Abstract

STUDIES ON THE HEAT STABILITY OF

EGG YOLK ANTIBODY (IgY)

Elizabeth A. Bobeck

Under the supervision of Professor Mark E. Cook

At the University of Wisconsin-Madison

Nutritionally and economically-important heat-labile proteins, including enzymes, hormones, and antibodies (Ab), lose a substantial amount of activity following industrial processing. Protection of these heat-sensitive bioactive molecules is needed in order to realize expanded markets for these biologics. Using a model of heat-labile proteins, Ab to phospholipase A_2, and a sensitive detection system for Ab binding (ELISA), a pilot steam chamber was designed and constructed to develop methods of encapsulating proteins. After modification of the pilot chamber, it was shown that water plays a key role in Ab destruction. Samples (Ab, trehalose-encapsulated Ab, and industry standard) dried with drierite before steam treatment retained 100% activity after 60 seconds in 92-93C in a sealed 15ml centrifuge tube, while samples not dried but sealed prior to steam treatment lost activity (Ab retained 72.24% activity, trehalose-encapsulated Ab retained 74.03% activity, and industry standard retained 42.26% activity). A hydrophobic protein matrix (HPM) was developed. 41.73% binding activity remained in 1% Ab in pasta matrix, 0.94% remained in Ab in egg matrix, and 4.5% remained in industry standard after 60s in 92-93C in unsealed containers. This HPM may protect against steam-induced losses by protecting Ab from water.

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STUDIES ON THE HEAT STABILITY OF EGG YOLK ANTIBODY (IgY)

by

Elizabeth A. Bobeck

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Nutritionally and economically-important heat-labile proteins, including enzymes, hormones, and antibodies (Ab), lose a substantial amount of activity following industrial processing. Protection of these heat-sensitive bioactive molecules is needed in order to realize expanded markets for these biologics. Using a model of heat-labile proteins, Ab to phospholipase A_2, and a sensitive detection system for Ab binding (ELISA), a pilot steam chamber was designed and constructed to develop methods of encapsulating proteins. After modification of the pilot chamber, it was shown that water plays a key role in Ab destruction. Samples (Ab, trehalose-encapsulated Ab, and industry standard) dried with drierite before steam treatment retained 100% activity after 60 seconds in 92-93C in a sealed 15ml centrifuge tube, while samples not dried but sealed prior to steam treatment lost activity (Ab retained 72.24% activity, trehalose-encapsulated Ab retained 74.03% activity, and industry standard retained 42.26% activity). A hydrophobic protein matrix (HPM) was developed. 41.73% binding activity remained in 1% Ab in pasta matrix, 0.94% remained in Ab in egg matrix, and 4.5% remained in industry standard after 60s in 92-93C in unsealed containers. This HPM may protect against steam-induced losses by protecting Ab from water.

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Chapter 1

Heat Stability of *Gallus domesticus* Immunoglobulin Y (IgY)

Abstract

Feed efficiency is a critical aspect of food animal production. In terms of feed additives, egg yolk antibodies are affordable and improve feed efficiency without introducing problematic antibiotics. The drawback of antibody feed additives is that they denature during the harsh feed pellet conditioning process, rendering them inactive. Two-hundred grams of feed was mixed with 1% dried egg yolk containing α-PLA_2 antibody. Two gram samples were steam treated in a newly designed, small-scale machine for 0, 10, 30, 60, 120 and 300 seconds. α-PLA_2 antibody in feed samples was measured by ELISA, and a baseline curve was developed to model the loss of antibody activity. At ten seconds of steam treatment, 82.65% of antibody remained, while only 21% remained after 300 seconds. This inexpensive model can be used to develop a standard for comparison of antibody protection methods during the pelleting process.

Introduction

Feed efficiency is an important topic in the food animal production industry, including broiler operations, non-egg laying chickens (*Gallus domesticus*) used for meat production and consumption. Current research has shown that if energy levels are maintained in relationship to essential nutrient levels in the food, the broilers will increase growth rate and feed efficiency as dietary energy is increased. This improvement is essential because modern broilers are bred to eat at full capacity and will eat until they burst regardless of the quality of food presented (Saleh et al., 2004). Because modern broilers have been bred for quick growth and weight gain to a market weight of 2 kilograms, a typical broiler is only raised for approximately 37 days before slaughter. In terms of broiler production, feed comprises 70% of total costs (Saleh et al., 2004). Because commercial farms typically raise over 1 million birds at a time, simply increasing feed efficiency, and thus taking one day or even one feeding out of the production cost of that flock, can save the producer millions of dollars and labor over the course of a year.

Genetics, antibiotics, probiotics, vitamin supplements, antibodies, and pelleting the feed are all ways producers decrease the time it takes an animal to reach market weight, thus reducing feed and overall cost. In terms of feed additives, antibodies increase feed efficiency without introducing harmful antibiotics and enhance the existing genetic capability of the animal. It should be noted that antibiotics are fed to the animals using levels below those of antibiotics used for therapeutic reasons (Cook, 2004). While it is true that certain strains of bacteria in animals have developed resistance to antibiotics, studies have shown that a 7% improvement in growth is seen when the chickens are fed antibiotics (Cook, 2004). It is true that increasing floor space per broiler and increased sanitation (bacteria-free environment) of facilities also help feed efficiency; however, these options are not feasible in today's commercial broiler production (Cook, 2004).

In terms of feed efficiency, pelleting reduces waste, separation of ingredients, balances nutrition, improves flavor retention, and requires less energy to consume. When pellets disintegrate and fragment, much of these qualities are lost because animals, like humans, will disregard the fines (broken pieces) just as humans regard the last broken bits of cereal or chips in a bag (Fairfield, 2003). Particular boundaries that depend on the type of feed being pelleted, as stated in Fairfield, 2003 and MacBain, 1984, must be met in order for the feed to be processed correctly; otherwise, as stated above, the pellet will disintegrate, causing it to be useless for the animal and a waste of money for the producer. Other factors, such as the source of the feed supply and relative humidity, affect the pelleting process, but these factors will not be discussed here.

It has been shown that antibodies work by targeting and inhibiting certain cytokines, such as Interleukin-1 (IL-1) which decrease appetite during immune stimulation (Klasing et al, 1987). If the chickens are not eating, they are not gaining weight. Immune stimulation also forces the chickens to spend extra energy to thicken the walls of the intestine while recruiting immune cells to combat bacteria colonization, thus they are spending less energy for growth and development (Cook, 2004).

The only drawback to the use of antibodies is that they denature during the harsh pellet conditioning process, rendering them useless (Denmat et al., 1999). Studies have been conducted to determine the heat stability of chicken egg yolk antibodies, but they provide insufficient data for any commercial applications. For example, Hou-Pin Su et al. (1999) conducted a study in which they tested the effects of heat on the stability of egg yolk antibody. They showed that 21-81% of antibody remains if the temperature of heat treatment was kept

under 70 degrees Celsius. This heat data is unsatisfactory because commercial pelleting procedures routinely heat the mixture to 180-190 degrees Celsius, and 70 degrees Celsius is considerably less. Also, 21-81% is too variable of a range in terms of actual antibody remaining. Results suggest this process would not be useful for any commercial use or even for establishing a target temperature at which the feed should be pelleted to improve the delicate antibody's survival.

Previous feed pelleting studies in our lab have been conducted but are incomplete due to the fact that it takes two tons of feed and two weeks to a month for a house of hens to produce the two to three pounds of egg yolk needed to conduct this type of study in actual pelleting mills. The research is time consuming and costly to the lab and to the mill, which must shut down for a day to run the experiment. In response to a need for studying ways to increase antibody in processed feed, a unique, small-scale machine was used to mimic the conditioning process of pelleting while staying within the operating parameters of the pelleting mill documented in Fairfield, (2003) and MacBain, (1984). This machine aided in the development of a denaturation curve which will serve as a standard to test the efficacy of various antibody protection methods.

Materials and Methods

This study was in conjunction with Professor Mark Cook, David Trott, and Mingder Yang, Department of Animal Science, Madison, WI, 53706. Students from the School of Engineering built a machine that will enable the testing of time and heat effects on chicken (*Gallus domesticus*) antibody stability in feed on a small scale. The machine consisted of a 6" x 24"x 12" rectangular chamber with tinted polycarbonate sides held together by aluminum angle iron. It contained eight one and one-half inch in circumference, corked slide-out trays for depositing

chicken feed and was connected to a small steam generator that provided temperature changes *(Figures 1-5)*. Temperature was regulated though the rate of inflow versus outflow of the steam. A thermometer equipped with a stopwatch placed in the center of the top of the chamber allowed for the regular checking of the temperature and the controlling of the time in the steam treatment for each trial.

Preliminary testing was done to establish a baseline for temperature and ability to hold temperature. In order to maintain a target temperature of 82-88 degrees Celsius, lower level slot two was blocked with a number 11 black cork and lower level slots three and four were blocked with number 13 black corks (slots numbered left to right from one to four). Incoming steam was set at a red calibration mark approximately one centimeter from the steam generator's off position. The position was also determined through preliminary testing in order for the steam generator to hold a constant temperature for approximately four to six hours.

Effects of time were investigated in order to develop a baseline antibody loss curve to later test antibody protection methods. Samples consisted of three two-gram feed samples containing α-PLA_2 antibody (diluted to a 1:100 α-PLA_2 to egg yolk ratio), with three samples each subjected to steam treatment for 10, 30, 60, 120, and 300 seconds, respectively. Hens were immunized with PLA_2 antigen and produced egg yolk antibody against PLA_2. The samples were then put into individual trays to freeze-dry for one day *(Figure 4*). Time needed to freeze-dry depends on time in steam treatment, as longer time intervals increase wetness of the feed sample.

After the completion of freeze-drying, each two-gram sample was diluted 1:6 in acidified PBS in order to extract antibody. A plain feed control and a zero-second PLA_2 feed sample control were also extracted in acidified PBS. All samples (20 in total) were further diluted 1:2000, and an Enzyme-linked Immunosorbent Assay (ELISA) was run to confirm any remaining antibody. ELISA involves coating a 96-well Maxisorp plate for at least 12 hours with 100 uL / well of solution containing 12 ml of a 50 uM $NaHCO_3$ buffer solution and 40 uL PLA_2 antigen (sigma P-6534 2.9 ug/ ul). After washing the plate, 175 uL/ well of 1% Bovine Serum Albumin (BSA) was added and incubated on a plate shaker for one hour. Upon removal of the blocking solution, 100 uL/ well of each sample was added to two separate wells for repeat measures. The samples were at a dilution of one part egg yolk to 2000 parts 1% BSA. Samples incubated for 15 minutes on a plate shaker and then were washed six times. Two uL of the secondary or detection antibody goat-anti-chicken IgG-Fc conjugated to horseradish peroxidase (HRP) was mixed with 10 ml 1% BSA and added 100 uL/ well. The secondary antibody was incubated for 15 minutes and then the plate was washed eight times. 125 uL/well of substrate solution containing 100 uL 3, 3', 5, 5'-tetramethyl benzidine (TMB), 128 uL of H_2O_2, and 19.74 ml of 0.05 sodium acetate were reacted with HRP enzyme to produce a color reaction (blue).

After five minutes, 50 uL/ well of 0.05M sulfuric acid stop solution was added to produce a color reaction (yellow). The plate was read on a plate reader to find the optical density of the sample, thus revealing remaining antibody, as described in Li et al. (1998). In this experiment, OD was measured at 450 nm because this is the wavelength at which yellow light is absorbed.

Titer was expressed in arbitrary units based on a standard curve from dilutions of egg yolk used in feed. Optical density readings for the positive control egg yolk were measured at dilutions of 1:2000—1:64000. The negative control was egg yolk from a hen not immunized against PLA_2. The negative control represents the non-specific binding of egg yolk antibody to antigen. All data was zeroed with a blank of 1% BSA.

As optical density goes down, dilution factor increases, but not linearly. The standard curve was made linear by taking the square root of arbitrary units as a conversion factor. Arbitrary units of the samples as calculated by standard curve equation were then squared to obtain the titer. In order to calculate the percentage of titer lost at each interval, the following equation was used: $100*(T_0 - T_X)/ T_0$, with T_0= titer at time zero and T_X = titer at 0, 10, 30, 60, 120, and 300 seconds. The student's t-test was also used to calculate the significance of results.

Results

The standard was made linear by taking the square root of arbitrary units: OD= .0109x -.0679, where x= square root of arbitrary units (*Figure 6*). This equation was used to convert OD of samples to arbitrary units as discussed in methods. For example, if a sample had an OD reading of 0.8, then x= (0.8+.0679)/.0109=79.6. The titer was calculated by converting the square root of arbitrary units, 79.6^2=6340 arbitrary units. This was done for all samples, followed by the averaging of the three samples at each time point (*Figure 7*). The titer of the samples clearly decreased as time in steam treatment increased. Variance was large as evidenced by the standard deviation equal to 0.205. OD readings at time 0 ranged from .59-1.23, indicating high variance in titer before any steam treatment. Despite the clear downward trend in titer, the small sample size

and the large variance resulted in not finding statistical significance between steam treatments, as measured by student's t-test.

Using the equation from the methods section, at 10 and 30 seconds of heat treatment, 82.65 and 81.74 percent of antibody remained, respectively. This was calculated at 10 seconds of steam treatment as follows: 100*(8066-6667)/8066= 82.65%. At 300 seconds, only 21 percent of antibody remained (*Figure 8*). In a previous experiment, it was determined that all antibody activity ceased around 300 seconds (data not shown). These results compared to results in a feed mill study where antibody titer remaining after ten seconds was 10-20%.

Discussion

Although the p-value for the samples was not statistically significant, results do show a downward slope in the loss of antibody as time in heat increases. Problems in the mixing process and concentration differences of antibody due to extra air and water weight led to this insignificant p-value. Results will be more statistically significant in the future as changes in mixing and concentration are implemented. In the commercial pelleting process, approximately 80-90 percent of the antibody is lost during the ten seconds of steam treatment for chicken feed. The steam machine used in this study does not show 80 percent loss of antibody until 300 seconds due to outside error, but it is still a viable way to test antibody stability (*Figure 8*).

The purpose of the standard is to quantify results in arbitrary units rather than specific units. Since the absolute amount of PLA_2 antibody in egg yolk is not known (PLA_2 is not a pure compound, it is simply ground up pig pancreas), a quantifiable standard does not exist. Arbitrary units are used in place of specific units.

Even though the graph shows a downward trend over time for the loss of antibody, further testing will be needed. Time 0 shows a high variability in antibody concentration (.59-1.23 OD readings), showing each sample started out with a different amount of antibody. Thus, samples for the rest of the trials may have started out with highly variable amounts of antibody, which accounts for higher than normal OD readings as time in steam increased and a standard deviation of .205. In the 120 and 300 second samples, it appears that the same amount of antibody is left. Had each sample started out with the same amount of antibody, there would have been much less variation and thus a cleaner trendline (*Figure 7*). Each sample within an interval may have lost approximately the same amount of antibody, but because each sample started out with a different amount, OD readings show varied results. This inconsistent amount of antibody in the untested samples is probably due to the mixing procedure. Although two grams of egg yolk antibody powder was slowly mixed into 198 grams of chicken feed (to obtain a 1:100 dilution) while using a Kitchen-Aid, this mixing was not thorough enough. In the future, a series dilution, possibly starting with a ten gram sample and adding two gram samples at a time, may be used to mix the egg yolk antibody powder more evenly.

Another way to reduce variance in further studies may be to increase trial samples to five per interval instead of three per interval. Earlier trials that used three gram samples proved to be highly variable in amount of antibody lost due to concentration errors. These earlier trials included taking one gram from the three gram sample to use for the ELISA assay. Because water tended to accumulate in samples as time in steam treatment increased, samples taken from longer intervals actually included more water weight and less antibody as compared to shorter time

intervals, thus leading to problems with concentration. This problem was fixed in later trials; samples were changed to two grams in order to be sure that the whole sample was being steam treated, and then the whole two gram sample was freeze dried to remove any excess water weight. The sample was then extracted in acidified PBS to pull out any remaining antibody. These changes gave more consistent results.

It was recognized that the antibody was nearly denatured by five minutes (79% lost, with a standard deviation of .205), but results do not clearly show how much has survived around one minute to two minutes in steam treatment. Further testing shall be done to establish the time at which steam denatures the antibody to establish a reliable curve for the one minute to two minute range. A reliable curve needs more measurements at multiple time points, for example, every 15 seconds. Once a repeatable control curve is established, antibody protection methods can be tested versus the control.

Future studies will involve testing of different methods to protect the antibody. The loss of antibody curve developed in this study may be used to test the efficacy of these protection methods, such as trehalose and encapsulation. Results show that the steam machine can be used as an accurate model of the commercial pelleting process.

Figures and Graphs

Figure 1; Front view of the feed steamer where all trials were conducted. The chamber contains individual sampling areas so samples do not contaminate eachother.

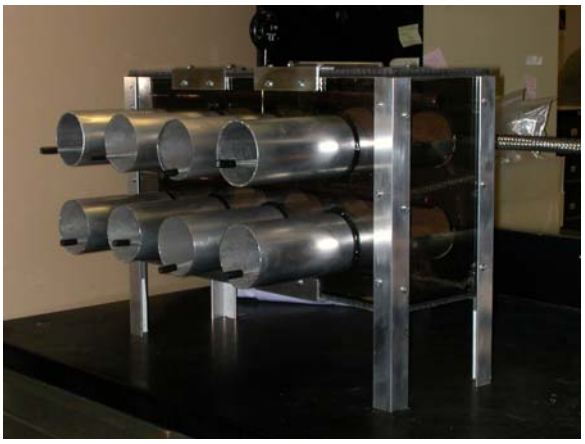


Figure 2; Side view of the feed steamer.

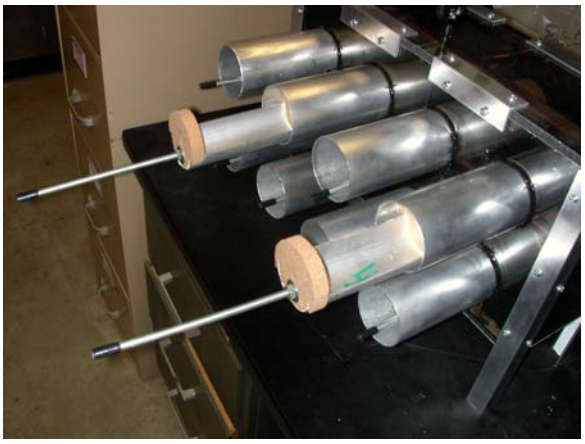
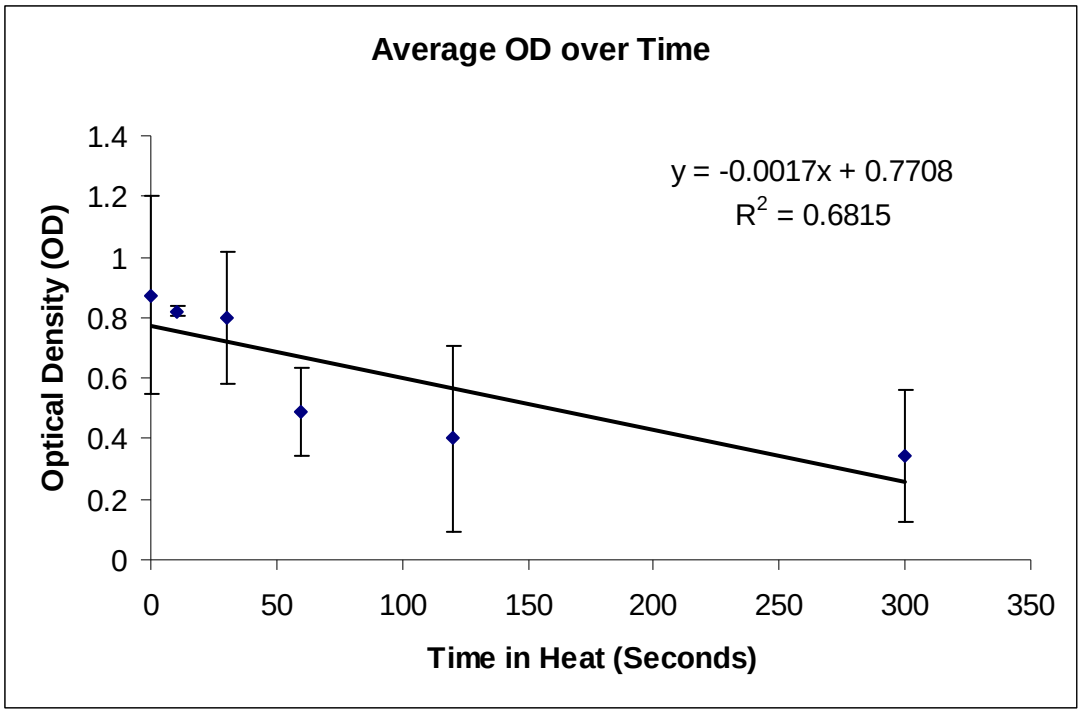


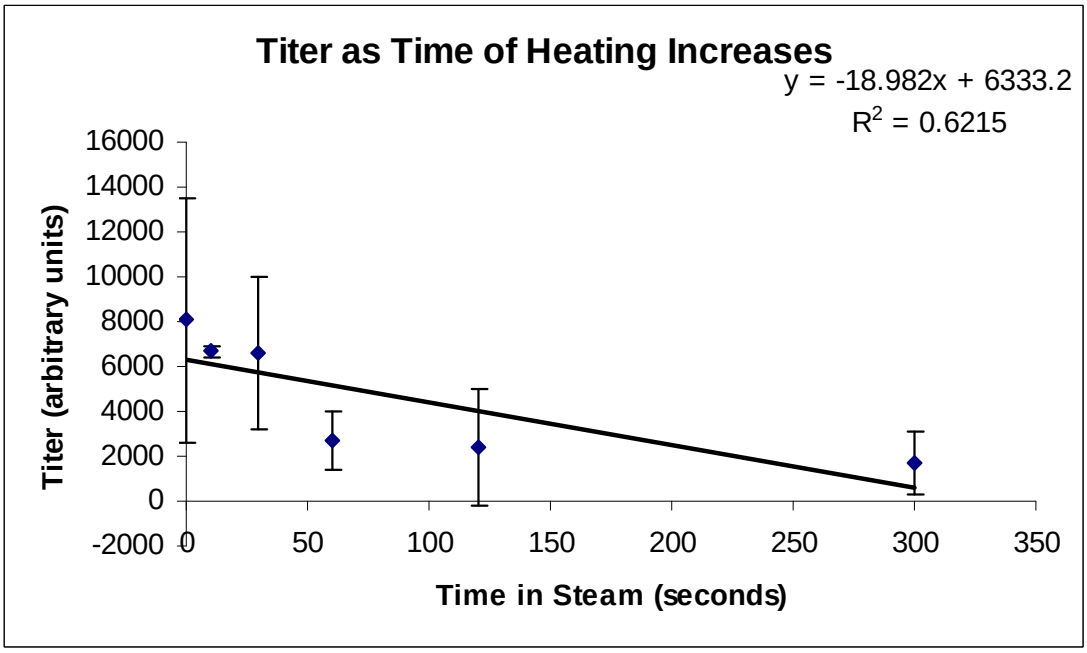
Figure 3; Side view with sampling troughs pulled out.

Figure 4; Individual sampling trough and freeze dry trays. Sampling troughs slide into the larger sampling trough as seen in figure 1.

Figure 5; Steam generator used in all trials.



Graph 1; Average optical density (OD) over time. Samples were steam treated for 0, 10, 30, 60, 90, or 300 seconds. As time in heat treatment increases, OD decreases.



Graph 2; Titer as time of heating increases. Although there is a downward trend, results were not statistically significant.

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Chapter 2

Egg antibody technology as a replacement for growth-promoting antibiotics

Abstract

Feed efficiency is a critical aspect of food animal production. In terms of feed additives to improve feed efficiency, egg yolk antibodies (Ab) are an affordable alternative method to improve feed efficiency without introducing problematic antibiotics. The drawback of Ab feed additives is the protein denaturation during the harsh process of feed pelleting, rendering them inactive. Ab thermostability can be improved through the use of trehalose, a sugar that has been proven effective in increasing protection by forming a glassy matrix around the material. In this study, trehalose was mixed with egg Ab powder to obtain six different percent trehalose groups; 0, 3, 6, 9, 12, and 16.2%, respectively. One gram samples were individually steam treated in a small-scale model of commercial steam treatment for 0, 10, 30, 60, 120 and 300 seconds at a temperature range of 82-88 degrees Celsius. α-PLA_2 Ab that withstood the treatment in the samples was measured by ELISA and six graphs were generated based on optical density (OD). This inexpensive model can be used to develop models for denaturation curves of various heat-sensitive feed additives, particularly Ab additives.

Introduction

In 1893, Klemperer discovered that immunized hens transfer "neutralizing proteins" to the egg yolk, meaning that antibodies present are (specifically IgY) in the yolk (Schade et al., 2001). Egg Ab research was not a main focus until issues of animal welfare became a topic of concern due to the publication of Russel and Birch's *The Principles of Humane Experimental Technique* in 1959, which discussed other methodologies of collecting Ab from other animals, such as rabbits. Because of this publication, researchers realized the importance and simplicity of Klemperer's results, which offered a possible alternative to antibiotic treatment in the production animal. This over a century old technology opened up a new area of egg Ab research. Technology steadily improved through the 1980's and has yielded improved conditions and technique involving laboratory animals and insight into the seemingly endless applications of IgY.

Feed efficiency, defined as pound of weight gained over pound of feed consumed, is an important topic in the food animal production industry, including broiler operations, which raise non-egg laying chickens (*Gallus domesticus*) for their meat. Current research has shown that if energy levels are maintained in relationship to essential nutrient levels in the food, the broilers will increase growth rate and feed efficiency as dietary energy is increased. This improvement is essential because modern broilers are bred consume feed beyond satiety regardless of the quality of food presented (Saleh et al., 2004). A typical broiler is raised for 37 days before slaughter because of selective breeding to quickly reach a market weight of 2 kilograms (4.4 pounds). Feed comprises 70% of total costs in broiler production (Saleh et al., 2004). Because commercial farms typically raise over one million birds at a time, simply increasing feed efficiency, and thus taking one day or even one feeding out of the production cost of that flock, can save the producer millions of dollars and labor over the course of a year. Although there are many ways of

improving feed efficiency, some are looked down on from the consumer perspective, and alternative methods are always being studied.

Genetics, antibiotics, probiotics, vitamin supplements, organic acids, antibodies, and pelleting the feed are all ways producers increase feed efficiency, thus decreasing the time and money spent in bringing an animal to market weight. Simply increasing floor space per broiler and improved sanitation (bacteria-free environment) of facilities also helps feed efficiency; however, these options are not feasible in today's commercial broiler production (Cook, 2004). In terms of feed additives, antibodies are a viable alternative to antibiotics. Ab have been shown to increase feed efficiency and enhance the existing genetic capability of the animal as effectively as the current sub-therapeutic levels of antibiotics used in animal industry through a different, more consumer acceptable mechanism (Cook, 2004, Doyle, 2001).

It has been shown that antibodies work by targeting and inhibiting certain cytokines, such as Interleukin-1 (IL-1) which decreases appetite during immune stimulation (Klasing et al, 1987). Therefore, an Ab to this cytokine would prevent the loss of appetite and increase feed consumption even during immune challenge. This immune stimulation also forces the broiler to spend extra energy to thicken the walls of the intestine while recruiting immune cells to combat bacteria colonization, thus spending less energy for growth and development (Cook, 2004). Utilizing this wasted energy for growth by using immune-suppressing antibodies will allow producers to save feed and money.

Consumers are beginning to demand antibiotic-free products and thus antibodies are gaining respect as an alternative solution to antibiotic use in animal feed. Many countries in the European Union have already outlawed the feeding of antibiotics at sub-therapeutic levels in an effort to decrease bacterial tolerance, as well as decrease human consumption of animal products spiked with antibiotic residue (Doyle, 2001). IgY antibodies, however, are susceptible to denaturation when subject to heat treatment during feed processing, which is an important process for feeds fed to particular species, such as swine (Denmat et al., 1999). Feed containing IgY is passed through a steam treatment for eight seconds in the pelleting process, basically rendering the Ab useless through protein denaturation. Antibodies are more likely to replace antibiotics if they are heat-stable and can function using current industry infrastructure.

Sugar has been shown to increase the stability of the proteins, such as Ab, when subjected to extreme heat, pH, and enzymatic testing (Kovacs-Nolan and Mine, 2004). Trehalose, a disaccharide, forms a glassy matrix around the protein and protects it from denaturation (Doyle, 2001). This matrix can be used to protect potential growth-promoting antibiotic replacements, including probiotics, immune modulators, organic acids, enzymes such as phytase, and many other feed supplements, from the heat treatment of the pelleting process. Trehalose has also been shown to be effective in the long-term preservation of biologically-active materials during storage (Worrall, 2005).

Large-scale studies involving one ton of feed and two to three pounds of egg Ab powder previously done in pelleting mills by members of the Cook lab have shown that trehalose is effective in protecting the Ab in the commercial pelleting process (data unpublished).

Commercial pelleting mills are currently using a 12% trehalose mixture to aid in the protection of feed additives as they encounter steam treatment. Because of the high cost of these large-scale studies, it is not financially feasible to run multiple studies to develop experimental denaturation curves and test protection methods. A simple, low-cost alternative on which multiple studies could be run is needed. A version of the steam treatment system was developed by Engineering students from UW-Madison to test protection methods on a small scale. This system will aid in the development of a model curve for the degradation of feed additives in steam treatment over time, as well as testing of protective methods such as trehalose.

Developing a model curve for Ab degradation is significant because the primary characteristic of any replacement for antibiotics in feed, in addition to growth promotion, is affordability and repeatability. If an additive is denatured in the processing of feed, cost is increased in order to obtain an effective level of growth promotion in the animal. Experimental curves of denaturation over time must be generated in order to test protection methods for growth-promoting additives. Denaturation curves can lead to adjustments in the amount of supplement to add to feed in order to get the desired growth-promoting results. Small-scale studies have yet to be conducted to determine the ideal percentage of trehalose to protect Ab during the pelleting process. The purpose of this study is to determine if 12% is ideal for protection, or if a lower percentage may be used.

Materials and Methods

This study was in conjunction with Professor Mark Cook and David Trott, Department of Animal Science, Madison, WI, 53706. Students from the School of Engineering built a model machine that will enable the testing of time and heat effects on chicken (*Gallus domesticus*) Ab

stability in feed on a small scale. The machine consisted of a 6" x 24"x 12" rectangular chamber with tinted polycarbonate sides held together by aluminum angle iron. It contained eight one and one-half inch in circumference, corked slide-out trays for depositing chicken feed and was connected to a small steam generator that provided temperature changes *(Figures 1-4)*. Temperature was regulated though the rate of inflow versus outflow of the steam. A thermometer equipped with a stopwatch placed in the center of the top of the chamber allowed for the regular checking of the temperature and the controlling of the time in the steam treatment for each trial.

Preliminary testing was done to establish a baseline for temperature and ability to hold temperature. In order to maintain a target temperature of 82-88 degrees Celsius, lower level slot two was blocked with a number 11 black cork and lower level slots three and four were blocked with number 13 black corks (slots numbered left to right from one to four). Incoming steam was set at a red calibration mark approximately one centimeter from the steam generator's off position. The position was also determined through preliminary testing in order for the steam generator to hold a constant temperature for approximately four to six hours. Previous studies have been conducted in order to establish a model curve for the denaturation of PLA_2. Results from this study concluded that the steam machine can be used as an accurate model of the commercial pelleting process.

Hens were immunized with PLA_2 antigen and produced egg yolk Ab against PLA_2. Individual one-gram samples were tested in a steam chamber and then diluted in 9ml acidified PBS in order to extract Ab. All samples (36 in total) were further diluted 1:2000, and an Enzyme-linked Immunosorbent Assay (ELISA) was run to confirm any remaining Ab.

ELISA involves coating a 96-well Maxisorp plate for at least 12 hours with 100 uL / well of solution containing 12 ml of a 50 uM $NaHCO_3$ buffer solution and 40 uL PLA_2 antigen (sigma P-6534 2.9 ug/ ul). After washing the plate, 175 uL/ well of 1% Bovine Serum Albumin (BSA) was added and incubated on a plate shaker for one hour. After removal of the blocking solution, 100 uL/ well of each sample was added to two wells for repeat measures. The samples were at a dilution of one part egg yolk to 2000 parts 1% BSA. Samples incubated for 15 minutes on a plate shaker and then were washed six times. Two uL of the secondary Ab, goat-anti-chicken IgG-Fc conjugated to horseradish peroxidase (HRP), was mixed with 10 ml 1% BSA and added 100 uL/ well. The secondary Ab was incubated for 15 minutes and the plate was then washed eight times.

Application of 125 uL/well of substrate solution (100 uL of 3, 3', 5, 5'-tetramethyl benzidine (TMB), 128 uL of H_2O_2, and 19.74 ml of 0.05 M sodium acetate) caused a color reaction (blue) due to the HRP enzyme. After approximately five minutes (reaction time varies per plate), 50 uL/ well of 0.05M sulfuric acid stop solution was added to produce another color reaction (yellow). The plate was read on a plate reader to find the optical density (OD) of the sample, thus revealing remaining Ab. OD was measured at 450 nm because this is the wavelength at which yellow light is absorbed. This sandwich ELISA procedure was adapted from ELISA protocol, as outlined in Schade, et al., to detect remaining Ab.

An initial experiment was conducted with 0% trehalose samples to test variation of the model system in generating a denaturation curve. Three two-gram samples were exposed to 0, 10, 30, 60, 120, and 300 seconds of steam treatment. ELISA (described previously) was used to measure loss of Ab activity as measured by optical density.

The positive control in steam treatment was 12% trehalose, as this is the percentage used commercially and this study involves testing other percentages against 12% to find an ideal percentage of trehalose. The negative control for ELISA was egg yolk from a hen not immunized against PLA_2. This negative control represents the non-specific binding of egg yolk Ab to antigen. All ELISA data was zeroed with a blank of 1% BSA.

Results

In an initial experiment with 0% trehalose, three samples at each time point were necessary to generate a reasonable denaturation curve due to the variance of sampling in the model system (Graph 1).

Optical density of samples was graphed against time in steam treatment (Graph 2). As percent trehalose increases, more Ab should remain in the sample and thus the OD reading should be higher. 9, 12, and 16.2% trehalose generated downward sloping denaturation curves with little or no outlying points. 3% showed a fairly good downward trend, with the exception of the 120 second data point. 0% was similar to 3% in that it showed a downward trend until 120 seconds, where the recorded OD was comparable to the OD of 0 and 10 seconds in steam treatment. This suggests that the same amount of Ab remained in the 0, 10, and 120 second trials for the 0% trehalose. 6% also had an elevated reading at 120 second reading. These results show that either there was a sampling problem with the 120 second steam treatment or there is something different going in the lower percent trehalose trials at the 120 second time interval that deserves further study.

Discussion

Although there was high variability in OD remaining, results do show a downward trend in the loss of Ab as time in heat increases. Results at 300 seconds suggest that higher percent trehalose affords more protection to the Ab. At 300 seconds, OD is highest at 16.2% and gradually drops to zero for readings after 9%. 6% trehalose is an outlier and can be disregarded due to the remaining downward trend of the other percentages at 300 seconds. 0, 3, and 9% show zero Ab activity at 300 seconds.

In the future, samples will be measured out and accordingly diluted depending on what percent trehalose they contain (0-16.2%) so they all start out with the same amount of Ab. As percentage trehalose increases, amount of starting Ab in sample decreases, thus producing bias in results as higher percentages of trehalose have less Ab to denature, and thus less to protect in the heating process. This will reduce error due to different amounts of Ab present in the starting material and also make comparison of Ab remaining easier during the process of analyzing data.

It is unknown what kind of sampling error produced the skewed results in the 120 second range of the lower percent trehalose samples. Error in samples could not be averaged out because only one sample was run per percent at each time interval in order to simplify the baseline study and get an idea of what results to expect.

Future studies will involve repeat measures of at least three samples at each percent and time interval in order to reduce error due to sampling and non-homogenous application of steam treatment. The machine will also be modified in order to ensure steam is reaching all samples in an even fashion and not contributing to error in final Ab readings. Although graph 2 generally

shows a downward trend over time for the loss of Ab, further testing will be needed to reduce error in the previously discussed areas.

Due to variation of response to steam treatment, it took three samples to generate a reasonable denaturation curve. The trehalose experiment only tested one sample at each percent trehalose and time interval in an attempt to generate baseline data. Repeating this experiment two more times and taking the averages of the experiments will generate a more reliable curve. Ideally, the model system could be modified to decrease variance to produce a reliable curve using only one or two samples per treatment time interval.

After identifying the ideal percent trehalose for commercial uses, further studies will involve testing of methods besides trehalose to protect the Ab. The denaturation curve developed in this study may be used to compare the efficacy of these alternative protection methods, as trehalose is the best method that is currently commercially applicable.

Acknowledgements
I would like to thank the Engineering students for their help in the development of the small-scale model for steam treatment, Vanessa Leone for editing and consultation, and Mark Cook and David Trott for their continuing knowledge and guidance in this series of studies on IgY heat stability and protection methods.

Figures and Graphs

Figure 1; Front view of the feed steamer where all trials were conducted. The chamber contains individual sampling areas so samples do not contaminate eachother.

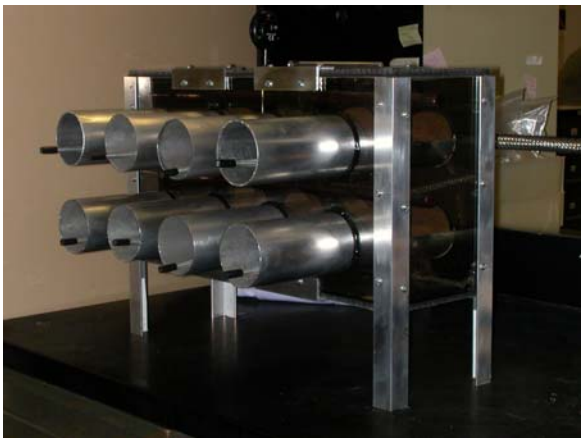


Figure 2; Side view of the feed steamer.

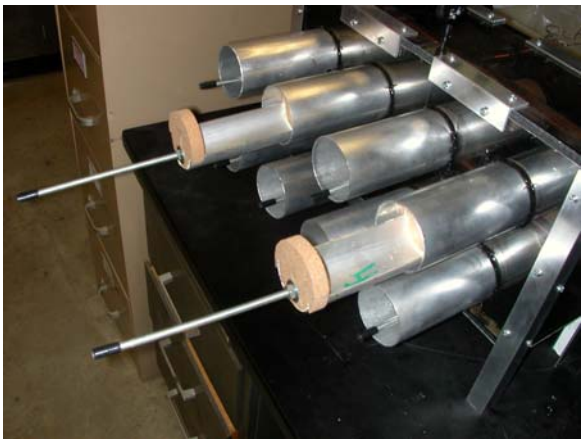
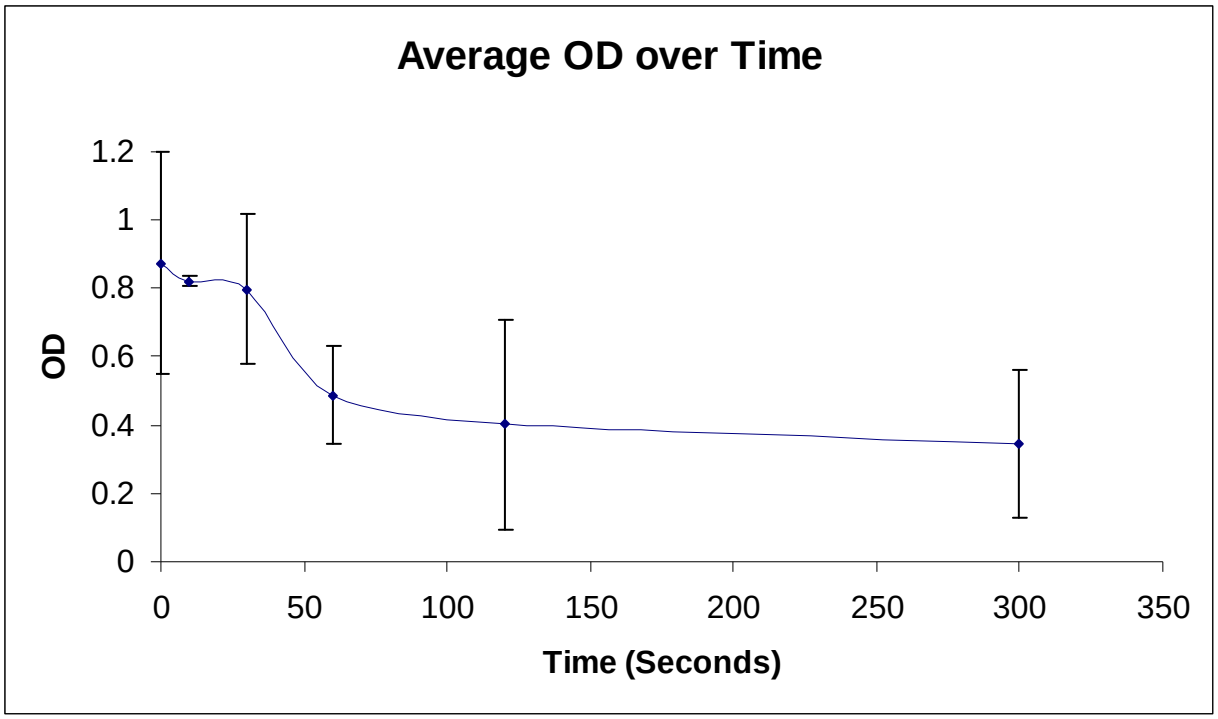


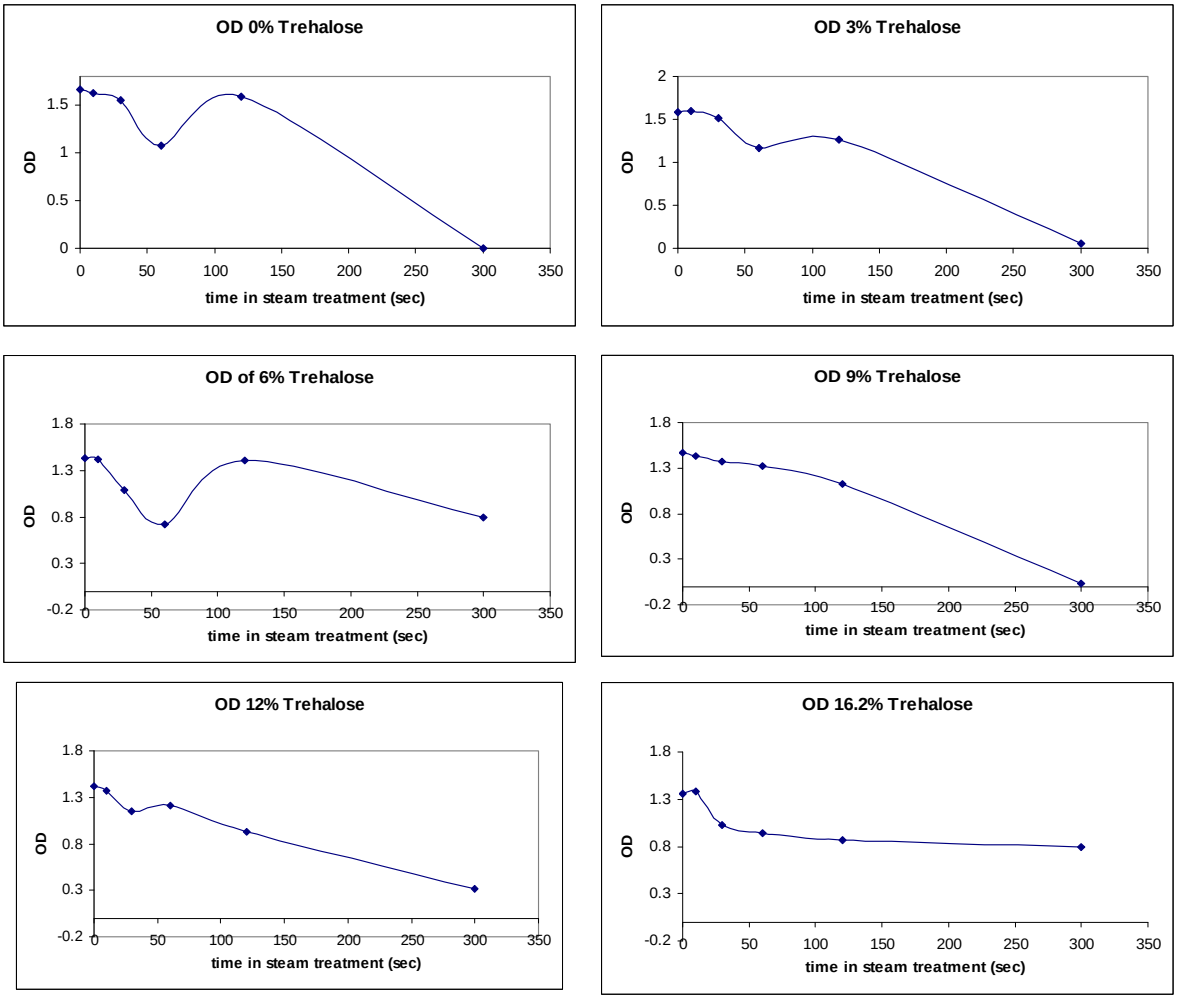
Figure 3; Side view with sampling troughs pulled out.

Figure 4; Individual sampling trough and freeze dry trays. Sampling troughs slide into the larger sampling trough as seen in figure 1.

Figure 5; Steam generator used in all trials.



Graph 1; Average Optical Density of IgY. Three two-gram samples containing 0% trehalose were steam treated for 0, 10, 30, 60, 120, and 300 seconds, respectively.



Graph 2; Optical Density vs. Time in Steam Treatment shown with individual graphs for ease of viewing. 12% trehalose was regarded as the control because this is the percentage currently used commercially.

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Chapter 3

Hydrophobic protein matrices (HPM) for stabilizing heat-labile proteins

Abstract

Thermostability is essential before bioactive proteins can receive broad industrial uses. While gene manipulation has increased thermostability of select proteins, others, such as polyclonal antibodies, require encapsulation technology. Polyclonal chicken egg antibody was used as a model system to devise new encapsulation matrices. A steam chamber model was developed to test thermostability, an enzyme-linked immunosorbent assay (ELISA) was modified to determine antibody activity remaining after steam treatment, and antibody binding degradation curves were generated. It was shown that if the protein was sequestered from water, thermostability can be maintained (100% binding activity remained after 60 seconds at 92-93C in steam treatment). A hydrophobic protein matrix (HPM) was developed to improve upon the current industry standard of trehalose-encapsulation for improved heat stability. After 60 seconds in 92-93C steam treatment, 41.73% binding activity remained in 1% antibody in pasta matrix, 0.94% remained in antibody in egg matrix, and 4.5% remained in trehalose industry standard. These findings show that a HPM may improve upon current industry practices.

Introduction

Thermostability is essential before bioactive proteins can receive broad industrial uses. A wide variety of protection methods, including inorganic salts, genetic engineering, and the exploit of bacterial thermal protective mechanisms, are in use today (Kumar & Nussinov, 2001, and Adams & Kelly, 1995). Encapsulation, however, is the only viable technology for the stabilization of select proteins (e.g. antibodies) (Haefner et al., 2005). This method has been used in the food industry to protect proteins from moisture, heat, and other extremes, in addition to masking unfavorable odors or tastes (Gibbs et al., 1999). Kovacs-Nolan and Mine (2005) have also developed a controlled-release technique of antibody (Ab) microencapsulation capable of surviving the gastrointestinal conditions with 80% of Ab activity retained.

Sugar has been shown to increase the stability of the proteins, such as Ab, when subjected to extreme heat, pH, and enzymatic testing (Kovacs-Nolan & Mine, 2004). Trehalose, a disaccharide, forms a glassy matrix around the protein and protects it from denaturation (Doyle, 2001, and Lins et. al., 2004). Lins et. al. (2004) has documented the ability of trehalose to provide heat stabilization and proposed a molecular mechanism for its stabilizing properties. This matrix can be used to protect proteins of interest, including probiotics, immune modulators, organic acids, enzymes such as phytase, and many other feed supplements, from the heat treatment of the industrial processing. Trehalose has also been shown to be effective in the long-term preservation of biologically-active materials during storage (Worrall, 2005). Previous studies from this lab (data not reported) have also shown the efficacy of trehalose in protecting Ab.

This study was conducted with two objectives; 1) Understand the role of water in protein thermostability, and 2) Improve upon current industry standards (trehalose encapsulation) by finding and testing alternative methods for protein heat stabilization.

Methods

This study was conducted in conjunction with Professor Mark Cook, Department of Animal Science, Professor Mark Etzel, Department of Food Science/ Chemical & Biological Engineering, and Mingder Yang, aOvaTech. In spring of 2006, the steam chamber was remodeled to improve steam distribution and temperature control in order to reduce sample variation. Collaboration with David Wahl, an engineering student, transformed the existing chamber, a 6" x 24" x 12" polycarbonate box with eight individual sampling spaces into a 12" x 24" x 12" polycarbonate box with a single sampling space (see figures 3 and 4.)

Objective 1: Determine water's role in protein thermostability.

Three samples per treatment (Ab in egg matrix, trehalose-encapsulated Ab, and trehalose industry standard) were placed in sealed 15ml centrifuge tubes and then steam treated at 92-93C. In a separate trial, five samples per treatment (Ab in egg matrix, trehalose-encapsulated Ab, and trehalose industry standard) were mixed with drierite in a 2:1 ratio in a closed 15ml centrifuge tube and allowed to dry for two days at room temperature. Samples remained sealed and were then placed in the steam machine at 92-93C for 60 seconds. Controls for each experiment (drierite or no drierite) were not steam treated.

After steam treatment, remaining Ab was extracted from all samples (control and steam treated) overnight using a 1:10 ratio of sample to acidified PBS (pH=5.5). Samples were then centrifuged and a portion of the supernatant was removed and diluted to 1:16000 in 1% BSA (bovine-serum albumin). Ab in the sample that withstood the heat treatment was measured by an enzyme-linked immunosorbent assay (ELISA), according to the described method in Bobeck et. al., 2006. Data were analyzed based on average optical density (OD) regression against control optical density and each sample was expressed as percent (of control) remaining after steam treatment.

Objective 2: Develop and test alternative heat-labile protein protection methods and compare to trehalose (current industry standard).

A hydrophobic matrix (HPM) consisting of semolina flour was developed to improve upon trehalose's heat protection capabilities. The HPM and food matrix were dried in a dessicator with drierite (within a heat oven maintained at 40C) for at least four days prior to addition of Ab in order to remove internal water. Spray-dried antibody (aOvaTech, 2006) was then mixed with the HPM at 1.0% and then extruded (pressed through a pasta machine). Ab was either extruded or not extruded. Trehalose-encapsulated Ab (aOvaTech, 2006) was mixed with food matrix at 1.0% and then placed in a dessicator (within a heat oven maintained at 40C) with the Ab/ hydrophobic matrix samples to dry for three days prior to steam treatment. Half-gram samples (two replicates per treatment) were weighed from the dried treatment groups and placed in seven-day pillboxes (open to steam treatment). Another set of half-gram samples (one sample per treatment) was weighed, placed in 15ml centrifuge tubes, and then sealed. Controls (no steam treatment) were left in the dessicator. Samples (in seven-day pillboxes or sealed centrifuge tubes) were placed in

the steam chamber for 60 seconds at 92-93C and then returned to the dessicator to dry for two days post-steam treatment.

Ab was extracted overnight using a 1:10 ratio of sample to acidified PBS (pH=5.5). Samples were then centrifuged and a portion of the supernatant was removed and diluted 1:8000 in 1% BSA (bovine-serum albumin). Ab in the sample that withstood the heat treatment was measured by an enzyme-linked immunosorbent assay (ELISA), according to the described method in Bobeck et. al., 2006. Data were analyzed based on average optical density (OD) regression against control optical density and each sample was expressed as percent (of control) remaining after steam treatment.

Results

Objective 1: Samples dried with drierite before steam treatment (Ab in egg matrix and trehalose-encapsulated Ab) retained 100% activity after 60 seconds in 92-93C in a sealed 15ml centrifuge tube. Trehalose industry standard retained 88.0% activity. Samples not dried with drierite pre-steam treatment are as follows; Ab in egg matrix retained 72.24% activity, trehalose-encapsulated Ab retained 74.03% activity, and trehalose industry standard retained 42.26% activity after 60 seconds in a sealed 15ml centrifuge tube at 92-93C.

Objective 2: As compared to control samples subjected to 60 seconds in steam treatment at 92-93C, samples containing 1% Ab in pasta matrix retained an average of 41.72% activity and extruded Ab in egg matrix retained an average of 2.23% activity. Antibody in egg matrix (not extruded) samples retained an average of 0.94% activity and trehalose industry standard samples retained an average of 4.49% activity. Sealed centrifuge tubes samples containing 1% Ab in

pasta, extruded Ab in egg matrix, non-extruded Ab in egg matrix, and trehalose industry standard all retained 100% Ab activity.

Discussion

Objective 1: Results show that sequestering the Ab from water is essential in maintaining Ab stability (Figure 1). In sealed centrifuge tubes at 92-93C, where heat was the only factor attributing to protein degradation, samples lost more activity than samples that had been dried with drierite prior to treatment. Samples that were not dried prior to steam treatment contained internal water from the Ab (approx. 3-5%) and food matrix (used in trehalose industry standard, approx. 12%). Even this small amount of water appeared to be sufficient to produce Ab denaturation (refer to Figure 1). Upon drying with drierite pre-steam treatment, samples retained 100% activity, with the exception of the trehalose industry standard samples. This food matrix may possess other intrinsic properties, such as chemical reactions, hydrophobic interactions, or molecular bonding, that may increase denaturation of the Ab when subject to steam treatment.

Ab in egg matrix and trehalose-encapsulated Ab performed similarly when sealed in centrifuge tubes. In comparison to previous experiments where trehalose-encapsulated Ab retained more activity than Ab in egg matrix alone (data not published), these data suggest that trehalose affords protection from water damage to the protein, but not necessarily heat damage. These data also suggest that if heat-labile products can be dried before heat treatment, the addition of trehalose may not be necessary to obtain heat stabilization in the protein.

While these studies (drierite vs. no drierite pre-steam treatment) are slightly different in format (number of samples) and dilution, they consistently show that water, both internal and external, contributes to antibody degradation.

Objective 2:

Allison et. al. (1998) state that dry proteins are theoretically more stable than those containing water, but also show that the drying process itself may create some damage. It has been shown in our lab that Ab undergoes the Maillard reaction when stored at 40C over a period of weeks to months, causing color change and protein damage (Rowe et. al, 2007). Ab used in this experiment, therefore, may have been further damaged during the three day drying process at 40C (post-steam treatment) before ELISA was conducted. It is unknown, however, how much damage was caused, but it appears to be minimal due to previous experiments with the Maillard Reaction where drying damaged proteins over a period of weeks.

Lins et. al., (2004) characterize the molecular mechanism for trehalose's protein stabilization properties, citing hydrogen bonding as a main source of increased stability. In both objective 1 and 2, trehalose-encapsulated Ab was able to retain more activity vs. Ab in egg matrix. The HPM (pasta) and extrusion may protect the Ab in a similar way, in addition to repelling water due to increased hydrophobicity (Haefner et. al., 2004). Bonomi et. al. (2004) showed that semolina flour, a component of the HPM, showed increased hydrophobicity and increased protein-affinity when mixed with water and extruded.

1% Ab in pasta matrix samples outperformed trehalose industry standard samples during 60 seconds of heat treatment, suggesting this pasta matrix (HPM) may improve on current industry standards for protein encapsulation (Figure 2). Extrusion in itself may afford protection to the Ab. Although considerably less than HPM and food matrices (which retained 41.72% and 4.49%, respectively, extruded Ab in egg matrix retained more activity than non-extruded Ab in egg matrix (2.23% extruded vs. 0.94% unextruded). These data were consistent with protein-stabilizing extrusion methods and results obtained by Haefner et. al. (2005), in which similar extrusion technology was used to stabilize heat-labile proteins.

Standard error between sample 1 and 2 for each treatment was also low, showing the steam chamber model has good repeatability (refer to Table 1). Ideally, there would have been more replicate samples in this study, but due to limited space in the sampling chamber, more replicates for all treatments were not possible. It should be noted, however, that there are probably many matrices other than this particular HPM that could afford increased protection to the Ab.

Conclusion

The stabilization of heat-labile proteins is important topic in industries, including food-additive, biotechnological, and pharmaceutical, where protein survivability and functionality are necessary. Improving upon current methods is essential to decrease protein loss, which translates to decreased financial loss and increased efficiency.

Further studies will involve making the Ab and matrix drying process more industrially-relevant. Alternate Ab protection methods based upon hydrophobic matrices will be developed (similar to studies done in Objective 2) and the protective matrices will be kept as dry as possible before

steam treatment. Further studies will also include the measurement of the vessel temperature (non-conducting plastic centrifuge tubes) post-steam treatment to determine if any loss of Ab can be attributed to vessel interference. The pasta (HPM) will undergo additional studies to understand its uses and limitations for industry, including ratio of Ab: HPM, particle size, and maximal protective temperature. Alternative HPM's will also be researched in order to improve upon this particular model.

Figures and Tables

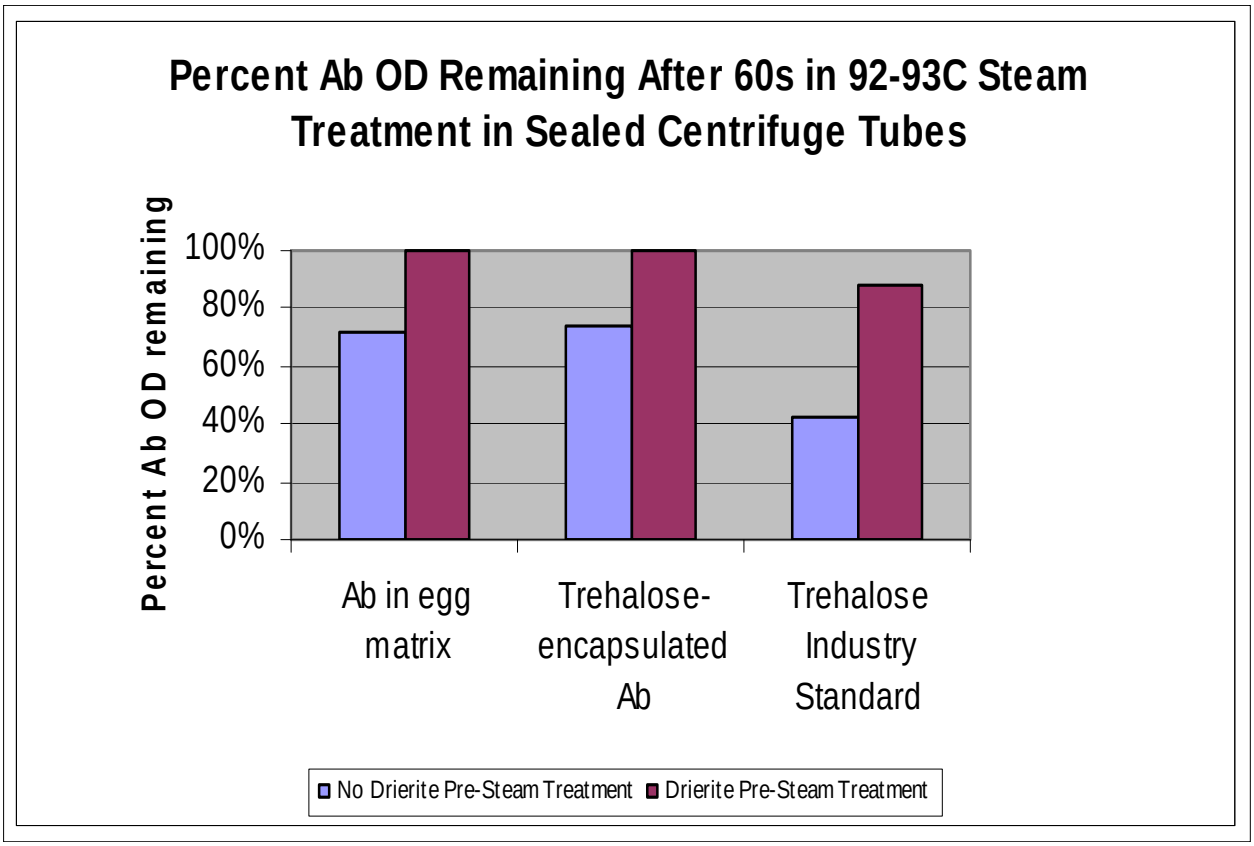

Figure 1: Percent of antibody activity remaining after 60 seconds in steam treatment. Samples remained sealed in centrifuge tubes during steam treatment and were dried with drierite pre-steam treatment or not dried pre-steam treatment.

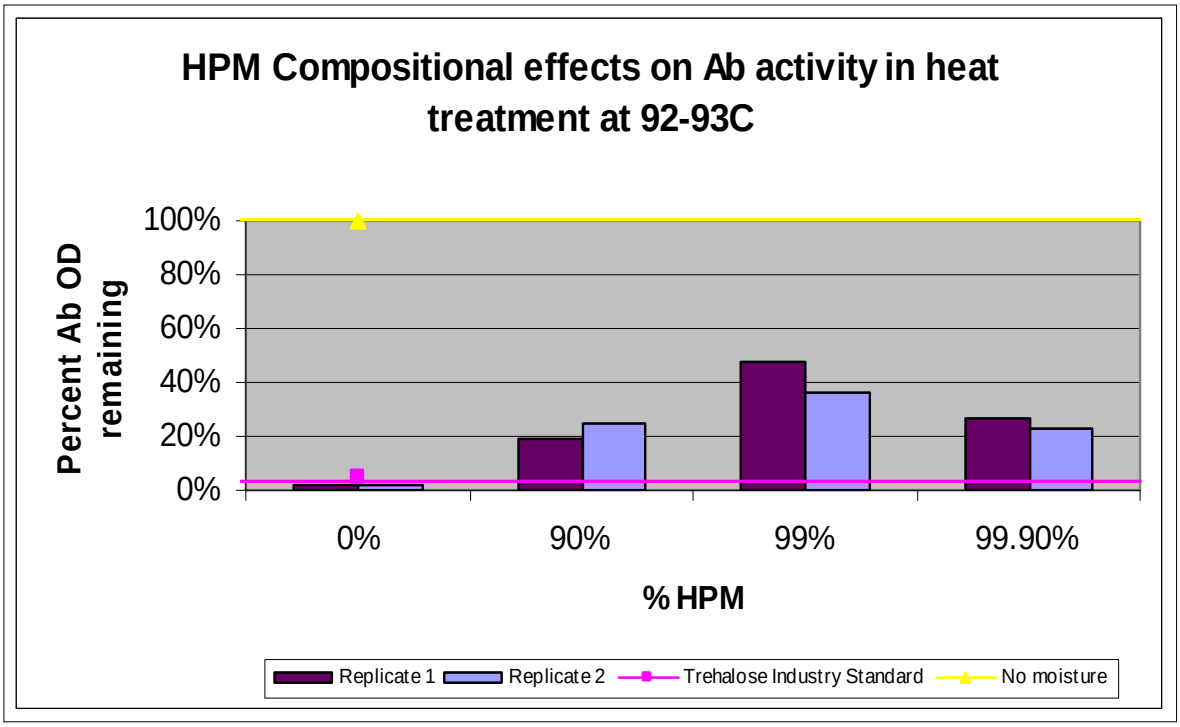

Figure 2: Percent of Ab OD remaining after 60 seconds in 92-93C steam treatment. Percent OD remaining is expressed as percent of control OD. No moisture denotes samples in capped centrifuge tubes, dried prior to treatment. These samples retained 100% activity after steam treatment, suggesting water's detrimental role in Ab inactivation. As % HPM (pasta) was increased, Ab activity post-treatment improved, with a maximum at 99% HPM. This HPM may improve on current industry standards for protein encapsulation.

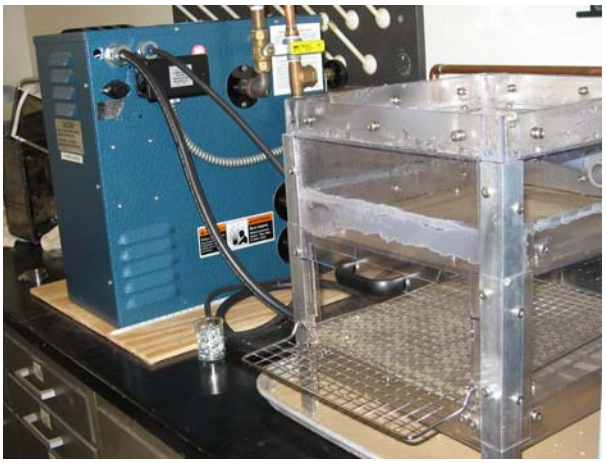

Figure 3: Steam generator and steam chamber used to produce steam and test samples for all trials.

Figure 4: Close-up of steam chamber. There are two levels; steam enters the chamber in the top level and is applied evenly to samples on the lower level via holes chamber divider. This division ensures even application and prevents condensation from dripping directly onto samples during steam treatment. Samples are placed on a wire cookie sheet and slide into the bottom level for treatment.

Treatment	Sample Average	Standard Error
1% Ab in pasta matrix (extruded)	41.72%	5.91%
Ab in egg matrix (extruded)	2.23%	0.10%
Ab in egg matrix (not extruded)	0.94%	0.10%
trehalose industry standard	4.49%	2.03%

Table 1: Standard error for Objective 2 samples.

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Heat Stability of *Gallus domesticus* Immunoglobulin Y (IgY)

THE UNIVERSITY of WISCONSIN MADISON

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Abstract

Feed efficiency is a critical aspect of food animal production. In terms of feed additives, egg yolk antibodies are affordable and improve feed efficiency without introducing problematic antibiotics. The drawback of antibody feed additives is that they denature during the harsh feed pellet conditioning process, rendering them inactive. Two-hundred grams of feed was mixed with 1% dried egg yolk containing α-PLA_2 antibody. Two gram samples were steam treated in a newly designed, small-scale machine for 0, 10, 30, 60, 120 and 300 seconds. α-PLA_2 antibody in feed samples was measured by ELISA, and a baseline curve was developed to model the loss of antibody activity. At ten seconds of steam treatment, 82.65% of antibody remained, while only 21% remained after 300 seconds. This inexpensive model can be used to develop a standard for comparison of antibody protection methods during the pelleting process.

Purpose of the Study

Can the steam treatment chamber made by engineering students be used to model the steam conditioning process of feed pelleting?

- Antibodies, useful alternatives to antibiotics in feed, denature during the harsh steam conditioning process of feed pelleting
- An inexpensive, small-scale model was needed to generate an antibody activity loss curve
- Antibody protection methods shall be compared against this curve to test efficacy

Materials and Methods

Students from the School of Engineering constructed an inexpensive, small-scale machine that enabled the testing of time and heat effects on the loss of α-PLA2 antibody in chicken feed (***Figure 1***). Two gram samples were each run at 10, 30, 60, 120, and 300 seconds in repeat measures. Samples were then freeze-dried to reduce error in concentration due to water accumulation in samples steam treated for longer intervals. α-PLA_2 antibody was extracted in acidified PBS and remaining α-PLA_2 antibody was detected by ELISA (***Figure 2***). Titer was expressed in arbitrary units based on a standard curve from dilutions of egg yolk used in feed. Optical density readings for the positive control egg yolk were measured at dilutions of 1:2000—1:64000. Titer and percent titer lost were calculated at each time interval.

Figure 1, small-scale steam treatment machine

Figure 2, holding trays for freeze-drying and heat treatment trials

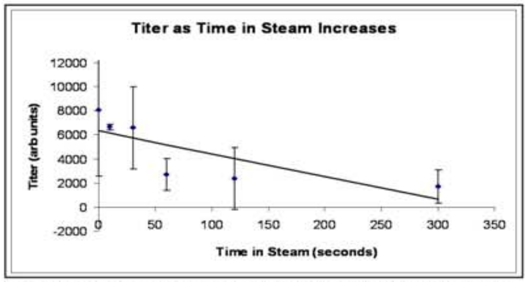

Figure 3, Titer as Time in Steam Treatment Increases-Arbitrary units of the samples as calculated by standard curve equation were squared to obtain the titer.

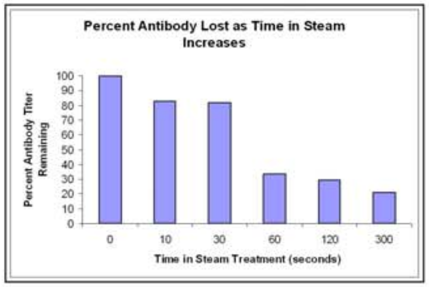

Figure 4, Percent Antibody Remaining After Steam Treatment-Percent titer at each interval was calculated using the following equation: $100*(T_0 - T_X)/T_0$ with T_0= titer at time zero and T_X = titer at 0, 10, 30, 60, 120, and 300 seconds

Results

- The variance of optical density was high, as evidenced by a standard deviation equal to .205 (***Figure 3***)
- At 10 seconds, 82.65% of antibody remained (***Figure 4***)
- At 300 seconds, 21% of antibody remained (***Figure 4***)

Discussion

- The p-value was not significant due to unequal distribution of PLA_2 antibody in samples before heat treatment and a small sampling size per time interval (three samples per interval)
- Further studies shall be done to generate a cleaner trendline in the 60—120 second range
- Despite the insignificant p-value, the titer shows a clear downward trend, indicating that the small-scale machine is an accurate representation of the commercial steam treatment process

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Elizabeth Bobeck would like to gratefully acknowledge David Trott for his patience in teaching ELISA and interpretation of results, and also Mark Cook and Mingder Yang for background information and mentoring during the study.

Heat Stability of *Gallus domesticus* Immunoglobulin Y (IgY)

Elizabeth A. Bobeck, David L. Trott, and Mark E. Cook

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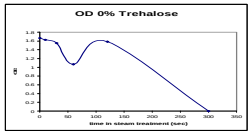
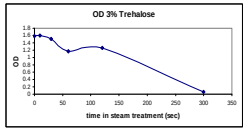
Abstract

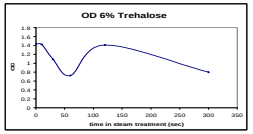
Feed efficiency is a critical aspect of food animal production. Egg yolk antibodies (Ab) are an affordable alternative method to improve feed efficiency without introducing problematic antibiotics. The drawback of Ab feed additives is protein denaturation during the harsh process of feed pelleting. Ab thermostability can be improved through the use of trehalose, a sugar that has been proven effective in increasing protection by forming a glassy matrix around the material. In this study, trehalose was mixed with egg Ab powder to obtain six different trehalose percent groups; 0, 3, 6, 9, 12, and 16.2%, respectively. One-gram samples were individually steam treated in a small-scale model of commercial steam treatment for 0, 10, 30, 60, 120 and 300 seconds at a temperature range of 82-88 degrees Celsius. PLA_2 Ab that withstood the treatment in the samples was measured by ELISA and six graphs were generated based on optical density (OD). This inexpensive model can be used to develop models for denaturation curves of various heat-sensitive feed additives, particularly Ab additives.

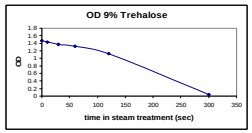
Materials and Methods

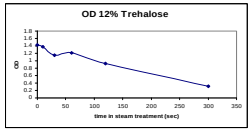
Students from the School of Engineering constructed an inexpensive, small-scale steam chamber that enabled the testing of trehalose's ability to protect antibody (Ab) in chicken feed (*Figures 1 and 2*). Hens were immunized with PLA_2 antigen and produced egg yolk Ab against PLA_2. To test the effects of varying percentages of trehalose on Ab stability, individual one-gram feed samples containing the same amount of Ab and varying amounts of trehalose (0, 3, 6, 9, 12, and 16.2%) were subjected to steam treatment in the chamber at 10, 30, 60, 120, and 300 seconds. Samples (36 in total) were diluted in 9ml acidified PBS to extract Ab and then further diluted 1:2000. The positive control in steam treatment was 12% trehalose (percentage used commercially) and this study involves testing other percentages against 12% to find an ideal protective amount of trehalose. Enzyme-linked Immunosorbent Assay (ELISA) was used to measure remaining Ab. Optical density (OD) readings for the positive control egg yolk were measured at dilutions of 1:2000—1:64000. The negative control for ELISA was egg yolk from a hen not immunized against PLA_2. This negative control represents non-specific binding of egg yolk Ab to antigen. All ELISA data was zeroed with a blank of 1% BSA.

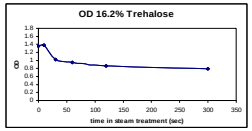










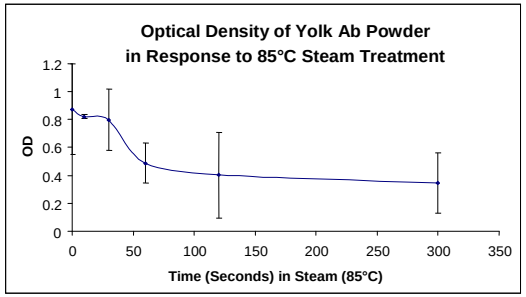


Graph 2, Optical Density Yolk Ab Powder with Trehalose. Separate graphs depict antibody activity dependent on trehalose percentage. 12% trehalose was regarded as the control because this is the percentage currently used commercially.

Purpose of the Study

Does 12% trehalose offer the optimum level of antibody protection? Can lower percentages of trehalose provide the same level of protection?

- **Antibodies, useful alternatives to antibiotics in feed, denature during the harsh steam conditioning process of feed pelleting.**
- **Sugar has been shown to increase the stability of the proteins, such as Ab, when subjected to extreme heat, pH, and enzymatic testing. Trehalose, a disaccharide, forms a glassy matrix around the protein and protects it from denaturation.**
- **Trehalose was mixed with egg Ab powder to obtain six different percent trehalose groups; 0, 3, 6, 9, 12, and 16.2%, in order to determine the most effective level of protection.**



Graph 1, Average Optical Density of IgY. Three two-gram samples containing 0% trehalose were steam treated for 0, 10, 30, 60, 120, and 300 seconds, respectively to generate a curve for comparison to varying percent trehalose curves.

Results

- **Without protection, loss of Ab increases as time in steam treatment increases.**
- **Results at 300 seconds suggest that a higher percent of trehalose affords more protection to the Ab in the feed; OD is highest at 16.2% and gradually drops to zero for samples with lower trehalose content.**
- **0, 3, and 9% show zero Ab activity at 300 seconds.**

Figure 1, Engineering students collaborated with Mark Cook's lab to build a small-scale steam treatment chamber in order to develop a curve modeling Ab denaturation.

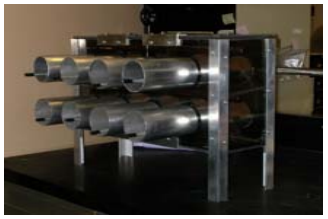


Figure 2, Side view of steam treatment chamber used in all trials.

Discussion

- **It is unknown what kind of sampling error produced the skewed results in the 120 second range of the lower percent trehalose samples. Error in samples could not be averaged out because only one sample was run per percent at each time interval in order to simplify the baseline study.**
- **Future studies will involve repeat measures of a minimum of three samples at each percent and time interval in order to reduce error due to sampling and non-homogenous application of steam treatment.**
- **The treatment chamber will be modified in order to ensure steam is reaching all samples in an even fashion and not contributing to error in final Ab readings.**

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I would like to thank the Engineering students for their help in the development of the small-scale model for steam treatment, David Trott and Mark Cook for their continuing knowledge and guidance in this series of studies on IgY heat stability and protection methods, and Vanessa Leone for consultation and editing.

Appendix C: Abstract, Heat Stability of *Gallus domesticus* Immunoglobulin Y (IgY), presented at Poultry Science Association 2006 Summer Meeting, Edmonton, Canada

W55 Methods in heat-stabilization of *gallus domesticus* Immunoglobulin Y (IgY). E. A. Bobeck*, D. L. Trott, M. E. Cook, and M. Yang, *University of Wisconsin, Madison.*

Post-pellet application of heat-labile protein (i.e. enzymes, antibodies, and peptides) has become the industry standard in recent years; however, heat-stabilization of these proteins would permit pre-pellet application. Development of a pilot steam conditioning system which models industry steam conditioning was necessary to create a laboratory model for testing encapsulation strategies and heat stability of proteins prior to large-scale production. Egg yolk antibody is an example of a protein increasingly being used in animal feeds. The objective of this project was to attempt to improve the heat stability of egg yolk proteins and to test a steam conditioning chamber engineered to model commercial conditioning prior to feed pellet manufacture. Ligand-specific whole egg liquid was mixed with no carbohydrate, 12% trehalose or 6% sorbitol plus 6% corn starch. Egg preparations were spray dried at 90C. The antibody powder was added to broiler feed and subjected to 10s of steam conditioning at 82C, then pelleted. Antibody was extracted from the feed and antibody ligand binding activity following encapsulation and heat treatment was assessed by a ligand-specific enzyme-linked immunosorbent assay (ELISA). Loss of optical density (OD) reading following heat processing relative to pre-processing was 87% for the control, 45% for trehalose and 84% for the sorbitol plus starch-treated egg products. In the trial involving an engineered pilot conditioner, ligand-specific egg yolk was mixed with 0 or 24% trehalose, freeze dried, and then mixed into feed. Feed samples were conditioned at 85C for up to 60s and then removed from the chamber. Ligand binding activity was assessed using ELISA. Loss of OD reading was similar between the two treatments at 10s (ca, 20%), 45 and 25% at 30s and 50 and 37% at 60s for the control and 24% trehalose treated egg yolk antibody, respectively. At this time, a direct comparison of antibody loss under commercial heat treatment of feeds is not readily piloted in a bench scaled conditioner, however, trehalose does appear to offer some heat stability to egg antibody in both the commercial and piloted conditioners.

Key Words: antibody heat stability, trehalose, IgY

Methods in Heat Stabilization of *Gallus domesticus* Immunoglobulin Y (IgY)

E. A. Bobeck*, D. L. Trott, M. E. Cook, and M. Yang, Department of Animal Sciences University of Wisconsin, Madison, WI, 53706

Abstract: W55

Post-pellet application of heat-labile protein (i.e. enzymes, antibodies, and peptides) has become the industry standard in recent years; however, heat-stabilization of these proteins would permit pre-pellet application. Development of a pilot steam conditioning system which models industry steam conditioning was necessary to create a laboratory model for testing encapsulation strategies and heat stability of proteins prior to large-scale production. Egg yolk antibody is an example of a protein increasingly being used in animal feeds. The objective of this project was to attempt to improve the heat stability of egg yolk proteins and to test a steam conditioning chamber engineered to model commercial conditioning prior to feed pellet manufacture. Ligand-specific whole egg liquid was mixed with no carbohydrate,12% trehalose or 6% sorbitol plus 6% corn starch. Egg preparations were spray dried at 90C. The antibody powder was added to broiler feed and subjected to 10s of steam conditioning at 82C, then pelleted. Antibody was extracted from the feed and antibody ligand binding activity following encapsulation and heat treatment was assessed by a ligand-specific enzyme-linked immunosorbent assay (ELISA). Loss of optical density (OD) reading following heat processing relative to pre-processing was 87% for the control, 45% for trehalose and 84% for the sorbitol plus starch-treated egg products. In the trial involving an engineered pilot conditioner, ligand-specific egg yolk was mixed with 0 or 24% trehalose, freeze dried, and then mixed into feed. Feed samples were conditioned at 85C for up to 120s and then removed from the chamber. Ligand binding activity was assessed using ELISA. Loss of OD reading was similar between the two treatments at 10s (ca, 20%), 45 and 25% at 30s and 50 and 37% at 60s for the control and 24% trehalose treated egg yolk antibody, respectively. At this time, a direct comparison of antibody loss under commercial heat treatment of feeds is not readily piloted in a bench scaled conditioner, however, trehalose does appear to offer some heat stability to egg antibody in both the commercial and piloted conditioners.

Objective, Experiment 1: Develop methods to protect $aPLA_2$ from denaturation during the harsh feed pelleting process and test these methods on the industrial level.

Hypothesis: The bioactivity of heat-labile protein loss during feed pelleting can be modeled.

Methods:

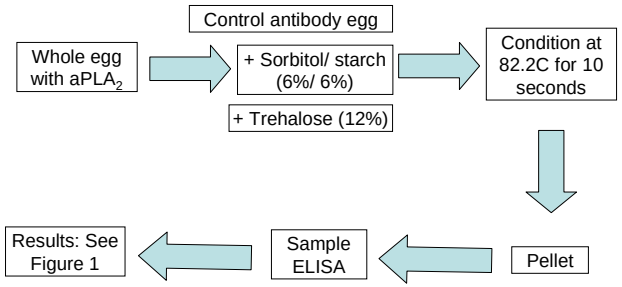
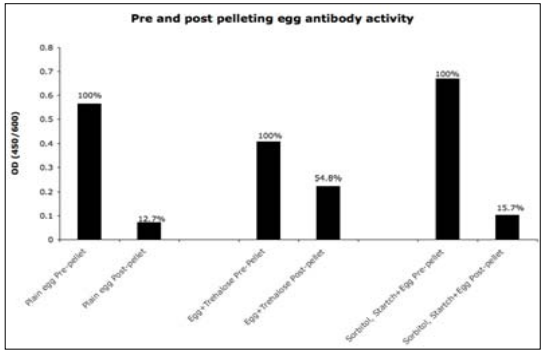


Figure 1, Effect of steam treatment in the three treatment groups, sorbitol/ starch (6% /6%), 12% trehalose, and plain on optical density of ligand binding activity.



Objective, Experiment 2: Build micromodel of industrial pelleting process and develop antibody denaturation curve in order to test antibody protection methods

Device: MBA Electric Steam Generator (Figure 2)

-Manufacturer Sussman Electric Boilers, Model MBA3

-Produces 9 lbs of steam per hour with a design pressure of 100PSIG and a max operating pressure of 85 PSIG

Chamber:

-6" x 24"x 12" rectangular chamber with tinted polycarbonate sides held together by aluminum angle iron (Figure 3)

-Eight one and one-half inch in circumference, corked slide-out trays for depositing chicken feed connect

Figure 2: Electric steam generator that provided steam in all trials

Figure 3: Pilot steam chamber, a micromodel of the steam conditioning process used commercially

Methods:

-Treatment: $aPLA_2$ whole egg with 0 or 24% Trehalose. Spray dried.

-Mixed with chick mash to satisfy 2 pounds of antibody per ton of feed

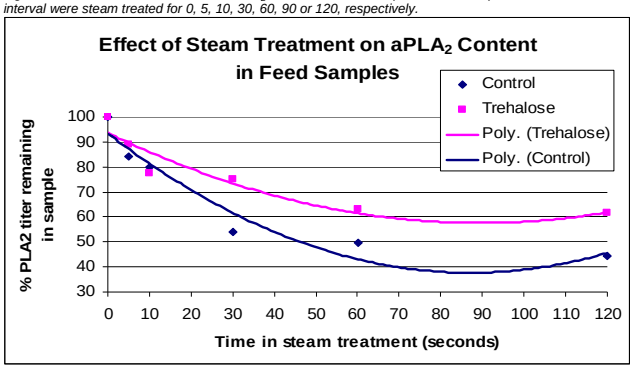
-Steamed in chamber 0-120 seconds

-ELISA performed to detect remaining antibody

Results:

-Loss of OD reading was similar between the two treatments at 10s (ca, 20%), 45 and 25% at 30s, 50 and 37% at 60s for the control and 24% trehalose treated egg yolk antibody, respectively. (See Figure 4)

Figure 4, Effect of Steam Treatment on PLA_2 Content in Feed Samples. Three samples at each time interval were steam treated for 0, 5, 10, 30, 60, 90 or 120, respectively.



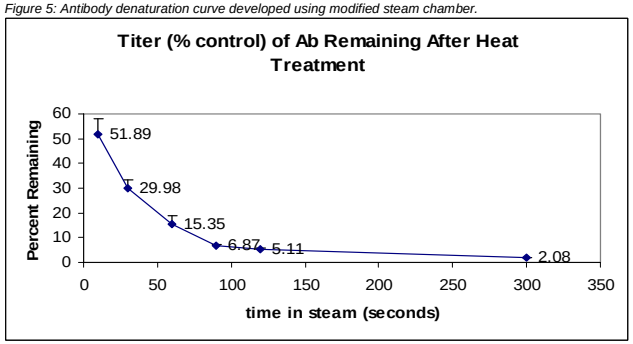
Objective, Experiment 3: Modify steam chamber to reduce variation between samples by applying the steam more evenly and efficiently in one sampling area instead of eight separate sampling units.

-$aPLA_2$ added to chick mash at a ratio of two pounds antibody per ton chick mash

-Samples steam treated 0-300 seconds

-Results: Less variation and more consistency in the antibody denaturation curve. (See Figure 5)

Figure 5: Antibody denaturation curve developed using modified steam chamber.



Conclusion: A bench model to study the denaturation of bioactive proteins has been developed. This instrument will provide a means for the rapid development of heat stabilization technologies.

Acknowledgements
I would like to thank the Engineering students of Professor Hoerr's class for their help in the development of the small-scale model for steam treatment, Vanessa Leone for editing and consultation, aOvatech, and the Hilldale Grant Committee.

Appendix E: Hydrophobic Protein Matrices for Stabilizing Heat-Labile Proteins, presented at the spring Undergraduate Research Symposium and Posters in the Rotunda, April of 2006

Hydrophobic Protein Matrices for Stabilizing Heat-Labile Proteins

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[1]Department of Animal Sciences, [2]Department of Food Science/ Chemical & Biological Engineering,
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Abstract:
Thermostability is essential before bioactive proteins can receive broad industrial uses. While gene manipulation has increased thermostability of select proteins, others, such as polyclonal antibodies, require encapsulation technology. Polyclonal chicken egg antibody was used as a model system to devise new encapsulation matrices. A steam chamber model was developed to test thermostability, an enzyme-linked immunosorbent assay (ELISA) was modified to determine antibody activity remaining after steam treatment, and antibody binding degradation curves were generated. It was shown that if the protein was sequestered from water, thermostability can be maintained (100% binding activity remained after 60 seconds at 92-93C in steam treatment). A hydrophobic protein matrix (HPM) was developed to improve upon the current industry standard of trehalose-encapsulation for improved heat stability. After 60 seconds in 92-93C steam treatment, 41.73% binding activity remained in 1% antibody in pasta matrix, 0.94% remained in antibody without a matrix, and 4.5% remained in 1% antibody in food matrix. These findings show that a HPM may improve upon current industry practices.

Introduction:

¤Recent interest in the industrial use of heat-labile substances, including enzymes, proteins, feed-additives, and probiotics, has increased

¤Activity of these heat-labile substances is destroyed during harsh heat treatments

¤Antibody (Ab) was used as a model to study heat stabilization techniques for heat-labile substances of industrial importance

Device: MBA Electric Steam Generator (Figure 2)
¤Manufacturer Sussman Electric Boilers, Model MBA3
¤Produces 9 lbs of steam per hour with a design pressure of 100PSIG and a max operating pressure of 85 PSIG

Chamber:
¤12" x 12"x 12" rectangular chamber with tinted polycarbonate sides held together by aluminum angle iron (Figure XX) CHECK MEASUREMENTS

¤Separate chambers for steam collection and application of steam to sample; divider made of polycarbonate with 1mm holes drilled every one inch for passage of steam between upper chamber (steam collection) and lower chamber (application to sample).

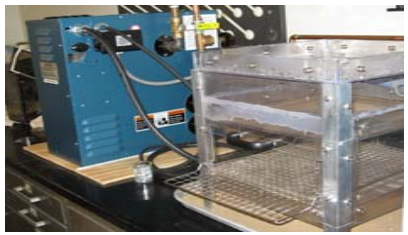

Figure 1: Steam generator and steam chamber used in all trials

Objective 1: To determine the role of water in protein thermostability

Methods:

One gram samples of the following treatment groups were placed in 15 ml centrifuge tubes with or without drierite:
• Ab in egg matrix
• Trehalose-encapsulated Ab in egg matrix
•Trehalose industry standard

→ Steam treat at 92-93C for 60 seconds

→ Extract Ab with acidified PBS (pH=5.5), centrifuge, and remove a portion of supernatant and dilute to 1:16000 in 1% BSA

→ Detect remaining Ab post-steam treatment with ELISA and analyze data; express as percent of control (no steam treatment) remaining

Results:

¤When samples were treated with drierite prior to steam treatment, 100% activity remained in Ab in egg matrix and trehalose-encapsulated Ab in egg matrix. 88% activity remained in trehalose industry standard.

¤When samples were not dried prior to steam treatment, 72% activity remained in Ab in egg matrix, 74% remained in trehalose-encapsulated Ab in egg matrix, and 42% activity remained in trehalose industry standard.

¤The loss in trehalose industry standard activity (dried prior to treatment) could be attributed to intrinsic matrix differences, such as hydrophobicity, chemical reactions, or molecular bonding.

¤Water plays a large role in Ab detriment during heat treatment

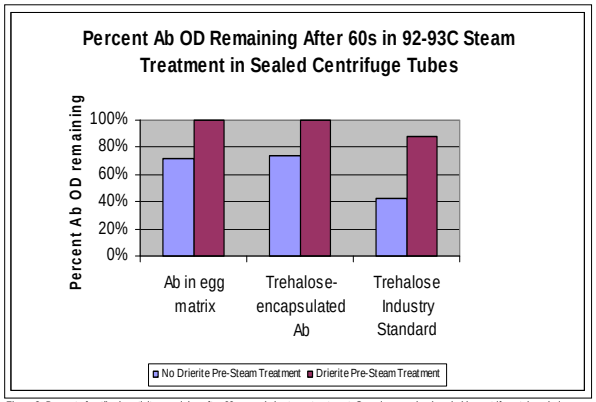

Figure 2: Percent of antibody activity remaining after 60 seconds in steam treatment. Samples remained sealed in centrifuge tubes during steam treatment and were dried with drierite pre-steam treatment or not dried pre-steam treatment.

Objective 2: To develop new encapsulate for heat stabilization

Methods:

¤Samples were prepared with Ab in egg matrix and 0%, 90%, 99%, 99.9% HPM, or trehalose industry standard

¤Half of these samples were placed in sealed 15ml centrifuge tubes

¤Half were left open to steam treatment in sampling boxes.

¤Steam treatment and analysis was analogous to objective 1

Results:

¤Trehalose industry standard retained 4.49% activity post-treatment, and capped samples (no moisture) retained 100% activity

¤0% HPM retained an average of 2.23%, 90% retained 21.60%, 99% retained 41.72%, and 99.9% retained 25% activity post-steam treatment for 60 seconds at 92-93C

Discussion:

¤HPM technology may be used to improve Ab stability during heat treatment

¤Both objective 1 and 2 showed that water may be one of the main variables in Ab denaturation during steam treatment; sequestering the Ab from water is key in maintaining activity

¤Matrix hydrophobicity and compression of Ab into small pellets may contribute to improved Ab activity post-steam treatment, as compared to industry standard

¤Future studies will explore the hydrophobicity of HPM and other matrices

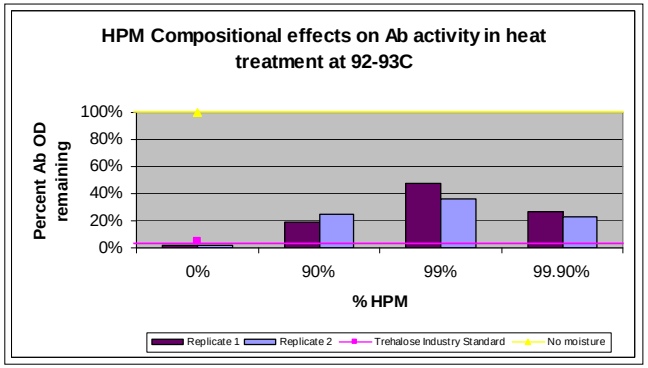

Figure 2: Percent of Ab OD remaining after 60 seconds in 92-93C steam treatment. Percent OD remaining is expressed as percent of control OD. No moisture denotes samples in capped centrifuge tubes, dried prior to treatment. These samples retained 100% activity after steam treatment, suggesting water's detrimental role in Ab inactivation. As % HPM was increased, Ab activity post-treatment improved, with a maximum at 99% HPM. This HPM may improve on current industry standards for protein encapsulation.

Acknowledgements:
Thank you to Mark Cook, Mark Etzel, David Trott for guidance and project development, and Mingder Yang for Ab supply.