Using Lipase to Improve the Functional Properties of Yolk-Contaminated Egg Whites

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ABSTRACT

Egg yolk contamination of egg whites continues to be a serious problem in the egg industry. The ability of egg whites to form stable and voluminous foams is greatly inhibited by accidental yolk contamination, even at extremely small levels. Experiments were conducted to determine if lipase can regenerate the functional properties of yolk-contaminated egg whites. Treatments included control, 0.2% yolk-contamination, and 0.2% yolk-contamination that was treated with lipase and colipase and heated at 37°C for 1 hour. Lipase from *Mucor meihei* and colipase from porcine pancreas were added to yolk-contaminated egg white samples to target and hydrolyze the triglycerides from egg yolk. Enzymatic hydrolysis was confirmed using thin-layer chromatography. Treatment of yolk-contaminated samples with lipase, colipase and heat yielded a drastic improvement in a number of the functional properties, including the final foam volume, foam capacity, and foaming power. These functional properties showed complete restoration to control levels. However, foam stability and foam drainage levels were not statistically different from yolk-contaminated samples that had not been enzymatically treated. Enzyme treated yolk-contaminated egg whites were also tested in an angel food cake system. There were three treatments, including an uncontaminated control, a contaminated control, and a lipase and colipase treated yolk-contaminated sample. Comparison between treatments was performed by volume analysis. The enzyme
treated yolk-contaminated egg whites performed similarly to uncontaminated control angel food cakes.

Keywords: eggs, yolk-contamination, lipase, colipase, functional properties
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# Table of Contents

ABSTRACT ................................................................................................................................... ii

Table of Contents .......................................................................................................................... vi

List of Tables ................................................................................................................................ xi

ACKNOWLEDGEMENTS ............................................................................................................ iv

INTRODUCTION .......................................................................................................................... 1

REVIEW OF LITERATURE ............................................................................................................ 3
  Eggs in the Food Industry ............................................................................................................. 3
  Use of Egg Whites in the Food Industry .................................................................................... 3
  Egg Quality and Acceptability ..................................................................................................... 3
  Implications of Yolk-Contaminated Egg Whites to Egg and Food Industries ......................... 5
  How Yolk Contamination Can Affect the Functional Properties of Egg Whites ..................... 6
  Type of Lipid in Yolk That Most Affects the Functional Properties of Yolk-Contaminated Egg Whites ......................................................................................................................... 7
  Anatomy of an Egg ....................................................................................................................... 8
  Components of Egg White .......................................................................................................... 9
  Components of Egg Yolk ............................................................................................................. 11
  Protein Foams ............................................................................................................................ 12
  Egg White Foaming Properties .................................................................................................. 13
  Effect of Temperature on Egg White Foam Formation ............................................................. 15
  Additives to Improve Egg White Foaming Properties ............................................................... 16
  Effect of Aging and Storage Environment on Eggs .................................................................... 16
  Surfactants ................................................................................................................................ 17
  Foam Capacity ............................................................................................................................. 18
  Foaming Stability ....................................................................................................................... 18
  Factors Affecting the Stability of Foams .................................................................................... 19
  Egg Microbiology ..................................................................................................................... 20
  Egg Pasteurization Methods and the Effect on Functional Properties ....................................... 23
  Lipases – Structure and Function ............................................................................................... 24
  Colipase .................................................................................................................................... 25
  Rate of Hydrolysis ....................................................................................................................... 26
  Mechanism of Hydrolysis – Lipase and Colipase ..................................................................... 27
List of Figures

Figure 1: Final foam volume of egg white samples. Treatments included a control, a 0.10% yolk-contaminated sample, and a 0.20% yolk-contaminated sample. When enzyme alone is added to yolk-contaminated egg whites and allowed to hydrolyze for 6 hours, no significant improvement in the final foam volume of the samples was observed........................................47

Figure 2: Effect of addition of lipase and colipase on the final foam volume of samples. Sample treatments include a control, 0.1% yolk-contamination, 0.1% yolk-contamination + enzyme but no colipase, and a 0.1% yolk-contamination + enzyme and colipase. Enzyme and colipase treated samples were allowed to hydrolyze at room temperature for three hours. .....................49

Figure 3: Thin Layer Chromatography analysis investigating the effect of colipase on the hydrolysis of triglycerides in yolk-contaminated egg white samples treated with lipase. The first four lanes of the TLC plate are lipid extracts taken from a yolk-contaminated sample that had not been treated with lipase and colipase. The next four lanes (5-8) are lipid extracts taken from a yolk-contaminated sample that had been treated with lipase and colipase and that had sat at room temperature for 6 hours ..................................................................................................... 50

Figure 4: Effect of egg yolk contamination and heating (37°C for 6 hours) on foam volume of beaten yolk-free and 0.2% yolk-contaminated egg whites..........................................................52

Figure 5: Thin Layer Chromatography analysis investigating the effects of heating on the rate of hydrolysis of lipase and colipase. The first four lanes of the TLC plate are lipid extracts taken from a heated sample that had been contaminated with yolk at the 0.2% level, but that had not been treated with lipase and colipase. The next four lanes (5-8) are lipid extracts taken from a heated sample that had been contaminated with egg yolk at the 0.2% level but that had been treated with lipase and colipase. Lanes 9-10 were spotted with standards, triglyceride and free fatty acid, respectively................................................................................................................ 54

Figure 6 Effect of duration of heating (37°C for 30 minutes, 45 minutes and 60 minutes) on the final foam volume of yolk-free and yolk-contaminated beaten egg whites.................................56

Figure 7: Results of final foam volume, reported in mL, for three different treatments (control, 0.2% yolk-contamination, and 0.2% + enzyme and colipase). All three treatments were heated for 1 hour at 37°C in a shaking water bath set to 85 rpm. Means having different letters were statistically different from one another (p< 0.05)......................................................................... 58

Figure 8: Results of foam capacity (%) for three different treatments (control, 0.2% yolk-contamination, and 0.2% + enzyme and colipase). All three treatments were heated for 1 hour at 37°C in a shaking water bath set to 85 rpm. Means having different letters were significantly different from one another (p< 0.05). ........................................................................59

Figure 9: Results for foaming power for three different treatments (control, 0.2% yolk contamination, and 0.2% + enzyme). All treatments were heated for 1 hour in a shaking water...
bath set to 85 rpm. Means having different letters were significantly different from one another (p < 0.05).

Figure 10: Results for foam stability for three different treatments (control, 0.2% yolk contamination, and 0.2% + enzyme). All treatments were heated for 1 hour in a shaking water bath set to 85 rpm. Means having different letters were significantly different from one another (p < 0.05).

Figure 11: Results for rate of foam drainage for three different treatments (control, 0.2% yolk contamination, and 0.2% + enzyme). All treatments were heated for 1 hour at 37°C in a shaking water bath set to 85 rpm. Means having different letters were significantly different from one another (p < 0.05).

Figure 12: Control egg white foam (Batch A) after beating but before the flour and sugar mixture was folded in. Foam was stiff, voluminous, and had a pearlescent sheen.

Figure 13: Control egg white batter (Batch A) when added to the baking pan. The egg white batter filled almost the whole volume of the baking pan.

Figure 14: Control angel food cake (Batch A) after cooling and removal from the baking pan.

Figure 15: Yolk-contaminated egg white foam (Batch A) after beating but before the addition of the flour and sugar mixture.

Figure 16: Yolk-contaminated egg white foam (Batch A) in baking pan. Volume of egg white foam is greatly reduced when compared to control levels.

Figure 17: Yolk-contaminated angel food cake (Batch A) after cooling and removal from baking pan.

Figure 18: Enzyme treated yolk-contaminated egg whites (Batch A) after whipping.

Figure 19: Enzyme treated yolk-contaminated egg white batter (Batch A) after addition to the baking pan.

Figure 20: Enzyme treated yolk-contaminated angel food cake (Batch A) immediately after baking.

Figure 21: Enzyme treated yolk-contaminated angel food cake (Batch A) after cooling and removal from pan.

Figure 22: Volumes of angel food cakes made from the first batch (Batch A) of egg whites. There were three different treatments: control, 0.2% yolk-contaminated egg whites, and 0.2% yolk-contaminated egg whites treated with lipase, colipase and heat. Volume measurements
were calculated according to the AACC Method for Determining Baking Quality of Angel-Cake Flour.

Figure 23: Volumes of angel food cakes made from second batch (Batch B) of egg whites. There were three different treatments: control, 0.2% yolk-contaminated egg whites, and 0.2% yolk-contaminated egg whites treated with lipase, colipase and heat. Volume measurements were calculated according to the AACC Method for Determining Baking Quality of Angel-Cake Flour.

Figure 24: Average volumes of angel food cakes from both batches (batch A and batch B) of egg whites. Volume measurements were calculated according to the AACC Method for Determining Baking Quality of Angel-Cake Flour.

Figure 25: Visual comparison of the uncontaminated control (on left) and the contaminated with enzyme treatment (on right). Both cakes were significantly similar in height, volume, and visual appeal.

Figure 26: Visual comparison of the contaminated with enzyme treatment sample (on left) with the contaminated control (on right). The enzyme treated angel food cake was significantly higher in volume, and appeared light and fluffy. The contaminated control was significantly smaller in volume, and appeared dense and tough.

Figure 27: Visual comparison of all three treatments: contaminated with enzyme treatment (left), uncontaminated control (center), and contaminated control (right).
List of Tables

Table 1: Angel Food Cake Ingredient List...........................................................................................................43

Table 2: Results of microbiological comparison between heated and non-heated egg white samples. Heated samples were heated in a shaking water bath for 1 hour at 37°C at 85 rpm. Following plating on tryptic soy agar, the plates were incubated for 48 hours at 35°C.................76
INTRODUCTION

Egg whites exhibit a number of important functional properties that are very useful to many sectors of the food industry. The most important of these functional properties is the ability of egg whites to form a stable and voluminous foam (Hammersnoj 2000). However, even slight amounts of yolk contamination in egg whites can negatively impact these functional properties, most especially the final foam volume, foam capacity, and foam stability. All of these functional properties are essential factors that determine the volume and quality of a number of baked goods commonly produced in the food industry, such as angel food cakes and other confections (Cauvain and Young 2006). Unfortunately, egg yolk contamination in egg whites is unavoidable in the egg industry, as the mechanical process that separates the egg yolk from the egg white often leads to inadvertent yolk contamination. While it is understood that even slight amounts of yolk-contamination can negatively impact the functional properties of egg whites and can result in a severe economic loss for the egg industry, very little research has been conducted to determine how these seemingly lost functional properties can be restored.

Because lipids in the egg yolk are responsible for negatively impacting the functional properties of yolk-contaminated egg whites, any method of restoring these functional properties should directly target the source of the problem – egg yolk lipids. Research has shown that yolk-contaminated egg whites can be centrifuged to remove the lipids, thus restoring the functional properties (Cotterill and Funk 1963). However, this method is not useful in an industrial setting due to the large volume of egg whites in
each industrial batch. Another way of directly targeting the lipids in egg yolk is to use enzymes, particularly lipase, which acts to hydrolyze the triglycerides from the contaminating egg yolk into free fatty acids. Although some research has been conducted in this area, the methods described were not directly applicable to the egg industry. Therefore, the purpose of this research was to develop a method that would improve the functional properties of yolk-contaminated egg whites quickly and effectively, and that could be applied directly to industrial scale batches of egg whites. Developing a method to regenerate egg white functionality would greatly benefit both the egg industry and the many sectors of the food industry that utilize foamed egg whites in food products.
REVIEW OF LITERATURE

Eggs in the Food Industry

Eggs of various avian species have played an extremely important role in the diet of the human race since prehistoric times (Yamamoto 1997), and the importance of hen eggs has only continued to grow from both nutritional and industrial standpoints. Today the majority of eggs produced commercially are from the hen species *Gallus domesticus* (Stadelman 1995). Although a large percentage of the eggs are intended for household use, a growing number are being processed exclusively for use in the food industry (Yamamoto 1997).

Use of Egg Whites in the Food Industry

Egg whites exhibit a number of functional properties that are useful to the food industry (Cauvain and Young 2006). While egg whites are sometimes utilized for their gelation properties, the most important and widely utilized functional property of egg whites is their ability to form a stable foam (Hammershoj 2000). Foamed egg whites are commonly used in such products as ice cream, breads, cakes, confections, and meringues (Alleoni 2004). Foamed egg whites are important as they add texture and mouthfeel to these products, due to the foam's ability to incorporate and retain air (Alleoni 2004).

Egg Quality and Acceptability

Although a number of factors affect egg quality, the most important variable is the flock-management practices that the egg farmer follows. Good flock-management practices help to ensure that a very high percentage of eggs laid will be of excellent quality. Examples of good-flock management practices are selecting the genetic strain
of birds which consistently produce high-quality eggs, providing high quality feed, and following good sanitation practices which help to ensure that the flock remains healthy and disease free (Stadelman 1995).

The handling of the eggs immediately after laying also greatly impacts their overall quality. It is imperative that the eggs are refrigerated as soon as laying occurs. It is also common practice among some farmers to oil the shells of the eggs immediately upon collection. Oiling seals the pores of the egg which helps to limit the loss of moisture and carbon dioxide. If oiling is delayed by even several days, the process is much less effective at preserving the quality of the egg (Stadelman 1995).

Although it is difficult to exactly define egg quality, it is generally considered to be those characteristics which directly impact the egg’s acceptability to the consumer. Eggs are separated and categorized into several quality grades based on size, weight, quality of the shell, and internal characteristics. Candling is a common method used to detect abnormalities or cracks in the shell of the egg, as well as to assess the condition of the albumen and yolk. Very detailed guidelines for egg grading have been laid out by the USDA, and can be found in the USDA Egg Grading Manual. Exact specifications for egg grading and quality can be found in Title 21 Code of Federal Regulations section 160, and Title 7 CFR section 59 (Stadelman 1995).

Shell quality is determined by observing a number of characteristics of the egg shell, including color, cleanliness, thickness and the presence or absence of cracks or other imperfections which might impact a consumer’s perception of the acceptability of the egg. Shell thickness is an extremely important characteristic, and should be at least
0.33 millimeters thick to ensure that the egg has a better than 50% chance of not breaking before arriving for sale at market. There are three possible classifications for egg shells: practically normal, slightly abnormal, and abnormal. Practically normal shells have a normal shape, texture and strength. Slightly abnormal eggs have a shell that is unusual in shape, which might lead to decreased strength. Abnormal eggs have a shell that is extremely deformed or weak due to thin spots and ridges (Stadelman 1995).

The quality of the albumen is measured using the Haugh unit, which relates the egg weight to the height of the thick albumen. In general, the higher the quality of the albumen, the higher the Haugh value. The quality of the yolk is measured using a number of characteristics including color, spherical roundness, and membrane strength. The spherical roundness of the egg yolk is expressed using the Yolk Index. Membrane strength is determined by the ability of the egg yolk to remain intact during mechanical breaking and separation of the yolk from the albumen. Blood spots are considered to be one of the most common major interior quality defects of eggs, and can be detected using the candling method (Stadelman 1995).

**Implications of Yolk-Contaminated Egg Whites to Egg and Food Industries**

Although egg whites have excellent foaming properties, their ability to form stable and voluminous foams is greatly inhibited by the presence of lipid, such as that found in egg yolk. Often the lipid is accidently introduced into the egg white during the mechanical separation of the yolk from the albumen (McGee 1997). As such, even the slightest amount of yolk contamination in an industrial scale volume of egg whites can completely destroy their ability to foam. Even a small amount of lipid contamination can
translate into a huge economic loss for any sector of the food industry that uses foamed
egg whites in their products. Unfortunately, on a commercial basis it is virtually
impossible to mechanically separate the yolk from the albumen without having slight
yolk contamination in the egg white fraction. Therefore, it is of particular importance to
study and understand the impact that yolk contamination can have on egg whites
(Yamamoto 1995).

From a microbiological standpoint, egg yolk contamination in egg whites can also
be extremely detrimental. Studies have shown that when egg yolk is introduced into the
albumen, bacteria tend to grow much more quickly. This is primarily due to the fact that
eyolk inactivates a number of the inherent antimicrobial properties of egg white, and
it also provides important nutrients that bacteria need to grow that are normally absent
in the albumen (Stadelman 1995).

**How Yolk Contamination Can Affect the Functional Properties of Egg Whites**

The most important functional property of egg whites is their ability to create a
stable foam. Therefore, understanding what factors can negatively impact the foaming
of egg whites, such as lipid from egg yolk, is extremely important. Yolk is an inhibitor to
albumen foaming (Matringe 1999), and even at concentrations as low as 0.5%, yolk-
contaminated egg whites will show a drastic decrease in their foaming properties. The
reasons behind this marked decrease are largely thermodynamic in nature, as lipids are
much more surface-active than proteins. Therefore, the lipid contaminant will adsorb to
the air-water interface before the protein (Alleoni, 2004), thus inhibiting the adsorption of
proteins during the process of foam formation. While this might not initially seem like a
problem, films created by lipids are not nearly as cohesive as those created by proteins.
Therefore, the lipid films are simply not capable of withstanding the internal pressures created by the foam bubbles. The bubbles fall apart as the mechanical agitation necessary for normal foam formation occurs. Without fail, egg white foam created without egg yolk contamination consistently displays better foaming and functional properties (Fennema 1996). Another theory as to why egg yolk negatively impacts the functional properties of egg white is that the triglycerides bind with ovomucin, which is the component in egg white that is responsible for stabilizing the foam. Studies have been conducted that show that heating of yolk-contaminated egg whites helps to improve their functional properties. It has been suggested that heating of the yolk-contaminated egg whites causes the triglyceride and ovomucin to dissociate either partially or fully, thus improving or restoring the functional properties (Stadelman 1995).

Several studies have been conducted where the yolk in yolk-contaminated egg whites was directly targeted or removed, and the functional properties of the egg whites were fully restored. Different methods have been used to remove the egg yolk, including centrifugation or hydrolysis of the lipids in the yolk by treatment with enzymes. In both cases, the functional properties of the egg whites were fully recovered (Cotterill and Funk 1963).

Type of Lipid in Yolk That Most Affects the Functional Properties of Yolk-Contaminated Egg Whites

According to Fennema (1996), it is the phospholipids of the egg yolk in particular that act to severely impact the foaming properties of egg whites (1996). However, according to Stadelman (1995), is it the triglyceride fraction of egg yolk which impacts the foaming properties of egg whites the most. Obviously, there is debate as to which
A hen egg consists of five broad components: 1) a cuticle layer, 2) pore canals, 3) a hard shell, 4) albumen and 5) yolk. The egg shell structure is complex, and consists of a calcium carbonate layer that is shrouded in a thin cuticle film, and two shell membranes. The cuticle layer covers the shell and pore canals, and is approximately 10 µm thick. The cuticle layer, which is composed of protein and trace amounts of lipids and carbohydrates, is the first line of defense for the developing embryo within the egg, as it offers the egg both moisture resistance (Stadelman 1995) and protection from invading microorganisms. Although the cuticle layer provides an excellent barrier, it is easily damaged and removed by washing or improper handling (Yamamoto 1997).

Beneath the cuticle layer there are between 10,000-17,000 pores which are unevenly positioned throughout the surface of the eggshell (Yamamoto 1997, Stadelman 1995). These pores are primarily responsible for allowing the developing embryo to both obtain oxygen and expel carbon dioxide from its microenvironment (Stadelman 1995). While the pores do allow for gas and moisture transfer, they do not allow for the passage of liquid water. These selective properties protect the developing embryo within the egg from a number of environmental and microbiological challenges (Yamamoto 1997). The eggshell, which has a highly complicated structure consisting of a number of crystalline layers, acts mainly to provide structural support to the developing embryo. The eggshell also acts to resist and repel water, an important property that ensures the safety and survival of the chick growing inside. If the shell,
along with the cuticle layer, did not act to both repel and resist water, the embryo would most likely perish if the egg became wet, as oxygen and carbon dioxide would be blocked from diffusing into and out of the egg (Stadelman 1995).

The shell membrane is composed of both an inner and outer layer, whose structures are similar to tightly interwoven threads. It has been postulated that the purpose of these membranes is to trap invading microorganisms within their finely woven nets, although the exact purpose of the membranes is still uncertain (Stadelman 1995). In the interior of the egg, the yolk is protected and surrounded by the albumen, which consists of a thin, thick, and chalaziferous layers. The chalaziferous layer (via the chalazae code) acts to protect the yolk by anchoring the yolk in the center of the egg. The egg yolk is further enrobed by a vitelline membrane. (Yamamoto 1997).

An egg contains three main components, which are water (75%), proteins (12%), and lipid (12%). If dissecting an egg based on weight, the egg is 9.5% shell, 63% albumen, and 27.5% yolk. Extremely small amounts of carbohydrates, minerals and pigments are present as well. Proteins are found most abundantly in the egg yolk (44%) and egg whites (50%).

**Components of Egg White**

Egg white, also called the albumen, is composed almost exclusively of water (88%) and protein (10.4%). Due to the fact that egg white contains a high percentage of water, the albumen accounts for approximately 60% of the entire egg by weight. Egg white is a complex mixture of over 40 different proteins, although only several of these are of direct importance to the functional properties of albumen. A large number of egg white proteins are also directly responsible for the natural antimicrobial systems that
occur in the egg. Those proteins which are directly involved in egg white functionality are ovalbumin, ovotransferrin (conalbumin) and ovomucin (Yamamoto 1997).

Ovalbumin, a phosphoglycoprotein (Stadelman 1995), is considered to be the major protein fraction in egg white albumen (approximately 50%). Although the exact function of ovalbumin is unknown, it has been suggested that ovalbumin is primarily involved in the coagulating and foaming properties of egg whites (Yamamoto 1997). Ovalbumin consists of three different components which differ only in phosphorous content: A1, A2, and A3 (Stadelman 1995).

Ovotransferrin, also known as conalbumin, is a heat sensitive glycoprotein that is found in two different forms (Stadelman 1995). One of the most interesting properties of conalbumin is its ability to bind and sequester metal ions. This property is what makes ovotransferrin an extremely important part of the egg antimicrobial system (Yamamoto 1997).

One of the many unique aspects of egg whites is the fact that there are two different types of albumen. Thick albumen, which is characterized by a high viscosity, has a four times higher level of ovomucin than compared to the thin albumen, which has a much lower viscosity. It is generally agreed that ovomucin is responsible for the gelation properties of egg whites (Yamamoto 1997).

Although egg white contains two types of albumen, there are four layers that occur naturally in the egg: the outer thin albumen, the thick albumen, the inner thin albumen, and the chalaziferous layer. Although the proportions of these different types
of egg whites vary widely according to breed, size of the egg, and environmental conditions under which the egg was laid, all eggs do contain these four layers.

Carbohydrates are present in egg whites, but are found only in extremely small amounts and in both free and bound forms. Glucose makes up the majority of the free carbohydrate fraction, whereas the bound carbohydrate is found mostly in glycoproteins. The mineral composition of egg white can vary widely, and depends on a number of different factors including diet, temperature, and the age of the bird that produced the egg (Stadelman 1995).

**Components of Egg Yolk**

According to Stadelman (1995) and Yamamoto (1997), the egg yolk can be described as a homogeneously emulsified fluid. Lipids make up the vast majority of the yolk, and include triglycerides (65.5%), phospholipids (28.3%), and cholesterol (5.2%). The phospholipids in egg yolk are composed of phosphatidylcholine (73%), phosphatidylethanolamine (15.0%), lysophosphatidylcholine (5.8%), sphingomyelin (2.5%), and lysophosphatidylethanolamine (2.1%). Phospholipids are unique molecules in that they contain both a hydrophobic and hydrophilic group. This property is what makes egg yolk such an excellent emulsifier, and is one of the reasons egg yolk is used so extensively in the food industry (Yamamoto 1997).

Egg yolk does contain protein, which is found primarily in the form of lipoproteins. The four main fractions of lipoproteins found in egg yolk are low density lipoproteins, high density lipoproteins, phosvitin, and livetin. Low density lipoproteins are the major protein found in egg yolk (65%), and are known for their excellent emulsification properties. High density lipoproteins are found primarily as a complex
with phosvitin, which contains the majority of phosphorous found in the egg yolk. Livetin, which is water soluble, contains the majority of the enzymes found in eggs, and includes α-amylase, cholinesterase, and phosphatase, among others (Yamamoto 1997).

Although small amounts of carbohydrates are present (only about 1% in the yolk), the majority are present as oligosaccharides bound to glycoproteins. The rest of the carbohydrate is found in the form of free glucose. Low levels of minerals and pigments can also be found, and these include phosphorous (the most abundant), carotenes and riboflavin. Carotenes are the pigments responsible for the yellow color of the egg yolk. As carotenes cannot be synthesized by the hen directly, the hen’s feed is the primary source for pigmentation in egg yolk (Yamamoto 1997).

**Protein Foams**

Protein foams are complex and dynamic colloidal systems that consist of an aqueous continuous phase and a gaseous dispersed phase. Proteins are the main surface-active agents that help in formation and stabilization of foams (Fennema 1996). There are many criteria that a protein must meet in order for it to be considered an effective foaming agent. Most importantly, the protein must quickly and efficiently adsorb to the air-water interface. Once adsorption occurs, the protein must be able to quickly unfold at the interfacial surface (Fennema 1996). In order for adsorption to occur, the proteins involved must unfold rapidly. The unfolding process is aided by mechanical agitation (often whipping or beating) that occurs during the foam creation process. During this process the disulfide bonds present within the structure of the egg white proteins are broken, which increases the surface hydrophobicity of the protein.
Thus, the proteins are better able to adsorb to the air-water interface (Hammershoj 2000).

**Egg White Foaming Properties**

The term functional properties refer to those characteristics of an egg that make them extremely useful ingredients in food products. Typically, these properties include coagulation, foaming, emulsifying and nutrient contribution. Although a number of foods can provide some or all of these properties, eggs are especially capable of producing excellent foams and emulsions, they coagulate easily, and they are also densely packed with a number of important proteins and other nutrients. Perhaps the most widely utilized functional properties of eggs whites are their intrinsic ability to produce stable and voluminous foams, which coagulate and set well when baked (Stadelman 1995).

When egg albumen is beaten, air bubbles become trapped and a foam is created. However, the process of egg white foam formation is extremely complex and involves a number of chemical interactions. Beating acts to unfold the albumen proteins, which allows them to aggregate at the liquid-air interface. These partially denatured proteins interact to create a stabilizing film around the bubbles as they are created through mechanical agitation. This process is absolutely essential to the formation and stability of the foam. The fact that egg white proteins have both hydrophilic and hydrophobic regions is an exceptionally important characteristic which directly helps in denaturation and adsorption to the liquid-air interface. As beating continues, trapped air bubbles become smaller and therefore increase in number. As more air is incorporated and the number of bubbles increases, the foam becomes stiff.
In culinary terms this stage is usually referred to as “stiff peaks”. Egg whites that have been whipped for an appropriate amount of time appear glossy and moist. Over-whipping or excess beating results in an extremely fragile foam that often appears dry (Stadelman 1995).

Egg albumen contains a variety of different proteins which act in tandem to help create the stable and voluminous foams which egg whites are known for. In particular, the globulins help facilitate foam formation by lowering surface tension. The ovomucin-lysozyme complex helps to provide stability for the newly created foam. Ovomucin in particular is the protein that when denatured associates to form the stabilizing film around the newly created air bubbles in the foam. When baked, the ovalbumin and conalbumin confer heat-setting properties. The protein chemistry of overwhipping egg whites is complex, but generally the ovomucin becomes oversolubilized which lowers the elasticity of the bubbles. When bubble elasticity is lost, the bubbles rupture much more easily (Stadelman 1995).

Measuring the functional properties of egg whites can be difficult, and often results vary according to the experimental set up, equipment, and lab environment under which the experiment was conducted. As such, functional property measurements conducted in different labs at different times and under different conditions can be difficult, if not impossible, to compare. Raw functional property measurements, as well as functional property measurements taken through incorporation of egg whites in a food system, are of equal importance and value, and are easier to compare. Typically angel food cakes are used for the purpose of comparing foam characteristics in food systems. A number of different raw functional
property measurements can be taken, including observing and comparing whip time, final foam volume and foam stability. When comparing functional properties in a food system, characteristics such as volume, texture, grain, and elasticity of the crumb can be used (Stadelman 1995).

The time that the egg whites must be whipped to achieve the stiff peak stage is easy to measure, but it is important to note that whip time alone does not give any information about the resulting foam. Whip time is defined as the time that is required to beat egg whites into a foam of a specific aeration. When attempting to compare functional properties of egg white foams, it is important that all of the samples be whipped for the same amount of time. Volume is a useful characteristic to compare, but can be difficult to accurately measure due to trapped air pockets, which can skew the results. Attempting to remove the air pockets can result in damage to the foam, so often depth measurements are the only way to measure final foam volume. When depth measurements are taken for final foam volume, the height measurement must be converted into volume, and is typically reported in mL. Stability of egg white foam is usually measured based on the amount of drainage that occurs over a specific amount of time. The drainage can be measured a number of different ways, including by volume and weight (Stadelman 1995).

**Effect of Temperature on Egg White Foam Formation**

Temperature is the primary environmental factor that most greatly affects the foam that is produced. In general, egg whites that are at room temperature foam much more quickly than egg whites which are at refrigerated temperatures. In general, higher temperatures reduce surface tension, which allows the foam to achieve greater volume
more quickly. However, the stability of the foam is not directly impacted by temperature (Stadelman 1995).

**Additives to Improve Egg White Foaming Properties**

There are a number of compounds that can be added to egg white which will directly impact the foam and functional properties. Glycerol, sugar and sorbitol all have the ability to positively impact the foam stability of egg whites by acting to increase the viscosity of the albumen. These compounds can also provide protection against heat (Stadelman 1995).

**Effect of Aging and Storage Environment on Eggs**

As an egg ages, a number of different chemical and physical changes occur that directly impact and alter the internal characteristics and functional properties of the egg. Those factors which directly impact these changes are time, temperature, humidity and handling. As an egg ages, and especially if the shell was not oiled immediately after laying, carbon dioxide is lost through the pores of the shell. This loss of carbon dioxide causes the pH of the albumen to rise quickly, which results in thinning of the egg white and the loss of yolk membrane strength. The pH of the albumen of freshly laid eggs is usually between 7.6 and 8.5. As the egg ages the pH rises to a maximum of 9.5, although the rate at which the pH increases is largely dependent on storage temperature. The pH of the egg yolk also changes as the egg ages. Immediately after laying, the pH of the egg yolk is usually about 6.0 but during storage increases to between 6.4 and 6.9 (Stadelman 1995).

The yolk membrane strength is reduced because water from the albumen naturally tends to migrate to the yolk as the egg ages. This stretches the membrane,
which drastically decreases the membrane strength, making it more susceptible to rupture. Lipids from the egg yolk also tend to migrate through the yolk membrane and into the albumen, although this usually only occurs after many weeks. Usually, fresh egg white contains only slight amounts of lipid, approximately 0.02%. However, when eggs are stored for a long period of time, the membrane surrounding the yolk can become weak. As a result, lipids from the yolk tend to migrate through the membrane into the albumen, thus causing a change in the foaming properties of the egg whites (Yamamoti, 1995). All of these negative changes can be delayed by refrigerating the egg at temperatures close to freezing (Stadelman 1995).

**Surfactants**

Surfactants, which are small surface-active molecules that consist of a hydrophilic head and a hydrophobic tail region (McClements 2005) are an essential component in the creation of foams (Fennema 1996). Among other things, the ultimate goal of a surfactant is to improve both foaming capacity and foaming ability (McClements 2005). Typically only a small amount of surfactant is needed. A concentration no greater than 0.1% is adequate (Fennema 1996).

The functional properties of surfactants are based on the structure of the hydrophilic and lipophilic regions (McClements 2005). There are several different categories of surfactants, and the particular category that a surfactant belongs to is determined by characterization of the hydrophilic head region. The different categories include nonionic, anionic, cationic (Fennema 1996), and zwitterionic (McClements 2005). It is interesting to note that most of the surfactants commonly used in the food industry are nonionic in nature (McClements 2005). Monoglycerides, Tweens and
polysorbates, among others, are examples of nonionic surfactants. Fatty acid salts and steryl lacylate salts are examples of anionic surfactants. An example of a zwitterionic surfactant, which has both a positively and negatively charged group, is lecithin, a compound found in the yolk of eggs (McClements 2005).

Surfactants can be characterized mathematically by a hydrophile-lipophile balance (HLB) value. HLB values can range from 1 to 40, where a small number implies the surfactant is more soluble in oil, and a large number implies the surfactant is more soluble in water. Therefore, a lower HLB value indicates the surfactant is best used when making water-in-oil emulsions, while a higher HLB value indicates the surfactant is best used when making oil-in-water emulsions. An HLB value of 7 indicates the surfactant is equally soluble in oil and water (Fennema 1996).

In general, proteins are the surfactants most commonly used, especially when making foams and oil-in-water emulsions. Proteins that have little secondary and tertiary structure are highly desirable because they are able to unfold quickly and more completely at the air-water interface (Fennema 1996).

**Foam Capacity**

Foaming capacity is a general term that describes the amount of interfacial area that can be created by a specific protein foam. Several quantitative methods are commonly used to express foaming capacity, most notably overrun (steady-state foam value) and foaming power (Fennema 1996).

**Foaming Stability**

Foam stability is the ability of a protein to stabilize foam against various environmental conditions that can lead to degradation and instability. Generally, the two
factors used to evaluate foam stability are evaluating the rate at which the foam volume decreases over time, as well as the amount of drainage (Yamamoto 1997). Foaming stability is often expressed mathematically as the time required for 50% of the liquid to drain from the foam. Foaming stability can also be expressed as the amount of time necessary for foam volume to decrease by 50% (Fennema 1996). Foam stability of egg white may also be evaluated in an angel food cake system by observing the volume of the final cake product as well as the fineness of the cellular structure in the interior of the cake (Yamamoto 1997).

**Factors Affecting the Stability of Foams**

Although egg whites are noted for their ability to form stable and voluminous foams, there are a number of environmental factors that can adversely affect both foaming capacity and foaming ability. Firstly, pH is important in determining the stability of a protein-based foam. It has consistently been found that protein foams are much more stable at the isoelectric point of the proteins involved in foam formation. At the isoelectric point the repulsive interactions between proteins are greatly reduced. This promotes protein-protein interaction and adsorption to the air-water interface in the form of a thick film. Furthermore, the reduction in protein repulsion allows for more protein to adsorb to the air-water interface. A pH at or near the isoelectric point will allow for improved foaming capacity and foaming ability. At pH values farther from the isoelectric point of the proteins involved in foam formation, the opposite is true and foaming capacity and foaming ability will be reduced. (Fennema 1996).

The presence of salts, such as sodium chloride, has been shown to increase foaming capacity and foaming ability. It is thought that the salt ions neutralize charges
between proteins involved in foaming (Fennema 1996). The addition of sugar to a protein foam will decrease foaming capacity but increase foam stability. The foaming capacity is reduced because the sugars will not allow the proteins to unfold as completely as they normally would at the air-water interface. As a result, the ability of the system to produce a voluminous foam is decreased. However, sugars also act to increase the bulk-phase viscosity of the system, which reduces the rate of drainage of the lamella fluid. This results in a very stable foam system (Fennema 1996).

Protein foams are exposed to a variety of environmental conditions which can make them unstable. These include gravitational forces which promote drainage of liquid from the foam layer and Ostwald Ripening, which is the process by which gas diffuses from the smaller bubbles to the larger bubbles in the foam system. Finally, coalescence of bubbles in the foam matrix can occur because of instability of the protein film that separates the air bubbles in the foam (Fennema 1996).

Egg Microbiology

Although it was initially thought that eggs were free from bacterial contamination, it is now well understood that the surface of the egg shell and potentially the interior of the egg are frequently contaminated by several types of microorganisms. Bacterial contamination of the egg shell typically occurs through direct contact with hen feces after the eggs are laid. *Salmonella* is the pathogenic organism that is most commonly associated with eggs, and can be found both on the interior and exterior of the egg. Bacterial contamination of the interior of the egg can occur through the mother's blood, or the ovary and oviduct before the egg is laid. In this case, pathogens are incorporated into the egg before shell formation is completed (Yamamoto 1997). Occasionally,
molds and fungi can colonize the surface of the egg and penetrate through the egg shell pores (Stadelman 1995).

Regardless of the method of contamination, eggs have a number of mechanisms whereby microbial invasion can be inhibited. Although bacterial contamination of the shell might occur, when the eggs defense mechanisms work in tandem they are quite capable of preventing microorganisms from migrating to the interior of the egg, which would jeopardize the health and safety of the developing embryo. The egg defensive mechanism works primarily through compartmentalization, meaning that microorganisms must pass through a number of barriers, physical and chemical, to reach the interior of the egg (Stadelman 1995). Eggs possess two types of defensive mechanisms to ward off bacterial invasion: structural and chemical. The structure of the egg is the first defense mechanism against bacterial invasion, and begins with the protection provided by the cuticle layer. The cuticle layer covers the thousands of pore canals that are present on the surface of the egg shell, thus preventing bacteria from penetrating through the pore canals into the interior of the egg. However, the cuticle can be easily compromised by exposure to hen feces, improper handling, washing (moisture), and improper storage conditions (Yamamoto 1997).

The egg shell itself acts as a mechanical barrier against microorganisms, and is an important part of the egg defensive system. Factors affecting the efficacy of the shell defensive mechanism include shell thickness and the condition of the shell itself. Thicker shells are better able to resist microbial invasion, and cracks or defects in the shell result in a decreased ability to ward off bacterial contamination. Furthermore, the number of microorganisms on the surface of the shell and the duration of contamination
directly impact the ability of the shell to act as an effective barrier against bacteria (Yamamoto 1997).

The egg shell membrane can be found on the inside of the egg shell, and is also a major part of the egg defensive mechanism. The structure of the membrane is similar to tangled threads, and it has been hypothesized that one of the functions of the membrane is to trap and sequester any microorganisms that successfully penetrate the cuticle and the shell. The egg shell membrane is the last structural defense mechanism. However, eggs possess a number of chemical defense mechanisms in the albumin, and these primarily include chemical factors found in conalbumin (ovotransferrin), ovomucoid, ovomucin, lysozyme, ovomacroglobulin, ovoinhibitor, cystatin, and a number of other enzymes (Yamamoto 1997). Aside from the chemical defenses of the albumin, the viscosity of the albumin itself offers some protection from microbial invasion. The high viscosity of the albumen helps to prevent the ability of invading bacteria to move freely through the egg to the yolk (Stadelman 1995).

Ovotransferrin (conalbumin) has the unique ability to bind metal ions, which acts to inhibit microbial growth by sequestering metal ions that are essential for bacterial growth and proliferation. When ovotransferrin complexes with metal ions, the ions are rendered unavailable to any microorganisms that have successfully invaded the interior of the shell. Therefore, ovotransferrin possesses an extremely powerful bacteriostatic capability. It has been shown that pathogenic *E. coli*, *Pseudomonas aeruginosa* and *Vibrio cholera* were all inhibited by ovotransferrin. However, several studies have shown that regardless of metal ion saturation, ovotransferrin exhibits a very strong antimicrobial nature. Therefore, it is possible that there are other factors, perhaps
conformational, that affect the antimicrobial ability of ovotransferrin besides the metal ion binding capability (Yamamoto 1997).

Ovomucoid, a protein found in egg albumen, is a powerful trypsin inhibitor. Lysozyme, also found in egg whites, is an enzyme that has a potent bacteriolytic action, as it catalyzes the hydrolysis of peptidoglycan, a component of the bacterial cell wall, which results in cell lysis. It is important to note that lysozyme only has lytic capabilities for gram positive bacteria, as the cell walls of gram negative bacteria have a polysaccharide layer which blocks access to the peptidoglycan portions of the cell wall. Ovomacroglobulin has the ability to inhibit viral hemagglutination, while ovoinhibitor acts as a trypsin inhibitor that inhibits bacterial and fungal serine proteases. A number of other defensive enzymes are also present in small amounts in the egg albumen, including phosphatase, catalase and glycosidase (Yamamoto, 1997). While the albumen possesses a number of chemical and enzymatic defenses, the egg yolk is essentially defenseless. Therefore, all of the egg defensive mechanisms, both structure and chemical, are united in the sole purpose of protecting the egg yolk (Stadelman 1995).

**Egg Pasteurization Methods and the Effect on Functional Properties**

Despite the excellent structural and chemical defenses that eggs possess, it is still possible for pathogenic bacteria to colonize the interior of an egg. To address these possible safety issues, the Egg Products Inspection Act was passed in 1970 by the United States Congress, requiring that all egg products be pasteurized. This law was designed to specifically address concerns of *Salmonella* contamination in whole eggs.
and liquid egg products. The standard pasteurization conditions in the United States which are set by the USDA, are 60 degrees Celsius for 3.5 minutes.

While it is of primary importance to ensure the microbiological safety of all egg products through pasteurization, it is essential to understand the potential negative effects that heating can have on both whole egg and liquid egg products. Heating has the ability to directly impact a number of the important functional properties of eggs, so a balance must be found between ensuring microbiological safety without sacrificing product quality and functionality. Luckily, *Salmonella*, the organism that is primarily targeted in pasteurization, is very easy to kill. Although Salmonella is typically considered to be a hardy microorganism, there are a number of chemical and environmental factors in eggs that render the *Salmonella* less able to withstand heating. Firstly, the pH of the albumen helps to reduce the ability of *Salmonella* to survive the pasteurization process. *Salmonella* typically are most resistant at a pH of 5 to 6, but as the typical pH of albumen is 9.1, their ability to survive in egg white is greatly reduced. It is interesting to note that significant yolk contamination in egg whites can actually increase the resistance of *Salmonella* to temperature inactivation, as the fats in the yolk offer some protective effects. The same can be said for whole egg products, which contain both egg white and egg yolk (Stadelman 1995).

**Lipases – Structure and Function**

Lipases (triacylglycerol acylhydrolase EC 3.1.1.3) are water soluble enzymes that hydrolyze lipids. Triglyceride lipases break down triglycerides into diglycerides, monoglycerides and free fatty acids. Lipases require a lipid-water interface for efficient
hydrolysis to occur (Børgstrom 1984). Lipases have been isolated from many sources including humans, animals, bacteria and fungi (Børgstrom 1984).

Lipase usage continues to increase every year. Many scientists agree that lipases are one of the most important and significant classes of enzymes (Woolley 1994). To date lipases are used extensively in the dairy industry to hydrolyze milk fat and to enhance the flavors of certain cheeses. Bacterial lipases have been used in the medical field to act as digestive aides, as flavor-modifying enzymes in the food industry, and as detergent additives in the cleaning industry (Woolley 1994). Bacterial lipases are without a doubt the most useful, as synthesis and purification is relatively simple and inexpensive. Furthermore, bacterial lipases are also more stable than those isolated from animals, plants or fungi (Børgstrom 1984).

The structures of lipase enzymes have been studied rather extensively. Lipase is composed of two domains – a large N-terminal domain and a smaller C-terminal domain. The N-terminal domain contains the active site and other important areas. The structure of lipase is unusual because the active site is hidden beneath a loop, sometimes called the “flap”. When the flap is positioned so that it covers the active site, the enzyme is in the inactive form. Most likely the flap covers the active site when the enzyme is in an aqueous environment and not attached to a lipid-water interface (Woolley 1994).

**Colipase**

Colipase is a single polypeptide chain that has the primary function of restoring the activity of lipase that has been inhibited by bile salts. While it is true that bile salts play a protective role by making the lipase more heat stable, too high a concentration of
bile salts results in total inhibition of the enzyme. This occurs because high concentrations of bile salts dislodge the enzyme from the lipid-water interface, stopping hydrolysis. Colipase, however, is capable of restoring hydrolysis by binding and anchoring the lipase to the interface (Børgstrom 1984).

Colipase has been isolated from a number of sources, including man, ox, horse, and chicken. In terms of its general structure, there are two regions on the molecule which contain a high concentration of molecular cross-linking. The great extent of cross-linking is what makes colipase heat stable (Børgstrom 1984).

**Rate of Hydrolysis**

While some varieties of lipase enzymes exhibit positional specificity, others do not. The difference arises from the particular source of the lipase, whether from the pancreas, fungi, or bacteria. Pancreatic lipase is known to act only at the 1 and 3 positions on a triglyceride molecule and not at the 2 position. Therefore, pancreatic lipase, along with many other sources of lipase, exhibit what is known as positional specificity of hydrolysis. Therefore, they have a preference for hydrolysis based on the position of the ester bond that is to be hydrolyzed (Whitaker 1972). However, there are some sources of lipase that do not exhibit this specificity, such as the majority of the bacterial lipases (Børgstrom 1984). Lipase assays have demonstrated that these sources of lipases will hydrolyze all three ester bonds at almost the same rate (Whitaker 1972).

Lipases also show differences in the relative rate of hydrolysis between triglycerides, diglycerides, and monoglycerides. Typically lipases hydrolyze triglycerides the most efficiently, followed closely by diglycerides. However, monoglycerides are
hydrolyzed much more slowly. Typically, monoglycerides are only hydrolyzed into free fatty acid molecules (glycerol) after all of the triglycerides and diglycerides have been hydrolyzed. Therefore, while lipase enzymes are capable of hydrolyzing triglycerides, diglycerides, and monoglycerides, the rate at which each of these compounds are broken down varies greatly according to lipase specificity (Whitaker 1972).

**Mechanism of Hydrolysis – Lipase and Colipase**

It is still not completely understood how lipase and colipase interact together at the lipid-water interface. There are two proposed mechanisms as to how this interaction takes place. Both mechanisms are equally probable, and it is possible that a combination of both theories accurately describes the actual chemical process. The first theory involves colipase binding to the lipase enzyme molecule, followed by binding of the lipase-colipase complex to the lipid-water interface. The colipase in this model serves to recognize the lipid substrate and anchor the lipase to the interface. The second theory involves a complex composed of three units (colipase, lipase and bile salts). This complex causes the unmasking of a recognition site on the colipase molecule that permits the three part complex to bind to the lipid-water interface. While this particular theory has not been completely substantiated, evidence does show that colipase undergoes a conformational change when it binds to bile salts (Børgstrom 1984).

A specific mechanism for lipid hydrolysis has been proposed. This mechanism involves the formation of a tetrahedral intermediate. In general terms, the mechanism proceeds as follows: the lipase enzyme binds the substrate via a non-covalent bond. The serine oxygen carries out a nucleophilic attack to form a tetrahedral intermediate.
Next, the ester bond on the substrate is cleaved. The tetrahedral intermediate breaks down to an acetyl-enzyme intermediate, followed by dissociation. A water molecule attacks the serine ester to form a deacylation intermediate. Finally, the acyl-enzyme is cleaved, the Ser152 leaving group is protonated, and the fatty acid dissociates (Woolley 1994).

**Emulsions**

A lipid-water interface is required for lipid hydrolysis to occur through lipase action. The primary way to create such an interface is through the formation of an emulsion. An emulsion is defined as a system composed of two immiscible liquids (usually lipid and water), where one of the two liquids is dispersed (in the form of extremely small droplets) in the other (McClements 2005). The liquid that makes up the small droplets in the system is termed the dispersed or discontinuous phase. The remaining liquid is termed the continuous phase. Therefore, in an oil-in-water emulsion, the oil would be the dispersed phase while the water would be the continuous phase (McClements 2005). In an emulsion the lipid phase is surrounded by a surface layer usually composed of amphipathic molecules (usually denatured proteins). The lipid phase is often composed of non-polar lipids like triglycerides. These lipids are not capable of forming a stable emulsion by themselves because they tend to coalesce. Coalescence is a thermodynamic phenomenon whereby the lipid droplets merge together to minimize the amount of lipophilic surface area that is exposed to the water (Børgstrom 1984).

Emulsions can be created in a number of ways, although usually some form of mechanical energy is used to disperse the lipid into small droplets within the continuous
phase. It is the role of the amphipathic molecules to form surface layers that surround the lipid droplets to help prevent lipid coalescence. Emulsions are excellent systems for monitoring lipid hydrolysis because the rate of lipid hydrolysis is determined by the total lipid surface area available to the enzyme. The greater the surface area of the lipid, the greater the rate of hydrolysis by the lipase. Emulsions are able to give a large lipid surface area in an extremely small volume (Børgstrom 1984).

**Emulsifiers**

An emulsifier is defined as a surface active molecule that adsorbs to the surface of small lipid droplets formed during mechanical agitation. Most emulsifiers possess both a polar and non-polar region in their chemical structure and are therefore considered amphipathic molecules. There emulsifier creates a protective layer around the lipid droplet that helps to prevent or lessen the amount of coalescence that occurs. Emulsifiers achieve this goal by not allowing the small lipid droplets to come close enough together to coalesce (McClements 2005).

A good emulsifier should possess the following properties: 1) an emulsifier should be able to readily adsorb to the lipid-water interface when an emulsion is created. 2) An emulsifier should stabilize an emulsion by reducing the interfacial tensions that result from lipid droplets being in close proximity to water and 3) the emulsifier should prevent the lipid droplets from merging together, which would destabilize the system (McClements 2005).

**Emulsion Stability**

When an emulsion of oil and water is created, the result is an extremely unstable system. The lipid droplets will coalesce with other lipid droplets until two distinct layers
are formed. The top layer will be the oil and the bottom layer will be the water due to inherent differences in density between the two liquids. This destabilization is a thermodynamic process as it is not favorable for oil droplets to have such extensive contact with water. Therefore, even when emulsifiers (stabilizers) are used, emulsions can be said to be kinetically stable, but thermodynamically unstable (McClements 2005). Yet another factor that leads to emulsion instability is the fact that emulsions are dynamic systems that are constantly in motion. Instability frequently occurs because of Brownian motion, gravity and mechanical stress (McClements 2005).

There are two environmental forces which act to destabilize an emulsion. These are gravity and droplet aggregation. Gravity can cause creaming and sedimentation, while droplet aggregation can cause flocculation and coalescence. Flocculation is defined as the process by which two or more droplets merge together to create an aggregate where the droplets retain their individual shape and integrity. Coalescence occurs when two or more droplets come together to form one large droplet. Coalescence is what leads to the formation of separate layers of lipid and water and complete destabilization of the emulsion (McClements 2005).

**Lipase Assays – Methods for Studying Lipase Enzymes**

There are three methods by which lipase enzymes can be studied. The first involves monitoring physical changes that occur in the system during hydrolysis. The second involves detecting changes in color (fluorescence) due to liberated products, and the third involves measuring the amount of product (diglycerides, monoglycerides, and free fatty acids) that are produced over a certain period of time. Although these three criteria might not seem very diverse, there are thousands of different lipase
assays and protocols that can be followed or modified to fit a certain scientific objective (Børgstrom 1984).

Deciding which particular assay to use and under what conditions to conduct the assay under are difficult questions that are frequently only answered through trial and error. Perhaps one of the most important criteria to look at is what substrate should be used in the assay. While a lipase enzyme may be able to hydrolyze a number of different compounds, some substrates are better than others. The process of choosing which substrate to use can pose a challenge to even the most seasoned enzymologist. However, many times the choice of which substrate to use is entirely dependent on the needs of the researcher. Despite the large variety of substrates, triglycerides are frequently used as they are quickly and efficiently hydrolyzed. Examples of common triglyceride substrates are triolein, olive oil, trioctanoin and tributyrin (Doolittle and Reue 1999).

Secondly, choosing the environmental conditions for the enzyme assay can be challenging as well. Deciding the optimum pH, optimum temperature, and optimum concentration of enzyme, substrate, emulsifier and colipase is not an easy task (Doolittle and Reue 1999). While there is some literature available, oftentimes an assay protocol must be modified to fit a scientist’s goals, equipment, and time availability. While the choice may be difficult, many times conditions such as temperature and pH are chosen by default due to the needs of the researcher and the conditions that are commonly found in the food industry on an industrial scale basis (Doolittle and Reue 1999).
While environmental conditions such as pH and temperature seem like obvious things to consider, equipment choices can be easily overlooked. For example, it is important to carry out the lipase assay in a glass container, as plastic containers can interfere with the action of the lipase. This is because the lipase-colipase complex will bind to the walls of the plastic reaction vessels. Furthermore, when agitating the emulsion system during the assay it is preferable to use a propeller stirrer instead of a magnetic stirrer (Doolittle and Reue 1999).

Once the substrate and environmental conditions have been chosen, a decision must be made as to how to go about analyzing and quantifying the success of the enzyme assay. This usually involves determining the amount of substrate that was successfully hydrolyzed. By determining the amount of substrate hydrolyzed a researcher can analyze whether or not the conditions of the enzyme assay were optimal. There are many different methods by which the products of an enzyme assay can be measured. Perhaps the most widely used method is the quantification of the amount of free fatty acids that are produced throughout the assay. There are several methods to measure these changes, one of the easiest being using Thin Layer Chromatography (TLC) to separate the products for analysis. TLC allows the researcher to observe trends, such as increasing amounts of free fatty acids, or decreasing amounts of triglycerides, over time (Doolittle and Reue 1999).

**Thin Layer Chromatography**

The science of chromatography allows for easy separation, isolation, identification and quantification of individual components within a certain mixture (Fried, 1986). While thin-layer chromatography is useful in dozens of different fields, it is
especially useful when attempting to quantify the products of enzymatic assays. The sample containing an unknown mixture of compounds is analyzed by being carried through a stationary phase via the upward migration of a mobile phase (solvent). Each component within the sample in question will move up the stationary phase at different rates, and therefore different lengths. The sample is applied or “spotted” in very small amounts on a sorbent layer that is usually supported by glass. The sorbent layer or stationary phase consists of silica gel, cellulose or alumina. After spotting the plate is then placed into a developing chamber which contains a solvent system. The solvent moves up the sorbent layer by capillary action. Each compound in the sample is allowed the same migration time, but each component will have different migration distances depending on a number of chemical factors. In most cases, detection reagent must be applied to the plate after development to make the separated compounds visible for quantification. Often the detection reagent is fluorescent in nature and must be viewed under UV light (Fried 1986).

**Measuring Quality of Baked Products**

The major characteristics, both external and internal, that are used to determine quality of baked products are size, volume, height, shape, crust type, color, crumb structure, softness, mouth-feel, taste and aroma. Of these, volume and height are of considerable importance. Height is regarded as the most useful measure of cake quality as the pan physically constrains the cake as it bakes, causing the batter to expand upwards. Therefore, slight differences in batter expansion or gas retention of the dough are easily observed by measuring the height of the cake. Volume is another invaluable method for determining quality differences between cakes, and can be
calculated using a simple mathematical equation. Density is another parameter that can be considered when comparing baked goods, and is defined as the mass of the product divided by its volume. Specific Volume (SV) is another measurement frequently used, and is defined as the reciprocal of the product density. Measuring the cake color is a common practice when assessing cake quality. Determining the color of the crust is quite simple and can be done using a colorimeter, as the surface texture of the crust has no effect on the color measurements. While a colorimeter gives three readings, L, a, and b values, the reading that is most important when analyzing the crust color is the red-yellow measurement. Measuring the crumb, or interior of the cake, is much more complicated, as the cellular structure of the crumb interferes with the colorimeter measurements. The crumb structure acts to cast shadows, which can give false readings on the colorimeter. The color spectrum that is of highest importance when analyzing the color of the crumb is the yellow to white region (Cauvain and Young 2006).

**Egg White in Angel Food Cake**

Liquid albumen is responsible for providing a number of important characteristics to non-fatted sponge cakes, such as angel food cake. Perhaps most importantly, foamed egg whites make a major contribution to batter aeration and structure formation, much more so than flour. The egg white proteins act to directly contribute to the strength of the baked cake, and they also impart flavor and moisture due to the high water content of egg whites (75%). Besides the negative impact that egg yolk has on the foaming properties of egg whites, egg yolk is not desirable in non-fatted white cakes
because the yolk would result in a yellow cake crumb, which is an undesirable color (Cauvain and Young 2006).

**Statement of Purpose**

Foamed egg whites are used extensively in the food industry in a number of important and popular products, such as ice creams, breads, cakes, confections and meringues. Foamed egg whites are incorporated into these products because of the many important functional properties that they impart, the most important being their ability to form a stable and voluminous foam (Alleoni 2004). However, even small amounts of egg yolk contamination in egg whites can drastically impair the final foam volume, the foam capacity, foaming power, foam stability and foam drainage rates of the foamed egg whites. Therefore, yolk-contamination of egg whites has a major economic impact to the egg industry and the food industry as a whole (Yamamoto 1995).

The purpose of this research was to develop a method that could be easily adapted for industry use, to improve the functional properties of egg whites that had been contaminated with egg yolk. In order for the method to be truly applicable to industry, the method needed to have conditions (time and temperature) that would be easily applied in industry. Furthermore, the procedure would need to be relatively straightforward, and the cost of the method would need to be low. The method would directly target the lipids in egg yolk, as they are the compounds which negatively impact the functional properties of yolk-contaminated egg whites (Matringe 1999).
MATERIALS AND METHODS

**Sample Preparation**

Fresh eggs that were no more than one to two days old were obtained from Dr. Paul Siegel of the Poultry Science Department of Virginia Tech. Immediately after delivery, a visual examination was conducted where any eggs that appeared cracked or in any other way abnormal were discarded. As the quality of eggs received were very high, only a small number of eggs were discarded. The remaining eggs were then carefully cracked by hand. After cracking, the interior of the egg was inspected, and any eggs that contained a blood spot were discarded. The egg white and egg yolk were separated into glass beakers. All glassware was washed thoroughly with soap and water, and rinsed with methanol and distilled water and dried before use. Aliquots of 50 g of egg white were added to 150 mL Erlenmeyer flasks. To prepare contaminated samples, a syringe with a blunt needle was used to dispense the egg yolk into the egg white sample. Fresh egg yolk was loaded into the body of the syringe, and yolk was added to the egg white sample drop by drop to reach the desired contamination level. For samples that were to be treated with enzyme, the lipase and colipase were added directly to the yolk-contaminated sample using a tared balance, and gently mixed thoroughly. Each batch of egg whites and egg yolks consisted of approximately 4 dozen eggs.

All samples were then labeled and covered with aluminum foil to prevent evaporation during heating. A shaking water bath (Shal.form, Precision Scientific, Serial #: 9402-305) was preheated to 37 degrees Celsius and set to shake at 85 rpm. Before
samples were heated, a calibrated thermometer was used to confirm the temperature of the water bath. Samples were heated individually and placed in a holding device inside the shaking water bath to ensure that they did not tip while shaking. Samples were left in the shaking water bath for exactly one hour, after which time they were quickly cooled in a cold water bath until they had reached a temperature of 23°C. A calibrated thermometer was used to check the temperature of the cooling sample periodically. When the sample had reached 23°C it was immediately removed from the cold water bath.

**Method for Whipping Egg White Samples**

A plastic bowl of known volume was used as a receptacle for whipping and gathering of functional property data. The bowl had been modified by drilling a 1.5 cm hole through the bottom of the bowl to allow for foam drainage during experimentation. A rubber stopper was sanded to the appropriate width and height so that the hole could be stoppered securely and easily removed for drainage measurements. The rubber stopper was inserted securely into place. The stoppered bowl was weighed, and the cooled sample was then poured into the bowl. The bowl containing the sample was weighed, and the height (cm) of the egg whites before whipping was measured. Later the height in cm was converted to volume using the above mentioned derived formula. Using a hand-held mixer with a whisk attachment (Michael Graves Design, Model M30, Minneapolis, MN) the egg white sample was whipped on the highest setting for exactly 75 seconds, so that the resulting foam was stiff but not dry. Immediately after whipping, the weight of the bowl containing the foamed egg white was recorded.
Determining Volume-Height Relationship in Plastic Bowl

To accurately measure the volume of egg whites after whipping, it was decided that the height in centimeters of the foam in the whipping bowl would be recorded and later converted to volume. Other methods of measuring the final foam volume were considered, but would have resulted in excessive manipulation and destruction of the foam. Therefore, it was necessary to establish a relationship between height and volume in the whipping bowl. This was done by incrementally adding 10 mL of water to the bowl, up to 800 mL. After each addition of 10 mL of water, a ruler was inserted to the bottom of the bowl, and the height of the water was recorded in centimeters. These values were then plotted using the statistical program JMP 6.0.2. An equation was derived using the Fit Polynomial Function relating height to volume in the bowl. The following formula was developed and used to convert the height of the foamed egg whites into volume:

Volume (mL) = -124.4072 + 128.34065(height in cm of foam) + 16.234377(height in cm of foam – 2.68261)² – 0.7548746(height in cm of foam – 2.68261)³

Enzyme and Colipase Concentration

Lipase from *Mucor miehei* was the enzyme used in this research experiment (Sigma-Aldrich, Saint Louis, Missouri). The enzyme was obtained in lyophilized powder form, with an activity level of approximately 4,000 units/ mg. The optimum pH for this enzyme is 7.7, and the optimum temperature is 37°C. One unit of the enzyme is capable of hydrolyzing 1.0 microequivalent of fatty acid from a triglyceride in 1 hour, at a pH of 7.7 and a temperature of 37°C. The colipase used in this research experiment (Elastin Products Company, Owensville, Missouri) was isolated from porcine pancreas.
The colipase was obtained in a partially purified, highly soluble (2% NaCl) lyophilized state.

The concentrations of both lipase and colipase used during experimentation were determined through trial and error. Egg white volume after whipping for 75 seconds was used as a gauge to determine whether the concentrations of lipase and colipase were high enough to successfully improve functional properties. If the final volume of the enzyme and colipase treated yolk-contaminated egg whites were not significantly improved so as to equal the control, the experiment was repeated with increased amounts of lipase or colipase.

**Determining Final Foam Volume of Egg White Samples**

To determine the volume of the foamed egg whites, a plastic spatula was used to gently and quickly level the surface of the foam without over-manipulation. A ruler was then inserted into the egg whites to the bottom of the center of the bowl and the height in centimeters of the foam was recorded. This measurement was later converted to volume using the equation mentioned previously.

**Description of Measurement Apparatus**

The bowl containing the whipped egg whites was covered in aluminum foil to prevent evaporation during drainage and carefully rested on a ring stand. A 50 mL graduated cylinder was placed onto a top-loading balance and the balance was tared. The tared balance and graduated cylinder were placed directly underneath the bowl, making sure that the mouth of the graduated cylinder was aligned directly beneath the stoppered hole. The rubber stopper was removed from the bowl, and a timer was started. After 1 hour, the weight and volume (in mL) of the liquid drainage was
recorded. The time it took for the liquid drainage to equal half of the weight of the original foam was the final measurement recorded.

**Determining Foam Capacity**

A mathematical method for calculating the foam capacity (%) is given by Janssen et al (1971).

\[
\text{Foam Capacity} \, (\%) = 100 \times \frac{\text{volume of foam}}{\text{volume of initial liquid phase}}
\]

Where the volume of foam is the final foam volume determined after whipping, and the volume of the initial liquid phase is the volume of egg whites in mL prior to whipping.

**Determining Foam Drainage Rate**

The following equation to determine the foam drainage rate was given by Janssen et al (1971).

\[
\text{Drainage} = (\text{volume of liquid phase after 60 min}) - (\text{volume of liquid phase after 30 sec})
\]

Where the volume of liquid phase is the volume in mL of drainage collected in the graduated cylinder during functional properties testing.

**Determining Foaming Power**

The following formula to determine foaming power was given by Fennema (1996).

\[
\text{Foaming Power} = 100 \times \frac{\text{volume of gas incorporated}}{\text{volume of liquid}}
\]
Where the volume of gas incorporated during whipping is the volume of the egg whites after whipping minus the volume of egg whites before whipping. The volume of liquid is the volume of egg whites in mL before whipping.

**Determining Foam Stability**

The following formula was given by Janssen et al. (1971) to determine the foam stability of foamed egg whites:

\[
\text{Foam Stability} \, (\%) = 100 \times \frac{\text{volume of initial liquid phase} - \text{volume of drainage}}{\text{volume of initial liquid phase}}
\]

Where the volume of the initial liquid phase is the volume in mL of the egg whites before foaming, the volume of drainage is the volume in mL of drainage collected after 1 hour.

**Verification of Enzymatic Hydrolysis**

To determine whether the enzyme was functional in the experimental system, the following protocol was used (Christie, 1982). A lipid extraction following the Bligh and Dyer (Christie 1982) method was performed on a yolk-contaminated sample and on an enzyme-treated yolk-contaminated sample. The resulting lipid extracts were analyzed using thin layer chromatography (TLC). A solvent system of 80 mL hexane, 20 mL diethylether, and 1 mL acetic acid was prepared and added to a glass developing tank that was presaturated and had a TLC plate in the back to ensure proper saturation. Three microliters of each sample was spotted onto a TLC plate. Each individual sample was spotted on four separate lanes to ensure reproducibility. Two standards were also spotted, including 1 microliter of glycerol trioleate and 2 microliters of palmitic acid. The TLC plate was developed in the solvent system, removed and allowed to dry in a
chemical hood. The TLC plate was sprayed with 2',7'-dichlorofluorescein (0.1% in ethanol), allowed to dry, and then analyzed under UV light. Pictures were taken for documentation purposes.

**Microbial Analysis**

An experiment was conducted to determine whether heating for 1 hour at 37°C impacted total bacterial counts of the samples. To ensure that organisms from the environment did not impact the microbial counts, all research was conducted in a class II Biosafety Cabinet. Eggs were immersed in a 70% ethanol solution for 10 seconds, then removed and allowed to dry. Wearing gloves that had been dipped in 70% ethanol solution, the eggs were cracked by hand, with the whites and yolks separated into two beakers that had been sterilized in the autoclave. Two non-heated samples were studied, a control and a 0.2% yolk-contaminated sample. Two heated samples were also studied, a control and a 0.2% yolk-contaminated sample. Samples were prepared in accordance with the previously mentioned protocol. The samples that were to be heated were placed in a shaking water bath for 1 hour at 37°C at 85 rpm.

Serial dilutions were carried out using 9 mL peptone blanks. All samples were plated onto Tryptic-Soy Agar (TSA) (Difco™, Sparks, Maryland) in triplicates using the spread-plate method. Samples that had been heated were plated out to a dilution of $10^{-4}$, while non-heated samples were plated out to a dilution of $10^{-3}$. Plates were inverted and incubated at 35°C. Colony counts were performed first at 24 hours, and again at 48 hours.
**Baking Analysis**

Three treatments were tested in an angel food cake system, including a control, a 0.2% yolk-contaminated sample, and an enzyme and colipase treated 0.2% yolk-contaminated sample. Samples were prepared in accordance with the previously mentioned protocol. Although 300 mL of egg whites were required for each cake, all samples were prepared in 50 mL aliquots to ensure the same heat transfer rate and treatment as in previous experimentation. Only the enzyme treated samples were heated in the shaking water bath. The baking protocol used was provided by Dr. Frank Conforti of the Human Nutrition Foods and Exercise Department of Virginia Polytechnic Institute and State University (Table 1).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cake Flour</td>
<td>1 cup</td>
</tr>
<tr>
<td>Confectioner’s Sugar</td>
<td>1 ½ cup</td>
</tr>
<tr>
<td>Egg Whites</td>
<td>1 ½ cup</td>
</tr>
<tr>
<td>Cream of Tartar</td>
<td>1 ¼ teaspoon</td>
</tr>
<tr>
<td>Salt</td>
<td>¼ teaspoon</td>
</tr>
<tr>
<td>Pure Vanilla Extract</td>
<td>1 ½ teaspoons</td>
</tr>
<tr>
<td>Almond Extract</td>
<td>½ teaspoons</td>
</tr>
</tbody>
</table>

Table 1: Angel Food Cake Ingredient List

A conventional oven, whose temperature settings had already been verified, was preheated to 190.5°C. On a sheet of wax paper, 1 cup of cake flour (Swans Down brand) and 1 ½ cups of confectioner’s sugar (Food Lion brand) was sifted together three
times. In a stand mixer, the 50 mL individual egg white samples were combined to reach 1 ½ cups, and were whipped using a whisk attachment until slightly foamy. Next, 1 ¼ teaspoons cream of tartar (Food Lion brand), ¼ teaspoon salt, 1 ½ teaspoons pure vanilla extract (Kroger brand), and ½ teaspoons almond extract (Sauer’s Brand) were added to the foamy egg whites. The egg white mixture was then beaten on high speed to the soft peak stage, after which time ¾ cups granulated sugar (Domino brand) was slowly added to the mixture, over a period of 30 seconds. The egg whites were then beaten until stiff and shiny peaks were formed and no trace of sugar was detected when rubbed between the fingers. The sifted cake flour and confectioner’s sugar mixture was gradually folded into the foamed egg whites by hand. The batter was then carefully added to an ungreased 10 inch tube pan with a removable bottom and a long narrow spatula was passed through the batter to remove air bubbles. Before the batter was added, the interior of the pans were lightly sprinkled with water. The pans were then placed into the preheated oven on the lowest rack, and baked until golden brown and springy to the touch, exactly 23 minutes.

Immediately after removal from the oven, the cakes were inverted onto a cooling rack and allowed to cool upside down in the pan for approximately 2 hours. After 2 hours, the cakes were gently removed from the tube pan and analysis using the AACC method 10-15 for determining the baking quality of angel-cake flour was carried out. A qualitative visual comparison was conducted.
RESULTS AND DISCUSSION

**Determining Type of Enzyme**

There are conflicting opinions among many in the scientific community as to whether it is the triglycerides or phospholipids in egg yolk which are more directly responsible for impacting the functional properties of yolk-contaminated egg whites. According to Fennema (1996), it is the phospholipids in egg yolk that primarily act to severely impact the foaming properties of egg whites. However, according to Stadelman (1995), it is the triglyceride fraction in egg yolk which impacts the foaming properties of egg whites the most. Obviously, there is debate as to which class of lipid is the most responsible for negatively impacting the functional properties of egg whites. Therefore, both lipase and phospholipase were considered as potential enzymes for use in this study. The mitigating factors influencing the decision as to which type of enzyme to use in this research were cost and availability. It was found that the particular variety of phospholipase that would most effectively hydrolyze the phospholipids in egg yolk was not commercially available, whereas lipase from a number of different sources was found to be readily available and relatively inexpensive.

Lipase was also considered for use in this research because experimentation had been conducted in the past that had used lipase to improve the functional properties of yolk-contaminated egg whites. Cotterill and Funk (1963) successfully developed a method whereby lipase from pancreatic sources was used to improve the functional properties of yolk-contaminated egg white. However, attempts to use lipase isolated from wheat germ to improve the functional properties of yolk-contaminated egg
whites were not successful. Murphy and Ubing (1959) also used lipase, isolated from the pancreas of hogs, to successfully restore functional properties to yolk-contaminated egg whites. No scientific publications were found detailing the successful use of phospholipase to improve egg white functionality, and ultimately the decision was made to use lipase in this study.

**Determining Conditions for Enzymatic Hydrolysis to Occur**

Initially, a number of different sources of lipase were tested. Ultimately, it was determined that lipase from *Mucor miehei* EC 3.1.1.3 (also known as triacylglycerol acylhydrolase or triacylglycerol lipase) would be used. This decision was based on its high activity level, optimum temperature requirements, and relatively low cost. The theoretical amount of enzyme needed to hydrolyze the exact amount of yolk lipid in the contaminated egg white samples was calculated based on the reported enzyme activity level. When no improvement in the final foam volume of the samples was observed with this concentration of enzyme, the amount of enzyme added was increased by a factor of 5. When still no improvement in the functional properties of the yolk-contaminated samples were observed, it became clear that the lipase enzyme was not functional in the experimental system (Figure 1). This was confirmed through thin layer chromatography analysis, which showed no difference in triglyceride and free fatty acid levels between the yolk-contaminated samples that had and had not been treated with lipase.
It became apparent that colipase, a protein cofactor, may be needed in order for hydrolysis to occur and for the enzyme to function properly. The concentration of colipase used was determined experimentally through trial and error. When enzyme and colipase were added to yolk-contaminated egg white samples and allowed to sit at room temperature for 3 hours, marked improvement in the final foam volume of the samples were immediately detected (Figure 2). It appeared that with the addition of colipase the enzyme was functional in the yolk-contaminated egg white system. Hydrolytic activity was confirmed through TLC analysis. Lipid extractions were performed on two samples, one that had been contaminated with egg yolk but that had not been treated with lipase and colipase, and the other that had been contaminated...
with 0.2% egg yolk but that had been treated with lipase and colipase. Both samples were allowed to sit at room temperature for 6 hours without mixing. The TLC analysis confirmed that the lipase, in the presence of colipase, was effective at hydrolyzing the contaminating triglycerides in egg yolk to free fatty acids. Although only a slight fraction of the triglycerides had successfully been hydrolyzed (Figure 3), the samples treated with lipase and colipase showed drastic improvements in their functional properties.

These results differ from those reported by Cotterill and Funk (1963), who were able to successfully use lipase, without the outside addition of colipase, to return the functional properties of yolk-contaminated egg whites to uncontaminated control levels. However, Cotterill and Funk (1963) chose to use a variety of lipase enzyme derived from pancreatic sources, while the variety used in this research experiment was derived from a bacterial source. This variation is most likely the result of the fact that a different source of lipase was used in each experimental study or that colipase was present in the pancreatic source of lipase used by Cotterill and Funk (1963).
Figure 2: Effect of addition of lipase and colipase on the final foam volume of samples. Sample treatments include a control, 0.1% yolk-contamination, 0.1% yolk-contamination + enzyme but no colipase, and a 0.1% yolk-contamination + enzyme and colipase. Enzyme and colipase treated samples were allowed to hydrolyze at room temperature for three hours.
Figure 3: Thin Layer Chromatography analysis investigating the effect of colipase on the hydrolysis of triglycerides in yolk-contaminated egg white samples treated with lipase. The first four lanes of the TLC plate are lipid extracts taken from a yolk-contaminated sample that had not been treated with lipase and colipase. The next four lanes (5-8) are lipid extracts taken from a yolk-contaminated sample that had been treated with lipase and colipase and that had sat at room temperature for 6 hours.

**Heating of Lipase and Colipase Treated Samples**

The amount of time that samples needed to be left at room temperature (6 hours) to ensure that sufficient hydrolysis occurred was longer than ideal. From an industry, microbiological and laboratory standpoint, it was obvious that this was too long of a period for samples to be left at room temperature. Cotterill and Funk (1963) successfully heated samples of lipase treated yolk-contaminated egg whites to 21.1°C and held them for a 24 hour period at this temperature. Cotterill and Funk (1963) did not report that any evaluation on the effects of heating egg white samples had been
conducted, although the samples used in that experiment were not heated any higher than room temperature. As samples in this study were to be heated to much higher temperatures, according to the optimum temperature requirements for the enzyme being used, it was important to determine what effects heating to 37°C had on the egg white samples. Testing was also conducted to determine whether heating of the yolk-contaminated samples that had been treated with lipase and colipase would result in hydrolysis occurring more quickly, allowing the amount of time for improvement in the functional properties to be decreased drastically.

It was imperative to determine whether or not heating by itself would impact the functional properties of yolk-free or yolk-contaminated egg whites in either a positive or negative fashion. Experiments were conducted to determine if there was any difference in the final foam volume between samples that had been heated, compared to identical samples that had not been heated. It was found that heating samples at 37°C for a period of up to and including 6 hours had no effect on the final foam volume of yolk-free and yolk-contaminated egg whites (Figure 4).

It is also important to note that Cotterill and Funk (1963) adjusted the pH of the egg white samples being tested. Egg white samples were adjusted to pH values ranging from 6.5 to 10.0 using 1N NaOH before incubation, but after the addition of enzyme. Unlike in this study, Cotterill and Funk (1963) did not measure the functional properties of enzyme treated yolk-contaminated egg whites using quantitative methods in the laboratory. All functional property measurements were taken by comparing the differences in angel food cake volume between samples. Cotterill and Funk (1963) found that as the pH of the egg whites was reduced to below 9.5, the functional
performance of the samples were impaired, as measured using angel food cake systems. Freshly laid eggs typically range in pH from 7.6-8.5, and after extended storage can rise to a pH as high as 9.5 (Stadelman 1995). It was decided not to adjust the pH of egg white samples in this particular research experiment, as the ultimate goal was to develop a method to improve the functional properties of yolk-contaminated egg whites that could be easily adapted to industry use. It was felt that a method that required pH adjustment of industrial batches of egg whites would be impractical and potentially detrimental from an industry standpoint.

Figure 4: Effect of egg yolk contamination and heating (37°C for 6 hours) on foam volume of beaten yolk-free and 0.2% yolk-contaminated egg whites.

In order to confirm the experimental results that indicated that the functional properties of yolk-contaminated egg whites that had been treated with lipase and
colipase would improve more quickly when heated, TLC analysis was conducted. Two samples were tested, the first being heated yolk-contaminated egg whites that had not been treated with lipase and colipase, and the second being heated yolk-contaminated egg whites that had been treated with lipase and colipase. Both samples were heated for 6 hours at 37°C in a water bath set to shake at 85 rpm. A shaking water bath was used to ensure that the samples were constantly, but gently, agitated. As can be seen from Figure 5, the heated sample contaminated with yolk at the 0.2% level that had been treated with lipase and colipase had almost all of the triglycerides from egg yolk hydrolyzed to free fatty acids. This confirmed the hypothesis that heating of the enzyme and colipase treated samples would increase the rate at which lipase hydrolyzed the triglycerides in the contaminating egg yolk. Unheated enzyme treated samples that had been left at room temperature for 6 hours showed only minimal hydrolysis, but when heated at 37°C for 6 hours, showed almost total triglyceride hydrolysis.
Once it was established that heating had no negative impact on the sample foaming properties and was effective at increasing the rate of enzymatic hydrolysis, the next step was to determine the exact duration of heating needed to achieve a quick and complete improvement in functional properties of yolk-contaminated samples. It was understood from previous experimentation that the functional properties of yolk-contaminated egg whites could be restored without complete triglyceride hydrolysis. Therefore, samples did not need to be heated for 6 hours, the time it takes for the
enzyme to hydrolyze almost all of the contaminating triglycerides. Heating times of 30 minutes, 45 minutes, and 60 minutes were tested to determine which heating duration would completely restore the functional properties of yolk-contaminated egg whites that had been treated with lipase and colipase. Final foam volume was the parameter used to assess improvement in functional properties. After 30 minutes, partial restoration of final foam volume was observed (Figure 6). After 45 minutes, the final foam volume was almost fully restored, although not completely or consistently (Figure 5 and Figure 6). After 60 minutes, the final foam volume was essentially completely restored (Figure 6). No research was conducted to compare the amount of triglyceride hydrolysis of enzyme treated yolk-contaminated samples that had been heated for 1 hour against identical samples that had been left at room temperature for 1 hour. However, previous results showed that without heating, functional property restoration of enzyme treated yolk-contaminated samples took approximately six hours. Therefore, it can be concluded that enzyme treated yolk-contaminated samples left at room temperature for one hour without heating would only show partial improvement in functional properties.
Figure 6 Effect of duration of heating (37°C for 30 minutes, 45 minutes and 60 minutes) on the final foam volume of yolk-free and yolk-contaminated beaten egg whites.

The concentration of lipase and colipase used throughout this experiment were determined through trial and error. As there is little scientific literature describing experimental conditions similar to this study, it was necessary to start by using only the theoretical enzyme concentration levels, and working upwards in concentration until quick, effective, and reproducible results were obtained that consistently demonstrated improvement in the functional properties of yolk-contaminated egg whites. Furthermore, as each source of lipase has different levels of activity, it was necessary regardless of the amount of published scientific literature to independently determine enzyme the required enzyme concentration based on the reported activity.

During testing to determine the optimal heating time for yolk-contaminated samples treated with lipase and colipase, it became apparent that it was in fact possible to use lipase, colipase and heat to fully restore the functional properties of yolk-
contaminated egg whites. Therefore, these observations were replicated numerous times, using eggs whose freshness and quality could be assured. Fresh eggs were obtained from the Virginia Tech Poultry Science Department as needed.

**Restoring Functional Properties of Yolk-Contaminated Egg Whites**

Three treatments were studied in this experiment: control, 0.2% yolk-contaminated, and 0.2% yolk-contaminated egg whites treated with lipase and colipase. Murphy and Uhing (1959) successfully improved the functional properties of egg whites that had been contaminated with egg yolk at the 0.03% level. Based on the success of their research, Murphy and Uhing (1959) patented their method. Cotterill and Funk (1963) experimented with egg yolk contaminated at 0.1% and 0.2% levels. It was decided that for this study the higher level of yolk contamination would be used, 0.2%.

As the ultimate goal of this study was to develop a method that was applicable to industry, it was important to target a relatively high level of yolk-contamination as it is likely that if a method was successful at improving the functional properties of egg whites that had been contaminated with egg yolk at a high level would also be successful at lower contamination levels. Functional properties measured included final foam volume (Figure 7), foam capacity (Figure 8), foaming power (Figure 9), foam stability (Figure 10), and rate of foam drainage (Figure 11).

As can be seen from Figure 7, it was concluded that treatment of yolk-contaminated samples with lipase, colipase and heat was effective at restoring the final foam volume to those equal to the control. Foam capacity was also fully restored to control levels through treatment of yolk-contaminated samples with lipase, colipase and heat (Figure 8). As can be seen from Figure 9, foaming power values for yolk-
contaminated samples were fully restored to control levels through treatment with lipase, colipase and heat. However, even with treatment of yolk-contaminated samples with lipase, colipase and heat, certain functional properties were not restored. The foam stability remained unchanged and was significantly different from the control (Figure 10). The rate of foam drainage remained unchanged and was also significantly different from the control (Figure 11).

Figure 7: Results of final foam volume, reported in mL, for three different treatments (control, 0.2% yolk-contamination, and 0.2% + enzyme and colipase). All three treatments were heated for 1 hour at 37°C in a shaking water bath set to 85 rpm. Means having different letters were statistically different from one another (p< 0.05).
Figure 8: Results of foam capacity (%) for three different treatments (control, 0.2% yolk-contamination, and 0.2% + enzyme and colipase). All three treatments were heated for 1 hour at 37°C in a shaking water bath set to 85 rpm. Means having different letters were significantly different from one another (p< 0.05).

Figure 9: Results for foaming power for three different treatments (control, 0.2% yolk-contamination, and 0.2% + enzyme). All treatments were heated for 1 hour in a shaking water bath set to 85 rpm. Means having different letters were significantly different from one another (p < 0.05).
Figure 10: Results for foam stability for three different treatments (control, 0.2% yolk contamination, and 0.2% + enzyme). All treatments were heated for 1 hour in a shaking water bath set to 85 rpm. Means having different letters were significantly different from one another (p < 0.05).

Figure 11: Results for rate of foam drainage for three different treatments (control, 0.2% yolk contamination, and 0.2% + enzyme). All treatments were heated for 1 hour at 37°C in a shaking water bath set to 85 rpm. Means having different letters were significantly different from one another (p< 0.05).
As can be seen from the data presented in Figures 7-11, treatment of contaminated samples with lipase, colipase and heat yielded a drastic improvement in a number of the functional properties. However, while most functional properties did show improvement with this treatment, some did not. While final foam volume, foam capacity (%), and foaming power all showed full restoration to control levels, foam stability and rate of foam drainage levels were not restored. While the average foam stability for treated contaminated samples is slightly higher than that of the untreated contaminated samples, there is no significant statistical difference between the two. Therefore, it can be concluded that treatment with lipase, colipase and heat does not help to restore the foam stability when compared to identical contaminated samples that were untreated with lipase, colipase and heat. A similar trend was observed when comparing foam drainage values between the enzyme treated contaminated samples and the untreated contaminated samples. While the average foam drainage value for the 0.2% + enzyme samples were slightly lower than the average drainage value for the 0.2% contamination samples, there was no statistically significant difference between the two. Although some functional properties were not improved with enzyme and heat treatments, a number of important functional properties were completely restored to control levels.

The exact chemical mechanism by which lipase is able to improve the functional properties of yolk-contaminated egg whites was not fully understood or investigated. To date, no research has been conducted which directly seeks to explain this
phenomenon, although several studies have demonstrated the ability of lipase to successfully improve the functional properties of yolk-contaminated egg whites.

**Angel Food Cake**

Although many functional properties of yolk-contaminated egg whites were restored to control levels through treatment with lipase, colipase and heat, some functional properties important to baking performance were not. Therefore, it was unclear how enzyme treated yolk-contaminated samples would perform in a food system. Of particular concern was the lack of improvement of the foam stability of enzyme treated yolk-contaminated egg whites, as all of the functional properties, including foam stability, play an important part in baking performance (Cauvain and Young 2006).

To investigate the performance of enzyme treated yolk-contaminated egg whites in a baking system, testing was conducted using an angel food cake application, which is a commonly used food testing system for egg whites. Cotterill and Funk (1963) used angel food cake as a model food system to evaluate and compare the functional properties of enzyme treated egg whites. Angel food cakes were also used to compare the differences in functional properties between the uncontaminated control, contaminated control, and enzyme and colipase treated samples. It was determined that an angel food cake system would be used in this current study to evaluate the efficacy of lipase from *Mucor miehei* to improve the functional properties of yolk-contaminated egg whites in a food system, based on previous research and literature review. Six angel food cakes were prepared, two per treatment, each cake being made from a different egg white batch (Batch A and Batch B). The formulation used to
prepare the angel food cakes was provided by Dr. Frank Conforti of the Human, Nutrition, Foods and Exercise Department of Virginia Tech.

The control angel food cakes were prepared according to the baking protocol mentioned previously. When beaten control egg whites from Batch A produced voluminous foams within 2 minutes of whipping. However, the second batch of egg whites took slightly longer to foam, about 3 minutes. The beaten control egg whites from both batches had a glossy pearlescent sheen (Figure 12). When the flour and confectioner's sugar mixture was folded into the foamed egg white batter, the egg whites maintained their high volume, stability and stiff peaks. When the batter was added to the baking pan, the batter filled almost the total volume of the baking pan (Figure 13). During baking, the control cakes from both batches rose quickly and browned evenly (Figure 14). However, the uncontaminated control angel food cake made from the second batch of eggs (Batch B) fell very slightly during baking. It was unclear as to what caused the control cake to fall, although most likely it was due to quality issues with the egg whites from the second batch (Batch B). It is important to note that the enzyme treated yolk-contaminated cake (Batch B) was made on the same day as the uncontaminated control cake (Batch B) that fell during baking as well.

The control angel food cakes took exactly 23 minutes to bake. Doneness was confirmed by testing the springiness of the baked angel food cake by touch. After removal from the oven, the cakes were left in the baking pan and turned upside down to cool for approximately 2 hours (Figure 14).
Figure 12: Control egg white foam (Batch A) after beating but before the flour and sugar mixture was folded in. Foam was stiff, voluminous, and had a pearlescent sheen.

Figure 13: Control egg white batter (Batch A) when added to the baking pan. The egg white batter filled almost the whole volume of the baking pan.
The angel food cakes containing contaminated egg whites were prepared according to the above mentioned baking protocol. Although the contaminated egg whites did successfully reach the foamy stage, it took approximately 3 times as long (6 minutes) to reach this stage than the control. This was most especially true for the second batch of egg whites. It was only as the beating continued and the cream of tartar and granulated sugar were added that the yolk-contaminated egg whites reached a stiff peak stage. As foamed yolk-contaminated egg whites are by nature highly unstable, the addition of stabilizing agents such as sugar, salt and cream of tartar was most likely responsible for the ability of the yolk-contaminated egg whites to successfully form a relatively voluminous foam. Even after extensive beating and the addition of the stabilizing agents, the contaminated egg white foam was greatly reduced in volume when compared to the uncontaminated control (Figure 15). Despite the
decreased volume, when the flour and granulated sugar mixture was folded into the yolk-contaminated egg white foam, the foam appeared to maintain volume and stability. When added to the baking pan, the yolk-contaminated batter only filled approximately 2/3rds of the volume of the pan (Figure 16). During baking the angel food cake did not rise as dramatically as the control and enzyme treated cakes, and after removal from the pan the yolk-contaminated cakes did not have the typical qualities and characteristics expected of angel food cakes. The top of the cakes were excessively flat, and the cakes appeared much denser than the light and fluffy control cake (Figure 17). This was especially true for the yolk-contaminated angel food cake made with egg whites from Batch A. While the yolk-contaminated angel food cake made with egg whites from Batch B did appear to be slightly less dense and not as flat, the volume was still much smaller than the uncontaminated cakes.

Figure 15: Yolk-contaminated egg white foam (Batch A) after beating but before the addition of the flour and sugar mixture.
Figure 16: Yolk-contaminated egg white foam (Batch A) in baking pan. Volume of egg white foam is greatly reduced when compared to control levels.

Figure 17: Yolk-contaminated angel food cake (Batch A) after cooling and removal from baking pan
The final angel food cake was prepared using yolk-contaminated egg whites that had been treated with lipase, colipase and heat according to the previously mentioned protocol. Without a doubt, the enzyme treated, yolk-contaminated egg whites performed dramatically better than the contaminated control, although egg whites from the first batch (Batch A) performed better than from the second batch (Batch B), based on final angel food cake volume and visual observation during the whipping and baking process. This is most likely due to variations in the quality of the egg whites used in each batch. Generally variations among cakes, especially cakes made on different days, are relatively normal and are to be expected.

Within seconds of whipping, the enzyme treated egg whites had begun to foam, and quickly produced a highly voluminous foam that appeared similar in volume, appearance and stability to the positive control foam (Figure 18). Whipping of the enzyme treated yolk-contaminated egg whites took approximately two minutes for Batch A and 3 ½ minutes for Batch B. The volume of the enzyme treated yolk-contaminated egg white foam from Batch B was not as high as from the first batch (Batch A). The enzyme treated egg whites retained their stability and volume when the flour and sugar mixture were folded in.

There was slight variation in the volume of the enzyme treated cakes after baking in the oven, with the angel food cake containing egg whites from batch A having a slightly higher volume than the angel food cake containing egg whites from batch B. This is most likely due to variations in the quality of the egg whites from the difference batches. Another factor contributing to the differences in volume between the two batches of egg whites is that the enzyme treated angel food cake made from Batch B
fell slightly during baking, and it was unclear whether this was a result of natural variation between cakes, quality of the eggs used, and humidity or human error.

It is probable that the variations seen between enzyme-treated cakes is a direct result of egg white quality, as each cake was formulated using different batches of eggs. It is likely that had the enzyme treated yolk-contaminated angel food cake made from Batch B not fallen during baking, the final volume of the cake would have equaled or exceeded the final volume of the uncontaminated control cake. However, in all cases, the enzyme-treated cakes showed dramatic improvement over the yolk-contaminated cakes. Improvement was noted not only in the final cake volumes, but also in appearance, fluffiness and springiness. The enzyme treated cakes had the light and fluffy appearance that is common in all angel food cakes, and appeared similar to the control cake in volume, texture, and fluffiness (Figure 19, Figure 20 and Figure 21).

Somewhat similar results were obtained by Cotterill and Funk (1963), who also found that enzymatically treated yolk-contaminated egg whites produced angel food cakes that were similar, but not equal, in volume to the uncontaminated control cakes. Cotterill and Funk (1963) baked angel food cakes containing both 0.1% and 0.2% yolk-contamination levels. The pH of the egg white batter was adjusted to range between 6.5-10.0. Cotterill and Funk (1963) found that for egg whites contaminated at the 0.1% and 0.2% levels and containing 0.03% lipase, angel food cakes of almost identical in volume to the control were produced at pH ranges of 8.0-9.5. Below 8.0 and above 9.5, the volumes of the angel food cakes were lower, but still similar, to the volumes of the uncontaminated angel food cakes. Angel food cakes contaminated at the 0.1% level and containing 0.05% lipase were found to be drastically smaller in volume than the
uncontaminated control angel food cakes, and were in fact more similar to the contaminated control.

Figure 18: Enzyme treated yolk-contaminated egg whites (Batch A) after whipping

Figure 19: Enzyme treated yolk-contaminated egg white batter (Batch A) after addition to the baking pan
Further quantitative analysis was conducted to compare the volumes of the angel food cakes made with the three different treatments (Figure 23, Figure 24).
Figure 22: Volumes of angel food cakes made from the first batch (Batch A) of egg whites. There were three different treatments: control, 0.2% yolk-contaminated egg whites, and 0.2% yolk-contaminated egg whites treated with lipase, colipase and heat. Volume measurements were calculated according to the AACC Method for Determining Baking Quality of Angel-Cake Flour.

Figure 23: Volumes of angel food cakes made from second batch (Batch B) of egg whites. There were three different treatments: control, 0.2% yolk-contaminated egg whites, and 0.2% yolk-contaminated egg whites treated with lipase, colipase and heat. Volume measurements were calculated according to the AACC Method for Determining Baking Quality of Angel-Cake Flour.
The results of the angel food cake baking experiment were dramatic and showed that the enzyme treated yolk-contaminated egg whites performed similarly to the uncontaminated control. Most importantly, enzyme treated yolk-contaminated angel food cakes consistently displayed higher volumes than the contaminated control cakes. When the volumes of the angel food cakes made with the three different treatments are compared, the uncontaminated control and enzyme treatments were highly similar, while the contaminated control treatment was not similar to both the positive control and enzyme treated samples (Figures 23, 24, 25 and 26). Therefore, it can be concluded that although laboratory results initially indicated that the enzyme treated samples had poor foam stability, in a food system the enzyme treated samples performed similarly to the uncontaminated control. Dramatic improvement in volume and visual appearance
were observed in the enzyme treated samples versus the yolk-contaminated samples. It was apparent that angel food cakes made with yolk-contaminated egg whites would not meet acceptability and quality standards in the food industry, as they were much lower in volume and appeared dense and tough. Without a doubt, the visual appearance of the contaminated cakes would have been obvious to consumers. Visually the enzyme treated cakes were voluminous, light and fluffy, and appeared similar to the uncontaminated control.

Although a sensory analysis was not conducted in this study, Cotterill and Funk (1963) did conduct a sensory analysis, where the flavor characteristics and acceptability were compared among the uncontaminated control cakes, contaminated control cakes, and enzyme treated yolk-contaminated control cakes. Sensory results showed that the flavor characteristics of the enzyme treated yolk-contaminated angel food cakes were highly undesirable, as they were particularly bitter. It is unclear what caused the bitterness in the enzyme treated angel food cakes, although it is possible that either the enzyme itself, or the hydrolytic products of the enzyme activity were responsible for the undesirable flavor of the enzyme treated yolk-contaminated angel food cakes in the Cotterill and Funk (1963) study.
Figure 25: Visual comparison of the uncontaminated control (on left) and the contaminated with enzyme treatment (on right). Both cakes were significantly similar in height, volume, and visual appeal.

Figure 26: Visual comparison of the contaminated with enzyme treatment sample (on left) with the contaminated control (on right). The enzyme treated angel food cake was significantly higher in volume, and appeared light and fluffy. The contaminated control was significantly smaller in volume, and appeared dense and tough.

Figure 27: Visual comparison of all three treatments: contaminated with enzyme treatment (left), uncontaminated control (center), and contaminated control (right).
Microbiological Analysis

A microbiological experiment was conducted to determine if heating of egg white samples for 1 hour at 37°C in a shaking water bath promoted bacterial growth. Furthermore, this analysis was conducted to determine if there was any possibility that bacterial growth and proliferation could be responsible for the changes in functional properties observed in the enzyme treated samples. Coterill and Funk (1963) did not conduct microbial analysis, and assumed that it was unlikely that bacterial proliferation had anything to do with the improvements of the enzymatically treated yolk-contaminated egg whites. Although samples in that particular study were essentially incubated at room temperature, they were held at room temperature for 24 hours, which could be enough time to allow for significant microbial growth. As can be seen from the data presented in Table 2, no microbial growth was observed in either the control or heated samples.

<table>
<thead>
<tr>
<th>Description</th>
<th>Dilutions</th>
<th>Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control No Heat</td>
<td>$10^{-1}$, $10^{-2}$, $10^{-3}$</td>
<td>&lt; $1.0 \times 10^1$ cfu/mL ESPC</td>
</tr>
<tr>
<td>Control Heated</td>
<td>$10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$</td>
<td>&lt; $1.0 \times 10^1$ cfu/mL ESPC</td>
</tr>
<tr>
<td>0.2% No Heat</td>
<td>$10^{-1}$, $10^{-2}$, $10^{-3}$</td>
<td>&lt; $1.0 \times 10^1$ cfu/mL ESPC</td>
</tr>
<tr>
<td>0.2% Heated</td>
<td>$10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$</td>
<td>&lt; $1.0 \times 10^1$ cfu/mL ESPC</td>
</tr>
</tbody>
</table>

Table 2: Results of microbiological comparison between heated and non-heated egg white samples. Heated samples were heated in a shaking water bath for 1 hour at 37°C at 85 rpm. Following plating on tryptic soy agar, the plates were incubated for 48 hours at 35°C.
CONCLUSION

The use of lipase to specifically target triglycerides in yolk-contaminated egg whites to restore a number of the functional properties was successful. Most importantly, heating of the enzyme treated yolk-contaminated samples made functional property improvement possible in a very short period of time. It can be concluded that, although the exact chemistry is unclear, the hydrolytic products of triglycerides are not detrimental to the final foam volume, foam capacity, and foaming power. While the foam stability and foam drainage rate were not improved to control levels, the angel food cakes produced with enzyme treated yolk-contaminated egg white samples were similar to the control.

Further research needs to be conducted to more extensively evaluate the exact chemical and thermodynamic relationship between the hydrolytic products of triglycerides and the air-water interface during foam formation. Additional baking tests should be conducted to more thoroughly evaluate the ability of enzyme treated yolk-contaminated egg whites to perform in food systems. The method developed as a result of this research should be tested on samples of larger volume, to more closely evaluate the applicability of this method to the egg industry.

The application of immobilized enzyme technology to this method would be of particular interest and is highly appealing due to the fact that the lipase enzyme can be reused, resulting in decreased costs. One of the major goals of the immobilized enzyme experimentation would involve determining how to immobilize colipase, along with the lipase enzyme.
REFERENCES


