protein may result in a physical interaction or complex that

stabilizes the protein system. The addition of anionic additives such as 2-decylcitric acid, sodium dodecyl sulphate (SDS), and lauric acid have been shown to improve the thermal stability of ovalbumin in weak alkaline pH regions.[15] Hegg and Löfqvist[15] speculated that the hydrophobic core of the protein may be stabilized by the interaction of the hydrocarbon chain of the additive strengthening its core. It has also been shown that adding an amphiphilic phospholipid along with a pre-heating step prior to sonication treatment improved the thermal stability of ovalbumin and can interact with proteins as shown with sodium caseinate.[16,17] However, the interaction and complex is dependent on pH and concentration of the phospholipid.

We proposed that pre-heating with sonication might be used in combination with other ingredients such as octenyl succinic anhydride (OSA) to improve the hen egg white protein's thermal stability through two simultaneous mechanisms. First, the OSA's eight carbon tail or hydrophobic groups may interact with egg proteins hydrophobic moieties, once exposed during pre-heating or sonication. Such complexing will delay further protein-protein hydrophobic aggregation, thus improve heat stability. Secondly, OSA's partially hydrolyzed dicarboxylic acid polar moiety may provide electrostatic repulsions among the proteins since it will be uniformly mixed in the protein system by sonication.[18] Ma and Holme[18] have shown that chemically modifying proteins with a dicarboxylic acid would improve heat stability. Therefore, the system created or ingredient interaction may prevent protein aggregation after heating the protein at high temperatures.

Our hypothesis is that the combination of pre-heating, sonication with the addition of OSA to fresh egg white (FEW) can improve the heat stability of an egg white protein dispersion by forming a heat stable complex. The objectives of this study are to study the effects of pre-

heating treatment with sonication and OSA addition on heat stability of the protein dispersion. No such study on egg white has been conducted for improving protein thermal stability.

Materials

Large grade A fresh eggs were purchased from a local supermarket (Ames, IA) with similar sell-by dates for each replicate. OSA was purchased from Sigma Aldrich (St. Louis, MO). All other materials and chemicals were purchased from Fisher Scientific (Pittsburgh, PA). The modification procedure was similar to the method of Ashokkumar et al.[7] with a difference in heating temperature and the addition of OSA.

Methods

Fresh egg white physical treatments

We designed this experiment to evaluate the effect of pre-heating and dosage level of OSA on improvement of protein thermal stability. Therefore, two sets of samples were created, one with different levels of OSA (5, 10, and 20% based on protein (dwb)) without pre-heating and the other with OSA at 2, 5, 10, and 20% with pre-heating treatment of egg white protein. Three replicates of these treatments were conducted.

The fresh eggs were broken and the white was separated from the yolk. The fresh egg white was then mixed with 0.02% sodium azide (w/w) and diluted in half with Milli-Q water to inhibit possible gelation during the pre-heating step. If the treatment was pre-heated, the mixed fresh egg white (FEW) were heated in a water bath set at 63°C for 5 minutes before sonication. OSA was added at 4 minutes during heating and the system was manually mixed. The sonication treatments were done in 150 mL-volume batches, using an ultrasound or sonicator (Fisher Scientific model 500 Ultrasonic Dismembrator, Pittsburgh, PA). The amplitude was set at 70%

with 30 seconds on and 10 seconds off up to 2 minutes. The different physical treatment combinations are outlined in Table 1.

Thermal stability evaluation and effect of pH adjustment on complex stability

In addition to a treatment comparison for the effect of pre-heating, another pair of comparisons was made to evaluate the effect of pH on the thermal stability evaluation. One set of samples was evaluated as-is. The other set was adjusted to pH 7 by using pH 7 buffer (made of 0.1M sodium phosphate) so the effect of pH change due to OSA addition can be removed. The treatments were repeated 3 times.

Thermal stability evaluation by measuring turbidity of protein after 75°C heating

A thermal stability evaluation based on turbidity after heating at 75°C for 30 minutes was conducted on the samples. The sample was diluted to approximately 1% protein or 1 mg / mL with Milli-Q water or pH 7 buffer and 5 mL of the dispersion was transferred to 15 mL Corning test tube. The centrifuge tube was placed in the shaking water bath set at 75 ± 2°C for 30 minutes. The transmittance of the FEW-OSA was measured using an ultraviolet-visible DU720 spectrophotometer (Beckman Coulter Inc., Brea, CA) at a wavelength of 600nm, according to a modified method of Zhang et al.[19] and Shimada and Matsushita.[20] Milli-Q water or the pH 7 buffer was used as the blank. All samples were evaluated twice for each treatment replicate. The turbidity value was calculated by subtracting the %T value from 100. The change in turbidity value was the difference between the turbidity values of after and before heating.

Thermal stability evaluation by turbidity and protein solubility at 95°C

A turbidity method was used to confirm and further test the heat stability of the protein-OSA complex at a higher temperature. The physically treated FEW was diluted to approximately 2.5% protein solution (w/w) or 25 mg / mL with pH 7 buffer. The diluted solution, 1 mL, was then transferred to a disposable glass tube containing 5 mL of heated 95°C pH 7 buffer (a final 0.4% protein concentration). The test tube was then heated at 95°C for 1 hour. The % transmittance of the treatments was measured at 600 nm with the turbidity value calculated as before. The concentration of soluble protein in supernatant was measured using the biuret protein assay to ensure that a decrease in turbidity was not due to a reduction in soluble protein.[21] Bovine serum albumin (BSA) was used to establish a standard curve and the percent protein retained in supernatant was calculated based on the measured absorbance values at 540 nm of the samples before and after heating. A solution not heated to 95°C was determined as the beginning turbidity value of the sample.

Thermal stability evaluation by measuring protein solubility at 75°C over time

A thermal stability evaluation modified from Ball and Winn[22] based on protein solubility heated at 75°C with samples taken over time was also conducted to determine thermal stability. This test was used to distinguish differences among treatments if possible. Again, the treatment was diluted to ~1% protein dispersion with pH 7 buffer solution. The diluted dispersion, 5 mL, was transferred to several 15 mL corning test tubes and capped. All the centrifuge tubes were placed in the shaking water bath set at 75 ± 2°C and samples were taken out at specific time points (0, 3, 5, 10, 15, and 25 minutes). Time 0 was the sample as-is that was not heated. After heating, the samples were allowed to cool before analysis. About 2 mL of the sample was

transferred to a 2 mL Eppendorf test tube for centrifugation at 12,000 *g* for 30 minutes. The bicinchoninic acid (BCA) protein assay was used to measure the protein content in the supernatant.[23,24] The absorbance value of the treatments was measured at 562 nm. BSA was used for developing a standard curve. The % of protein retained was then calculated based on the measured protein values of the original dispersion and that of the supernatant.

Thermal stability evaluation by measuring turbidity after 121°C heating for 20 minutes

An extreme thermal stability evaluation mimicking autoclave or sterilization conditions was conducted on the samples. The sample was diluted to approximately 2.5% protein with pH 7 buffer and 5 mL of the dispersion was transferred to glass test tubes. The glass tubes were autoclaved and held for 20 minutes after the temperature had reached 121°C. The samples were then allowed to cool to room temperature before analysis. The % transmittance of the FEW-OSA complexes was measured at a wavelength of 600 nm. All samples were tested twice for each treatment replicate.

Evaluation of protein OSA complex by Raman spectroscopy

DXR Raman microscope spectroscopy (Thermo Scientific, Waltham, MA) was used for Raman spectra acquisition with 780 nm excitation at 10 mW, 10× objective, and 25 μm slit. The laser exposure time was 20 s and spectral resolution was 2.5–4.6 cm^{-1}. The OMNIC™ suite (Thermo Scientific, Waltham, MA) was used for data processing. The spectra were baseline-corrected and smoothed. An iterative polynomial background removal algorithm was implemented to remove background fluorescence from the Raman spectral data. Spectra area normalization (whole spectrum from 300-3000 cm^{-1} wavenumber) and specific peak

normalization (phenylalanine peak at 1002 cm^{-1}) were used for data preprocessing. The protein structure changes were elucidated based on the wavenumber and ratio of wavenumbers corresponding to known protein structures listed in Table 2. The method was modified from Zhang et al.[19] and Wang et al.[25]

Statistical analyses

All treatments were conducted in triplicates and each sample was measured in duplicates for the analytical assays, except for the 121°C thermal stability assay. Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC). The data was blocked by treatment replicates and evaluated using PROC GLM, PROC MIXED, or PROC GLIMMIX with Tukey adjustment to determine if there was any significant difference among the treatment means with a p-value of 0.05. Comparisons were made between the control treatment and the physical treatments with OSA added, with and without pre-heating treatment.

Results and Discussion

Thermal stability evaluation of different physical treatments of FEW

Effect of 75°C heating on thermal stability of modified FEW

When evaluating the FEW treatment for improvement in thermal stability, the initial, after heating, and the actual change in turbidity value should be used as good indicators for protein aggregation. It is challenging to compare between the treatments since its initial and the change in turbidity after heating could vary depending on other factors besides heat, such as protein concentration, salt, and pH of the protein dispersion.[20, 26-28] Thus, the change in % turbidity value was used as another indication for thermal stability improvement. Improvement in thermal stability is shown if this value decreases or is more negative in value.

Figure 1 shows the % turbidity before and after heating at 75°C for 30 minutes. It was difficult to determine if any improvement occurred between the control and treatments since the control also decreased in turbidity after heating in the unadjusted pH treatments. The OSA treatments seemed to have little change in % turbidity before and after heating. Since the protein concentration was ~1.0%, it should be high enough to observe some aggregation after heating. Shimada and Matsushita[26] found no random aggregation formation at 0.1%, but aggregation did occur with egg albumen when heated at 2.5% of protein concentration. It is critical to note that the pH values among the control and OSA treatments did vary. The pH values of samples were obtained before dilution with Milli-Q water. The control sample had a pH of about 9, while the increasing addition of OSA decreased the pH of the dispersion. The higher pH value can explain why the control sample % turbidity was lower after heating compared to same treatments because proteins are more soluble in alkaline pH.[29,30] In addition, ovalbumin has been known to convert to the more heat stable form of S-ovalbumin at a pH 9-10 when mildly heated.[31]

The concentration of OSA added with or without pre-heating also has an effect on the turbidity of the protein dispersion. The treatments are most likely to be more turbid due to the pH reduction towards 4.6, the isoelectric point of ovalbumin (54% and major egg white protein). Among the treatments, the 5% OSA (5NH, no pre-heating or 5H, with pre-heating) treatments, with a pH value closest to pH 7 had the best improvement in turbidity change %. Those treatments with a higher pH value were statistically not different from the control in change. Pre-heating did not lead to any difference.

The pH 7 adjusted samples showed that with an increasing concentration of OSA there was a trend for increasing improvement in turbidity after heating compared with the control (Figure 1). The turbidity change of 20NH is statistically different from 20H treatment (8.0

compared to -18.0, respectively) showing evidence that the addition of pre-heating to the physical treatment combination may have a positive effect as observed in whey proteins with pre-heating and sonication.[7] The pre-heated treatments showed a larger improvement with higher OSA levels compared to the non-preheat treatments. The high standard deviation is a concern that may have been caused by the variability in protein among egg white batches, but in general a positive trend was observed. The difference in results between the no pH adjustment and pH 7 adjusted samples shows that pH of the dispersion has an impact on the protein's thermal stability. Hegg and Löfqvist[15] determined that at pH between 3 and 10, ovalbumin would aggregate with optimum aggregation at pH 5.5. The pH-adjusted treatments indicate that the pre-heating step might be beneficial at higher levels of OSA addition. Further thermal stability evaluations were conducted to confirm these results.

Effect of 95°C heating on thermal stability of modified FEW

The results of the higher temperature thermal stability evaluation are shown in Figure 2. When the pH was adjusted to pH 7, the higher level of OSA (20% OSA) showed the most improvement overall and was significantly better than the control treatment. The solubility of the protein was measured to confirm that the improvement observed in turbidity was not due to the protein precipitating out of solution after heating. The pre-heating treatments had higher turbidity values after heating than the no pre-heating treatments at similar levels of OSA, indicating pre-heating may not be necessary. Even though the solubility of the protein initially for the 20H compared to the 20NH treatment were less and significantly different from each other (data not shown), the concentration of soluble protein after heating was not significantly different. The change in protein % (percent soluble protein after / percent soluble protein before x 100, with

100% indicates little precipitation occurred) was close to 100 and not significantly different. The turbidity change between 20NH and 20H treatments were also not significantly different. The thermal stability evaluation test at 95°C showed similar trends of improved thermal stability as the 75°C thermal stability testing.

Effect of 75°C heating over time on thermal stability of modified FEW

Even though the higher concentrations of OSA showed better-improved thermal stability, it would be difficult to have consumer acceptance at very high levels of food additive use. Consumers may be adverse to a higher concentration of OSA added to egg white. Therefore, we like to identify the minimum OSA to achieve a reasonable stability. We conducted another thermal evaluation. The heating temperature was set at 75°C to mimic the process for high temperature short time pasteurization conditions used in the dairy industry based on the U.S. Food and Drug Administration Pasteurized Milk Ordinance, but we extended to 25 minutes of heating. Figure 3 shows the % protein retained after heating and centrifugation at 0, 3, 5, 10, 15, and 25 minutes.

The control treatment had the lowest concentration of soluble protein after heating for 25 minutes. The concentration of soluble protein in control was significantly different compared to the other treatments after 5 minutes of heating. The only treatments that showed a change in protein solubility after heating over time were the control and 2H treatment, which were lower in soluble protein. The protein solubility of all the other treatments did not change statistically over time. Some of the mean values showed a decrease in solubility, but due to the large standard deviation, there were no significant reductions. The test was not able to determine at what level of OSA addition was optimal for functionality since no significant difference was shown.

Overall, all the treatments showed an improvement compared to the control treatment even at the 2% OSA addition level with pre-heating.

Effect of commercial sterilization condition on heat stability of modified FEW

A higher heating temperature and a more concentrated protein (~2.5%) was used to further observe any differences among treatments and to investigate if producing a shelf-stable egg protein liquid would be possible. The protein content cannot be too high because it can lead to the formation of a gel after heating.[32] Figure 4 shows the % turbidity of the protein after autoclave. After heating, the higher level of OSA addition showed an increasing level of improvement and the treatments with pre-heating were statistically better than the non-pre-heating treatments at similar OSA levels. The test supports the hypothesis that the pre-heating step is beneficial with increasing concentrations of OSA. The minimum level of OSA needed for non-pre-heating treatment is about 5% addition. While with pre-heating, it could be reduced to 2% depending upon the increase in functionality that is needed.

Structure evaluation of modified FEW by Raman spectroscopy

When proteins are heated, its native protein structure changes. As the protein unfolds, several specific functional groups are expected to change and can be detected by Raman spectroscopy. Painter and Koenig[33] used Raman and showed that the changes in ovalbumin protein (54% of egg white) spectra were very similar to egg albumen spectra. After ovalbumin was heated or denatured there was a major change in the intermolecular β-sheet region or amide I, and amide III wavenumber of 1665 cm^{-1} and 1254 cm^{-1}, respectively. The disulfide region was found near wavenumber 500 cm^{-1} or between 510-550 cm^{-1}, but the signal was not strong enough to observe a change in ovalbumin. These three specific wavenumber areas are of interest in

protein aggregation. The other regions of interest are the tryptophan, tyrosine environment, CH deformation around tryptophan, and amide I (antiparallel β-sheet formation) regions.[14,19,25,33] The reference wavenumber areas are based a study by Wang et al.[24] and Zhang et al.[19] for modified whey proteins and Painter and Koening[33] evaluating egg white (Table 2). If a peak in that area was observed to be close to those wavenumber areas, it was taken as the functional group of the protein.

The Raman curves or profiles among the control, 20NH, and 20H could be very similar since the type of modification is not chemical. However, changes in intensity are anticipated due to structural conformation change. The principle component analysis (PCA) of the spectra was conducted to observe the existence of any difference in structure among the treatments. The PCA plots of unheated and heated products both show that the spectra of the Control treatment was more different from the 20NH and 20H treatment (Figure 5, a and b). Figure 6, a through c show that there is a difference between unheated and heated control (part a) and 20NH treatment (part b). However, 20H PCA plot (part c) shows no significant difference in spectra, indicating that the structure of the treated protein dispersion may be more rigid or resistant to change. The plots match what was observed for the average spectra in Figure 7 (part a to c). It is obvious that differences in the unheated vs. heated spectra of the control treatment exist, and there was some difference of 20NH treatment. There were some visible changes in spectra after heating compared to unheated in certain wavenumber areas. However, the 20H treatment seems to not have any noticeable change compared to the heated to non heated spectra. The partially glycosylated whey proteins investigated by Wang et al.[25] were shown to be more heat stable compared to the unmodified whey protein isolate (WPI), and when control was heated, there was a major shift in the β-sheet regions. However, the glycosylated protein was shown to be rigid or

resistant to change in structure and the spectra change before and after heating, which is similar to what was observed for the 20H treatment.

The average spectra of each treatment were separated based on unheated (Figure 8, a to d) vs. heated (Figure 9, a to d) with specific values of wavenumber as listed in Table 2. There are specific changes in functional groups with some shifting from the reference values when examining the actual spectra. The disulfide bond regions, for example, seems to show a peak at 494 cm^{-1}, close to the 508, 538, and 542 cm^{-1} based on previous research.[25] All three treatments showed an increase in intensity in disulfide after heating, especially the control. The increase in disulfide bonds could have been due to the partial unfolding of the sulfhydryl groups, which formed more disulfide bonds after heating. There was a similar trend in values observed for the tryptophan region (hydrophobic amino acid) at 750 cm^{-1} after heating. The 20H and 20NH had a higher intensity value for the 850 / 825 cm^{-1} region before heating of the samples compared to the control, indicating the microenvironment around tyrosine was more polar or charged that could be due to the addition of OSA. A similar trend was observed at 1322 cm^{-1} indicating that a change to the CH bonds caused by tryptophan was being deformed before heating. Zhang et al.[19] observed a decrease in intensity at 1335 cm^{-1} when WPI was heated, indicating exposure of hydrophobic groups to the outer surface, but the modified and heated egg white treatments in our study showed a increase in intensity. This may indicate OSA interacting with the buried hydrophobic amino acids possibly stabilizing the proteins hydrophobic center. There was not much change in the Amide III, β-sheet formation region, but a change was observed for the Amide I, antiparallel β-sheet region for the 20NH and 20H treatments. All these values indicate that the protein is unfolding after heating. At the same time, there are some changes occurring to the modified protein comparing the treatments to the control dispersion before heating as

indicated by the tyrosine environment, CH deformation caused by tryptophan, and antiparallel β-sheet wavenumber intensity. After heating, an increase in intensity for all treatments occurred, with a higher increase in intensity occurring for control.

Zhao et al.[14] chemically succinylating spray-dried egg white with succinic anhydride showed that the chemical succinylation marker bands with Raman were around 1737 and 1420 cm^{-1}. However, no peak was observed at 1737 cm^{-1} and a distinct peak does not exists around 1420 cm^{-1}. The control and physical treatments' spectra intensity around 1420 cm^{-1} does change after heating compared to the control treatment. Since the pH was < 7 after the combination physical treatment, any chemical reaction by OSA should have been inhibited. This suggests that the protein is likely only physically modified.

Possible mechanisms of the formation of a heat stable protein complex

From the thermal stability evaluations, there was an improvement by the physical treatments compared to the control FEW due to a change in protein physical structure as indicated by Raman spectroscopy. Based on the results, we speculate the possible mechanisms for thermal stabilization of the protein, as illustrated in Figure 10. We suggest that not only did the dicarboxylic acid become hydrolyzed, providing electrostatic repulsions (higher intensity value for 850 / 825 cm^{-1} indicating that the microenvironment around tyrosine was more polar), but the hydrophobic tail of OSA may have stabilized the protein hydrophobic core (increase before and after heating at 1322 cm^{-1} compared to the control treatment showing more CH deformation caused by tryptophan, meaning less hydrophobicity on surface of protein) both leading to the suppression of protein aggregation when heated. The pre-heating, sonication, and OSA additive all play a role in forming the thermal stable complex.

Once the proteins are partially denatured with pre-heating, sonication helps to disperse aggregated proteins. The modification process involves lower pre-heating temperature than whey proteins for the process because egg white proteins are more heat sensitive than most other proteins. The addition of the amphiphilic OSA further improved the stability of the system shown with the lower turbidity values at higher heating temperature. The OSA added was not only more soluble but was simultaneously uniformly mixed with the proteins that were partially unfolded after sonication and pre-heating treatment. Sonication solubilizing or dispersing OSA is an important consideration because it exposes charged or anionic groups throughout the dispersion immediately. The pH of the solution decreased, due to the partially hydrolysis of the OSA (~14% hydrolyzed as quantified by titration). The OSA would provide the electrostatic charge needed to prevent aggregation at alkaline pH range (relative to pI) of the protein since proteins carry a negative charge at pH values above its isoelectric point. This may be the same mechanism of action suggested when dextran sulfate was added to β-lactoglobulin.[34] However, with the addition of OSA, its hydrophobic moieties may help interact with other hydrophobic groups of the partially unfolded protein, further strengthening the hydrophobic core and making the protein more resistant to change in structure when being heated. When the protein is further heated at elevated temperatures, less precipitation occurs resulting in a more stable protein system. Previous research by Vardhanabhuti et al.[34] suggested a complex or coacervate may be forming.

More research is needed to further optimize the process to determine what interactions are occurring at molecular level, and to show the formation of coacervates and mechanism of the process. It is hypothesized that the heat induced aggregate formation may have changed from coagulum (random aggregation) to an organized type of aggregation (soluble linear aggregates)

due to improved molecular interaction between protein and OSA. Investigations are needed to determine if the hydrophobic moieties of the OSA groups are playing an important role in inhibiting the formation of hydrophobic protein-protein interactions during heating.

Conclusions

The combination of pre-heating, OSA, and sonication showed an improvement in thermal stability of the protein dispersion. The 20NH treatment was the best treatment when the protein dispersion was heated to 121°C for 20 minutes. The pre-heating step is beneficial when an increase in heating temperature was used for thermal stability evaluation. The specific mechanism why the physically modified protein becomes less turbid with heating compared to its initial turbidity has not been tested. Although a strong physical interaction between the protein and OSA was shown by Raman spectroscopy, the mechanism needs further elucidation. The physically modified FEW could increase the use of egg proteins in the beverage or food processing industry, because such protein is more heat stable and forms a transparent dispersion after heating. A consumer friendly, thermal stable egg white protein ingredient could potentially increase the demand for eggs and their uses in the food processing industry.

Acknowledgments

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Abbreviations Used

BCA, bicinchoninic acid; BSA, bovine serum albumin; FEW, fresh hen egg white or fresh egg white; H, with pre-heating; N, no pre-heating; OSA, octenyl succinic anhydride; PCA,

principle component analysis; SDS, sodium dodecyl sulphate; Son, sonication or sonicated; WPI, whey protein isolate.

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Table 1. Treatments and acronyms for the evaluation of the effect of OSA concentration and pre-heating on protein thermal stability

OSA Concentration (% relative to protein)	Pre-heating Step	Treatment Abbrevations
0	No	Control
5	No	5NH
10	No	10NH
20	No	20NH
2	Yes	2H
5	Yes	5H
10	Yes	10H
20	Yes	20H

Table 2. Effect of physical modification on structural spectra of proteins (Control, 20NH, and 20H treatment) unheated and heated at 75°C for 20 minutes, at 5% protein concentration.

Wavenumber (cm^{-1})*	Actual Wavenumber (cm^{-1})	Structure	Control unheated	Control Heated	20NH unheated	20NH heated	20H unheated	20H heated
508,538,542	494	Disulfide bonds (SS)	0.16 ± 0.02	0.67 ± 0.16	0.20 ± 0.03	0.27 ± 0.05	0.20 ± 0.03	0.21 ± 0.04
759, 763	750	Tryptophan (hydrophobic amino acid that is normally buried)	0.14 ± 0.02	0.35 ± 0.07	0.17 ± 0.01	0.23 ± 0.04	0.18 ± 0.00	0.18 ± 0.00
850/830	850/825	Environment of Tyrosine (if its ionic or not)	1.34 ± 0.05	1.48 ± 0.09	1.75 ± 0.03	1.69 ± 0.13	1.62 ± 0.03	1.58 ± 0.08
1230-1240**	1240	**Amide III, β-sheet formation**	0.17 ± 0.02	0.24 ± 0.01	0.18 ± 0.01	0.24 ± 0.01	0.17 ± 0.03	0.25 ± 0.02
1335[x]	1322	CH deformation, Tryptophan	0.68 ± 0.08	2.04 ± 0.43	0.95 ± 0.10	1.22 ± 0.09	0.94 ± 0.05	1.03 ± 0.08
1450	1450	Amide II	0.66 ± 0.03	0.90 ± 0.14	0.99 ± 0.04	0.99 ± 0.06	1.04 ± 0.01	1.02 ± 0.06
1668	1668	**Amide I, Antiparallel β-sheet formation**	1.14 ± 0.03	1.74 ± 0.30	2.07 ± 0.10	2.44 ± 0.14	2.16 ± 0.04	2.41 ± 0.06

All values are the means ± standard deviations of three replicates. Intensity units are arbitrary units.
*Wavenumber values from Wang et al., 2013; [x]Wavenumber value from Zhang et al., 2012; ** Wavenumber values from Painter and Koenig, 1976.

Figure captions:

Figure 1. Effect of FEW physical treatment on thermal stability of a) no pH adjustment, b) pH 7 adjusted (1% protein) systems evaluated as % turbdidty change after heating at 75°C for 30 min (n=3). All values are the means ± standard deviations of three replicates; different letters among bars or in the column represent significant differences ($P<0.05$). Refer to Table 1 for treatment acronym definitions.

Figure 2. Effect of FEW physical treatment combinations on thermal stability by turbidity and the calculated change in protein solubility after 95°C and 1 hr heating of pH 7, ~0.4 % protein dispersins (n=3). All values are the means ± standard deviations of three replicates; different letters among bars or in the column represent significant differences ($P<0.05$). Refer to Table 1 for treatment acronym definitions.

Figure 3. Effect of FEW physical treatments on % protein retained in dispersion after heating 1.0% protein at 75°C over various times at pH 7 (n=3). Refer to Table 1 for treatment acronym definitions.

Figure 4. Effect of FEW physical treatment cobminations on thermal stability of 2.5% protein evaluated as % turbidity change after heating at 121°C for 20 minutes at pH 7 (n=2). All values are the means ± standard deviations of two replicates; different letters among bars or in the column represent significant differences ($P<0.05$). Refer to Table 1 for treatment acronym definitions.

Figure 5. Principle Component Analysis (PCA) graph of 5 % protein concentration at pH 7 a) unheated and b) heated 75°C for 30 minutes. (Control blue, 20NH green, and 20H red).

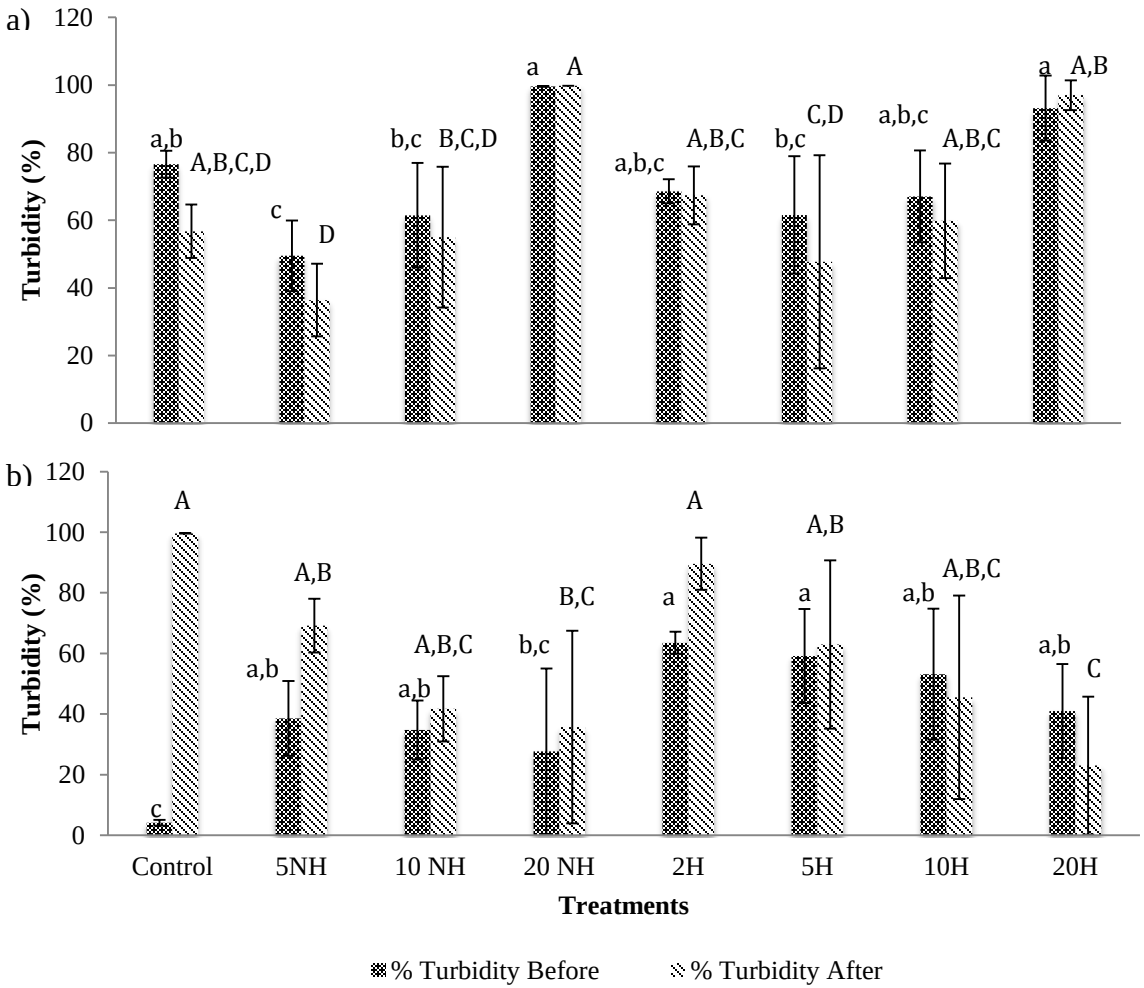
Figure 6. PCA graph of pH 7 adjusted 5 % protein concentration a) Control, b) 20NH, and c) 20H treatments unheated and heated at 75°C for 30 min spectra. (blue unheated, red heated).

Figure 7. Raman spectrums of a) Control, b) 20NH, and c) 20H treatment before and after heating 75°C for 30 min at 5 % protein concentration. (blue, unheated and red, heated spectra)

Figure 8. Spectra of unheated pH 7 adjusted, 5% protein concentration treatments; Control (blue), 20NH (green), and 20H (red) imposed on one another. Wavenumber (cm^{-1}) regions separated based on functional groups; a) disulfide, b) tryptophan and tyrosine, c) amide III and β sheet formation; CH deformation around tryptophan, and d) amide II; amide 1 region.

Figure 9. Spectra of heated pH 7 adjusted, 5% protein concentration treatments; Control, (blue), 20NH (green), and 20H (red) imposed on one another. Wavenumber (cm^{-1}) regions separated out based on functional groups; a) disulfide, b) tryptophan and tyrosine, c) amide III and β sheet formation; CH deformation around tryptophan, and d) amide II; amide 1 region.

Figure 10. Suggested mechanism of FEW protein-OSA complex formation.



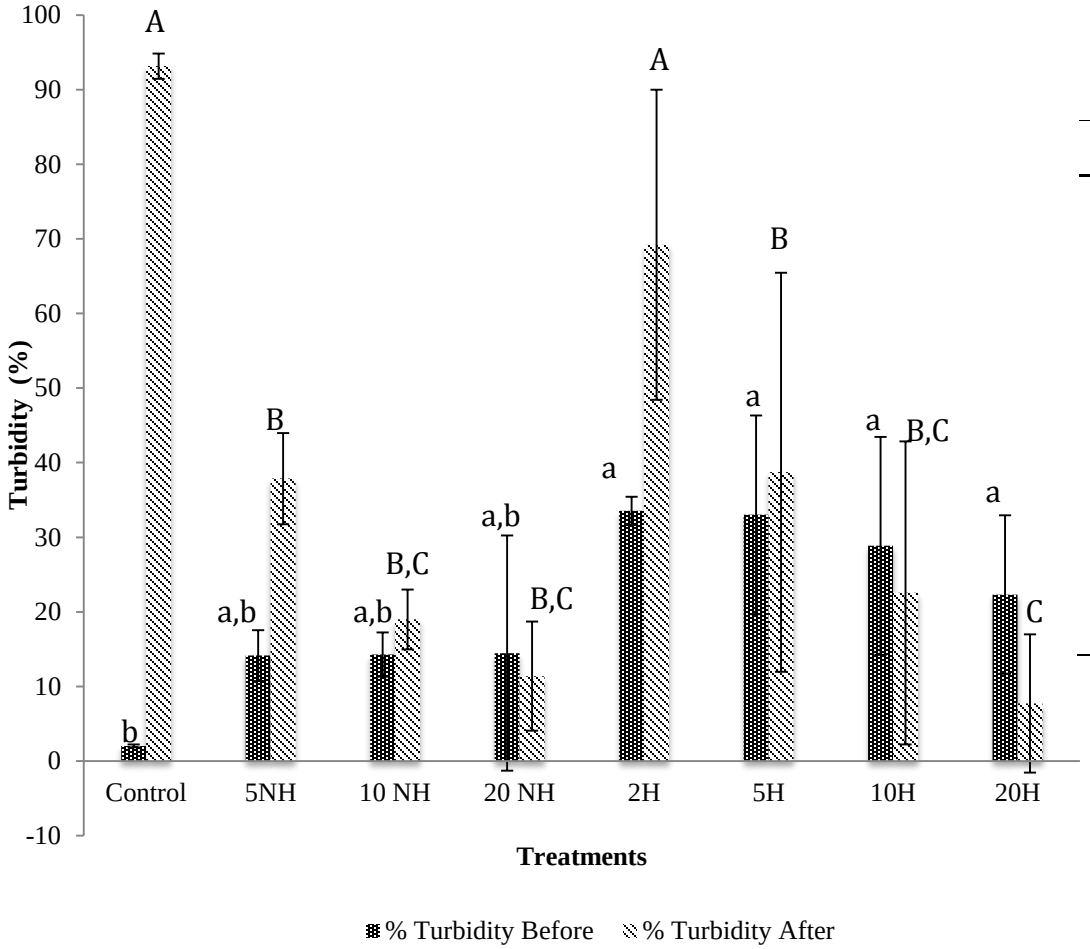
No pH Adjustment

Treatment	Change	pH determined
Control	-19.9 ± 11.9^{c}	9.06-9.13
5NH	-13.1 ± 0.8^{bc}	6.86-7.05
10 NH	-6.5 ± 6.0^{abc}	5.82-6.14
20 NH	0.2 ± 0.1^{ab}	5.52-5.60
2H	-1.2 ± 5.9^{ab}	7.58-8.02
5H	-13.9 ± 14.6^{bc}	6.96-7.44
10H	-7.3 ± 3.3^{abc}	6.10-6.95
20H	3.8 ± 5.2^{a}	5.55-5.96

pH adjusted to 7

Treatment	Change
Control	95.5 ± 1.0^{a}
5NH	30.6 ± 4.0^{b}
10 NH	7.0 ± 1.1^{c}
20 NH	8.0 ± 4.6^{c}
2H	26.1 ± 10.3^{b}
5H	3.8 ± 12.6^{c}
10H	-7.6 ± 12.0^{cd}
20H	-18.0 ± 7.4^{d}

Figure 1. Effect of FEW physical treatment on thermal stability of a) no pH adjustment, b) pH 7 adjusted (1% protein) systems evaluated as % turbdidty change after heating at 75°C for 30 min (n=3). All values are the means ± standard deviations of three replicates; different letters among bars or in the column represent significant differences (P<0.05). Refer to Table 1 for treatment acronym definitions.



pH adjusted to 7

Treatment	Change	Change in Protein%
Control	91.1 ± 1.6^{a}	97.03 ± 11.85
5NH	23.7 ± 3.0^{bc}	99.99 ± 6.68
10 NH	4.7 ± 1.7^{cd}	102.65 ± 0.17
20 NH	-3.1 ± 8.5^{cd}	100.90 ± 0.31
2H	35.7 ± 20.9^{b}	110.64 ± 9.52
5H	5.7 ± 13.5^{cd}	104.06 ± 3.63
10H	-6.3 ± 5.7^{a}	101.32 ± 1.67
20H	-14.6 ± 1.4^{d}	98.78 ± 0.96

Figure 2. Effect of FEW physical treatment combinations on thermal stability by turbidity and the calculated change in protein solubility after 95°C and 1 hr heating of pH 7, ~0.4 % protein dispersins (n=3). All values are the means ± standard deviations of three replicates; different letters among bars or in the column represent significant differences (P<0.05). Refer to Table 1 for treatment acronym definitions.

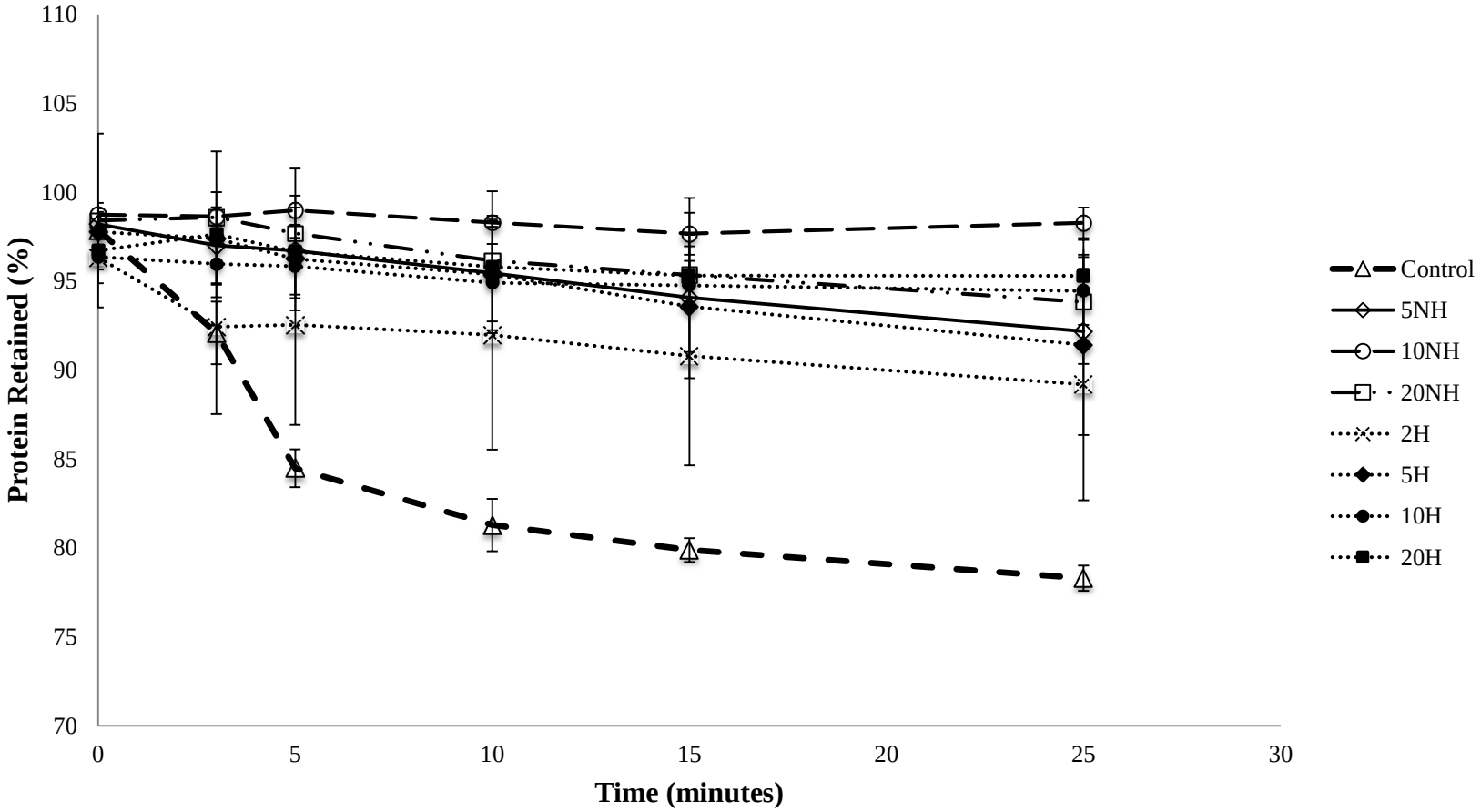
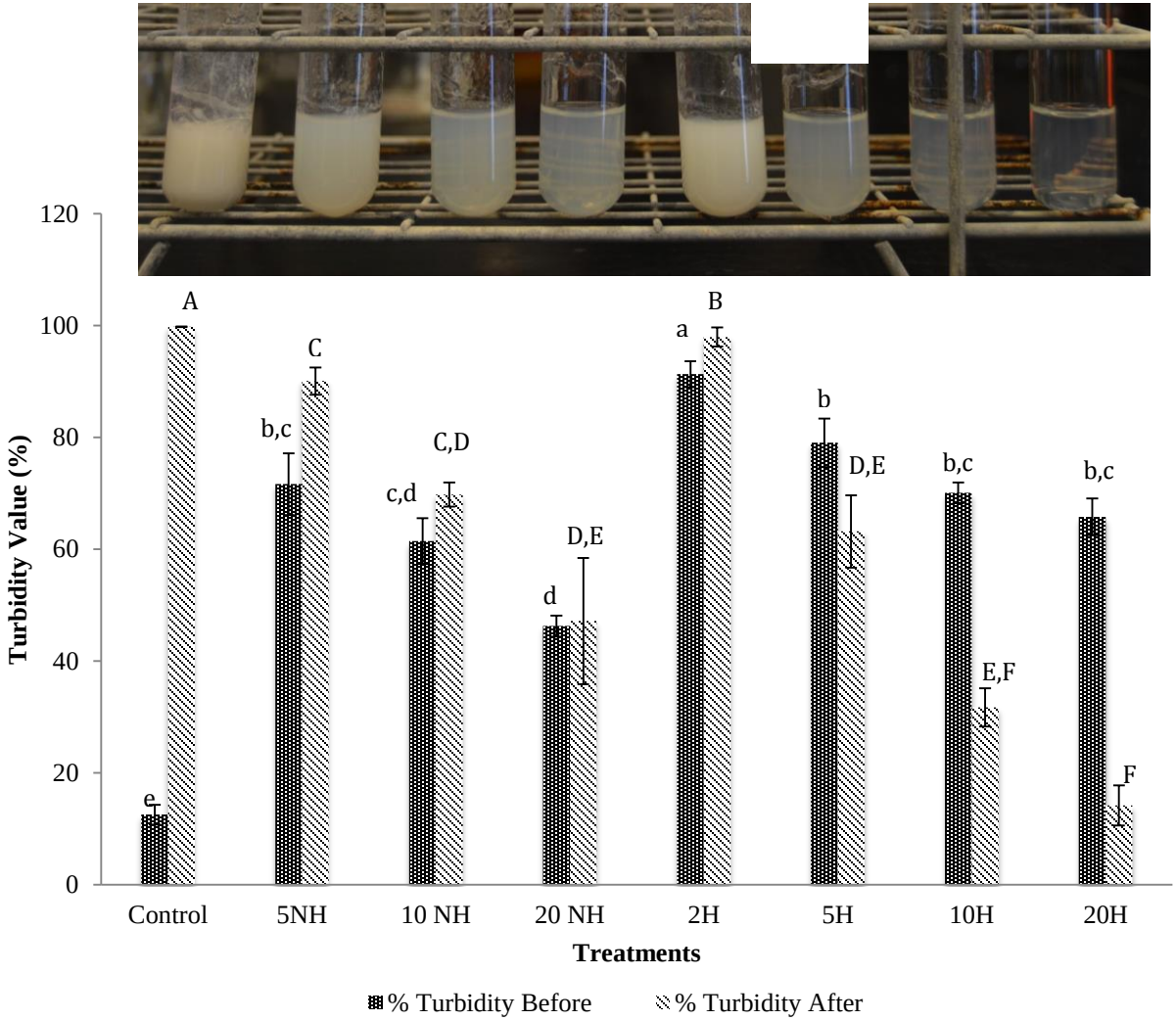


Figure 3. Effect of FEW physical treatments on % protein retained in dispersion after heating 1.0% protein at 75°C over various times at pH 7 (n=3). Refer to Table 1 for treatment acronym definitions.

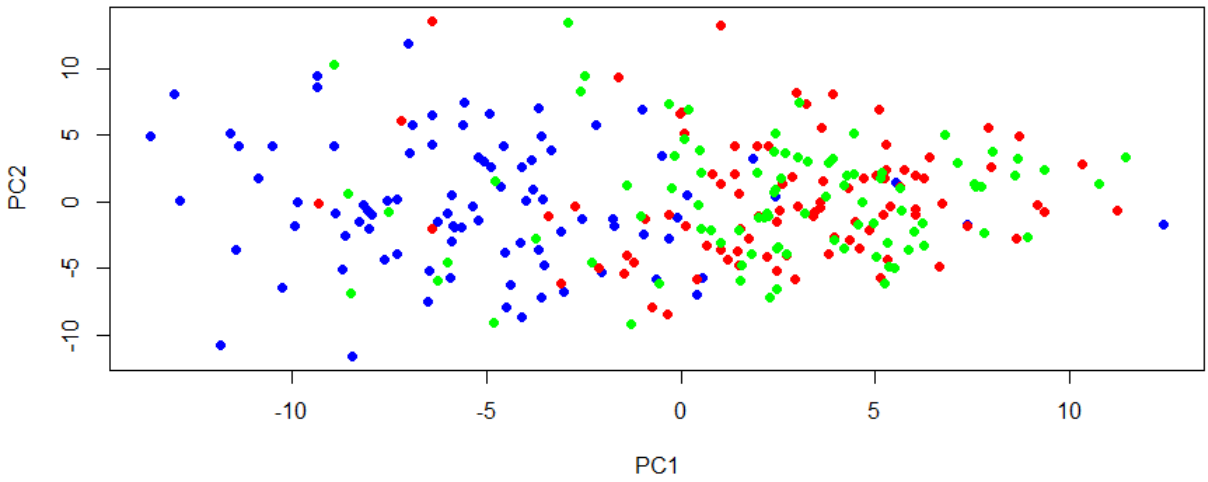


pH adjusted to 7

Treatment	Change
Control	87.2 ± 1.7^{a}
5NH	18.4 ± 3.1^{b}
10 NH	8.3 ± 1.9^{b}
20 NH	0.9 ± 9.4^{bc}
2H	6.7 ± 0.6^{b}
5H	-15.9 ± 2.1^{c}
10H	-38.4 ± 5.3^{d}
20H	-51.6 ± 0.3^{d}

Figure 4. Effect of FEW physical treatment cobminations on thermal stability of 2.5% protein evaluated as % turbidity change after heating at 121°C for 20 minutes at pH 7 (n=2). All values are the means ± standard deviations of two replicates; different letters among bars or in the column represent significant differences ($P<0.05$). Refer to Table 1 for treatment acronym definitions.

a) Control, 20NH, and 20H, all unheated



b) Control, 20NH, and 20H, all heated (75°C for 30 min)

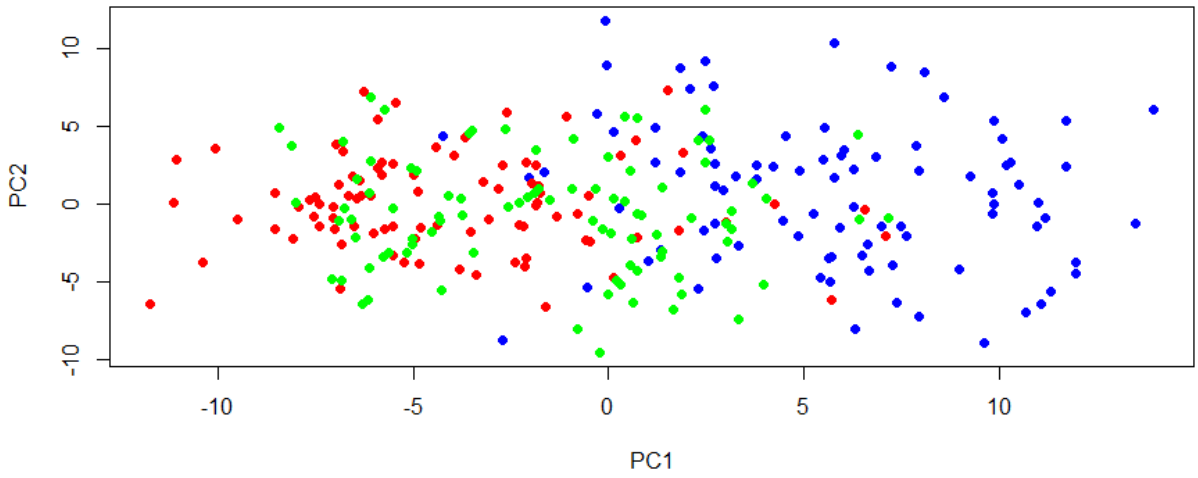
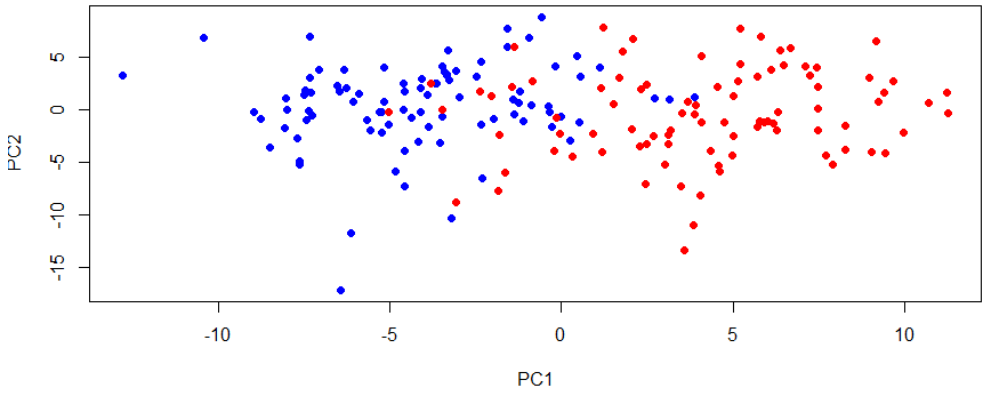
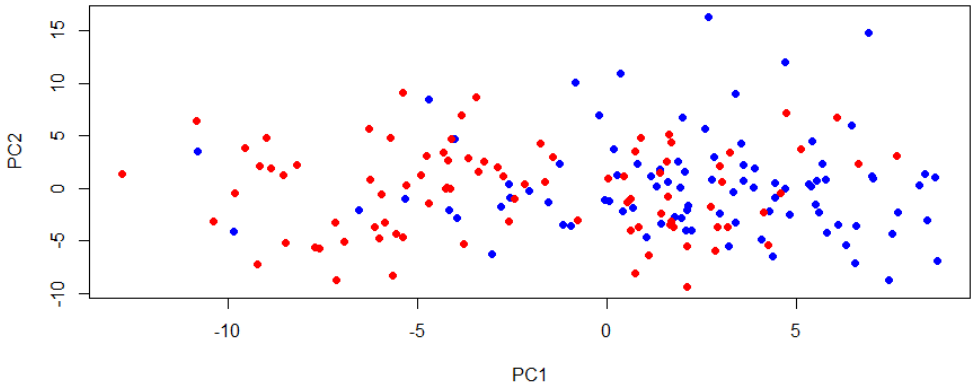


Figure 5. Principle Component Analysis (PCA) graph of 5 % protein concentration at pH 7 a) unheated and b) heated 75°C for 30 minutes. (Control blue, 20NH green, and 20H red).

a) Control before and after heating



b) 20NH before and after heating



c) 20H before and after heating

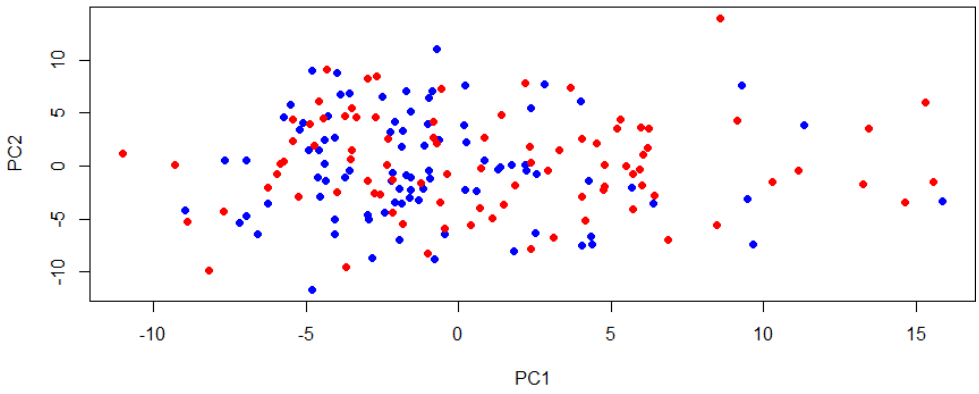


Figure 6. PCA graph of pH 7 adjusted 5 % protein concentration a) Control, b) 20NH, and c) 20H treatments unheated and heated at 75°C for 30 min spectra. (blue unheated, red heated).

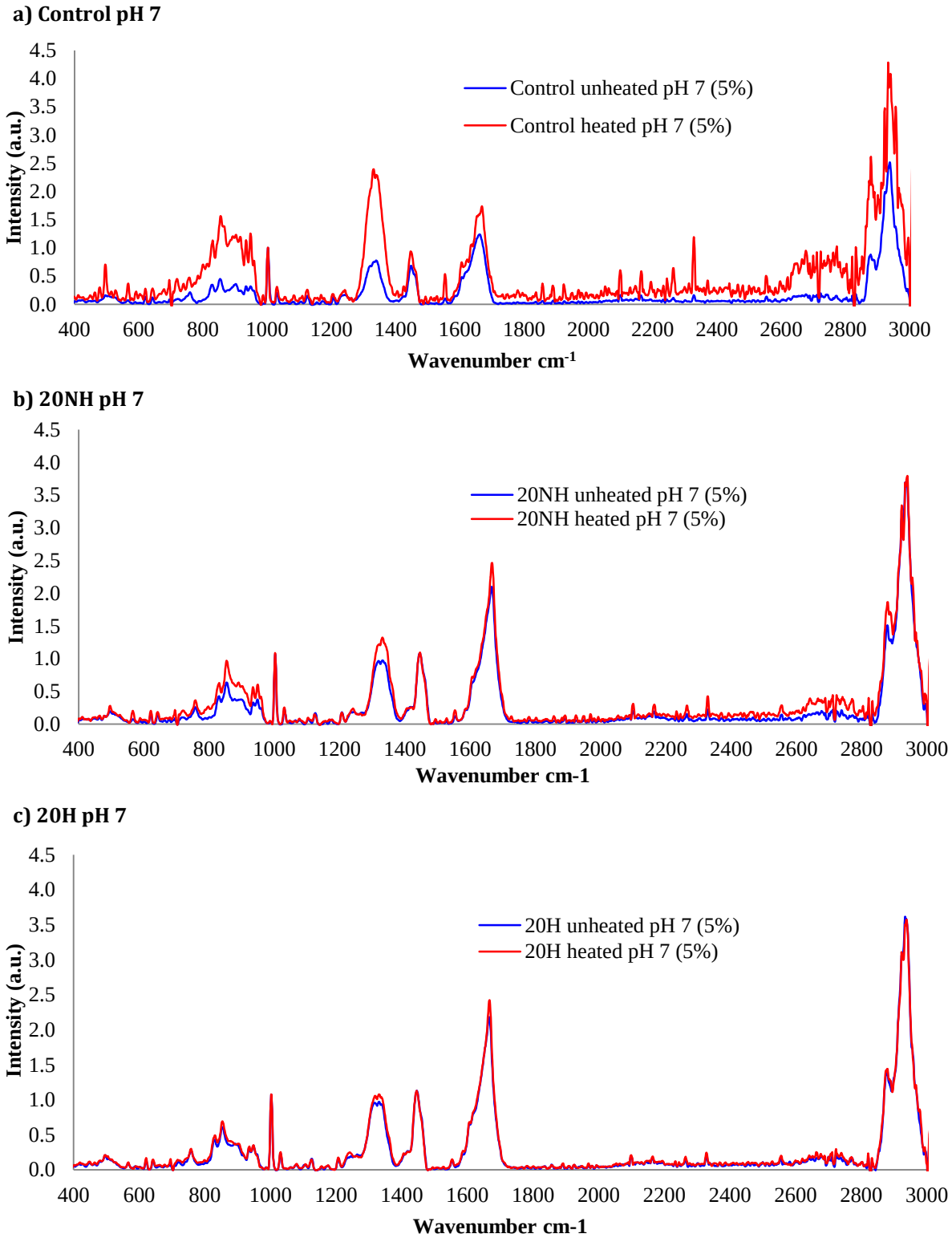


Figure 7. Raman spectrums of a) Control, b) 20NH, and c) 20H treatment before and after heating 75°C for 30 min at 5 % protein concentration. (blue, unheated and red, heated spectra)

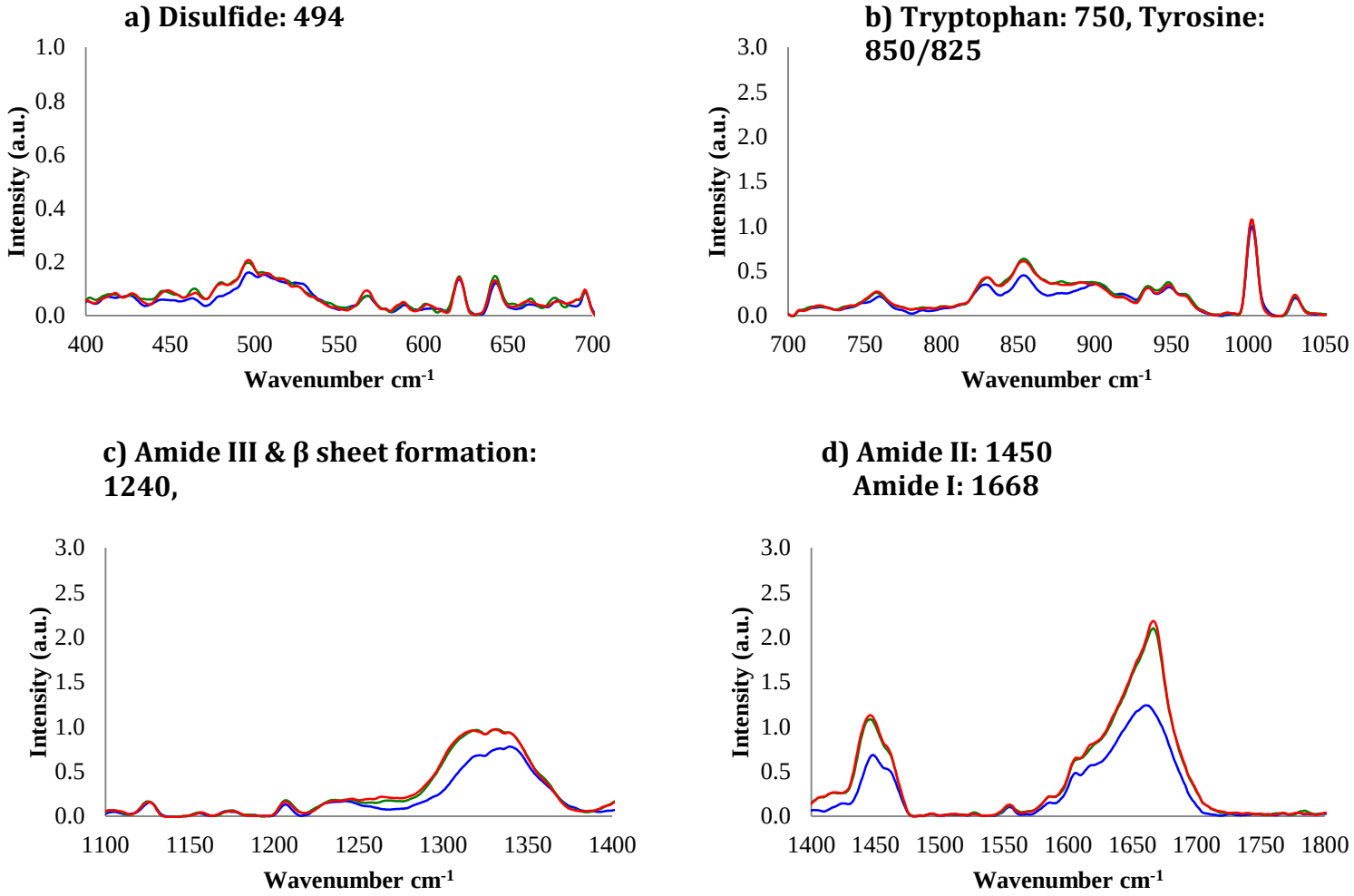


Figure 8. Spectra of unheated pH 7 adjusted, 5% protein concentration treatments; Control (blue), 20NH (green), and 20H (red) imposed on one another. Wavenumber (cm^{-1}) regions separated based on functional groups; a) disulfide, b) tryptophan and tyrosine, c) amide III and β sheet formation; CH deformation around tryptophan, and d) amide II; amide 1 region.

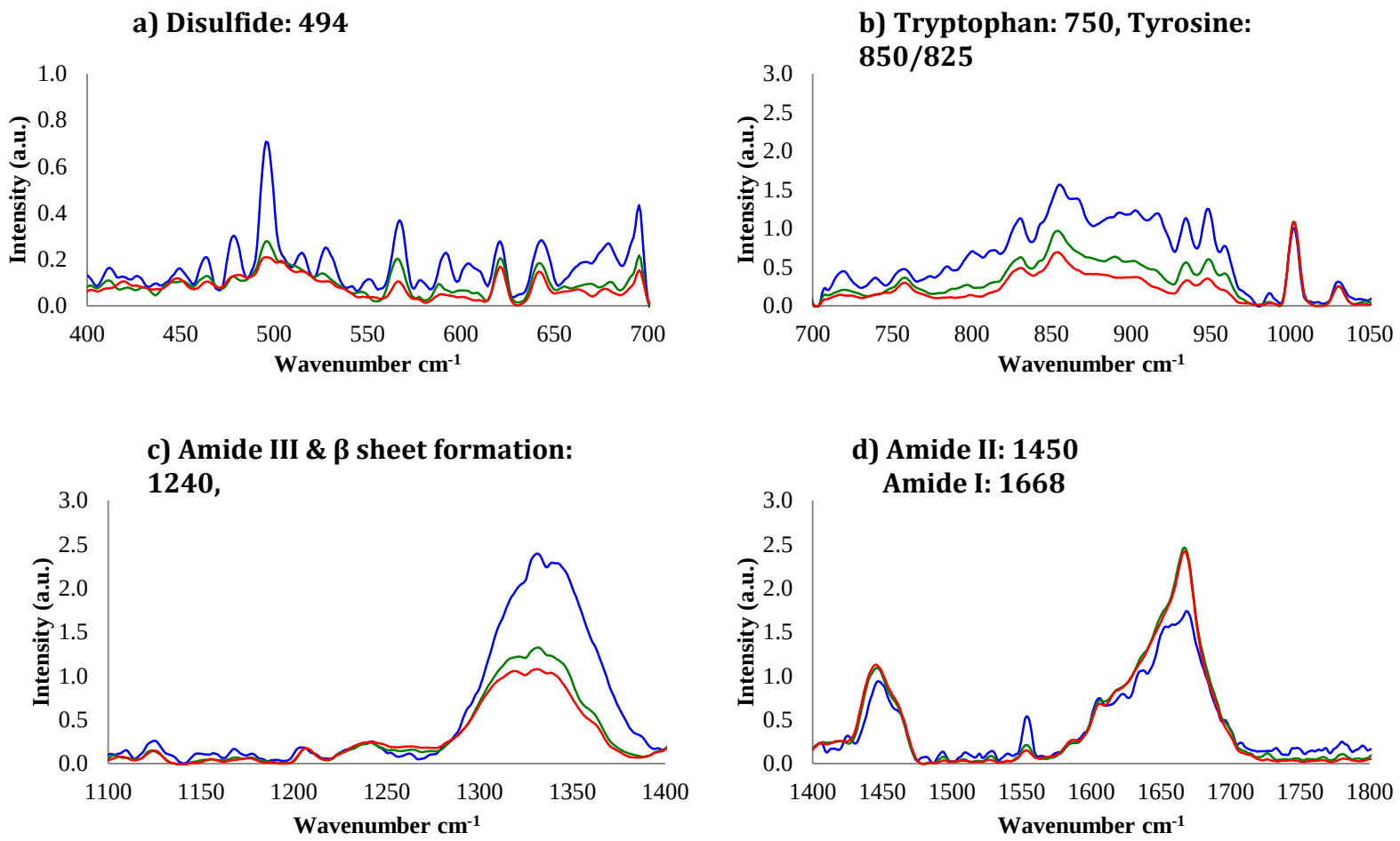


Figure 9. Spectra of heated pH 7 adjusted, 5% protein concentration treatments; Control, (blue), 20NH (green), and 20H (red) imposed on one another. Wavenumber (cm^{-1}) regions separated out based on functional groups; a) disulfide, b) tryptophan and tyrosine, c) amide III and β sheet formation; CH deformation around tryptophan, and d) amide II; amide 1 region.

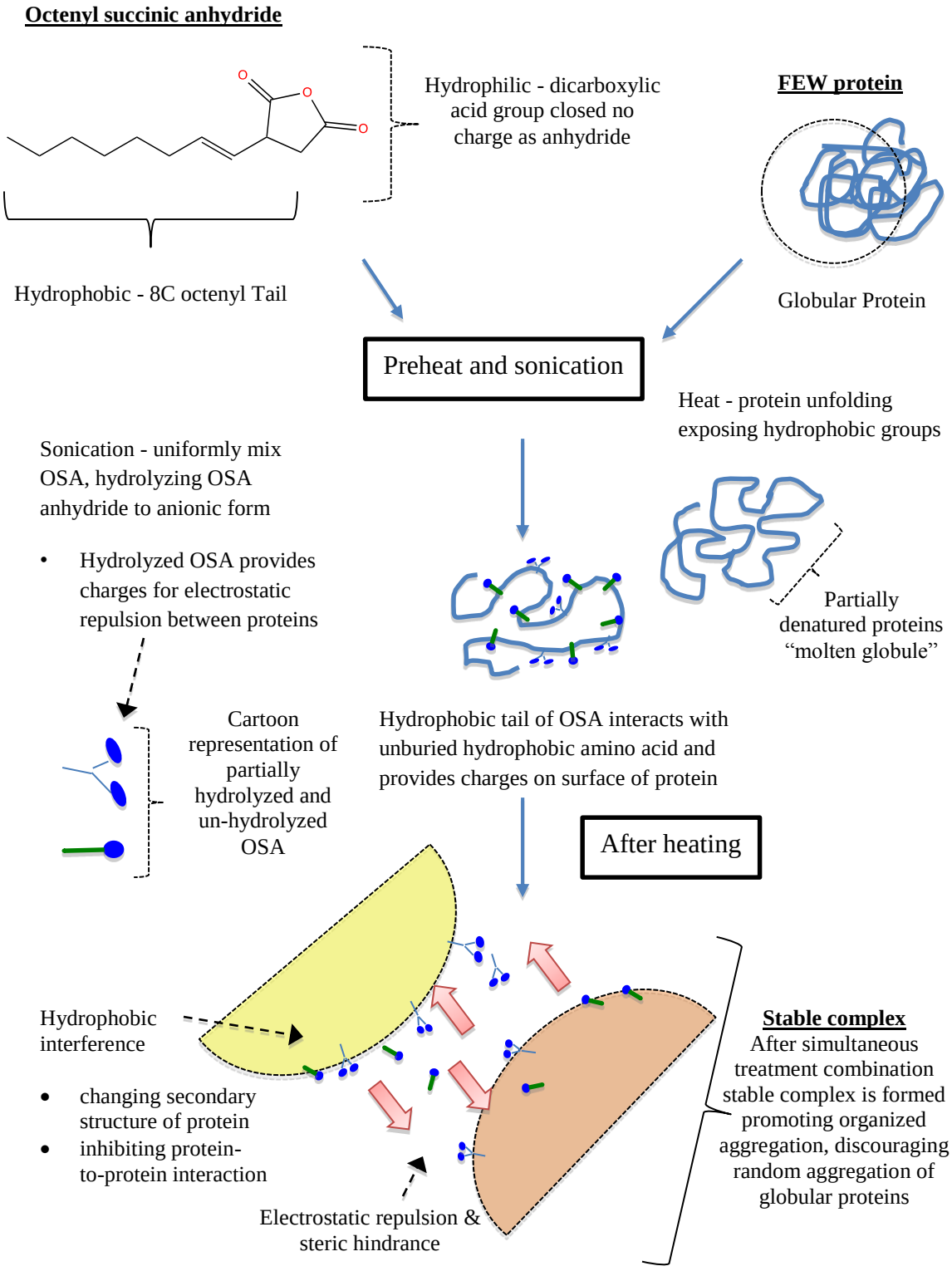


Figure 10. Suggested mechanism of FEW protein-OSA complex formation.

CHAPTER 5: GENERAL CONCLUSIONS

The focus of this dissertation was the modification of egg white proteins to make it more thermally stable. By improving its sensitivity to heat it may lead to an increased use of egg whites in the food industry and in new applications. Egg albumen is an economical source of animal protein and it is high in protein quality. Egg white is already a good emulsifier, foaming, and gelation agent that is mostly used as-is in meringues, or angel food cakes.

Chemical modification using the Maillard reaction and dry heating is an effective method to modify egg white proteins. Dry heating is already a step used in the production of egg white powder, but research is needed to determine these dry heating conditions when different anionic carbohydrates are conjugated to proteins. Our study revealed that a molar ratio of 1:1 free amine of egg white protein to the reducing end of hydrolyzed carboxymethylcellulose (HCMC) was effective in glycating and increasing egg albumen's thermal stability at 75°C and 95°C stability evaluation. The optimal dry heating time was between 2 to 7 days based on free amine and turbidity. At 5:1 molar ratio, thermal stability also improved. Sonication was not useful in glycating protein at the 5:1 molar ratio. The natural glucose found in albumen was also shown to interfere with the glycation reaction.

Octenyl succinic anhydride (OSA) modification of egg white proteins was shown to be effective in improving the thermal stability of egg albumen. Different starting products were modified, i.e.; dehydrated egg white powder (EWP), fresh egg white (FEW), and commercial fresh egg white protein (CFEW). Our study showed that the degree of OSA succinylation was different for each starting product. The use of sonication pretreatment was

effective in improving the reaction efficiency and a lower concentration of OSA used for the same degree of reaction and functionality. The 10% OSA CFEW showed the best property when evaluated at 95°C compared to 75°C for turbidity and protein solubility. However, when the same treatment was spray dried the improvement was lost. This shows the importance of sonication after chemical modification and spray drying conditions in final product functionalities.

Different combinations of physical treatments using pre-heat, sonication, and the addition of OSA were effective in improving protein thermal stability. Our study showed that with pre-heating and higher levels of OSA addition an increase in protein stability was observed when evaluating at temperatures of 75, 95, and 121°C. The optimal level of thermal stability improvement would depend upon what temperature or processing conditions are required for the modified egg white. Raman Spectroscopy was used to characterize the 20% OSA physical treatments with pre-heating and without, before and after heating. An increase in intensity of the β-sheet region after heating occurred similar to other studies. However, the increase in antiparallel β-sheet formation after modification without heating shows a change in secondary structure had occurred. It showed that there were some physical interactions and that this complex structure of the modified protein did not significantly change after heating, as indicated by the spectra of the modified protein. We speculate that the modification may have promoted the globular proteins to aggregate as linear soluble aggregates after heating. Characterization of this complex needs to be further investigated to fully understand the mechanism.

All three studies provided different types of modification that are potentially solutions to improve the thermal stability of egg white proteins as a whole system. Each modification

can be further optimized with more in-depth studies on its mechanism of action. The physical treatment shows the most promise if the concentration of OSA can be further reduced through process optimization since the type of modification is more acceptable to consumers.