IMAC
PIONEERS IN DAIRY & FOOD TECHNOLOGY
International Media And Cultures

6/1/2006

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To: Ms. Felicia Billingslea
Director, Food Labeling and Standards
Food and Drug Administration
5100 Paint Branch Parkway (HFS-820)
College Park, MD 20740

Dear Ms. Billingslea,

Subject: Petition submitted pursuant to section 403 (w) (6) of the Food Allergen Labeling and Consumer Protection Act.

Re: Allergen labeling exemption for IMAC media with enzyme treated soy solids as one of the ingredients.

Pursuant to section 403 (w) (6) of the Federal Food Drug, and Cosmetic Act (FDCA), International Media and Cultures, Inc. (IMAC), submits the enclosed petition requesting exemption from the labeling requirements of section 403 (w) (1), established by the Food Allergen Labeling and Consumer Protection Act (FALCPA), for IMAC media with enzyme treated soy solids as one of the ingredients.

Earlier we have submitted for notification under section 403 (w) (7) of the Food Allergen Labeling and Consumer Protection Act. The notification was designated FALN 003 and the assigned docket No. 2005 FL-0488. The submission was on November 23, 2005. The notification was objected mainly on the basis it should have been submitted under section 403 (w) (6) but not under 403 (w) (7). Also, F.D.A. (Dr. Gottleib) pointed out the additional information, additional tests, clarification of statements needed with regard to the tests submitted in the notification, which were required to qualify for the exemption. Following F.D.A. guidelines and taking into account other criteria F.D.A. used for objecting the other notifications, this petition is being submitted for 21 USC 343 (w) (6) / 403 (w) (6) with all the required information and updates.

Also, please be noted that according to F.D.A. letter (e-mail) sent to Debbie Barry on October 7, 2005, stating that F.D.A. has not determined if starter growth media is considered part of FALCPA 2004. We have ample data (analytical, food challenge tests, statistical data, research data) to prove that cheese made with culture prepared using IMAC starter media with enzyme treated soy solids (as one of the ingredients), does not have any soy antigens and thus does not cause any allergenic response that poses a risk to human health. Since F.D.A. is giving

2006FL-0238 CP1

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provision to get an exemption under section 403 (w) (6), we have elected to submit this petition requesting exemption from the labeling requirements of section 403 (w) (1).

We are submitting the details on test procedures, analytical test results and supporting data to prove that cheese made using IMAC media with enzyme treated soy solids (as one of the ingredients), followed by fermentation does not have any soy antigens and thus does not cause an allergenic response that poses a risk to human health. Thus we request that IMAC media with enzyme treated soy solids which will be used to grow starter cultures and thus to manufacture cheese, be exempt form the labeling requirements of section 403 (w) (1), pursuant to section 403 (w) (6).

An original with three additional copies are enclosed for F.D.A. review. Please feel free to contact me at 303-337-4028 ext. 104 (office), or 303-944-5215 (cell) or drreddy@askimac.com. Please confirm the receipt of this petition and the allocation of starting date of the petition. Also, kindly inform us which internet site we have to look into regarding posting of this petition.

I want to thank you very much.

Yours Sincerely,

Mali Reddy

Mali Reddy, D.V.M., Ph.D.

PETITION FOR EXEMPTION FROM FOOD ALLERGEN LABELING ON THE FINISHED CHEESE, WHEN IMAC (INTERNATIONAL MEDIA AND CULTURES) MEDIA WITH ENZYME TREATED SOY AS ONE OF THE INGREDIENTS USED TO PREPARE CHEESE CULTURE TO MAKE CHEESE, UNDER 21 USC 343 (W) (6) / 403 (w) (6) FOR EXEMPTIONS FROM FOOD ALLERGEN LABELING. *

SUBMITTED BY:
INTERNATIONAL MEDIA AND CULTURES, INC.
1250 S. PARKER ROAD
DENVER, COLORADO 80231

* Earlier it was submitted as notification pursuant to section 403 (w) (7) of the food allergen labeling and consumer protection act. The notification has been designated FALN 003 and the assigned docket No. 2005 FL-0488. The submission was on November 23, 2005. The notification was objected, mainly on the basis it should have been submitted under section 403 (w) (6), but not under 403 (w) (7). Also, F.D.A. (Dr. Gottleib) pointed out the additional information needed with regard to the tests submitted in the notification, additional tests and clarification of statements, required to qualify for the exemption. Following F.D.A. guidelines and other criteria F.D.A. used for objecting the other notifications, this petition is being submitted for 21 USC 343 (w) (6) / 403 (w) (6) with all the required information and updates.

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2. SUMMARY

The food allergen labeling and consumer protection act of 2004 (title II of public law 108-282), requires all the food manufacturers to identify the presence of the eight major food allergens in their food label (22). Under this act, the common or usual name of the ingredient must include the food source from which the major food allergen is derived. The law is enacted to help the public identify the major allergens in the foods. It is to protect the public health because according to section 202 (1) (A) (B) approximately 2% of adults and 5% of infants in the U.S. suffer from food allergies and roughly 30,000 individuals require emergency room treatment and 150 people die, because of allergic reactions to food. Also, nationally the number of recalls rose to 121 in 2000 from about a 35 a decade earlier. However, according to FDA (51), it is estimated that only 0.2% of children and adults in the U.S. are allergic to soy. Based on this estimate, soy allergy appears to be less prevalent than allergies to other major food allergens, according to FDA. This law should help people to identify the allergenic ingredients in a food so that they do not consume such foods. Congress should be applauded for the passage of FALCPA, to eliminate unscrupulous manufacturers in the commerce, who do not have any respect to the safety of the public.

Congress should also be applauded for giving opportunity (by law) to the food industry to submit petitions under 403 (w) (6), to exempt a food ingredient described in section 201 (2) from the allergen labeling requirement, provided the petitioner provides scientific evidence (including the analytical methods used to produce the evidence) that demonstrates that such food ingredient, as derived by the method specified in the petition, does not cause an allergic response that poses a risk to human health.

Earlier on November 23, 2005, we have submitted a notification pursuant to 403 (w) (7) of the food allergen labeling and consumer protection act. The notification has been designated FAL 003 and was assigned docket No. 2005 FL-0488. The notification was objected by FDA mainly on the basis of submission under wrong category. According to FDA's objection letter (21), it is stated as follows: "FALN 003 argues that cheese and other dairy products made with IMAC starter culture

media may be safely consumed by soy allergic consumers. However, under 403 (w) (7), a notifier must demonstrate that the ingredient is the subject of the notification does not contain allergic protein. There is an alternative process (a petition process under section 403 (w) (6) by which a person may request that an ingredient be exempt from the allergen labeling requirements in section 403 (w) (1). The standard for exemption under this alternative process is that the petitioner must provide scientific evidence (including the analytical method used to produce the evidence) that demonstrates that the food ingredient (as derived by the method specified in the petition) does not cause an allergenic response that poses a risk to human health.

I want to thank FDA for pointing us in the right direction. FDA (Dr. Gottlieb, Deputy Commissioner for medical and scientific affairs) has raised several questions regarding the test details we have not submitted in the notification. He had an excellent point. We did not know how much detail was required by FDA. We are submitting this petition with all the details to demonstrate that the food ingredient we have used in our starter medium, upon the enzyme hydrolysis and fermentation, when used to manufacture cheese, does not cause an allergenic response that poses a risk to human health. We have also taken into account FDA objections on other notifications submitted under 403 (w) (7). The methodology we have used, the choice of the analytical tests, and the human food challenge test, we have conducted pursuant to this petition should be satisfactory to FDA. The previous objections of FDA (which are posted on internet) on FALN 001 and FALN 002 are summarized in the next section to compare the test procedures implemented by various notifiers (19,20).

3. Letter in response to objection regarding starter growth media FALN No.003 (docket No. 2005 FL-0488) Posted February 21, 2006 (not submitted before)

Dear Dr. Gottlieb,

I have received your communication regarding our docket 2005 FL-0488 (21). In the first place, I want to thank you very much for pointing out the pertinent points as it relates to the objection. Although we have all the data, we did not present it to make it clear. We are submitting the answers to clarify your objection, for further clarification. In your communication, page 2 paragraph 3, states that under section 403 (w) (7), a notifier must demonstrate that the ingredient that is the subject of the notification does not contain allergenic protein. Alternatively, a notification may contain a determination by FDA that the ingredient in question does not cause an allergic response that poses a risk to human health, under a pre-market approval or notification program under section 409 of the act. You have also stated that, there is an alternative process (a petition process under section 403 (w) (6) by which a person may request that an ingredient be exempt from the allergen labeling requirement in section 403 (w) (1). The standard for exemption under this alternative process is that the petitioner must provide scientific evidence (including the analytical method used to produce the evidence) that demonstrates the food ingredient (as derived by the method specified in the petition) does not cause an allergic response that poses a risk to human health. Importantly, FALN 003 was submitted as a notification under 403 (w) (7) and thus the notification must demonstrate that the ingredient does not contain allergenic protein.

Dr. Gottleib, thanks for pointing out the mistake we made. We should have submitted our petition under section 403 (w) (6) to demonstrate such food ingredient (as derived by the method specified in the petition) does not cause an allergenic response that poses a risk to human health. It makes sense because, the "soy solids" we have used in the procedure were coated with a high concentration of using turbulizer.

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got both my M.S. and Ph.D. Degrees at Iowa State University in dairy microbiology, specializing in various starter cultures and their bacteriophages. For your reference I am enclosing my resume (31). I was the first person in the U.S. to work on bacteriophages affecting streptococcus thermophilus and lactobacillus strains which are used in Italian type cheeses. Due to this research, starter medium to inhibit such phages, was developed. For my research work on dairy starter cultures, I was awarded Richard M. Hoyt memorial award (1974) by the United States National Milk production federation of Washington, D.C, at the annual conference of the American Dairy Science Association.

You have also raised a question, what are the effects of different cheese making conditions on the degree of proteolysis? Age reflects the degree of proteolysis, more age, more proteolysis. This is why, protein is broken down less in 6 months old cheddar than 18 months old cheddar. No matter what cheese it is, the coagulating enzyme used is rennet, (which is a proteolytic enzyme). The rennet will cleave both the milk proteins as well as soy proteins. It is a well known and established fact in the literature. In terms of degree of proteolysis, cheese starter cultures proteolyzes soy protein as efficiently as the milk protein, casein. We are enclosing our experimental data, to clarify this issue in this petition.

To answer your other questions, i.e. although the request for exemption under FALCPA refers to use of starter cultures grown in IMAC starter culture medium in cheese and other dairy products, the notification provides no information or data on the use of starter cultures in other dairy product other than cheeses. I totally agree with you. We have never used IMAC media in products such as sour cream, buttermilk, and yogurt. Consequently, it must be restricted to cheese only.

To answer the question on characterizing the starter media, we did not elaborate much because of the scope of notification. Generally the lactic type of cultures,

lactococcus lactis var lactis, and lactococcus variety cremoris require more phosphate in the medium because of their least calcium dependency for growth. High phosphate salts chelate the divalent ion calcium, so that phage cannot adsorb on to the bacterial cell wall. If you use more phosphate salts, you have to use more protein or hydrolyzed protein to support the growth of bacteria. In the first place, such phosphates are used in the media to slow down the bacteriophage. Unfortunately, phosphates are hard on the growth of bacteria because they are chelants. Phosphate tolerance of starter cultures vary from strain to strain, even though they belong to the same genus and species. Because of this variance in phosphate tolerance, the composition of various media varies. For example, Italian cheese type media needs phosphate up to 10 to 12%, whereas cheddar type media requires up to 18 to 20%. Accordingly, the protein (stimulant) has to be varied.

The starter bacterial cultures derive their nitrogen source from protein in the starter medium through proteolysis. To give an idea, if raw milk has over million organisms per ML, with a titratble acidity of 0.18, such a milk cannot be used for cheese making because protein is broken down. Where as, in bulk starter culture, the concentration of micro-organisms can get up to 100x107 to 10x109 per gram with a titratable acidity of 1.0 to 1.8. You can imagine how much protein is broken down to supply amino acids for the growth and multiplication of starter bacteria. The broken down protein is utilized by starter bacteria to build their enzymes, cell membranes and cellular structures. Generally starter bacteria go through 24 to 26 log cycles during the starter manufacture. A gram of liquid starter (7% solids reconstituted medium) will have 0.07 grams of total solids, of which protein will be a maximum of .021 grams or 2.1mg. It is calculated on the basis of dried starter culture medium having 30.0% protein. Twenty one milligrams of protein will be utilized to build 2 billion to 5 billion micro-organisms per gram. At that rate, each organism will end up using .000004 micro-grams of protein. Starter micro-organisms are biologically very active and their generation time is only 20 to 30 minutes. This is the reason why they can out perform the

growth of any pathogenic organisms, due to their extensive ability to break and utilize complex proteins, shorter generation time, and their inherent genetic ability to produce organic acids to lower the pH, and to produce other biological compounds which will reduce the O/R potential of the growth medium. This is also the reason why dairy starter cultures can transform harmful allergens to non-allergens. We are attaching the data on the growth and proteolysis by various starter cultures in IMAC media, in this petition.

Such starter culture when inoculated into cheese milk, will also go through 5 to 6 logs of growth. During this growth period some of the peptides, which come from liquid starter culture, will be further broken down to simpler amino acids due to liberation of exo and endo peptidases from the inactivated starter cultures during cheese making (44). The starter protein is completely broken down and gets rebuilt into different microbial proteins. During this process, perhaps some of the proteins present in starter media, which are antigenic to induce allergy in humans may be rendered non allergenic. The activity of the starter cultures, to break proteins and peptides is further augmented with the aid of rennet enzyme used in the cheese making. It is so true in the case of dairy lactic cultures because their casein (milk protein) breaking enzymes are plasmid controlled. As long as the organism can utilize simpler peptides and amino acids, the plasmids or extra chromosomal genes will not be switched on to manufacture proteolytic (caseolytic) enzymes. Even if there are any allergenic peptides left in the starter culture, they will be broken down to simpler amino acids during cheese making. This is the reason why, we could not detect any soy allergens in the cheese, using IMAC starter medium with enzyme (protease) treated and fermented soy solids. Under the circumstances, the allergenic proteins are rendered harmless, which is evidenced by the, fluro enzyme immunoassay, food challenge tests, and the wide statistical data. Turbulizing or micro-coating the individual protein units with proteolytic enzyme followed by fermentation is the main reason for the success of IMAC medium, to convert allergenic protein into harmless end product. This is evidence by 99.5% proteolysis at the end of enzyme hydrolysis

and culturing. Such medium, which is enzyme treated and cultured does not alter the quality of cheese in terms of taste, flavor, and characteristics such as stretch, mouth feel, and melt etc.

The reason we did not give the exact composition of the starter media in the notification because it is proprietary to each company. We are only asked to list the ingredients. However, I will give the approximate percentage of the ingredients. No starter media will have over 30 to 38% protein on a dry basis. If protein is too low, the cultures will not grow good because they need peptides and amino acids to grow and multiply. These proteins can be of milk origin, soy origin or combinations. Each media has to have significant amount of carbohydrate source, i.e. milk sugar and dextrose etc. The carbohydrate varies from 15 to 40% depends on the concentration of culture required. All these figures are well established in the art. In addition, starter media needs minor minerals such as magnesium, manganese, ferrous salt, etc. Also, media requires anti-caking agents such as cellulose, or corn flour or silicon dioxide to prevent clumping of the medium in the bags. Some media needs dispersing agents. If you add all these ingredients (in the dry media), it comes to 90% to 93% solids, depends on the moisture in the ingredients.

When once the intact protein is broken down in the starter tank, it is very hard to distinguish the origin of the protein. Since the notification is on allergen issue, we have tried to determine the presence of allergens in the cheese. According, to several allergy doctors (M.D.'s), the allergen detection (antigen) is what is required more than the peptide length etc. Even if the test is negative for antigens, they still would like to see if wide range of people who consume such product do not react to it (food challenge test). Finally, food challenge test is the proof of the pudding. That is the reason why we have given the amount of cheese made using our IMAC media and number of people who consumed it without any complaints. We were very proud to submit that data with facts and figures.

With regard to the last issue in your letter, i.e. specific details of the "allergy test", we are submitting all the details you have requested. We have submitted an article on Dolen, to give the principle behind the EIA enzyme immunoassay test. We are submitting all the details your have requested and you will be pleased. The origin of the serum used in our test was of human origin and was obtained by the National Jewish Hospital, Denver, Colorado. Infact, the allergy test was conducted at the National Jewish Hospital Immunology Lab.

With regard to your question regarding tests conducted by regulatory agencies, we at IMAC have listed soy as an ingredient in the starter medium. We told the plant people to point out the ingredients on media to the regulatory people. Several media samples were taken from the plant and also samples of cheese made with such media, for allergen testing by the Regulatory agencies. The regulatory agencies have indicated that they will get back to the plant people with results, indicating if results are positive for allergens they will be informed for further actions. Until they hear from agencies, they have asked the plant people to continue what they are doing. Plants never heard from them. Like any other government agencies, no news is good news. If government agencies found allergens in the cheese, plant would hear about it. Just like FDA notification, absence of objection in 90 days, is an indication that the ingredient is exempt from declaration according to FALCPA 2004. We were all taking the same approach. If we submit something and if we do not hear any response, for a long period of time and sometimes forever, it is taken for granted there are no problems or allergens in the cheese. Plant people knew the cheese was negative for soy antigens because they checked qualitatively using ELISA test (although FDA does not recognize this test fully). We were told all along, (and in act it is true) that starter media is a non-standard of identity product and it does not have any standards in the 21 CFR. It is true because the ingredients used in starters are not allowed in the standard of identity cheeses. Consequently, to eliminate confusion, the starter ingredients are not listed on the cheese label. It was perfectly ok with the government. With the introduction FALCPA 2004, the

major allergens issue came up. IMAC was able to gather all the information to submit to FDA the intent of FALCPA 2004 act is to protect the consumer. However, it also gave a provision that if the ingredient is rendered non-allergenic, it can get exemption. In our case, the soy protein is rendered non-allergenic. However, we did not submit all the details of the test we ran because we did not know how much detail was required. It was our fault. Now that FDA raised an objection on our notification, (mainly due to submission under wrong category) we will submit the information in detail. Dr. Gottlieb, if the allergen is absent in cheese and if we declare it like it has allergen, it is deceiving the consumer. Under worst conditions, only thing cheese manufactures has to do is to declare it as soy that is used in the cheese culture, on the finished cheese labeling. With that declaration, label will satisfy FALCPA 2004 act. However, customers will be more confused than before. We thought we would submit our petition, since the allergen is inactivated. On the same lines, several people in the industry are perplexed about yeast extract issue. Yeast extract may be a minor allergen. However, if the yeast is grown on wheat, which is a major allergen, it will have to conform to FALCPA 2004 law. It must be declared as wheat next to cheese culture. Nobody is talking about this. IMAC is the only starter company who came forward for notification. In fact, the IMAC procedure of treating the starter media with protease enzyme and then fermenting with culture, will also inactivate any wheat allergens present in the yeast extract. Although we did not check for wheat allergens in the media, it should work. If not, it is a step better than, not using protease enzyme.

Another issue is wheat grown spore cultures, used in blue cheese. Nobody's talking about this. Is this the reason why FALCPA 2004 did not consider starter as ingredients in the law? Please refer to the FDA letter of October 7th (23,24). If you check US dairy cheeses for wheat allergens, I am sure you will find them because of the yeast extracts in the starter medium. The only other alternative to eliminate these problems is to go back to non-fat dry milk base media. The problem here is controlling the phage and thus slows starters. Slow starters or

dead starters create public health hazards in terms of proliferation of deadly pathogenic bacteria in the cheeses. Meanwhile, we at IMAC ran several studies, chemical (proteolysis) immunological test (skin prick test, and fluro enzyme immunoassay) and food challenge experiments to prove to ourselves and the industry that the finished cheese made with IMAC starter (with hydrolyzed and fermented soy solids) did not excite any allergic reactions in soy sensitive human beings. I am sure U.S.D.A. did enquire with local health departments if they received any complaints from the public on the finished cheeses. It is a well known fact that the United States, FDA, and Department of Agriculture does an excellent job of monitoring the products to safe guard the health of the people. Also, as a reputable industry supplier, IMAC would not dare to supply anything to harm public health. The quality control departments of the cheese plants constantly monitor the customer complaints both in terms of quality as well as safety. They maintain a log for all the lot numbers of cheese. This is part of the recall procedure and the plant must be ready to act on. The recall procedures must be in place in every production facility. Every lot number of the media will be identified with the cheese produced, and such cheese with specific lot numbers will always be on records to identify the customer who purchased the product. The customer's inturn monitor the lot numbers to identify the end user of such product. All these procedures are more strictly followed in the industry, especially with homeland security, bioterrorism precautions and procedures. As a supplier, our shipments will only be accepted with the certificate of analysis. The U.S. system of monitoring the safe material movement from ingredients suppliers to manufacturers to end users, with the intervention of government agencies is flawless and commendable. These days, no way an unscrupulous ingredient will get through the system. We are also submitting food challenge test results of cheeses (made with IMAC starter media) fed to control group of people. The experiments were also conducted i.e. food challenge human experiments, using the laboratory simulated cheeses. Although these trials were done on limited people, the results proved that the cheese made with IMAC media with soy solids, (which is enzyme treated and then fermented), did not

induce any allergy even in the soy sensitive people. According to physicians who treat allergies, the best testimonial is the data we have gathered for the past 5 years i.e. 650,000,000lbs of cheese made with IMAC media (with enzyme hydrolyzed and fermented soy solids) fed to over 500,000,000 people with no single allergy complaint.

I hope, I have answered all your questions and concerns. Under the circumstances, we sincerely request that, you grant us exemption perhaps under 403 (w) (6) that food ingredient (soy solids in IMAC media and method of preparation of starter, cheese etc.) does not cause an allergenic response that poses a risk to human health, provided you are satisfied with our experiments and answers for your questions. Since we have submitted the information on November 23, 2005, we want our petition to be treated as an extension, if possible only. Please feel free to call me at 303-944-5215 (cell) or 303-337-4028 ext. 104 (office), for further questioning. I am also enclosing the letters we have sent to you and Ms. Geraldine June of FDA on 2/21/06 with regard to extension on notification. Upon understanding the details of your communication, we have decided to submit for petition under 403 (w) (6).

I hope, I have clarified several of these issues. Dr. Gottleib, you have brought up some very good points in the technicality of submission. The starter media, unlike ingredients such as hydrolyzed soy solids or extensively hydrolyzed casein solids etc., are exclusively used for fermentation only, along with other ingredients. They are not used anywhere else in the foods. They may have to be considered not as ingredient free from allergen as in the case 403 (w) (7), but as a food ingredient which does not cause an allergenic response that poses a risk to human health as in 403 (w) (6), especially on the basis of experimental evidence and extensive statistical data we have gathered, i.e. 650,000,000 pounds of cheese made using IMAC media with soy solids (using IMAC starter make procedure) and fed to over 550,000,000 people in the U.S. with no single complaint for a period of 5 years. We have even given the details on plant

names, geographic location, and telephone number of the contact person etc. All along we made a sincere effort to monitor diligently for any complaints or problems. I come from a medical background, and I was very particular about the health and hazard issues. We came to a firm conclusion through experimental results and statistical data that the cheese made with that IMAC media with soy solids (with IMAC procedure) does not cause any allergenic response that poses a risk to human health. Consequently, according to FDA suggestion, we have decided to submit (with more experimental data and finer details of the test methods), this petition under 403 (w) (6) for exemption.

Yours Sincerely,

Mali Reddy, D.V.M., Ph.D.

Note: This letter was not submitted before. It is attached in this petition.

IMAC International Media And Cultures
PIONEERS IN DAIRY & FOOD TECHNOLOGY

4

02/21/06

To:
Dr. Scott Gottlieb, M.D.
Deputy Commissioner for Medical and Scientific Affairs.
Food and Drug Administration.
5100 Paint branch Parkway
College Park, MD 20740.

From:
Dr. M.S. Reddy
IMAC, Inc.
1250 S. Parker Rd.
#203
Denver, CO. 80231.

Re: Docket number: 2005FL-0488

Dear Dr. Gottlieb,
We have received your letter by fax indicating that IMAC petition FAL 003submitted for exemption from food allergen labeling has been objected due to the lack of sufficient data with regard to Quantitive IgE assay demonstrating that the soy solids do not contain allergenic soy protein, no description of the methodology, detection limits, origin of sera etc. etc. We will be submitting all these details with the test run at National Jewish Hospital's immunology lab in Denver, Colorado. We will also be submitting other detailed information per your request. We could not give all the minute details because of the trade secrets involved, and also because of the posting of the notification. Now that you are requesting the details, we will be more than glad to submit to you. At this stage only thing we can tell you that the cheese is negative for soy allergens and thus does not cause an allergenic response that poses a risk to human health. Respectively we will be submitting the details of your request ASAP. Meanwhile, we request an extension on our petition with a written confirmation.

We are very happy that you have pointed out what you want. Now we can provide you the required details. Please be noted that according to FDA letter sent to Debbie Barry by Graciela Iguina on October 7th 2005, stating that FDA has not determined if starter growth media is considered part of FALCPA 2004. We did not have to file for notification as there are no set standards for the starters in the 21 Code of Federal Regulations, however, we have submitted for 21 U.S.C. 343(W)(7) because we had data

to demonstrate allergens are absent in the cheese. Now that you need the details on how the immuno lab at the national Jewish hospital ran the tests, we will be more than glad to submit the details of the test data to you. It is to be noted that all the starter companies are using yeast extract that might have been grown on wheat that is a major allergen, without having to declare wheat as an allergen on the cheese label. The same thing applies to blue cheese mold starters grown on wheat-based media.

Sincerely yours,

Mali

Dr.Mali Reddy. DVM, PhD.

Cc: Debbie Barry (F&A Dairy)
Dr. Ron Harbeck (Immunology Lab. National Jewish Hospital)
Geraldine June (FDA)

IMAC International Media And Cultures
PIONEERS IN DAIRY & FOOD TECHNOLOGY

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2/21/2006

From: Dr. Mali Reddy
IMAC 1250 S. Parker Rd., Ste. 203
Denver, CO 80231

To: Geraldine June
5100 Paint Branch Parkway
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Ms. June,

We are submitting our response to your letter dated 2/21/05, with regard to our petition FAL 003, docket No.2005 FL-0488. We are requesting an extension to submit the details that are requested. We appreciate Dr. Gottlieb's comments and it will help the situation, with the details we are going to submit. Meanwhile, we want to ask you a basic question i.e. According Ms. Graciela Iguina letter dated October 7th, Starter Media is not considered as part of FALCPA 2004. If so, do we have to declare any thing? Please comment.

What is the status on declaring yeast extract in starter medium as wheat? At present nobody is doing it. Does it mean it is ok to use wheat based yeast extract in the medium, without having to declare it? Who checks all the cheese for this allergen? Also, the wheat grown starter cultures (mold cultures) are not declared as wheat on blue cheese label. Is it because FDA has not determined if starter media for growing starter is not part of FALCPA 2004, according Ms. Graciela Iguina of FDA? Please comment. The main reason we are sending this letter is to request an extension to our petition, so that we can answer the questions raised on objection. Please give us a written confirmation.

Yours Sincerely,

Mali Reddy

Mali Reddy, D.V.M., Ph.D.

Cc: Dr. Scot Gottlieb, FDA
Ms. Debbie Garry, F&A Dairy
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6. FDA RESPONSE TO FALN 001

The following conclusions were drawn with FDA communication entitled "agency response letter objection regarding extensively hydrolyzed casein FALN No. 001(docket No. 205 N-0416)" (19).

1. It has been documented in the prior literature that "extensively hydrolyzed casein" did induce allergencity, which is evidenced by clinical reactivity in humans. According to FDA, an ingredient that does not contain allergenic protein would not be expected to provoke clinical reactivity in allergic individuals (page 1 of reference 19).
2. Extensively hydrolyzed casein has some amount of allergenic protein, consequently some milk allergic individuals exposed to "EHC" exhibited allergenic responses. In other words, FDA has pointed out that clinical evidence of allergic response to a food or food ingredient strongly suggests that allergenic protein is present in that food (page 2 of reference 19).
3. Extensively hydrolyzed casein is considered as "hypo allergenic ingredient". The American academy of pediatrics (AAP) defines "hypo allergenic" to indicate 95% confidence that 90% of allergic individuals will not react to a given formula. FDA is correct in saying that hypo allergic ingredient cannot be considered non allergenic because non allergenic protein should not exhibit any clinical symptoms of allergy in humans. However, the notification clearly stated that some individuals showed clinical symptoms of allergy when challenged with extensively hydrolyzed casein (page 2 of reference 19).
4. Crossed radio-immuno-electrophoresis (CRIE), study showed that IgE produced by milk allergic individuals does bind to EHC. The same authors stated that "the lack of CRIE scores does not necessarily reflect a complete lack of allergenicity". According to some other authors Dean et.al 1993 (1), Hoffman and Sampson 1997 (2), Docena et.al 2002 (3), the extensively hydrolyzed casein did bind to IgE in vitro, indicating it has antigenicity to elicit allergic responses. Conclusion, CRIE may not be valid to demonstrate lack of allergenic protein (page 2 of reference 19).

5. Skin prick tests are generally considered an indicator of the presence of IgE rather than an absolute diagnosis of clinical allergy. According to Bock 1989 (4), Saylor and Bahna 1991 (5), Sampson et.al 1991 (6), Schwartz 1992, Hill et.al 1995 (8), that extensively hydrolyzed casein, gave positive results with the skin prick test. Conclusion is skin prick test is not an absolute test to determine lack of antigenicity or allergenic protein (page 2 and 3 of reference 19).
6. According to FDA, animal studies of immunogenicity (sensitization of guinea pigs), may inform, but currently cannot replace, clinical evidence, because no validated animal model of allergenic potential in humans exists at the present time (Kimber et.al 2003) (9). FDA is taking the position that the best test to prove lack of allergenicity is the human challenge test (page 3 of reference 19).
7. Extremely small peptides are not immunogenic. Larger peptides with 10 amino acids or more may bind to antibodies and induce allergenic reactions in animals. There are very limited studies in the literature regarding antigenicity of peptides with 10 amino acids or more (Makinen-Kiljunen et.al 1993) (10). The only way to answer this puzzle is to use human antibodies and see if they bind to antigens. If they did not bind means, that the peptide fraction is non antigenic, even if it has 10 amino acids or more. The best test is to use human antibodies and also demonstrate such protein does not induce clinical symptoms in humans.
8. According to FDA, the "ELISA" test does not directly measure the proteins allergenicity in humans, especially when primary antibodies from another species (in this case, rabbit) are used, but they can be very sensitive assays for the detection and measurement of protein. In other words, FDA strongly recommends or prefers use of human antibodies in the vitro diagnostic test, to determine the allergenic antigen proteins.

7. The following conclusions were drawn with FDA communication entitled "agency response letter objection regarding extensively hydrolyzed casein FALN No. 002 (docket No. 2005 FL-0434)":

1. Extensively hydrolyzed casein had 1.7% of peptides with molecular weights in the range of 1200 to 1500 Daltons, (11), which corresponds to approximately 9 to 15 amino acids in length and therefore may represent immunogenic protein. The polyclonal antibodies are used in ELISA test to detect intact casein. However, the notifier did not demonstrate the efficacy of these antibodies for detecting partially degraded, but still allergenic protein. In other words, irrespective of the length of peptides, FDA wants a demonstration using human specific antibodies to prove that partially degraded peptides are non antigenic (20).
2. In vivo immunological assays in animals may be used to describe immune responses to specific proteins. However, according to Kimber et.al 2003 (9), these animal models have methodological flaws and are not widely recognized as sufficient scientific evidence that a protein is not immunogenic in humans.
3. According to Cordle et.al 1994 (12), a negative result would not prove hypoallergenic performance, using the animal studies for establishing the absence of allergenic protein in an ingredient. Also, the notifier did not demonstrate that extensively hydrolyzed casein does not bind IgE in sensitive individuals, according to FDA. Also, according to Schwartz et.al 1991 (13), and Ragno et.al 1993 (14), IgE binding does not necessarily predict clinical reactivity, and it only suggests that the protein present in the ingredient may be allergenic.
4. Hypoallergenic does not mean non allergenic. According to FDA, the notifier acknowledges that there are a very small number of exquisitely sensitive milk allergic infants who will not be able to consume a hypoallergenic formula, made with extensively hydrolyzed casein. FDA concludes it is evident on the basis of human studies that extensively hydrolyzed casein induces allergenic response in sensitive individuals and thus contains allergenic protein (20).

It is crystal clear from the FDA objection, that molecular weight alone or chain length of the peptide alone or number of amino acids in a peptide chain alone or lack of IgE binding to the peptides alone does not necessarily reflect a particular protein is absolutely allergen or non allergen. The best proof is challenging humans with a suspected allergen and see if it excites clinical symptoms. Of course, in addition to that, FDA insists that reasonable scientific evidence must be presented to prove a particular protein is a non allergen.

According to the notification FALN No. 002 (20) extensively hydrolyzed casein is used as a final ingredient in the formula at 17.5%. If it is going to be used as is in the final preparation, It must be non-allergenic because it is not going to be treated any further before ingestion. Assuming alimentum powder is diluted at the rate of 10% solids and fed directly to the babies, 100 grams of such liquid will have 1.75 grams of casein hydrolyzate. Whereas in our petition, soy solids in the starter medium with 30% soy protein, when diluted at 10% solids will have 3.0% protein. However, 1 gram of such liquid will have .03 grams of proteolyzed soy protein, which is added to 100 grams of milk. Perhaps, a maximum of .001 grams of such soy protein will end up in 10 grams of the finished cheese. However upon enzyme hydrolysis and fermentation 99.5% of the soy protein is hydrolyzed or proteolyzed resulting in a maximum 0.015% of the intact soy protein in the finished starter culture. Modified fluro enzyme immunoassay further proved that no soy antigen was detected in the finished cheese made with such starter. This is due to further enzymolysis due to rennet and proteolysis due to starter cultures in the cheese (in the later part we have presented a detailed explanation of this phenomenon). Whereas, 10 grams of alimentum of FALN No. 002, will have 1.75 grams of casein hydrolyzate which does not go through any more enzymatic treatment or proteolysis before consumption. Consequently, even if it has traces of detectable antigen, it will induce allergenicity in babies. This is the reason why FDA is insisting absolute lack of antigenicity if an allergeric protein has to pass as non allergen for 403 (w) (7). Whereas in our case, 10 grams of cheese will only have .001 grams of enzyme hydrolyzed and fermented soy solids. Even at 3% inoculation of starter into cheese milk, 10 grams of cheese will have a maximum of .003 grams of enzyme hydrolyzed and fermented soy solids. Even that .003 grams will go through heat denaturation, enzyme hydrolysis (protease) and fermentation (starter culture) in the starter tank, and in the cheese vat it will have to go through enzyme hydrolysis due to rennet action and further culture fermentation. Fermentation in the starter tank, induces proteolysis due to proteases of the starter culture. In addition, peptides will be broken down to smaller peptides due to bacterial endo

proteases. Such smaller peptides will be transported into the starter cells and gets metabolized to simpler amino acids due to intracellular exo and endo peptidases. These resultant amino acids will be utilized by starter cells to build up their enzymes and cellular structures for further metabolism and growth. The peptides are further broken down due to the powerful enzymatic activities of the liberated exo proteases such as amino peptidases, carboxyl peptides and di-peptidases of the lysed starter cultures during cheese making and curing (44,45). In additium, the rennet enzyme will be continuously proteoyzing the proteins during and after cheese making (42,44). At the end of these treatments, none of the original soy protein (especially allergens) will be present in the cheese, which is evidenced by proteolysis tests, fluro enzyme immunoassay, clinical trials, and wide range study of feeding 500,000,000 people with over 600,000,000lbs. of cheese made with enzyme hydrolyzed and fermented soy solids based starter culture, with not even a single complaint from health departments, customer, consumers or any regulatory agencies or from any research and quality control departments of the cheese producing facilities. It is to be noted that according to the latest communication from FDA (51), it is currently estimated that 0.2% of children and adults in the U.S. are allergic to soy (soy implies untreated or unaltered native soy). Based on this estimate, soy allergy appears to be less prevalent than allergies to other major food allergens. The research and Q.A. departments constantly monitor and test the products and they do inform and check with the government field inspectors. Consequently, our petetion has better validity to indicate that soy solids used in the IMAC starter media will not induce allergencity in humans. They are rendered non allergenic due to enzyme hydrolysis and fermentation both during the starter preparation and cheese making. These test results are presented in the later chapters of this petition.

8. PURPOSE OF THE PETITION

The purpose of this petition is to provide scientific evidence that cheese made with IMAC starter media, (which has soy as one of the ingredients), manufactured and prepared according to the method specified in this petition, does not cause an allergenic response that poses a risk to human health. The IMAC starter medium (with soy solids as one of the ingredients) is enzyme hydrolyzed and then fermented in the starter tank. After culturing, it is used as an inoculant to manufacture cheese. During the cheese make procedure additional enzyme hydrolysis of the starter proteins take place due to the use of normal cheese coagulant (rennet) and also due to extensive breakdown of peptides with the aid of extra and intracellular starter culture enzymes. Because of the sequence of these reactions, the soy allergens are rendered non allergenic and thus does not induce any allergy even in soy sensitive human beings. We are respectfully submitting detailed experiments, results, methodology of testing, food challenge tests, in vitro test, and the vital statistical data to demonstrate the absence of allergens in the cheese made with IMAC media. We are confident that FDA will be pleased with the details and grant us the exemption. This exemption will serve as a good purpose to strengthen the intent of the act (FALCPA 2004) (22).

9. RATIONALE FOR EXEMPTION

In the United States, for the past few centuries (starting 1600) the cheese is being manufactured using starter cultures. These starter cultures were isolated from the nature. They are considered GRAS, (generally regarded as safe), according to the U.S. FDA. These starter culture organisms are non toxic, non pathogenic and they can out compete spoilage and pathogenic organisms which can harm human being. Starter cultures were traditionally grown in milk media i.e. high heat treated cow's milk. Later, the industry has recognized that due to the day to day variance of composition of milk because of feeds, breed of cattle, and presence of inhibitory compounds etc, the fresh milk was not a good reliable medium to grow the starter cultures. In order to standardize this practice and to assure healthy starter cultures, dairy industry has shifted to usage of standardized reconstituted non fat dry milk (15%), to grow the starter cultures. The idea here is that such a powdered milk, can be tested ahead of time to check for the presence of inhibitory compounds, (which will inhibit the growth of healthy starters) to eliminate the starter failure.

A few years later, with the growth and mechanization in the dairy industry, the successful cheese making became hard due to frequent failure of the starter cultures. Later it was discovered during 1930's that starter cultures were destroyed due to infection by bacterial viruses i.e. bacteriophage, when grown in milk based media. Milk has high concentration of readily available calcium which helps the bacteriophage to infect and destroy the dairy starter cultures. To obviate this problem, the United States Department of Agriculture came up with an idea around 1960's to use phosphates in the bacterial medium i.e. Milk, to retard the bacteriophage. The idea here is to tie up the readily available calcium with phosphate so that bacteriophage cannot infect and destroy the starter cultures. Even though, the use of phosphate salts are not legal to use in the standard of identity cheeses, as outlined in the U.S. code of Federal Regulations, U.S. government granted an exemption to use such chelating chemicals in starter medium, without having to declare on the finished cheese. Consequently, according to the code of Federal Regulations, there are no set standards for either starter cultures or starter media (48). Also, the ingredients in the starter cultures or media do no have to be declared on the finished cheese label (47).

Thus, the ingredient label on cheese reads like this: milk, cheese cultures, calcium chloride, salt, and enzymes. Even though starter media has non fat dry milk, whey, whey protein concentrate, ammonium salts, potassium salts, calcium salts, phosphates, citrates, yeast extract or autolyzed yeast extract, magnesium salts, hydrolyzed vegetable proteins, soy solids, lecithin, cellulose, corn flour, silicon dioxide, starch (anticaking agents), (thickening agent), magnesium sulphate, manganese chloride, ferrous sulpate etc., none of the ingredients in the starter media, other than the non fat fry milk are approved to be used in the standard of identity cheese products. However, these ingredients are essential in the synthetic starter medium to support the growth and protect starter cultures. By law, these ingredients have to be listed on cheese label along with the starter cultures. Since it will only confuse the consumers, U.S. government has elected to exempt the listing of ingredients present in the starter cultures on the cheese label. This exception is given only to the starter medium and cultures, because during the preparation of starters, there is thermal, enzymatic, and acid hydrolysis changes which will render these ingredients harmless. This is the reason why, even though majority of the yeast extract or autolyzed yeasts extract is made using wheat grown yeast (brewers yeast), still it is not declared on the cheese label. According to FALCPA (2004) the labeling requirements for major allergens is established. The FALCPA amended the FDCA and deems a food misbranded "if it is, or it contains an ingredient that bears or contains, a major food allergen" unless the food is labeled in accordance with one or two labeling options (FDCA 403 (w) (1)). The first option is placing the word "contains" followed by the name of the food source from which the major allergen is derived immediately after or adjacent to the ingredient statement. The second option is including the name of the food source from which the major allergen is derived in a parenthetical note following the ingredients listing in the ingredient statement, except that such parenthetical listing is not necessary when the name of the food source appears as part of the listing of another major allergen in the food product.

A "major food allergen" is defined in the FALCPA as milk, egg, fish, crustacean shell fish, wheat, peanuts, and soy beans (FDCA 210 (qq)). A major food allergen also includes a food ingredient that contains protein derived from a food specified in paragraph (1), with the exception of highly refined oils and

ingredients that are exempt under the petition and notification procedures found in the stature (FDCA 201 (qq) (2)).

According, to FALCPA although yeast extract is not considered as a major food allergen (although it is an allergen), it will be considered as a major food allergen if yeast is grown on wheat and thus may have residual (irrespective of amount) wheat protein due to procedures of making yeast extract. Consequently yeast extract or whole cell yeast or autolyzed yeast extract should be labeled as wheat with in parenthesis, unless it is proven beyond doubt that such preparation using the specified methods and approved testing procedures proves to be non allergenic in human, through petition or notification. Majority of the yeast preparations are derived from brewers yeast which use wheat as a base. However, in the case of starter medium, yeast extract is an essential ingredient and nobody is declaring it as wheat or may contain wheat next to cheese cultures on the retail cheese label. Perhaps, it is due to the procedures used in starter making, culturing and cheese making, which will render wheat allergens into non allergens in the finished cheese. On this basis only, even soy solids were allowed in the starter medium. Soy solids are used as stimulants in the starter medium, just like yeast products. Tryptic soy agar (a universally approved and used in every bacteriology lab), to enumerate lactic cultures uses soy protein to support the maximum growth of these micro-organisms. Lactic cultures grow luxuriously in the soy based media.

Because, of these ambiguities, Ms. Debbie Barry of F&A Dairy of California, (who has manufactured over 200,000,000lbs. of cheese using IMAC media with soy solids as one of the ingredients along with yeast extract) wrote to FDA to inquire if soy solids present in culture have to be declared on cheese label next to cultures. This communication was sent to FDA on October 6, 2005 (23). Ms. Barry's letter to FDA read as follows, we are a cheese manufacturer, our starter media base is of hydrolyzed soy. We have tests to prove that there is no allergen in our product after processing. The question is, do we need to declare soy and if so, where can I send our research in order to prove no allergen exits? Thanks for you time. The reply was given by FDA to Ms. Barry on October 7, 2005 (24). The FDA reply read as follows: subject: Re: allergen labeling starter (growth) media; FDA has not determined if growth media used to make enzymes

are considered part of an ingredient under the food allergen labeling and consumer protection act of 2004 (FALCPA).

According to our legal counsel interpretation of the FDA letter, starter media is not considered as part of FALCPA 2004, and its status interms of declaration did not change, unless otherwise, it is amended later by FDA to consider under FALCPA. This is especially true because we have the test data to prove that no soy allergens are present in the cheese made with such starter medium, which has soy as one of the ingredients. In the past five years, over 600,000,000lbs. of cheese was manufactured in the United States, using IMAC media with soy solids as one the ingredients, and fed to over 500,000,000 people with not even a single complaint from health departments, clinics or customers etc. Perhaps, according to FDA, procedures used to make starters and cheese may alter some of the allergenic proteins (present in starters) to non allergenicity. Thus, it does not warrant declaring on cheese label next to starter cultures, provided these ingredients come from starter cultures only. According to a recent communication from FDA (51), it is currently estimated that 0.2% of the children and adults in the U.S. are allergic to soy (which is untreated or unaltered). Based on this estimate, soy allergy appears to be less prevalent than allergies to other major food allergens. Earlier we have submitted our starter make procedures to government officials and we did not receive any negative comments.

We are very confident that using our methods, that such soy solids used in IMAC media, when processed as starter cultures, and used in cheese making, did not have any soy allergens in the finished cheese and thus does not pose any risk to the human health.

Even though FDA has not determined if starter media as part of ingredient of FALCPA 2004, we have decided to submit a notification under 403 (w) (7). We have elected to do this because we have test data to prove non allergenicity of our product in the finished cheese. The mistake we made (which was pointed out by FDA) was that we should have submitted under 403 (w) (6), because it had to do with the finished cheese, rather than the starting ingredient (21).

We are respectfully submitting our petition on IMAC starter media with enzyme treated soy solids as one of the ingredients, along with detailed methods and testing procedures to FDA, to obtain an exemption under 403 (w) (6) from FALCPA. We are very confident and positive that our petition confirms to the requirements of FDA for exemption under 403 (w) (6).

10. EVOLUTION OF STARTERS, STANDARD OF INDENTITY OF STARTER CULTURES & MEDIA, GENESIS OF USE OF HYDROLYZED SOY SOLIDS IN IMAC MEDIA, STARTER MEDIA INGREDIENTS THAT ARE POTENTIAL ALLERGENS

10.1. *Evolution of Starters:*

Starter cultures are used in the cheese or dairy food manufacturing for the following purposes (44,45,46,32,27):

1. Production of lactic acid to assist in the coagulation by rennet.
2. Reduction or elimination of pathogenic bacteria.
3. Flavor production.
4. Proteolysis to improve the digestibility and nutritive value.
5. Reduction of allergenicity and/or intolerance of food, through proteolysis and/or enzyme hydrolysis of disaccharides.
6. Control of moisture in the cheese.
7. Reduction of lactose to reduce lactose intolerance.
8. Reduction of simple sugars, to improve baking characteristics of cheese on pizza and other baked foods.
9. Starter cultures have therapeutic effect, such as: reduction of cholesterol, cancer in humans, etc.
10. Preservation of the dairy products.

10.2 Standards of identity of starter cultures and media:

The starter cultures used must be safe, suitable, non pathogenic and must be GRAS (generally regarded as safe), according to the code of Federal Regulations.

The starter medium used to grow starter cultures must be made of food grade nutrients and chemicals that support the growth of starter cultures. The following ingredients are commonly used in the starter media in the United States:

1. Non-fat dairy milk---source of protein and carbohydrate.
2. Whey solids-source of protein and carbohydrate.
3. Hydrolyzed vegetable protein (HVP)-source of nitrogen and stimulant and improves cell numbers through protein buffering.
4. Autolyzed yeast extract---source of protein and stimulant (brewers and bakers yeast).
5. Whole cell yeast (generally substrate for growing yeast is not identified, it could be molasses and/or wheat and/or barley)-source of protein and stimulant (brewers and bakers yeast).
6. Soy solids (Hydrolyzed soy solids)-source of nitrogen and stimulant, improves starter cell numbers through buffering.
7. Yeast autolysate-(generally substrate for growing yeast is not identified, it could be molasses and/or barley and/or wheat solids)-source of nitrogen, and stimulant (brewers and bakers yeast).
8. Starch (source not identified)-thickening agent and suspender. Potato is an allergen.
9. Maltodextrin-bodying agent, nutrient, source of carbohydrate and also suspending agent.
10. Casein (rennet casein and/or acid casein)-source of protein, stimulant, and improves bacterial cell numbers because of protein buffering.
11. Casein hydrolyzate (rennet casein and/or acid casein)-source of protein, peptides, amino acids, and stimulates growth and thus improves bacterial cell numbers.
12. Whey protein concentrate (35, 50, 80% protein)-source of protein and stimulant, and helps to build cell number because of buffering.
13. Acid whey-source of carbohydrate and stimulant.

14. Delactosed whey-source of carbohydrate and stimulant
15. Sodium citrate (chemical)-buffer, chelating agent to reduce calcium to protect bacteria from phage. It also serves as substrate to produce flavor.
16. Disodium phosphate (chemical)-buffer, chelating agent, to reduce calcium to protect bacteria from phage.
17. Monosodium phosphate (chemical)-buffer, chelating agent, to reduce calcium to protect bacteria from phage.
18. Diammonium phosphate (chemical)-buffer, chelating agent to reduce calcium to protect bacteria from phage.
19. Magnesium phosphate (chemical)-buffer, phage protectant, used in internal media.
20. Glycero phosphate (chemical)-buffer, phage protectant, protects the bacterial cells from acid injury.
21. Lecithin-minute amount used as emulsifier, protects the bacterial cells from acid injury by protecting membranes.
22. Magnesium sulfate (chemical)-used as a minor mineral to stimulate bacteria.
23. Manganese chloride (chemical)-mineral supplements to help the growth of starter bacteria.
24. Guar gum (hydrocolloid)-suspending agent.
25. Ammonia gas-used as a neutralizer liquid starter to limit the acid injury to the bacteria in the liquid starter. Toxic if inhaled.
26. Ammonium hydroxide (50% conc. chemical)-used as neutralizer to reduce the acid injury to the starter bacteria. Toxic due to corrosiveness.
27. Sodium hydroxide (50% conc. chemical)-neutralizer, to reduce acid injury to the starter bacterial cells. It causes severe burns, if spilled on skin.
28. Potassium hydroxide (50% conc. chemical)-neutralizer to reduce acid injury to the starter bacterial cells. It causes severe burns, if spilled on skin.
29. Demineralized whey-nutrient.
30. Whey Permeate-nutrient and mineral supplement, (a waste product produced after removing protein and lactose off the whey).

31. Ferrous sulfate (chemical)-mineral stimulant.
32. Sodium aluminum phosphate (chemical)-buffer, chelating agent, to protect starter bacteria from phage.
33. Capsule encapsulating chemicals-to prevent solubilization at alkaline pH, used in internal media.
34. Silicon dioxide (chemical)-anticaking agent to prevent clumping of the starter media ingredients.
35. Cellulose-anticaking agent to prevent clumping of the starter media ingredients.
36. Corn flour and cellulose-anticaking agent to prevent clumping of the starter media ingredients.
37. Potato starch-anticaking agent to prevent clumping of starter ingredients. Although potato derivatives are not major allergens, they are registered as allergens.
38. Aluminum silicate-anticaking agent to prevent clumping
39. Hydrochloric acid (chemical)-used to lower pH of the starter.
40. Phosphoric acid (chemical)-used to lower pH of the starter.
41. Citric acid (chemical)-used as a substrate for flavor compounds.
42. Bread and crackers made of wheat and other grains are used as substrates to grow mold starters such as Penecillium roquefortii and camembertii. The mold hyphae and their spores are then dried and ground along with their substrate and added to the milk or cheese curd to make blue cheese.

None of the above mentioned starter ingredients except for non-fat dry milk are an approved ingredient to be used in the standard of identity cheeses. Since all these ingredients are allowed and are commonly used in the manufacture of starter cultures, according to 21 CFR there is not set standard of identity for starter cultures or media, and consequently they are exempted from listing on the finished cheese label (47,48).

However, U.S.D.A. has set rules not to exceed the usage of starter i.e. 3.0%, to eliminate unscrupulous acts (47). Apparently at 3.0% level, these non-standard of identity products do not pose a threat. Also, the heat treatments, and fermentation (enzymolysis and acid hydrolysis) given during starter making and

subsequent enzyme additions, fermentations, and aging encountered in cheese making, render these ingredients harmless and thus does not warrant declaration on the cheese label. Consequently, none of these ingredients have to be declared on the cheese label. Only declaration to all these ingredients is "cheese cultures". The reason for this type of declaration is very simple. Starter cultures, when grown in milk, or other milk solids such as whey, are susceptible to phage infection. A phage is a virus that kills the bacterial cultures. U.S.D.A. came up with an invention that was patented and was given as a non royalty free invention to the industry to protect the starter cultures with use of chelating salts in the starter medium. Unfortunately, these salts are not to be used in the standard of identity products such as cheese etc. However, an exception has been made. However, these chemicals (chelating agents) when used in the starter medium the starter bacterial growth was slow even though they were protecting bacterial cells from phage. Consequently, all other stimulants such as yeast extract, autolyzed yeast extract, whole cell yeast, hydrolyzed vegetable proteins, hydrolyzed soy solids, hydrolyzed casein, etc. were allowed to be used in the starter medium to stimulate the starter bacteria so that they can grow well in the presence of chelating salts such as phosphates and citrates. None of theses stimulants were to be used in the standard of identity cheeses as ingredients. To alleviate this, U.S. government made an exception to starter cultures and media and allowed all these ingredients to label as "cheese cultures" on the finished cheese, without having to declare these ingredients. This exception is given only to the starter medium and cultures, because the treatment given to manufacture starter renders these ingredients harmless. Consequently, there are no set standards for the starters in the code of Federal regulations (48), as long as the ingredients are listed on the starter medium bags for verification by the government agencies and also by the Kashurath organizations.

10. 3 Genesis of use of hydrolyzed soy solids in IMAC media:

About 10 years ago, dairy industry personnel start complaining about phosphates in the starter medium for two reasons.

1. Reduction of cheese yield because of the emulsification effect of phosphates on casein. Dr. Gary Richardson, Professor of Food Science, at the Utah State University reported this incidence.
2. Since phosphates are used at such high concentrations in the medium, cheese plants kept complaining about their sewer bills. City sewage department charges the plants on the basis of phosphate concentration in the plant discharge. Request came from Ms. Becky Delzer, (Graduate of University of Wisconsin) Manager, Dairy farmers of America plant at Juda, Wisconsin, and other cheese plant managers across the country.

At the request of the dairy industry, IMAC started a research project to come up with a solution to reduce the concentration of phosphates in the starter medium and at the same time reduce phage incidence. After few years of research, IMAC found that enzyme treated soy solids decrease the use of phosphates in the starter medium. At the same time, the usage of yeast extract is also reduced. Several people in the industry prefer hydrolyzed soy solids to yeast solids, ever since FDA announced the soy solids ability to decrease heart diseases, which is a major cause of death in the United States. The reason hydrolyzed soy solids help reduce the high use of phosphates in the medium is that they have inherent phosphate in the matrix. According to the literature and practicing allergy Physicians, yeast extracts, whole cell yeast, and autolyzed yeast extracts etc. have histamine, which excite hyperallergenic reaction. IMAC has thoroughly studied over years, and developed the methods to hydrolyze the soy solids without imparting soy smell to cheese and at the same time making them non-allergenic. IMAC succeeded in accomplishing both and used hydrolyzed soy solids as an ingredient in the starter medium. Excellent quality cheese was made and the failure of starters have been significantly reduced. We have labeled enzyme treated soy solids on our starter medium and we

have informed F.D.A. and U.S.D.A. personnel through cheese plant management. Government agencies took the samples of cheese made with soy as an ingredient in the starter and apparently found no allergens or any health hazardous compounds to warrant declaration on cheese. We were told, as long as we use them in the starter medium, we were in compliance. Apparently, because the treatment we gave to prepare the medium and subsequent fermentation will render the hydrolyze soy proteins non-allergenic. Over 600 million pounds of cheese manufactured and distributed throughout the United States and over 500 million people consumed the cheese and dairy product made with IMAC starter medium with hydrolyzed soy as an ingredient, with no single complaint in terms of allergens. Thus we have accomplished the decreased use of phosphates in the starter medium to reduce the environmental pollution and to improve the quality of highly functional starter. Lately phosphates have also been blamed for the high rise of osteoporosis disease and for excess obesity in the United States. IMAC made a significant progress in this arena through research and development to reduce usage of phosphates in the starter medium. Enzyme treated soy solids are only one of the ingredients in IMAC medium. We are listing our starter making procedure, culture preparation, geographic distribution of cheese made with IMAC starter medium with enzyme treated soy solids etc. It is conceivable from the report that enzyme treated soy solids used in the starter medium, and thus used in cheese making are rendered non-allergenic. We are also supplying the references of the plants, and medical authorities we have consulted on this subject. It took us almost 10 years to verify this fact, through laboratory and field trials. We want to thank the Dairy industry for asking us to help. MAC is proud to help and service U.S. Dairy industry through research and development, by conforming to the regulations outlined in code of Federal regulations of the United States of America.

10.4 Starter media ingredients that are potential allergens:

1. Yeast Extract

In the dairy industry, it is a common practice to use autolyzed yeast and/or autolyzed yeast extract and/or whole cell yeast as one of the ingredients in the starter medium to grow the starter bacterial cultures. The grown starter medium that has yeast derivatives (autolyzed yeast and/or autolyzed yeast extract and/or whole cell yeast) is added to the milk to make cheese. Let us look into the manufacture of yeast extracts. According to the health authorities, yeast is an allergen, however, it is not a major allergen (top 8). Yeast is grown on either barley and/or wheat, during the manufacture of beer. The yeast cells are separated from beer (brewers yeast) and are used to manufacture yeast extract. Wheat is used in the beer manufacturing to aid in the head retention and also as a nitrogen source to grow yeast. If wheat is used to grow yeast, the subsequent yeast extract made of such wheat-impregnated yeast must also have wheat allergens. Since wheat is a major allergen (22), the yeast extract must also be declared as an allergen on the cheese label. Unfortunately, yeast extract is used in every brand of starter medium in the U.S. that is subsequently used to make cheese. Apparently the allergens in the yeast extract must have been rendered non-allergenic with the procedures employed in the culture growth manufacturing such as heat treatment, fermentation (enzymolysis and acid hydrolysis). The same will be true with the hydrolyzed soy solids used as stimulants in the starter medium. Over 600 million pounds of cheese is made and fed to over half a billion people, without any complaints. If hydrolyzed soy solids have to be declared on the label of the finished cheese along with cheese starters cultures, yeast extract produced by growing yeast on wheat based or wheat contaminated medium must also be declared because wheat is a major allergen according to FALCPA 2004 act. Unfortunately these stimulants, either yeast extract and or hydrolyzed soy solids are essential for the growth of starter cultures in the medium, especially phage resistant medium without which any active starter cannot be made. If starter is not active the resulting cheese will be of inferior quality. Thus, we request, honorable Secretary to exempt declaring the enzyme treated soy solids used as an ingredient in the starter medium in the finished cheese. It should be treated just like yeast extract or whole cell yeast, which are used as stimulants in the starter medium, to make cheese. Of course, we are

providing scientific data to prove the non allergenicity of such solids in the finished cheese.

2. Mold Starters

Similar scenario exists with blue cheese mold starters. Blue cheese is made using approved mold starters such as Penecillium Roquefort and Penicillium Camermbertii. These mold starters are grown on moistened bread made of wheat. After complete growth on bread, the entire bread is crumbled along with mold mycelia and spores. The entire preparation is called mold starter and is used to inoculate either milk or cheese curd to manufacture blue cheese and blue type cheeses such as Roquefort, Gorgonzola, Stilton etc. Camerbert and Brie cheeses are also made using mold as starter. Molds are used in these cheeses to impart characteristic mold flavor. Hence, wheat is a major allergen, such starters must also be declared as having wheat as an ingredient on the cheese labels. However it is not declared. Also, for the times immemorial, this practice is followed throughout the world and several millions, if not billions of pounds of cheese is made and fed to people. To date nobody complained about allergic responses in these mold fermented cheeses due to wheat solids. Apparently the wheat allergens also rendered non-allergenic with the growth of starter molds and later due to rennet enzyme, and lactic culture fermentation in milk during cheese making and ripening and acid in cheese due to both lactic acid and free fatty acids. Once again it goes to prove that the allergenicity of proteins apparently gets inactivated when they are fermented with dairy starter cultures.

3. Lecithin

It is a phospholipid. It is one of the starter medium ingredients like yeast extract and hydrolyzed soy solids. It is used in limited amounts. It is an emulsifier. It protects the bacterial cell walls from acid liberated by the bacteria. It is soy derivative. However, it undergoes all the treatments with the rest of the ingredients of the starter medium and renders non-allergenic. It is also to be noted that according to a recent communication from FDA (51), it is currently estimated that 0.2% of the children and the adults in the U.S. are allergic to soy (which is untreated or unaltered). Based on this estimate, soy allergy appears to be less prevalent than allergies to other major food allergens, according to FDA.

According FDA there are only few allergy related complaints related to soy lecithin, despite its wide spread use in the U.S. food industry. Consequently April, 2006 FDA has given a special consideration and limited exemption depends on the mode of usage of soy lecithin (51).

Dairy starter cultures are associated with plant and vegetable matter in nature. Their proteolytic enzymes apparently inactivate allergens provided the allergenic protein is denatured with heat and/or acid. Hydrolyzed vegetable proteins, soy solids have been used as starter culture growth stimulants. They did not emit allergic reactions, because of denaturation of proteins due to enzymolysis and acid hydrolysis during starter fermentation process as well as the process in the cheeses manufacturing (rennet enzyme, bacterial enzymes, acid production, and O/R reduction potential etc.).

11. IMAC (MEDIA) PRODUCT SUMMARY

IMAC produces several media as outlined in the IMAC brochure.
They are eliminate medium (Italian type), eradicate medium (American type) and enhance medium (Specialty culture medium). The IMAC brochure is enclosed for reference (25).
Eliminate medium: This medium is designed for growing streptococcus thermophilus (coccus) and lactobacillus bulgaricus (rod) cultures, to be used in the manufacture of Italian type cheeses such as mozzarella, provolone, parmesan, and romano etc. It can also be used in Swiss cheese manufacture. Two types of eliminate media are made, one series does not have enzyme treated soy as a one of the starter ingredients and the other one does not. The soy solids are either enzyme hydrolyzed and spray dried or enzyme treated with high concentration of enzyme by coating the enzymes using lecithin as a base with the aid of turbulizer. Eliminate media, in addition to high heat treated soy solids has whey solids, non fat dry milk, autolyzed yeast extract, glucose, mono and disodium phosphate salts, anticaking agents, and other minor minerals such as magnesium sulphate, manganese chloride and ferrous sulphate, as stimulants. The medium is dry blended using the above specified ingredients and coated with protease enzyme (neutrase) (17) and cellulase enzyme (celluclast) (18), prior to bagging.

Industrial culture preparation using IMAC media:

The cultures medium (eliminate) is reconstituted at 7% to 12% solids using tap water at 60F to 80F. The medium is generally made at a quantity of 500 or 1000 or 2000 gallons. The medium is cooked to 180F to 190F. Generally it takes a minimum of 1 hour 15 minutes, to 1 hour 45 minutes before the temperature reaches to 60C (140F). On average, the medium will be held a minimum of 1 hour before the temperature reaches to 120F. Plants are instructed to maintain this protocol when using IMAC media. The nutrase enzyme starts to proteolyze the soy proteins immediately after reconstitution at temperature of 50F. The maximum activity of enzyme is at 110F to 120F. Enzyme continues to act until the temperature reaches to 160F, (which is the pasteurization temperature), after which the enzyme gets deactivated. At this stage, protein hydrolysis due to proteolytic enzyme is 54%, according to dye binding test. Similar pattern is

obtained with cellulase enzyme. This enzyme will break cellulose in soy to fermentable sugars and also it will reduce the viscosity of the medium to enhance denaturation of proteins. Proteins start to get more dentured with high heat treatment, until temperature reaches to 185F to 190F, and also during holding at that temperature for 45 minutes to 1 hour. Then the medium is cooled to 108F to 113F (set temperature) and inoculated with the starter cultures strains of streptococcus thermophilus and lactobacillus bulgaricus. These organisms have symbiotic relationship and they grow fast in the culture medium. Generally it takes 8 to 10 hours of incubation compared to 16 hours with lactic cultures. Liquid starter media can be neutralized (while the culture is growing) at pH 5.2 to 6.2 using sodium or potassium or ammonium hydroxide, to improve the growth. This is called one step neutralization. It can also be neutralized two to three times. If 15% solids are used, the medium does not need neutralization because of the buffering. Yet some plants prefer to neutralize the starter, even if they are using high percentage solids. This is mainly to eliminate acid injury to starter cultures. With the eliminate medium, external pH control system can also be used to improve the cell numbers, using gaseous ammonia injection. IMAC also has another version called eliminate internal media, which does not need any neutralization even with low percentage solids. All these variations are practiced in the plants according to their requirements. The USDA and local dairy state inspectors monitor these systems. All these systems have to confirm to OSHA and Kashurath requirements. After the completion of growth, the medium is cooled to 40F to 60F, and held at that temperature until it is used in the cheese vats. Generally the pH of the cultured medium before adding to the cheese milk is anywhere from 4.6 to 5.0, depends on the type of neutralization used. Generally neutralization procedures, through improved bacterial cell numbers and proteolytic enzymes, tend to proteolyze the proteins of the starter medium more than the conventional procedure of non neutralization. According to the proteolysis data, cultures proteolyze 46% of the starter proteins. Thus, greater than 99.5% of the soy protein is proteolyzed in the starter culture (due to enzyme hydrolysis and fermentation), prior to use in the cheese milk. All this data is presented in this petition.

The above culture is used to make cheese using standard procedures outlined by code of Federal Regulations of the United States of America (49). During

cheese making, a proteolytic enzyme rennet is used to proteolyze and thus coagulate milk protein i.e. casein. This enzyme also proteolyzes the soy protein and its peptides (data is presented in this petition). Also, the starter culture added to cheese milk will proteolyze casein and starter solids (soy solids etc.) to simple peptides and amino acids, with the aid of starter extracellular proteases and intracellular exo and endo peptidases.

Eradicate medium:

It is designed to grow lactic cultures for making cheddar and other American variety of cheeses. One version of this medium has enzyme treated soy solids as growth stimulants along with whey solids, non fat dry milk, yeast extract or autolyzed yeast extract, glucose, Di and mono sodium phosphates, anticaking agents to prevent sticking, minor mineral stimulauts such as magnesium sulphate, manganese chloride and ferrous sulphate etc. The eradicate medium has more phosphates than the eliminate medium (Italian type) because the phages (viruses) which attack the lactic cultures do not require much calcium to adsorb to cell walls of the bacterium. Consequently, this American medium needs more stimulants to grow. These are all the known principles in the starter industry. The reconstitution, heating and holding temperatures are same as eliminate media. The set temperature is from 85F to 95F because the lactic cultures are mesophiles and they cannot withstand high temperature. They are more proteolytic than the thermopilic starters. These media can also be prepared at different solids, using one step or multiple step neutralizations, external pH control systems using ammonia gas, and internal pH control systems with more inherent buffers.

Enhance Medium:

This is used to grow additional cultures or secondary flavor producing cultures to manufacture either Italia type or American type cultures. The primary media is intended to grow the principle acid producers. If the cheese requires more flavor, these flavor type cultures cannot be grown along with the primary media because of the incompatibility in the associate growth patterns of the starter cultures. This medium has variations in the buffer levels and flavor precursors such as citrate salts to accommodate the growth of secondary starter cultures intended for special functions such as flavor, reduction of browning etc. Again these are all

well established procedures in the dairy industry and are approved by USDA (47).

No matter how many types or kinds of starters are used to make a particular variety of cheese, the total usage of the culture into cheese, cannot exceed 3% according to USDA (47). They (USDA) strictly follow these guidelines. For example: Swiss cheese is made using four different cultures i.e. streptococcus, thermophilus (coccus), and lactobacillus bulgaricus (rod), grown in eliminate medium, lactic cultures grown in eradicate medium and propionic acid cultures grown in sodium lactatic broth or non fat dry milk fortified with yeast extract. Generally starter media are used at 0.5% to 1.25% inoculation rate into cheese milk (47). If the starter is too slow due to phage or due to unknown inhibitors in the milk, starter usage may be more to finish up the cheese making successfully. It happens occasionally, as in the case of any fermentation processes.

Whenever enzyme treated soy solids are used in the starter medium, the percentage usage of phosphates can be significantly reduced. Cheese plants prefer to use fewer phosphates in the starter medium, because the sewer discharge will have fewer phosphates due to discharge of starter solids upon washing. They get fined severely by the city municipalities on the basis of concentration of phosphates in the discharge. The department of natural resources (DNR) does not like the discharge of phosphates onto the soil, because of eutophia, which will reduce oxygen in the air and water. Cheese plants that discharge more phosphates onto the ground get severely punished by the law. Unfortunately some amount of phosphate salts have to be used in the starter medium to protect the cultures form bacteriophage infections.

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Generally proteins are hydrolyzed using one of these three procedures (43):

1. Chemical hydrolysis: Acid hydrolysis is used to produce commercial protein hydrolyzates. In this instance proteins are heated at 110C in 6N HCL for 24 hours. However, acid hydrolysis has disadvantage in that it takes long time and also it destroys tryptophan, glutamine, and asparagines and other amino acids. Such a nutritionally deficient hydrolyzate does not support the growth of micro-organisms to the fullest extent. Also, acid hydrolysis results in the production of humin or humin like material which will impart chemical and physical defects in cheese. These types of hydrolyzates are good to use as flavor enhancers in food, but not as fermentation bases.
2. Alkaline hydrolysis: This type of hydrolysis is used as an alternative to acid hydrolysis in certain application. It can be achieved using sodium or potassium hydroxide with heat. The main disadvantage with this system is that it induces racemization and also it will completely destroy certain amino acids. Consequently, such a hydrolyzed soy protein is unsuitable because it will not support growth of starter cultures. Perhaps, it may be alright in other food applications.
3. Enzymatic hydrolysis: It is the most ideal way to hydrolyze proteins. Enzyme hydrolysis gives high yield of peptides and avoids non-hydrolytic alteration or destruction of end products. There are a number of proteolytic enzymes available in the market. It is the matter of choice for a particular application. Papain is widely used for proteolysis. However, in the modern, industrial applications microbial proteases are preferred because of their wide Ph optima, substrate specificity, and high activity. This is the reason why dairy starter culture proteases work excellent with soy proteins.

It is well known in the literature that proteolyitc enzymes can be used at a rate of 360:1 protein substrate to enzyme ratio to induce proteolysis at the room temperature to 37C in 1 to 6 hours time. It produces only up to 50 to 70% of the

hydrolysis. Further hydrolysis should be accomplished using peptidases and more especially amino peptidases. Using this two stage enzymatic procedure, the complete protein hydrolysis can be obtained almost to the extent of acid hydrolysis, without destroying the amino acids or altering the physical and chemical characteristics of the finished product (42).

In IMAC medium, the soy solids (proteins) are treated with protease enzyme through micro-coating the enzymes on to substrate with sufficient protease amount. Since the soy solids are at 8% or $\geq$ 8% moisture, and the mode of application of enzymes is in liquid phase, the protease enzyme will start proteolyzing the protein at the room temperature. In addition to the liquid phase enzyme, a small fraction of freeze dried protease enzyme is also included in the media. Freeze dried enzyme is not turbulized and is granular. It will stay in the matrix until it is reconstituted into liquid starter. Even the turbulized protease will work faster along with freeze dried protease enzyme on starter proteins, when once it is reconstituted with water. These enzymes work at a relatively faster rate in the liquid starter media because of optimum pH and temperatures maintained during starter manufacturing. These enzymes work until temperatures get up to 160F and then they start getting deactivated. At this stage, perhaps already over 50% of the soy protein is hydrolyzed. Our experiment proved that 54% of the soy protein is hydrolyzed with such enzyme treatment. After the starter tank is heated, and cooled to room temperature, the appropriate starter culture is inoculated. Once again, the starter culture proteolyze the intact proteins further to simpler peptides and amino acids. Our experiments proved cultures proteolyze 46% of the soy protein. It is clearly written in the literature and well established in the art that microbial protease works better than the vegetable or animal proteases. The cheese starter culture organisms have proteases (which induce proteolysis), extra cellular peptidases, and intracellular peptidase enzymes (exo peptidases such as amino peptidase, carboxyl peptidase and di-peptidase etc.). The extra cellular peptidase breaks the peptides to amino acids, during the growth of the culture. As the organisms go through several generations of growth, some of the organism die and liberate the powerful

intracellular enzymes, especially amino peptidase enzymes. These enzymes convert peptides to simpler amino acids, much more actively that the starter extra cellular peptidases. These peptidase enzymes are quite stable under reduced conditions, which exist in the grown starter culture.

When such a starter is added to the cheese milk, the protease enzyme once again activates at the ripening temperature of the cheese milk and starts proteolyzing the proteins and peptides of both the starter as well as milk further. Simultaneously, the rennet enzyme added to the milk, as a coagulant, will also start proteolyzing the proteins, thus inducing further degrees of proteolysis. As the cheese making progresses, the extra cellular peptides will start hydrolyzing the peptides further. After the cooking step and after salting during cheese making, some of the starter organisms will be killed due to heat, salt, and acid in the cheese, liberating the most powerful intracellular exo and endo-peptidases such as amino peptidases etc. which will further break peptides to amino acids. This is where, the flavor of the cheese start to develop. The sequence of protein hydrolysis during starter preparation, culturing, and cheese making, is as follows:

1. Proteolysis in the dry starter medium due to addition liquid proteolytic enzyme.
2. Proteolysis in the liquid starter medium due to added proteolytic enzymes proper time, temperature, and pH.
3. Proteolysis in the liquid starter due to culture growth (20 to 24 cycles of growth).
4. Action of bacterial peptidases in the starter culture.
5. Breakdown of protein with the introduction of another proteolytic enzyme (rennet), during cheese making.
6. Proteolysis due to starter culture growth during the cheese making (protease and exo peptidases).
7. Liberation of endo peptidases from starter cultures, after cooking and salting step during the cheese making. These endo peptidases further cleave longer peptides to smaller peptides and amino acids.

8. Continued action of rennet enzymes, bacterial protease, and peptidases during cheese ripening.

A detailed description of these sequences is presented earlier in tables. Through starter culture addition, a small amount of protein is added to the cheese milk and even that gets converted to amino acids. The final broken down peptides and amino acids in cheese are indistinguishable from which source they came from. Because of this complex thermodynamic effect, the soy proteins are rendered non-allergenic in the cheese (this is the reason why we have elected to check allergen in the cheese). It goes to prove why, not even a single complain came from the field, when 650,000,000lbs. of cheese (made with enzyme treated and fermented media with soy) was fed to over 500,000,000 people in the past 5 years in the United States alone. It is very hard to determine and distinguish, the peptides and amino acids of soy in the cheese, because the peptides and amino acids are also produced due to casein breakdown. However, human studies and antigen detection test, proved beyond doubt that such an allergenic proteins are denatured and hydrolyzed due to added proteolytic enzymes (protease and rennet), and proteases, peptidases, and amino peptidase of starter cultures. It is surprising to note in our study that, when the protease enzyme is not used to hydrolyze the soy protein in the starter medium, bacterial fermentation alone did not inactivate all the allergens in the cheese, (although most of them are inactivated). Surprisingly, even such cheese did not induce any allergic reactions in the soy allergic people. It only indicates that such a weekend antigen perhaps is easily hydrolyzed in the human body due to further proteolysis in the stomach and in the upper gastro-intestinal tract, rendering itself into non-allergen. However, the current IMAC systems of making starter, using soy solids treated with proteases enzyme (proteolyzed in the dry starter medium plus proteolyzed during starter making), starter culture growth in such starter medium, with further proteolysis and peptide hydrolysis due to starter microbial proteases, peptidases (extra cellular and intracellular), and exo proteases such as amino peptidases, carboxyl peptidases and di-peptidases, and further growth of such starter culture

during cheese making inducing more proteolysis, followed by proteolysis due to rennet, and peptide hydrolysis due to liberation of endo and exo peptidases of starter cultures, deactivates the soy allergens in the finished cheese. This entire sequence makes the soy proteins non allergenic and thus will not induce allergy in soy sensitive human beings. It is to be noted, that starter is only a small fraction of ingredient in the cheese making. It is not a major contributor of nutrients. The average industrial starter usage is 0.25% to 0.75%, more in the tune of 0.5%. The only safe way to use soy solids as stimulants for growing starter cultures is through IMAC system of treating with proteases, culturing, and then using such culture in cheese making. Even if yeast extract happen to have some allergens in the starter medium (wheat or yeast proteins), IMAC system should inactivate such allergens, so that the cheese is safe to consume.

16. THE FOLLOWING EXPERIMENTS WERE CONDUCTED CHRONOLOGICALLY, TO PROVE THE NON ALLERGENICITY OF CHEESE MANUFACTURED USING THE IMAC MEDIUM WITH ENZYME TREATED SOY SOLIDS AS ONE OF THE INGREDIENTS

16.a The ability of different cheese starter cultures to grow (proteolyze and metabolize) on soy protein vs. casein as a main source of protein in the starter media and their subsequent growth and acid production in milk (activity test):

Three different starter culture media were formulated using three different sources of protein as sole source of protein. Medium 1 has soy protein, medium 2 has soy protein treated with protease and cellulase enzyme, and medium 3 has sodium caseinate (purified milk protein-casein). Since soy protein concentrate has 60% protein, it is formulated in the media to arrive at 30% protein. Since sodium caseinate has 85% casein, it is used at 35% concentration to arrive at 30% protein. The soy protein concentrate was treated with protease enzyme to arrive at roughly 0.1% protease and .02% concentration of cellulase enzyme. The later preparation was referred to as enzyme treated soy protein. The compositions of three media are as listed in tables 1, 2 and 3.

Table 1: The composition of medium with soy protein as sole source of protein.	
Dry Ingredient	**Percentage**
Soy protein concentrate	50
Mono sodium phosphate	1.97
Di-sodium phosphate	2.0
Lactose	24
Glucose	17
Magnesium sulphate	.01
Magnesium chloride	.01
Ferrous sulphate	.01
Calcium chloride	2.0
Autolyzed yeast extract	3.0

Table 2: The composition of medium with protease and cellulase enzyme treated soy protein as sole source of protein.	
Ingredient	**Percentage**
Enzyme (protease and cellulase) treated soy protein Concentrate	50
Mono sodium phosphate	1.97
Di-sodium phosphate	2.0
Lactose	24
Glucose	17
Magnesium sulphate	.01
Manganese chloride	.01
Ferrous sulphate	.01
Calcium chloride	2.0
Autolyzed yeast extract	3.0

Table 3: The composition of medium with sodium caseinate as sole source of protein.	
Dry Ingredients	**Percentage**
Sodium caseinate	35
Mono sodium phosphate	2
Di-sodium phosphate	2
Lactose	39
Glucose	17
Magnesium sulphate	0.01
Manganese chloride	0.01
Ferrous sulphate	0.01
Calcium chloride	2.0
Autolyzed yeast extract	3.0

All the media were reconstituted at 7% solids in distilled water. The pH was adjusted to 6.5 and the liquid medium was dispersed in 100ml quantities into bottles. These bottles were heated to 180F to 185F, and held at that temperature for 45 minutes. The bottles were cooled to appropriate incubation temperature. Thirteen one hundred ml bottles were prepared out of each medium. Thus a total of 39 bottles were prepared. Bottles of each of medium were inoculated with individual lactic culture. A total of three single strains lactic cultures, one strain of lactococcus lactis var lactis, 2 strains of lactococcus lactis var cremoris were inoculated in duplicates into six bottles (100ml medium/bottle). These lactic cultures were designated A, B and C. Three different media were designate 1, 2 and 3. Milk grown lactic cultures were inoculated into the heat treated medium at the rate of 1%, and the bottles were incubated at 90F, for 16 hours. At the end of the incubation, pH was determined. Also, the cultured media were plated on tryptic soy agar and the plates were incubated at 32C for 48 hours, to determine the total count of bacteria. The smears of the media were prepared by staining with methylene blue to determine the density, chain lengths, and morphology. To determine the activity tests, the cultures grown in test media, were inoculated at the rate of 2% into sterile 11% reconstituted non-fat dry milk. The inoculated milk was incubated at 90F for 45 minutes. Then the inoculated milk was gradually heated up to 102f with in a span of 30 minutes (this is comparable to cooking in the cheese vat). After it attained 102F, it was held at 98F for an additional period of 2 hours and 15 minutes. At the end of this period, pH was determined. Also, simple staining procedure was used to determine the morphology of grown starter cultures.

Similar experiments were conducted with media 1, 2 and 3, which were reconstituted at 7% solids. Here three sets of different streptococcus thermophilus (coccus) and lactobacillus bulgaricus (rod) were prepared. Each set of mixed culture was inoculated into heat treated media at 1% level and the media was incubated at 108F for 8 hours. A the end of the incubation, pH was determined. The cultures were plated on tryptic soy agar and incubated 4 days at 37C, to determine the total bacterial count. Also, microscopy was conducted to determine the coccus to rod ratio i.e. streptococcus thermophilus to lactobacillus bulgaricus ratio.

To determine the activity of the culture, autoclaved 11% solids reconstituted non-fat dry milk was inoculated with culture at the rate of 2.0%. The bottles in duplicates were incubated at 90F for 45 minutes. At the end of the ripening period, the culture inoculated milk was heated to 113F in a span of 30 minutes (this is comparable to cook temperature of Italian type cheeses). After it attained, 113F, the bottles were held at that temperature for an additional 2 hours. At the end of the incubation, pH was determined. Also, microscopy of the activity milk was conducted to determine the coccus to rod ratio.

Table 4: The pH of the grown cultured medium, total microbial counts, activity of the culture (determined as pH), and microscopy of lactic culture-1 (lactococcus lactis var lactis), lactic culture-2 (lactococcus lactis var cremoris) and lactic culture-3 (lactococcus lactis var creamoris), grown in medium A (soy protein as sole source protein), medium B (enzyme treated soy protein as sole source of protein), and medium C (casein milk protein, as sole source of protein).

Lactic culture used	Medium used	Medium pH after 16 hour of incubation	Total viable bacterial count at the end of growth in the medium	Activity test results expressed in Ph units	Microscopy of the cultured medium
Lactococcus lactis var lactis-1	A	4.62	180x107	5.59	Healthy chains and few singles
	B	4.52	200x107	5.45	Healthy chains and few singles
	C	4.58	150x107	5.54	Healthy chains and lot of singles
Lactococcus lactis var cremoris-2	A	4.73	190x107	5.48	Healthy chains and few singles
	B	4.64	260x107	5.39	Healthy chains and few singles and pairs
	C	4.75	220x107	5.48	Healthy chains and few singles
Lactococcus lactis var cremoris-3	A	4.70	180x107	5.64	Healthy chains and few singles
	B	4.65	200x107	5.56	Healthy chains and several singles and pairs
	C	4.75	160x107	5.68	Healthy chains and few singles only

Table 5: The pH of the grown culture medium, the total microbial counts, activity of the culture (determined as pH), activity of the culture (determined as pH), and microscopy of Thermophilic lactic culture-1 (streptococcus thermophilus ST-1 and lactobacillus bulgaricus LB-1), thermophilic lactic culture-2 (streptococcus thermophilus ST-2 and lactobacillus helveticus LH-2) and thermophilic lactic clulture-3 (streptococcus thermophilus ST-3 and lactobacillus lactis LL-3) grown in medium A (soy protein as sole source of protein), medium B (enzyme treated soy protein as sole source of protein) and medium C (casein-milk protein, as sole source of protein).

Coccus & Rod Culture used	Medium used	Medium pH after 8 hours of incubation	C:R ratio after cultured	Total viable bacterial counts at the end of culturing	Activity test results expressed in pH units	C R ratio in the activity milk (cheese)
Streptococcus thermophilus strain St-1 + lactobacillus bulgaricus, strain LB-1	A	5.0	7:1	120x107	5.18	50:1
	B	4.94	5:1	150x107	5.0	30:1
	C	4.96	7:1	130x107	5.12	50:1
Streptococcus thermophilus strain St-2 + lactobacillus helveticus strain LH-2	A	4.70	1:1	190x107	4.72	30:1
	B	4.62	1:1	220x107	4.69	20:1
	C	4.64	1:1	210x107	4.69	20:1
Streptococcus thermophilus strain St-3 + lactobacillus lactis strain LL-3	A	4.86	4:1	150x107	5.0	50:1
	B	4.77	2:1	180x107	4.88	30:1
	C	4.81	3:1	160x107	4. 97	40:1

a All the results presented are averages of two duplicate samples

Results presented in table 4 clearly indicate that lactic cultures such as lactococcus lactis var lactis and lactococcus lactis var cremoris utilize soy protein and casein as sole source of protein. It was known before that these organisms do utilize casein as sole source, of protein. Prior literature indicates that starter cultures do grow well in the soy milk. The question here is that do they grow equally well using soy protein as a sole source of protein in comparison to milk protein, in starter medium. A question raised by FDA (Dr. Gottleib) was that how do we know that protein is broken down by various starter cultures, and do we know that all strains can utilize such protein and could their be strain variation in terms of proteolysis and how does cheese make conditions employed in different cheese alter such proteolysis? This question has been answered in this experiment. We have to use soy protein and casein as sole source of protein in media. An organism can only grow in such a medium provided it has enzymatic system to break the protein down to polypeptides, and amino acids. If proteases, peptides, and amino peptidases are not produced by starter culture, such protein cannot be broken down to simpler amino acids, and consequently such culture cannot grow and multiply to get to a concentration of 2 billion organisms/gram of the liquid medium. Unless, organisms are in healthy condition, they will not produce much acid upon transfer into milk. We have used activity test (which is comparable to cheese making), to check the biological activity of the culture grown in media with soy protein vs. casein as sole source of protein. We have included enzyme treated soy protein also to check the growth patterns of the starter cultures. Results clearly proved that lactic cultures utilize soy protein as sole source of protein to grow and multiply. Their efficiency of utilizing soy protein as sole source of protein is in par with casein (milk protein). If the protein cannot be utilized as sole source of protein, such protein cannot be broken down (proteolyzed) and thus presents problems in the cheese. Also, FDA's (21) question has been answered here i.e. Various strains of lactic cultures (determined on the basis of their phage sensitivities) did utilize soy protein as sole source of protein. The extent of utilization as evidence by their growth and ph lowering ability were similar. They are comparable to casein. How does various conditions effect the growth and activity of different starter strains? This

is where, the art of the cheese making comes in. Cheese plants adjust the conditions of cheese making to arrive at the same pH, prior to packing the finished product. Consequently, no two cheese plants have same conditions of cheese making. However, they alter their make procedures, to arrive at the same end product, in terms of chemical specs, rheological specs, and micro-biological specifications.

Experimental results using high temperature lactic acid organism such as streptococcus thermophilus, strains of lactobacillus bulgaricus, lactobacillus helveticus, and lactobacillus lactis on their ability to utilize different proteins are presented in table 5. Here again, these organisms utilized soy protein as sole source of protein. Their ability to utilize soy protein is as efficient as milk protein, (casein). The activity test (comparable to Italian type cheese making) results indicate that these organisms grew slightly better in soy protein base medium, compare to casein base medium. It indicates that they can proteolyze the soy protein down to simpler peptides and amino acids to grow and multiply to the highest concentrations in the starter medium as well as in the cheese milk. The results also are clear that the strain variations in terms of their growth ability during starter make as well as cheese make, are minimal. We made our best effort to answer this question. The results are presented in table 5. We had to run these basic experiments to answer FDA's question. It is obvious from the results that mesophilic lactic cultures (used in a variety of American type cheeses) as well as high temperature lactic acid producing starter cultures (used in Swiss and Italian type cheese, such as mozzarella, provolone, Romano, and parmesan etc.) grew well using soy protein as a sole source of protein, in the starter indicating that these organisms will proteolyze this protein as efficiently as they can proteolyze casein (milk protein). Also, the strain variations of these cultures were minimal indicating the uniformity of their growth pattern.

16.b The effect of rennet (milk coagulating enzyme used in cheese making) on proteolyzing the enzyme treated vs. non enzyme treated soy solids of the IMAC media, as evidenced by the growth of mutant culture:

The following experiments were conducted to check the effect of rennet (coagulating enzyme used in cheese making), on enzyme treated vs. non enzyme treated soy solids of the starter medium, upon the growth and acid production of proteinase negative mutant of dairy starter culture, streptococcus lactis var lactis. This mutant was obtained by high heat treating the lactic culture and subsequently plating and picking the mutants using the surface plating followed by velveteen plating on both tryptic soy agar and similar agar with soy protein concentrate as source of protein, (without any other nitrogen source). These are standard bacteriological techniques used in the research microbiology laboratories. This particular mutant strain lacks the ability to utilize intact soy protein but it can efficiently grow in the presence of simple peptides, and amino acids (autolyzed yeast extract and casamino acids). In other words, it can only grow on already proteolyzed protein. We have used this culture, to check if rennet can proteolyze the soy protein. Also, to check if rennet can further proteolyze soy peptides to amino acids to assist the growth of protease negative mutant culture. The purpose of this experiment is to demonstrate that the soy proteins and proteolyzed soy peptides of starter culture medium can be further broken down to simpler peptides and amino acids during cheese making. Once the fermented starter culture is added to the cheese milk, it is hard to trace the breakdown pattern of proteins and peptides of the starter origin in cheese. To answer the question, "does soy proteins and peptides go through further proteolysis due to rennet, during cheese making or not"? We have conducted this experiment.

Two different media were formulated as per the compositions outlined in table 1 and table 2. The medium composition with no enzyme treatment to soy solids is referred to as medium "X". Whereas, the medium with enzyme treatment to soy solids is referred to as "Y". The enzyme (protease) was coated on to the soy solids to arrive up to 0.1% of the total solids in the medium "Y". Both the media were reconstituted at 10.0% solids in distilled water and the initial pH was adjusted to 6.3. They were held at 37C for 1 hour and then heat treated to 190F

and held at that temperature for 1 hour. After cooling the medium, they were dispensed in 100ml quantities into previously sterilized bottles. To one bottle of medium "X", 1ml of 1% filter sterilized rennet was added, the other bottle did not have rennet. Both the bottles were incubated with 1ml of proteinase negative mutant culture, and were inoculated for 16 hours at 32C. Similar treatment was given to medium "Y" i.e. One bottle had rennet, other did not have rennet and both were inoculated with mutant culture and incubated for 16 hours. At the end of the incubation all the four bottles were checked for pH, flavor, and growth and morphology using microscopy. The results are presented in table 3. The results clearly indicate that non enzyme hydrolyzed soy protein could not be utilized optimally by mutant culture because it lacked protein breaking enzymes. However, when rennet enzyme was added to non enzyme treated soy protein, the mutant culture grew, indicating that rennet did proteolyze the soy protein to release peptides and perhaps few amino acids to help growth of the mutant. When, enzyme treated soy protein was used in the medium, the mutant grew to get pH down to 5.10, indicating that peptides and amino acids liberated off the soy protein supported the growth of the culture. When such already enzyme treated soy protein concentrate medium was further inoculated with rennet enzyme, the mutant grew to the fullest extent (pH 4.75), indicating that the enzyme hydrolyzed soy proteins, further broke down to simpler peptides and amino acids. Consequently, mutant grew much better than it did in the enzyme hydrolyze soy protein only (without rennet). It definitely proves, rennet enzyme used in cheese making had significant proteolytic effect on soy proteins as well peptides produced off the soy proteins. The significance of this experiment is vital because, the rennet enzyme will be intact and active in the cheese curd, even after the cheese is manufactured and it continuously proteolyse the peptides along with the bacterial exo and endo peptidases. This explains why, the cheese made, using enzyme hydrolyzed and fermented soy solids as an ingredient in the culture medium, did not have any soy allergenic antigens (tested by enzyme immunoassay) or did not excite any allergic reactions, in human food challenge tests.

Table 1: Composition of the medium (medium "X") with non enzyme hydrolyzed soy solids as the sole source protein and glucose as the sole source of carbohydrate.

Dry Ingredient	Percentage
Non enzyme treated soy protein concentrate	50
Monosodium phosphate	1.97
Di-sodium phosphate	2.0
Glucose	44.0
Magnesium sulphate	.01
Manganese chloride	.01
Ferrous sulphate	.01
Calcium chloride	2.0

Table 2: Composition of the medium (medium "Y") with enzyme treated soy protein concentrate as the sole source of protein and glucose as the sole source of carbohydrate.

Dry Ingredient	Percentage
Enzyme treated soy protein concentrate	50
Monosodium phosphate	1.97
Di-sodium phosphate	2.0
Glucose	44.0
Magnesium sulphate	.01
Manganese chloride	.01
Ferrous sulphate	.01
Calcium chloride	2.0

Table 3 a: Effect of rennet (proteolytic cheese enzyme) on non enzyme treated soy solids of the starter medium to support the growth of proteinase negative cheese culture.

pH, smell and microscopy of the non enzyme treated medium with soy solids, after 16 hours incubation:

Incubated without rennet at;						Incubated with rennet at;					
0 hours			16 hours			0 hours			16 hours		
pH	Smell	Micro-scopy	pH	Smell	Micro-scopy	pH	Smell	Micro-scopy	pH	Smell	Microscopy
6.30	No fermentation smell	ND (a)	5.80	No fermentation smell	Very few coccus organisms here and there	6.30	No fermentation smell	ND	5.20	Culture fermentation smell	Coccus organisms in chains

Table 3 b: Effect of rennet (proteolytic cheese enzyme) on non enzyme treated vs. enzyme treated soy solids of the starter medium to support the growth of proteinase negative cheese culture.

pH, smell, and microscopy of the enzyme treated medium with soy solids, after 16 hours incubation:

Incubated without rennet at;						Incubated with rennet at;					
0 hours			16 hours			0 hours			16 hours		
pH	Smell	Microscopy	pH	Smell	Micro-scopy	pH	Smell	Micro-scopy	pH	Smell	Microscopy
6.30	Slight broth smell	ND	5.10	Culture fermentation smell	Coccus organisms in chains	6.30	Slight broth smell	ND	4.75	Distinct culture fermentation smell	Coccus organism in chain and pairs (good growth)

(a) ND-not determined

16.c The effect of enzyme treatment vs. non enzyme treatment of starter media (with soy solids), followed by culturing upon proteolysis, and subsequent addition of such cultures upon proteolysis in the finished cheese:

Laboratory test starter medium was formulated to arrive at 40% protein, of which 30% was soy protein and 9% was of milk origin, 1% was of yeast origin (yeast extract). The rest of the ingredients were phosphate salts, sweet whey solids (lactose sugar), glucose, and minor minerals such as magnesium sulphate, manganese chloride, and ferrous sulphate. Medium composition was adjusted to satisfy the growth of both the American type cheese starter cultures, such as strains of lactococcus lactis var lactis, lactococcus lactis var cremoris, and lactococcus lactis var lactis subspecies diacetilactis, and high temperature Italian type starter cultures, such as, strains of streptococcus thermophilus, lactobacillus bulgaricus, lactobacillus helveticus, and lactobacillus lactis.

The test medium was divided into two fractions. The fraction one, was treated by coating with protease enzyme suspended in lecithin. The enzyme was coated uniformly to arrive at 0.08% enzyme in the medium. A freeze dried protease powdered enzyme was also included in the medium to arrive at 0.02% of the total mass. This fraction of the medium was referred to as enzyme treated medium. The similar media composition with no enzyme treatment is referred to as non-enzyme treated medium. It is clearly stated in the literature that dairy starter cultures grow well in soy milk or soy solids medium (15). It is well known in the microbiology and bacteriology arena that an excellent growth medium for dairy starter cultures is tryptic soy agar. It is also very well documented in the literature that rennet enzyme used in the cheese making, will proteolyze soy proteins (16). The soy solids consistently outperform milk solids interms of not only supporting the growth of bacteria but also in retarding the proliferation of bacteriophages. The bacteriophage infections have devastating effect on the economics of U.S. dairy and cheese industry. The intent of this experiment is to study the extent of proteolysis of soy proteins in the starter medium with and without the aid of protease enzyme treatment, and also its effect upon further proteolyse due to growth of various commonly used cheese starter cultures in such media. The intent here is that if the soy proteins are hydrolyzed to simpler peptides and amino acids, the allergenicity of such protein will be reduced or

eliminated. Both the enzyme treated as well as non enzyme treated starter medium was reconstituted to arrive at 7% solids and was held at 97F for 45 minutes, and then cooked to 185F and held at that temperature for 1 hour. In the cheese plant situation, it takes a minimum of 1½ to 2 hours to get up the temperature of liquid media to 185F to 190F. Generally the medium is held between 85F to 140F for at least 1 hour. In the laboratory situation, the medium will be cooked faster. In order to compensate that, we have standardized the starter make procedure in the laboratory to mimic the practical conditions of the commercial plant. It correlated well with the industrial operation. After the medium was heat treated, it was cooled to set temperature. One set was inoculated with Italian cheese type starter cultures i.e. TL-206, which is mixture of strains of streptococcus thermophilus, lactobacillus bulgaricus, lactobacillus lactis and lactobacillus helveticus. The other set was inoculated with mixed lactic culture having strains of both lactococcus lactis var lactis and lacococcus lactis var cremoris. The thermophilic cultures were incubated at 110F, whereas lactic cultures were incubated at 90F until pH of the cultures came down to 4.5 to 4.75 pH range. In this experiment, cultures were not neutralized during growth. Neutralization will definitely improve the proteolysis. Intent here is to study the degree of proteolysis under the minimal conditions. After the growth, standard laboratory tests were performed to check for the growth, activity and morphology of the organisms, to eliminate the possibility of contaminants in the medium. The cultured medium was also checked for smell and taste to eliminate the abnormal fermentation. Starter was also plated to check for absence of coliforms, and yeast and molds. Some of these contaminators can induce proteolysis, which will induce error in the experiment. The culture was centrifuged at low speed (850 G force) for 30 minutes. The sediment will have the non proteolyzed and larger water insoluble peptides, whereas the supernatant will have water soluble peptides and amino acids. The supernatant was analyzed for protein. The difference between the total proteins in culture minus total protein in the supernatant is the non proteolyzed protein. The following tests were conducted on the controls as well as enzyme treated and non enzyme treated culture media grown with lactic cultures (American cheese type) and high temperature lactic acid cultures (Italian cheese type):

1. Total protein analysis of the culture using dye binding.
2. Total protein analysis of the supernatant of the centrifuged culture.

Known assumptions or facts in protein chemistry:

1. Protein in the supernatant is the soluble protein, which is simpler short chain peptides such as and DI and TRI peptides.
2. Longer chain peptides and proteins will be in the sediment.

The results presented in table 1, clearly depicts that protease enzyme treatment of starter medium with soy solids decrease the total protein by 20%, compared to the control without enzymatic treatment. However, this test cannot determine the degree of protein breakdown. The 20% reduction in protein is due to liberation of amino acids and few peptides, which cannot be bound to the dye used in Udy dye binding test cultures. Both Italian and American cultures did not induce any significant reduction in the total protein concentration as detected by dye binding test. However, with this test we could not determine the degree of breakdown in the protein. The test results presented in table 1, clearly proved that proteolytic enzyme treatment decreased the protein concentration by 20%, by converting that much protein to non dye detectable amino acids and peptides.

The protein, and long chain peptides present in the culture (pH 4.5 - 4.7) go into the sediment. Whereas, the smaller peptides which are soluble in water, stays in the supernatant. The measure of the protein in the supernatant depicts the concentration of small chain water soluble peptides (TRI peptides or greater). The results presented in table 2, clearly proves that culturing alone, even without enzyme treatment induced 46.46% proteolysis, evidenced by the production of water soluble peptides. Whereas, enzyme treatment of the medium prior to heat treatment, followed by culturing resulted in 99.60% proteolysis, evidenced by the production of water soluble peptides, possibly TRI peptides. Similar results were obtained with American type cheddar cultures. This experiment clearly proved that the treatment of starter medium (with soy proteins as major source of protein) with the aid of proteolytic enzyme treatment followed by culturing with cheese starter cultures, proteolyzed 99.60% of the protein. Protein determinations, using aforementioned procedures, of cheese manufactured with conventional starter culture with no soy protein and cheese manufactured with enzyme treated and then cultured starter culture with soy protein, revealed that (although not significant) the later cheese had more water soluble peptides (TRI peptides) than former. These results are presented in table 3. The more water

soluble non-centrifugeable protein means more simple peptides, which is an indication that the proteolysis is carried to the satisfaction point. Allergy in human beings is due to large indigestible protein molecules, which are recognized as antigens. If the protein is broken down sufficiently, the antigencity is reduced and thus making a particular food non-allergenic. This is especially true with foods consumed through normal gastro intestinal tract rather than other routes of administration such as enteric feeding or intravenous feeding etc. It should be pointed out at this stage that we have thoroughly proved that the proteolytic enzyme treatment of starter medium alone proteolyzed the soy protein by 53%, where as cultures proteolyzed 47%, resulting in a total of 99.5% reduction of protein to simpler water soluble peptides (TRI). When such medium is used in the cheese making, at a rate of 1% to 2%, the peptides and left over minute amount of residual protein will be further broken down by the proteolytic enzyme rennet used in the cheese making. In addition to the rennet enzyme, the starter cultures will multiply again during the cheese making, breaking down the peptides further to simpler amino acids. Consequently, allergenic proteins will be rendered non-allergenic. We have repeated these tests several times in our laboratory and the results were highly reproducible. This goes to prove why, 600,000,000lbs. of cheese made with enzyme treated and then cultured starter media with soy protein, fed to over 500,000,000 people did not register even a single complaint in the field for the past 5 years. Even if there was a single complaint, health departments in that particular area would have known about this and must have informed the cheese producers. However, not a single complaint was registered.

Principle of Protein and Proteolysis measurement:

Protein measurements are made by mixing weighed samples with a known volume of reagent dye solution containing acid orange-12 dye. Proteins in the sample react with the dye to form a precipitate. The remaining unreacted dye concentration is inversely proportional to the protein content of the sample. A Udy colorimeter is used to measure the dye concentration can be set to display the percent protein directly. The Udy colorimeter is described in more detail in the measurement section (35,36,37). The following paragraphs give additional information about the chemistry of the reaction.

The U.S.A. dye, acid orange-12, is used because it, 1) binds strongly with proteins, 2) has an excellent color/protein sensitivity ratio, 3) gives clear, stable solutions, and 4) is easily purified for use as its own primary standard. It is essentially non-hydroponics in pure form. Dyes which have been tried by others do not have these properties and so have limited use. Acid orange-12 reacts with the three basic amino acid (BBA) functional groups on proteins. The strong electrostatic bond forms an insoluble protein dye complex. This precipitate is easily filtered or centrifuged so that the remaining dye concentration can be measured color metrically. Solutions of the dye have broad absorption peak at 482nm and follow Beer's Law of optical absorption.

Proteins from a natural source have an essentially constant ratio of basic amino acids to total amino acids or protein. This is genetically controlled by the organism. Each basic site originating from lysine (amino group), arginine (quanidino group) or histidine (imidazole ring) binds one molecule of dye. Measuring both Kjeldahl total nitrogen and equipment dye concentration on a large number of samples of a given commodity, permits the calculation of a regression equation. From this, a dye binding capacity (DBC) value for that specific protein system is established. This is a genetic constant for the given protein system, and does not change.

The DBC constant incorporates an average non-protein nitrogen (NPN) value since it is derived from the correlation with numerous Kjeldahl total nitrogen measurements. Individual samples may have appreciably different NPN levels. A total nitrogen measurement for such a sample would be less indicative of the true protein value. Once the DBC is established for a given protein system, the dye-binding method of estimating protein is independent and absolute. It is not responsive to variations in NPN.

When the ratio of BBA to protein is not genetically controlled, such as in random mixtures of commodities, or when the percentage of NPN changes, as in the ultra filtration concentration of whey proteins, protein measured by dye binding may not correlate as well with Kjeldahl total nitrogen measurements. Denaturation of lysine by overheating or Maillard type reactions will also lower the correlation between protein measured by dye-binding and Kjeldahl total nitrogen.

The protein dye reaction requires some controls over certain factors that influence the equilibrium dye concentration. Temperature of the sample dye mixture has a small but significant effect on the degree of protein dye association. Different ions and buffer systems have potentially large effects on the DBC value. A mass action effect is significant at low dye concentrations. Mass action and ionic effects are negligible or absent entirely in the normal concentration range used for the buffer system now used in Udy Reagent Dye Solution. Starch from some commodities binds a small, but significant, amount of dye. This does not cause loss of accuracy because the starch content and its dye binding are essentially constant. Correlations coefficients over 99% are normally obtained between equilibrium dye concentration and total nitrogen measurements.

The Udy dye binding method is being used on a large number of protein systems. These include, but are not limited to, all grains, oilseeds, forages, legumes, and meat and dairy products. Proteolysis in dairy products (cheese, yogurt, starter media etc.) can also be studied using the Udy dye binding method due to the fact that as the larger protein complexes breakdown from the inferent enzyme degradation, heat denaturations etc. they will show decreases of true protein levels using the Udy dye binding method (over a given time or due to experimentally treated conditions). An explanation of this is the Udy Reagent dye binds with the larger protein complexes (larger than 3 amino acids units). So, as the protein complexes breakdown to smaller polypeptide chain lengths, the ratio of large readable complex proteins to the unreadable amino acid units change. The result is a decrease in protein read on the Udy protein colorimeter for the given product (detected proteolysis).

Apparatus and Reagents:

Udy model MKP protein analyzer including an Udy dairy tester (colorimeter) along with standard Reagents and reference dye solutions.

Babcock centrifuge for spinning the liquid culture medium

Initial set-up:

1. Drain bottle and tubing attach the end of the drain tube (feed tubing up through the bottom of the unit) to the covet tip. It may be necessary to moisten the tip of the covet with water to make the attachment easier. Wipe off any fingerprints on the covet with a soft tissue or a soft cloth.
2. After the tubing is attached, seat the covet stop making sure that the scribe mark on the top of the covet faces the front.
3. Connect the power supply to the unit and plug the power supply transformer into the appropriate outlet. Make sure the unit switch is in the off position before plugging in the unit.
4. Turn on and warm up the unit for 30 minutes before operating.
5. Follow the operating instructions below.

Protein test set-up:

1. Fill the covet with de-mineralized water. Start the flow through process by gently squeezing the drain bottle. Place your finger over the vent hole of the drain bottle cap and release.
2. Place colorimeter switch to **CAL** position.
3. Adjust **ZERO** potentiometer to read .000 on meter.
4. Fill the covet with ".660" standard reference dye solution.
5. Adjust **GAIN** potentiometer to read .660 on the meter.
6. Place the colorimeter switch to percentage protein position.
7. Place **MENU** switch on appropriate channel No. 1, 2, 3, or 4 for raw or homogenized milk, starter etc.
8. Filter sample (25-30 drops or until stable) into covet and read percentage protein or use (in the Cal position) calibration chart supplied by Udy Corp. *(See sample prep).
9. For doing proteolysis studies compare protein differences between treated and untreated samples (can also compare as product ages).

Sample prep. Milk, starter media, cheese, supernatant etc.:

1. Warm sample to 37C and mix.
2. Weigh out 2.06 grams (2.0ml) of milk, starter media etc. sample into a tared 60ml polyethylene sample bottle.

3. Dispense 40.37grams (40ml) Reagent dye solution into the sample bottle. Cap the sample bottle and shake at least 15-20 seconds.
4. Adjust the temperature of the solution to 25C.
5. Add the filter Cap assembly to the sample bottle. Filter 24-30 drops into the cuvet. Read in the Cal mode use percentage protein=6.10 (1.257 reading).

For Cheese:

1. For cheese products blend 3 minutes with a 10 fold water dilution and refer to step 2 but use 4.12grams, read in the Cal mode use percentage protein=31.82.
2. Use Babcock centrifuge for spinning supernatant from step 6, proceed to step 2 but use 10.00grams, read in the Cal mode use percentage protein=5.281 (0.960 reading).

Table 1: The effect of enzyme treatment on starter media (with soy proteins) vs. non-enzyme treatment, on proteolysis after culturing with Italian type cheese cultures (streptococcus thermophilus and strains of lactobacillus), and American type cheese cultures (lactococcus lactis var lactis and lacotcoccus lactis var cremoris).

No.	Sample description	% of total protein (including proteins plus peptides)	% reduction in total protein using orange-12 dye (measurable proteins and peptides)	Presence of contaminants Coliforms	Presence of contaminants Yeast and Molds
1	Non-enzyme treated IMAC starter media, prior to culturing (control)	3.080	0	ND	ND
2	Enzyme treated IMAC starter media, prior to culturing (control)	2.477	19.58	ND	ND
4	Enzyme treated IMAC starter media, after culturing with 206 (Italian cheese starter culture)	2.470	19.80	ND	ND

5	Non-enzyme treated IMAC starter media after culturing with ML-6 (American type cheese starter)	3.013	2.17	ND	ND
6	Enzyme treated IMAC starter media, after culturing with ML-6 (American type cheese starter)	2.464	20.0	ND	ND

ND-None detected.

Table 2: Effect of enzyme treatment on starter media (with soy proteins) vs. non-enzyme treatment, on degree of proteolysis (determined by soluble peptides vs. non soluble proteins) after culturing with Italian type cheese cultures (streptococcus thermophilus and strains of lactobacillus), and American type cheese cultures (lactococcus lactis var lactis, and lactococcus var cremoris).

No.	Sample description	% total protein	% of smaller peptides (protein)	% of proteins and long – peptides	% of total proteolysis (measure of conversion of proteins to simpler peptides)	% of proteolysis due to culturing	% of proteins due to enzymatic treatment
1	Non-enzyme treated IMAC starter media, after culturing with 206 (Italian type cheese culture)	2.97	1.38	1.59	46.464	46.464	0
2	Enzyme treated IMAC starter media, after culturing with 206 (Italian type cheese culture)	2.47	2.46	.01	99.595	46.44	53.155
3	Non-enzyme treated IMAC starter media, after culturing with ML-6 (American type cheese culture)	3.03	1.47	1.56	48.514	48.154	0

4	Enzyme treated IMAC starter media, after culturing with ML-6 (American type cheese culture)	2.46	2.45	.01	99.593	48.154	51.43

Table 3: Effect of conventional cultured starter media (no soy proteins) vs. enzyme treated and cultured starter media (with soy solids), upon the liberation of simpler peptides (indication of degree of proteolysis) in the finished mozzarella cheese.

No.	Sample description	% total protein in cheese	% simpler peptides in cheese	% of simpler peptides in the cheese protein matrix a	Contaminates in cheese Coliforms	Contaminates in cheese Yeast and mold
1	Cheese made with conventional starter culture grown on media with no soy solids	28.96	1.0	3.46	ND b	ND
2	Cheese made with starter culture grown on enzyme treated media with soy solids	27.58	1.33	4.82	ND	ND

a Higher % of simpler peptides in the cheese is an indication of more proteolysis.
b ND-none detected.

17. Detailed experimental design, test procedures and test results of the modified unicap fluoro enzyme immunoassay to prove the inactivation of soy allergens in cheese made with variations of IMAC starter media:

The test for the presence of soy allergen in raw materials and in cheese was conducted at the diagnostic allergy laboratory which is connected with the allergy immunology research division of nationally recognized National Jewish Hospital (34) of Denver, Colorado, under the supervision of Dr. Ron Harbeck, Professor University of Colorado, Medical Center. The detection of allergens in raw materials and in cheese was conducted by enzyme immunoassay. The immunoassay was conducted using suspected allergenic source material bound to matrix. Known, soy allergy positive patient serum was added. Following incubation and washing, the unbound soy antibody will end in the elluant. The elluant is added to the immuno cap with soy antigen, and incubated. An enzyme labeled antihuman IgE antibody, specific for soy, was added. After incubation and washing, enzyme substrate was added. The response was measured using flouroscopy and the results were calculated in terms of presence or absence of allergens in the cheese. The method used is the unicap fluoro enzyme immunoassay (39) and the details of the test with the modifications are as presented in the succeeding chapters of this petition.

Details of the immunoassay test procedures and philosophy behind the tests are presented in a publication entitled "The Diagnostic Allergy Laboratory" by Dr. William K. Dolen. The publication is attached along with our notification (29).

Principle and the procedure of the modified Unicap (fluro enzyme immunoassay):

1. The liquified cheese sample (suspected for the presence of soy antigens) is added to immuno sorbent matrix and then incubated with the known human serum with specific soy antibodies (IgE).
2. After incubation, it is washed with buffer and the eluate is collected. If soy antigen is present in the cheese samples, it will tag on to the specific soy antibody present in the human serum and thus cannot be eluted upon washing, from the immuno absorbent matrix. On the other hand, if soy allergen is absent in the liquid cheese sample, the specific soy antibody (IgE) in the human serum will not attach to the immuno absorbent matrix and upon washing, it will end up in the eluate. It will be treated more like suspected serum with specific soy antibody in unicap test. Hereafter, the test procedure is same as unicap procedure, as outlined in unicap manual.
3. The eluate from step 2. is added to the immuno cap which has covalently coupled soy allergen, and thus allowing IgE from eluate to react with soy antigen.
4. After washing away non specific IgE, enzyme labeled antibodies against IgE is added to form complex.
5. After incubation, unbound enzyme anti IgE is washed away and the bound complex is then incubated with a developing agent.
6. After stopping the reaction, the fluorescence of the eluate is measured.
7. The lower response value indicates the presence of specific soy antigen in the cheese sample. The higher response value indicates the absence of specific antigen in the cheese sample.
8. The results of modified immuno cap test results will be completely opposites of the immuno cap test results. With the immuno cap, higher the fluorescence response value in the eluate, the more specific IgE present in the serum. Whereas, with modified immuno cap, higher the fluorescence response value in the eluate, the less specific antigen present in the suspected liquid cheese sample. Such procedures are valid and can only be done in a nationally recognized medical research center (29).
9. Our interest in this petition is to check for the presence or absence of soy antigens in the cheese. However, we have constructed enzyme

9. Our interest in this petition is to check for the presence or absence of soy antigens in the cheese. However, we have constructed enzyme immunoassay chart table showing the test results and their corresponding quantities of soy protein present in 1000mg of cheese using three test points. It is to be noted that the chart will give only quantity of soy protein not the quantity of allergenic antigen in the cheese. It is also possible to plot and determine the exact concentration of allergens in the cheese sample using the chart into account. Our aim here is qualitative determination i.e. allergens is present or absent.
10. The details of the tests, reagents used, precautions, handling of the immunocap, indications for instability, instrumentation, procedure, calibration, and limitation of the procedures etc. are presented in the unicap literature (39). In addition, a pictorial presentation of modified immuno cap (fluoro enzyme immunoassay test) is presented to show the pattern with positive sample, negative sample and test sample, later in this petition.

The modified fluro enzyme immunoassay was conducted on the following test samples prepared according to the procedures outlined in this communication:

1. Positive control

Starter Media formulated with high heat treated (heat hydrolyzed) Soy Protein with low PDI (protein dispensability index 20), without enzyme treatment, was reconstituted at 10% level using 2% sodium citrate solution and was immediately chilled. The enzyme immunoassay, result of 29 to 21 kilo units/liter is considered negative for soy allergens. A result closer to zero kilo units/liter is considered positive for the presence of soy allergens. High heat treated, low PDi soy protein, used without any protease enzyme treatment or fermentation(in the medium), used as a positive control, exhibited 5 kilo units/liter, indicating the presence of soy allergens and which further confirmed the validity of our test.

2. Test materials

The above specified Media composition, with heat hydrolyzed Soy Protein (but no protelytic enzyme added) was reconstituted at 15% solids level, heat treated to 185 F and held at that temperature for 45 minutes, cooled to 110F, inoculated with Streptococcus thermophilus, and Lactobacillus bulgaricus, incubated for 8 hrs, cooled to 40F. Such Starter was inoculated at 1% level into pasteurized milk and Mozzarella cheese was manufactured as per the standard of identity procedures, outlined by Food and Drug Administration's CFR 21 (49). The cheese was reconstituted at 10% level using 2% sodium citrate using the procedures outlined in the standard methods for the examination of dairy products. The sample was tested for Soy Allergens using the modified unicap fluoro enzyme immunoassay. A result of 29to21 kilo units/liter is considered negative for soy allergens. A result closer to zero kilo units/liter is considered positive for the presence of soy allergens in the cheese. The cheese made (using starter media with low PDi soy protein, with no proteolytic enzyme treatment, prior to fermentation, liquified, heat treated, cooled and fermented and then used to make cheese), exhibited 12 kilo units/liter, indicating the presence of soy allergens (at significantly lower level) in the cheese. The results revealed that soy allergen was detected in the cheese. However, the level is significantly lower than the starter medium. It indicates that heating of the starter medium, fermentation with starter cultures, significantly reduced that allergen level.

However, allergen was still detected in the cheese at a significantly lower level in comparison to the positive control, confirming some more proteolysis is required, with the addition of proteolytic enzyme, prior to fermentation process, to inactivate soy allergens totally.

3. Test material

The current IMAC starter medium, with heat hydrolyzed soy protein, which has predominantly protease and, small amounts of cellulase enzyme absorbed on to the media ingredients with lecithin as a base in the composition, was reconstituted at 17.5% solids level, heated to 185F and held at that temperature for 45 minutes. The medium was cooled to 110F, and inoculated with strains of Streptococcus thermophilus and Lactobacillus bulgaricus. The incubation was continued for 8 hours. At the end of the incubation, the starter was cooled to 40F. It was inoculated into pasteurized milk at the rate of 3% (maximum allowable usage accepted by U.S.D.A.) and mozzarella cheese was manufactured using the standard of identity procedure outlined in Food and Drug Administration 21 CFR (49). The cheese was reconstituted at 10% level, using 2% sodium citrate as outlined in the standard methods for the examination of the dairy products. The sample was tested for the soy allergens. A result of 29to21 kilo units/liter is considered negative for the presence of soy allergens. A result closer to zero kilo units/liter is considered positive for the presence of soy allergens in the cheese. The cheese (using IMAC starter which we are submitting for petition under 21 USC 343 (w) (6), with low PDI soy protein, which is treated with proteolytic enzyme and cellulase enzyme, liquified, heat treated, cooled, fermented, and then used to make cheese) exhibited 29 kilo units/liter, indicating the total absence of soy allergens in the cheese. The results revealed that cheese did not have any soy antigens indicating that heat treatment of soy solids, enzyme hydrolysis (proteolytic enzymes), and heat denaturation during starter making, fermentation and thus further hydrolysis of the proteins due to starter bacterial enzymes, proteolysis due to rennet enzyme in cheese and fermentation in cheese, totally inactivated the soy allergenic antigen. The cheese was free from soy allergens. This test was reproduced to verify the absence of allergens in the cheese. Elisa test (qualitative) also proved negative for the presence of soy antigens in cheese. The details of the test are presented in the ELISA brochure (38). However, due to deficiencies in Elisa testing,

according to the manufacturers, as well as FDA, we have elected to use unicap fluoro enzyme immunoassay with modifications using human IgE antibody, as a better test.

4. Negative control

Starter medium with no soy proteins (soy protein replaced with milk protein and yeast extract) was reconstituted at 10% level, heat treated at 185F, cooled to 110F, inoculated with strains of Streptococcus thermophilus and Lactobacillus bulgaricus, incubated for 8 hours, cooled to 40F. This starter was inoculated into pasteurized cheese milk at 1.25% level (normal level, although 3% inoculation is maximum allowed according to U.S.D.A.) (47) and the mozzarella cheese was manufactured using the standard of identity procedure as outlined in Food and Drug Administrations, 21 CFR (49). The cheese was reconstituted at 10% level using 2% sodium citrate solution, as per the procedure outlined in the standard methods for the examination of the dairy products. The sample was analyzed for the presence of soy antigens using the procedure outlined earlier. The enzyme immunoassay result of 29to21 kilo unit/liter is considered negative for the presence of soy allergens. A result closer to zero kilo unit/liter is considered positive for the presence of soy allergens in the cheese. The cheese made (using starter medium with no soy protein, which is liquified, heat treated, cooled, fermented, and then used to make cheese) exhibited 21 kilo units/liter, indicating the absence of soy allergens in the cheese. As expected, the results revealed, the absence of soy allergens (antigens) in the cheese. It confirmed the validity of test procedure, to eliminate the test interferences.

Enzyme immunoassay clearly indicated that soy allergens are deactivated in the cheese made using IMAC medium, (which we are submitting for petition), with hydrolyzed soy protein (thermal hydrolysis), fermentation (enzyme hydrolysis and proteolysis) due to the starter bacterial enzymes, and partial acid hydrolysis due to the production of lactic acid by starter cultures, proteolysis due to the addition of rennet (proteolysis) enzyme used in the cheese making and further proteolysis due to the growth of starter cultures during cheese making. Consequently the soy allergens are not present in the cheese made with IMAC starter media with enzyme hydrolyzed and fermented soy solids as starter stimulants. This is further confirmed by the absence of complaints on the cheese made by using

IMAC media for the past five years i.e. roughly 600,000,000 lbs. of cheese manufactured using IMAC media (eliminate, enhance, and eradicate). Cheese was consumed, by over 550,000,000 people (all regions, age groups, gender etc) in the U.S., without any allergy complaints. It totally confirms the fact that the cheese made with IMAC media is safe and is free from soy allergens.

Although F&A Dairy of California, Inc., a cheese manufacturer based in Newman, California received a communication from FDA stating that the starter growth media used in the cheese making is not determined as part of an ingredient under FALCPA of 2004 (correspondence from F&A Dairy of California, Inc., and FDA is enclosed) (23,24), since we have data to prove that cheese made using IMAC media with enzyme hydrolyzed and fermented soy solids does not have any soy allergens in cheese, we have elected to submit the petition under 403 (w) (6) for exemption.

To comply with petition requirements we are here with submitting the research data with detailed experimental design and test procedures used to confirm the absence of soy allergens in the cheese manufactured using IMAC starter growth media with enzyme hydrolyzed and fermented soy solids.

PICTORIAL PRESENTATION OF MODIFIED IMMUNO CAP (FLUORO ENZYME IMMUNOASSAY TEST USED IN THIS PETITION)

■■■■→Soy antigen in cheese

■■■■→Soy antigen in immuno cap

▒▒▒▒→IgE (soy antibody in the human serum)

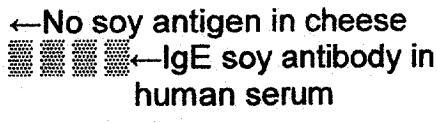
▒▒▒▒ →Enzyme labeled antihuman IgE antibody (IgE specific monoclonal B-galactosidase conjugated antibody).

☺☺ ☺ ☺→Enzyme substrate (4-methylumbilliferyl-B-D-galactoside)

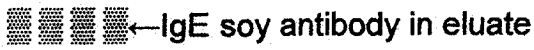
☹☹ ☹ ☹→Substrate upon hydrolysis (4-methylumbelliferon-generates fluorescence)

Negative Test: (If cheese did not have any soy antigens).

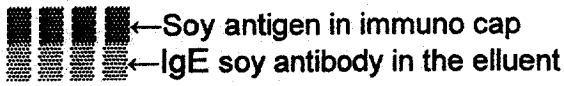
Step 1. Addition of cheese sample (with no soy antigens) to soy Antibody (IgE) in human serum, and upon incubation.



Step 2. Upon washing with Buffer, unbound IgE will end up in eluate

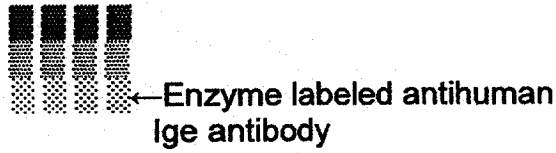


Step 3. Soy antigen in immuno cap and soy antibody (IgE) in the eluent, upon incubation.



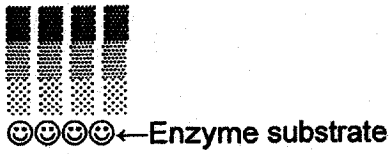
Step 4. Upon washing with buffered detergent.

Step 5. Addition of enzyme labeled antihuman IgE antibody, and upon incubation

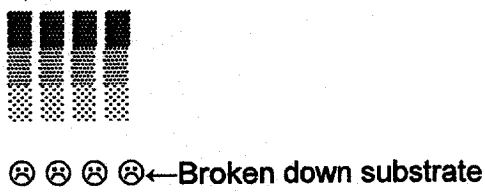


Step 6. Upon washing with buffered detergent

Step 7. Addition of enzyme substrate at at "0" time



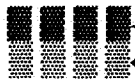
After incubating for a specified length of time



The reading of fluorescence will be high i.e. >21 kilo units/liter, indicating that IgE concentration is high. IgE concentration is high because, the cheese did not have any soy antigen to bind the human IgE.

Positive Test: (If cheese has soy antigens)

Step 1. Addition of cheese Samples (with soy antigen) to soy antibody (IgE) In human serum, and upon Incubation


→Soy antigen in cheese
←IgE soy antibody in human

Step 2. Upon washing with Buffer, no IgE will end up in eluate because it is Tagged on to soy Antigen and formed a stable complex

No IgE soy antibody in eluate

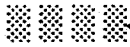
Step 3.

←No soy antibody (IgE) in eluate, Consequently, it won't bind to immuno cap antigen.

Step 4. Upon washing with detergent

←No soy antibody is left

Step 5. Addition of enzyme labeled antihuman IgE antibody


←Enzyme labeled antihuman IgE antibody. It will not tag on

because there is no human IgE

Step 6. Washing with detergent

←Nothing left, all enzyme labeled antibody IgE is washed out.

Step 7. Addition of Enzyme substrate At "0" time

←No enzyme labeled antihuman IgE
☺ ☺ ☺ ☺←Enzyme substrate

⇩

After specified length of incubation

☺ ☺ ☺ ☺←Enzyme substrate still intact because there is no enzyme to brake the substrate

Results: The fluoroscopy reading will be closer to 0 indicating no enzyme activity took place, and there is no detectable human IgE. Most of the human IgE was bound to soy antigens in the cheese. It indicates that soy antigens are present in the cheese.

Test Samples:	No soy protein or soy protein with no potential antigen present in cheese 0mg of soy protein/1000mg	Soy protein with potential soy antigen present in cheese, in small amounts 1.4mg of soy protein/1000mg	Soy protein with potential antigen present in cheese in large amounts, 30mg of soy protein/1000mg
Step 1: Addition of cheese sample to soy antibody (IgE) in human serum, upon incubation			
Step 2: Upon washing with buffer, unbound IgE will end up in eluate.			No human IgE in eluate
Step 3: Soy antigen in immuno cap and soy antibody (IgE) in the eluent, upon incubation			No human IgE present
Step 4: After washing with detergent wash			No human IgE present
Step 5: Addition of enzyme labeled antihuman IgE antibody, and upon incubation			Enzyme labled antihuman IgE antibody will not tag on because there is no human IgE
Step 6: After washing with detergent			No enzyme labeled antihuman IgE antibody present
Step 7: Addition of enzyme substrate at "0" time			

Test Samples:	No soy protein or soy protein with no potential antigene present in cheese in large amounts 0mg of soy protein/1000mg	Soy protein with potential soy antigene present in cheese, in small amounts 1.4mg of soy protein/1000mg	Soy protein with potential antigen present in cheese in large amounts, 30mg of soy protein/1000mg
Upon incubation	☹ ☹ ☹ ☹	☹ ☹ ☺ ☺	☺ ☺ ☺ ☺
Fluoroscopy results	>21 kilo units/liter	12 kilo units/liter	5 kilo units/liter
Presence of soy antigens in cheese	-	+	+

The following conditions have been verified with enzyme immunoassay used in our study:

1. Verification of binding of the immunosorbent matrix to soy allergens present in cheese sample.
2. IgE antibodies present in human serum exhibited good affinity for binding to soy allergen epitopes present in the cheese sample.
3. Antihuman IgE antibody tagged to enzyme is of mouse origin.
4. Enzyme activity is measured using flouroscopy.
5. No interference was observed with the sodium citrate used (to liquefy the cheese), interms of enzyme activity during enzyme immunoassay test.
6. A positive and negative control has been included in the test, to verify the results of the test sample.
7. Reproducibility of the test results were excellent.
8. Test was conducted at the National Jewish Hospital Immunology Diagnostic Center, which is recognized nationally (34).

The commercial manufactures supply allergen disc specific for soy allergens was used to bind IgE. In this case we have to use cheese solution as an allergen by binding it to immunosorbent matrix. Also, the positive serum for IgE is of human origin this was obtained by the National Jewish Hospital. Consequently, we had to design the test using known positive and negative cheese controls with soy antigens to verify the test samples of cheese. According to FDA (40), EIA tests

must include positive and negative controls to verify assay performance. We would like to present our results as qualitative rather than quantitative because we are interested in knowing only the presence or absence of soy antigen in the cheese. This is also, in accordance with FDA, even though we can determine the quantity of allergen present (40).

Quantitative mathematical model showing the amount of intact soy proteins and amount of peptides produced during starter make and their fate during subsequent cheese making:

1. Dry starter media has 30% soy protein.
2. If it is reconstituted at 10.0% solids in the liquid medium, the percentage of protein in the liquid starter is 3.0%.
3. Upon enzyme hydrolysis, 54% of the protein is proteolyzed i.e. 1.62% in the liquid starter out to 3.0%. The starter cultures will proteolyze 46% of the protein i.e. 1.38% out of 3.0%. The total protein proteolyzed is greater than 99.5% to peptides. However, we do not know the chain length of peptides. According to the literature since denatured protein was used as a substrate, the peptides should be mostly medium and short chained. Udy test can detect peptides up to 3 amino acids only.
4. If such starter with 3.0% peptides are inoculated at the rate of 1.0% into the cheese milk, the percentage of soy peptides in the milk are i.e. .03%. In other words, every 100 grams of milk will have .03 grams of soy peptides.
5. Generally cheese yield is 10% of the milk used, since most of the water soluble peptides and the amino acids drain out at various stages of cheese making. If we assume that a maximum of 10% of the soluble peptides stay in the cheese curd, then the total concentration of peptides in the cheese is .003 grams. Therefore, each gram of cheese will have 3mg. Even if 100% soluble peptides stayed in cheese curd, it amount to only 30mg/gram of cheese. These soy peptides will be proteolyzed to a certain extent by the rennet enzyme used in the cheese making. We have demonstrated this through experiments. Since these peptides are readily available in milk, starter cultures during ripening further break them down to simpler peptides. Such peptides get transported into the starter cells and gets further metabolized intracelluarly to amino acids (44). The endo peptidases and the liberated exo peptidases from the starter injured or dead cultures may break them down to simpler peptides, or to amino acids. Apparently the peptide length must be significantly small, and non allergenic because it did not attach to human IgE and thus could not be detected in the enzyme immunoassay. Such cheese did not excite any

allergic reactions in soy sensitive human beings in the double blinded placebo controlled food challenge tests. The best clinical field test is, 600,000,000lbs. of cheese made using such starter media (with enzyme hydrolyzed and fermented soy solids) was fed to over 500,000,000 people in the U.S. without a single registered allergy complaint in the past 5 years.

The quantitative mathematical model showing the fate of non proteolyzed soy protein present in the starter culture, when used in the cheese making:

1. 10% starter solution has 3% protein, of which 99.595% is proteolyzed. It means intact soy protein in the medium is .012%. Please look into our proteolysis data.
2. 100 grams of liquid starter will have .012 grams of intact soy protein.
3. If the starter is used at the rate of 1% into milk, the amount of intact soy protein in 100 grams of milk will be around 0.00012 grams.
4. Assuming that .00012 grams of soy protein is ended up in 10grams of cheese, it accounts for .0012% i.e. 10 grams cheese will have .0012 grams of soy protein or .12mg of soy protein.

In other words 1000mg of cheese may have a maximum of .012mg of intact soy protein, if nothing is proteolyzed after adding to the cheese milk. However, we have proved beyond doubt that soy proteins are proteolyzed by the rennet used in cheese making. Also, we have demonstrated that cheese cultures also proteolyze the soy protein (roughly 45%). Between the rennet and starter cultures, the residual intact soy protein of .012mg/1000mg cheese, will be broken down to simpler peptides and then down to amino acids through the action of exo proteases of the starter cultures i.e. amino peptidases and carboxyl peptidases and di-peptidases. Apparently none of the allergenic soy protein is intact in the cheese, which was proven by enzyme immunoassay (negative for allergenic protein) and also with the human clinical test (negative for any clinical symptoms), and also the fact that 600,000,000lbs. of cheese made using starter (with enzyme hydrolyzed and fermented soy solids) fed to over 500,000,000 people in the U.S. for the past 5, years with out a single allergenic complaint.

18. Details of the analytical test procedures and food challenge tests used, to prove the absence of soy allergenic antigen in the finished cheese:

18.1. Unicap fluoro enzyme immunoassay, detailed methodology, ingredients, and specifications details are already presented in the previous chapter (39).

18.2. Proteolysis determination (Udy dye binding), detailed methodology, ingredients, and specifications (35,36,37).

18.3. Food challenge test for checking allergic response in humans using chesse made with starter media which has non enzyme treated soy solids vs. cheese made with starter media which has enzyme treated soy solids vs. cheese made with starter media which has no soy solids as an ingredient.

18.4. The effect of growing lactic cultures, and coccus and rods using enzyme treated soy protein as sole source of protein, followed by making laboratory controlled simulated cheese products, (cheddar and mozzarella), and feeding such products to soy sensitive human subjects, to see if allergen exists in the cheese product.

18.5. Practical food challenge test of over 600,000,000lbs. of cheese (made using enzyme hydrolyzed and fermented IMAC starter media with soy solids as one of the ingredients) fed to over 500,000,000 people in the U.S. without a single complaint.

18.6. Testimonials from qualified physicians and medical professors.

18.1 UNICAP FLUORO ENZYME IMMUNOASSAY (39)

1. Positive control

IMAC starter medium without enzyme treatment (which has 30% soy protein on a dry basis) was reconstituted at 10% solids. At this concentration, theoretically 1 gram or 1000mg of liquid medium should have the following level of soy protein:

1. Starter medium has 30% protein on a dry basis.
2. If it is reconstituted at 10% level, 100 grams of the reconstituted medium should have 3.0% soy protein.
3. 1 gram or 1000mg of such liquid medium will have .03 grams of soy protein, 30mg of soy protein/1000mg of liquid medium.
4. The unicap fluoro enzyme immunoassay (EIA) results revealed 5 kilo units/liter.

2. Test material (cheese)

Starter medium with 30% soy protein reconstituted at 15% solids without enzyme hydrolysis but fermented with high temperature lactic culture, and then used in cheese making at the rate of 1%, should end up with the following amount of soy protein in the cheese:

1. 1 gram of liquid starter medium will have 45mg of soy protein.
2. It is proven that, if soy protein is not hydrolyzed with enzyme, but fermented with starter culture only, the soy protein will be proteloyzed to 46% of the original amount. The fermentation will reduce the intact soy protein down to 24.3mg/1 gram of liquid starter.
3. 1 gram of liquid starter with 24.3mg of soy protein is inoculated into 100 grams of cheese milk (i.e. 1.0%), the resultant milk will have 0.243mg intact soy protein per ml of milk
4. Assuming that a standard yield of 10% is obtained upon cheese making, 100 grams of milk should yield 10 grams of cheese. Since protein recovery is 90% during cheese making, the finished cheese should have 21.87mg of soy protein/10 grams of cheese i.e., 2.18mg/1 gram of cheese or 2.18mg/1000mg of cheese.
5. Rennet hydrolysis and further starter culture growth during cheese making, theoretically may reduce the intact soy protein to a minimum of

50% of the inoculated amount. Consequently, the final amount of intact soy protein left in the cheese should be roughly around 1.4mg/1000mg of cheese. The unicap fluoro enzyme immunoassay results, using such cheese, gave a reading of 12 kilo unit/liter. Mathematically we can equate the enzyme immunoassay reading to 1.4mg soy protein per 1000mg of cheese.

Hypothetical situation:

1. If we assume that the starter culture (test material), did not hydrolyze the soy protein in the starter medium and also if we assume that the intact soy protein is not further broken down by rennet or starter cultures during cheese making, the following could have been the amount of soy protein left in the cheese:

1. 1 gram of liquid starter medium will have 45mg of soy protein.
2. The resultant cheese made with such starter at 1% level, should have 40.5mg of soy protein/10 grams or 4.05mg of soy protein/1 gram of cheese.
3. Using the charts (EIA), we have developed the enzyme immunoassay results should have been around, 11 kilo units/liter, which equates to 5.52mg of soy protein/1000 mg of cheese.

2. If we assume that the soy protein is hydrolyzed using enzyme and it is further hydrolyzed by starter culture, and also if we assume that the resultant soy protein is further proteloyzed by rennet and starter culture during cheese making, the following could have been the amount of soy protein left in the cheese:

1. 1 gram of liquid starter medium will have 45mg of soy protein.
2. The enzyme will hydrolyze 54% or the soy protein, and culture will hydrolyze 45.5% of the soy protein in the liquid culture. In total only 0.5% of the soy protein will not be hydrolyzed. The intact soy protein left in the starter medium should be roughly around 0.225mg/1 gram of starter.
3. If such medium is inoculated into cheese milk, and assuming 90% of the intact protein is retained in the cheese matrix, cheese should have around 0.20mg/10 grams or .02mg/1 gram.
4. Assuming 50% of such protein is further proteolyzed due to rennet action and starter culture fermentation during cheese making, the resulting

amount of soy protein left in the cheese should have been around .01mg per/1000mg of cheese.

5. The enzyme immunoassay result should have been close to 21kilo units/liter, indicating not a detectable level of soy protein which will induce allergy, is left in the cheese.

3. Test materials (cheese)

IMAC medium with soy solids (30% on a dry basis), reconstituted at 17.5% solids, hydroylyzed and fermented, and then used in the cheese milk at 3.0% inoculation rate, should end up with the following amount of soy protein in cheese:

1. 1 gram of liquid starter will have 52.5mg of soy protein.
2. However, upon enzyme hydrolysis (54% proteolysis) and fermentation (45.5%), the intact unhydrolyzed soy protein left in the starter medium should be 0.5% i.e., .262mg/1000mg of starter.
3. Upon 3% inoculation into milk, 90% of such intact protein (0.78mg) will end up in the cheese matrix. Thus it amounts to, 0.70mg of soy protein/10 grams of cheese or .07mg/1000mg of cheese.
4. Enzyme hydrolysis and culture hydrolysis in the cheese vat may reduce it further to a minimum of 50%. Thus in this case, we may end up with .035mg/1000mg of cheese. However, enzyme immunoassay results (28 kilo units/liter), using human serum with soy antibodies, revealed no soy antigens were in the cheese. It indicts that the soy protein is completely hydrolyzed to a non detectable level of the smallest peptides or amino acids in the cheese matrix.

4. Negative control (cheese)

Cheese made with no soy protein in the starter medium, theoretically should be negative for the enzyme immunoassay and infact it was negative. The test result was 21 kilo units/liter.

Hypothetical situations:

1. If the same cheese, as is in example 3, made without enzyme hydrolysis (starter with 30% soy protein reconstituted at 17.5% solids, not enzyme hydrolyzed but fermented, and inoculated into cheese milk at the rate of 3.0%), the amount of soy protein retained in the cheese will be as follows:

1. 1 gram of liquid starter will have 52.5mg of soy protein.
2. Fermentation alone will break it to 46% thus leaving 54% of the protein intact in the starter medium. It amounts to 28.35mg of intact soy protein present in 1 gram of the liquid starter medium i.e. 85mg of intact protein/3 grams liquid starter.
3. Assuming 90% of such intact protein is retained in the cheese matrix, the amount of soy protein in 10 grams of cheese will be 76.54mg/10 grams or 7.65mg/1 gram of cheese.
4. Assuming a minimum of 50% of such protein is further broken down to smaller peptides due to rennet activity and starter culture activity during cheese making, 3.82mg of soy protein will be retained in 1 gram of cheese i.e. 3.82mg/1000mg of cheese. Using the chart, the enzyme immunoassay test results should have been close to 11 kilo units/liter.

2. Let us assume that in the liquid starter medium of example 3, neither the enzyme nor the culture proteolyzed any soy protein. Let us also assume the starter soy protein is not broken down further due to rennet enzyme and/or culture growth during cheese making. The resultantant cheese made of such starter at 3.0% level should have the following amount of intact soy protein:

1. 3 grams of liquid starter will have 157.5mg of soy protein.
2. The resultantant cheese made with such starter at 3% level should have 141.75mg of soy protein/10 grams or 14.17mg of soy protein per gram of cheese.
3. Using the enzyme immunoassay chart, we have developed, the results should have been around 9 kilo units/liter.

The enzyme immunoassay did not detect any soy protein (allergy inducing) in the cheese, made using IMAC medium with enzyme hydrolysis and fermentation. It confirms that the soy protein is completely hydrolyszed to simpler peptides (perhaps shorter than 8 amino acids) to amino acids in the cheese. It is further confirmed with the food challenge tests. It is next to impossible to determine the number of amino acids in soy peptide chain in the cheese because it is intermingled with milk proteins. The only way to determine is to check for preserve or absence of anigenicity of such peptide using enzyme immunoassay.

Table 1: Enzyme immunoassay chart table showing the test results and their corresponding quantities of soy protein.	
Detectable level of soy protein in cheese	Enzyme immunoassay results
46.32mg/1000mg	1kilo units/liter
42.4mg/1000mg	2 kilo units/liter
38.16mg/1000mg	3 kilo units/liter
30mg total soy protein/1000mg	5 kilo units/liter
25.92mg/1000mg	6 kilo units/liter
21.84mg/1000mg	7 kilo units/liter
17.76mg/1000mg	8 kilo units/liter
13.68mg/1000mg	9 kilo units/liter
9.6mg/1000mg	10 kilo units/liter
5.52mg/1000mg	11 kilo units/liter
1.44mg/1000mg	12 kilo units/liter
1.29mg/1000mg	13 kilo units/liter
1.14mg/1000mg	14 kilo units/liter
0.99mg/1000mg	15 kilo units/liter
0.89mg/1000mg	16 kilo units/liter
0.74mg/1000mg	17 kilo units/liter
0.59mg/1000mg	18 kilo units/liter
0.44mg/1000mg	19 kilo units/liter
0.29mg/1000mg	20 kilo units/liter
0mg/1000mg	21 kilo units/liter
ND/1000mg	>21 kilo units/liter <30 kilo units/liter

ND-none detected

a The test tolerance is to detect 0mg to 46.32mg of soy protein (which can induce allergy) per 1000mg of cheese.

b The enzyme immunoassay chart is constructed using 3 set points of, 30mg of soy protein/1000mg
5 kilo, units/liter 1.4mg of soy protein/1000mg - 12 kilo units/liter, and 0mg of soy protein/1000mg - 21 kilo units/liter.