

ORIGINAL SUBMISSION



# VENTRIA BIOSCIENCE

---

June 22, 2005

REC'D JUN 23 2005

Robert L. Martin, Ph.D.  
U. S. Food and Drug Administration  
Center for Food Safety and Applied Nutrition  
CFSAN/OO/OFAS/DBGNR  
HFS-255  
Harvey W. Wiley Federal Building  
5100 Paint Branch Parkway  
College Park, MD 20740-3835

Dear Dr. Martin,

We wish to notify you that Ventria Bioscience has determined that lysozyme (human) derived from rice is "generally recognized as safe" (GRAS) for use as an ingredient in functional foods and beverages and medical foods and as a replacement for hen egg white lysozyme in the food industry. Accordingly, lysozyme (human) derived from rice is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act.

Enclosed please find an original, two copies and one electronic copy on disk of the document used by Ventria Bioscience to make its GRAS determination. As directed by the agency, the information is formatted in accordance with proposal 21 CFR 170.36(c) (62 Federal Register 18937 (April 17, 1997)).

The data and information that serve as the basis for this GRAS notification will be sent to the FDA upon request or are available for the FDA's review and copying during business hours at the office of Delia Bethell, Ventria Bioscience, 4110 N. Freeway Blvd., Sacramento, CA 95834, telephone: 916-921-6148 ex 21, fax: 916-921-5611, email: [dbethell@ventria.com](mailto:dbethell@ventria.com).

Sincerely,

Delia R. Bethell, Ph.D.  
Vice President for Clinical Development

Cc: Scott Deeter  
President & CEO



# VENTRIA BIOSCIENCE

June 22, 2005

Robert L. Martin, Ph.D.  
U. S. Food and Drug Administration  
Center for Food Safety and Applied Nutrition  
CFSAN/OO/OFAS/DBGNR  
HFS-255  
Harvey W. Wiley Federal Building  
5100 Paint Branch Parkway  
College Park, MD 20740-3835

Dear Dr. Martin,

We wish to notify you that Ventria Bioscience has determined that lysozyme (human) derived from rice is "generally recognized as safe" (GRAS) for use as an ingredient in functional foods and beverages and medical foods and as a replacement for hen egg white lysozyme in the food industry. Accordingly, lysozyme (human) derived from rice is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act.

Enclosed please find an original, two copies and one electronic copy on disk of the document used by Ventria Bioscience to make its GRAS determination. As directed by the agency, the information is formatted in accordance with proposal 21 CFR 170.36(c) (62 Federal Register 18937 (April 17, 1997)).

The data and information that serve as the basis for this GRAS notification will be sent to the FDA upon request or are available for the FDA's review and copying during business hours at the office of Delia Bethell, Ventria Bioscience, 4110 N. Freeway Blvd., Sacramento, CA 95834, telephone: 916-921-6148 ex 21, fax: 916-921-5611, email: [dbethell@ventria.com](mailto:dbethell@ventria.com).

Sincerely,

Delia R. Bethell, Ph.D.  
Vice President for Clinical Development

Cc: Scott Deeter  
President & CEO

**GENERALLY RECOGNIZED AS SAFE (GRAS)  
NOTIFICATION FOR LYSOZYME (HUMAN)  
DERIVED FROM RICE**

Prepared by

Delia R. Bethell, Ph.D.  
Vice President for Clinical Development

Ventria Bioscience  
4110 N. Freeway Blvd.  
Sacramento, CA 95834  
Telephone: 916.921.6148  
Fax: 916-921-5611  
[dbethell@ventria.com](mailto:dbethell@ventria.com)

June 22, 2005

**Contains No Confidential Business Information**

## TABLE OF CONTENTS

TABLE OF CONTENTS.....	1
LIST OF TABLES.....	3
LIST OF FIGURES .....	4
I. GRAS EXEMPTION CLAIM.....	5
A. Name and Address of Notifier .....	5
B. Common or Usual Name of GRAS Substance.....	5
C. Conditions of Use.....	5
D. Basis for GRAS Determination.....	5
E. Statement of Availability of Data and Information.....	6
II. DESCRIPTION OF SUBSTANCE.....	7
A. Physical and Chemical Composition .....	7
B. Molecular Biology of Production.....	8
Conclusion .....	15
C. Manufacturing Process .....	16
1. General Description of the Production Process .....	16
2. Finished Product Specifications.....	18
Conclusion .....	19
III. USE AND CONSUMER EXPOSURE .....	20
A. Consumer Safety .....	20
1. Use of lysozyme in animals and humans.....	20
2. Animal Studies.....	20
4. Infant Formula Supplementation Studies.....	21
5. Pediatric Dosing of Lysozyme .....	21
6. Adult Dosing of Lysozyme .....	22
B. Consumer Exposure .....	22
1. Background exposure to lysozyme in saliva .....	22
2. EDI of rhLF from uses proposed by Ventria.....	23
Conclusion .....	26
IV. INTENDED EFFECTS.....	27
A. INTRODUCTION.....	27
B. ANTIMICROBIAL STUDIES.....	27
C. ANTI-INFLAMMATORY STUDIES.....	28
V. SAFETY ASSESSMENT .....	30
A. Substantial equivalence of lysozyme (human) derived from rice to native human lysozyme.....	30
1. Biochemical Equivalence.....	30
2. Activity Equivalence.....	39
3. Safety Equivalence .....	42
4. Allergenicity .....	45
5. Autoimmune disease and lysozyme .....	52
6. Amyloid formation and lysozyme.....	52
7. Studies in animals and humans with oral delivery of human lysozyme (human) derived from rice.....	53

Conclusion .....54  
8. Expert Panel Consensus Statement .....55  
VI. REFERENCES .....58  
APPENDIX A .....63  
    CSFII Food Codes Included in EDI Analysis for Ventria Proposed Uses .....63  
APPENDIX B .....66  
    Processing Chemicals and Materials .....66

## LIST OF TABLES

Table 1.	Lysozyme Levels in Human Exocrine Secretions .....	8
Table 2.	Batch results from recombinant human lysozyme purification .....	17
Table 3.	Manufacturing specifications for recombinant human lysozyme .....	18
Table 4.	Data on feed efficiency of LZ 159 rice fed to broiler chicks .....	21
Table 5.	Comparative intakes of egg white and rhLZ from cheese and meat uses (consumers).....	24
Table 6.	Average daily intake of lysozyme from proposed uses (500 mg/serving) .....	25
Table 7.	Category contribution: Average daily intake of lysozyme from proposed uses (500 mg/serving).....	25
Table 8.	Estimated rhLZ intake from ORS enriched at 0.2 mg/mL.....	26
Table 9.	Comparison of physical, chemical and biochemical properties of native human lysozyme and lysozyme (human) derived from rice .....	30
Table 10.	Comparison of amino acid composition of recombinant human lysozyme (rhLZ) comparison to native human lysozyme (hLZ) .....	31
Table 11.	Activity of recombinant human lysozyme compared with human lysozyme .....	39

## LIST OF FIGURES

Figure 1.	Human Lysozyme .....	7
Figure 2.	Figure Lysozyme Protein by ELISA in the Maturing Grain .....	11
Figure 3.	Northern Blot Analysis of Maturing Seed in LZ159 Rice .....	12
Figure 4.	Northern Blot Analysis of Various LZ159 Plant Tissues .....	13
Figure 5.	Tissue Specificity Studies of rhLZ .....	14
Figure 6.	Lysozyme Expression in LZ159 Rice .....	15
Figure 7.	Purification of recombinant human lysozyme .....	17
Figure 8.	Glycan analysis of rhLZ and hLZ .....	33
Figure 9.	SDS-PAGE of recombinant human lysozyme and control human lysozyme .....	34
Figure 10.	MALDI mass spectrum of native and recombinant human lysozyme .....	35
Figure 11.	pH stability of recombinant human lysozyme and native human lysozyme activity .....	36
Figure 12.	Thermal stability of recombinant human lysozyme and native human lysozyme .....	37
Figure 13.	Digestion of human lysozyme from breast milk, recombinant human lysozyme and hen egg white lysozyme using the ILSI protocol .....	38
Figure 14.	Specific activity of recombinant human lysozyme (rhLZ), human lysozyme (hLZ) and hen egg white lysozyme (cLZ) .....	39
Figure 15.	Bactericidal activity of rhLZ and hLZ .....	40
Figure 16.	Bactericidal activity of recombinant human lysozyme (rhLZ) .....	41
Figure 17.	Cross-reactivity of anti-hLZ antibody and anti-cLZ antibody to recombinant human lysozyme (rhLZ), native human lysozyme (hLZ) and hen egg white lysozyme (cLZ) .....	44
Figure 18.	Percent inhibition ELISA (mean $\pm$ SD) for 30 egg lysozyme positive sera .....	49
Figure 19.	ELISA inhibition for 2 egg lysozyme positive sera with inhibition by human recombinant lysozyme at high concentration .....	51

## I. GRAS EXEMPTION CLAIM

### A. *Name and Address of Notifier*

Ventria Bioscience  
4110 N. Freeway Blvd.  
Sacramento, CA 95834

Contact: Delia Bethell, Ph.D.  
Vice President for Clinical Development  
Telephone: 916-921-6148 ext. 21  
Facsimile: 916 921-5611  
Email: dbethell@ventria.com

### B. *Common or Usual Name of GRAS Substance*

Lysozyme (Human) Derived from Rice is the common or trade name for the enzyme lysozyme, E.C. 3.2.1.17, isolated from transgenic rice. The published literature may also refer to lysozyme as muramidase, mucopeptide N-acetylmuramylhydrolase, and mucopeptide glucohydrolase (Osserman, Canfield et al. 1974).

### C. *Conditions of Use*

The nutrient properties of lysozyme have many potential physiological benefits. Ventria Bioscience intends to add lysozyme (human) derived from rice to functional foods and drinks at levels not to exceed 500 mg/serving and to medical foods at levels not to exceed 0.5 mg/mL. Since human lysozyme is more active than hen egg white lysozyme, the lysozyme (human) derived from rice may serve as a replacement for hen egg white lysozyme in the food and beverage industries at levels not to exceed those affirmed in FR FDA 03/13/98 PR 63 FR 12421 for cheese and GRN 000064 for processed meats. Lysozyme (human) derived from rice may also be used as a processing aide in the wine and beer industries following appropriate regulatory approvals.

### D. *Basis for GRAS Determination*

The GRAS determination for lysozyme (human) derived from rice is based on scientific procedures.

Ventria Bioscience is providing this notification that the intake of lysozyme (human) derived from rice from its intended use is safe and also Generally Recognized as Safe (GRAS) under the Federal Food Drug and Cosmetic Act. This document establishes the substantial equivalence of lysozyme (human) derived from rice to human lysozyme. It also demonstrates that the safety of lysozyme is generally recognized.

Information on lysozyme was reviewed with respect to background information and biological studies *in vitro* and *in vivo* in animals and humans. The sources of information included studies identified through searches of online bibliographic systems, such as PubMed from the National Library of Medicine.

Determination of the GRAS status of lysozyme (human) derived from rice as a food ingredient has been made by a panel of experts qualified by scientific training to evaluate food safety and selected for their expertise in issues related to allergic and immunologic issues.

#### ***E. Statement of Availability of Data and Information***

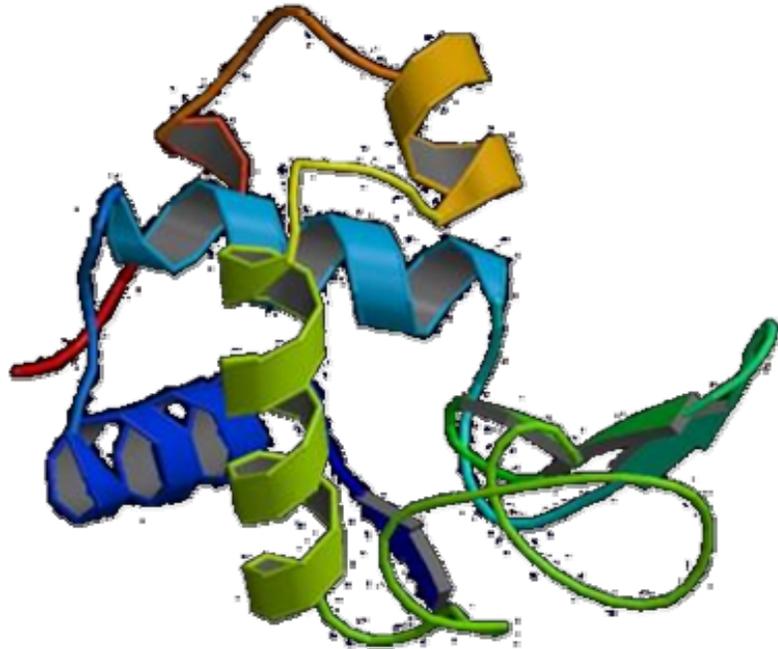
The data and information that serve as the basis for this GRAS notification will be sent to the FDA upon request or are available for the FDA's review and copying at reasonable times at the office of

Ventria Bioscience  
Delia Bethell, Ph.D.  
Vice President for Clinical Development  
4110 N. Freeway Blvd.  
Sacramento, CA 95834  
Telephone: 916-921-6148 ext 21  
Fax: 916-921-5611  
Email: [dbethell@ventria.com](mailto:dbethell@ventria.com)

## II. DESCRIPTION OF SUBSTANCE

### A. *Physical and Chemical Composition*

Human lysozyme is present in human biological fluids and secretions (milk, tears, saliva, genital, nasal and bronchial secretions) as well plasma and circulating neutrophils. Lysozyme is an enzyme protein (EC 3.2.1.17) of 130 amino acids with a molecular weight of 14.5 kD (Canfield, Kammerman et al. 1971; Jolles and Jolles 1971). The natural substrate of lysozyme is the high molecular weight, insoluble peptidoglycan polymer (GlcNAc-MurNAc)<sub>n</sub> found in most bacterial cell walls. Lysozyme catalyzes the hydrolysis of  $\beta$ (-1,4-) linkage between N-acetylglucosamine and N-acetylmuramic acid in the bacterial cell wall. The enzyme lyses primarily gram positive and a few gram negative bacteria or induces their aggregation (Witholt, Heerikhuizen et al. 1976). In addition, human lysozyme is capable of modulating the functions of human polymorphonuclear leukocytes (PMN) during inflammation (Gordon, Douglas et al. 1979).



**Figure 1. Human Lysozyme**

Lysozyme is a single polypeptide chain. The cDNA was isolated from a human placenta library. There is only one human lysozyme gene and it has been assigned to chromosome 12 (Peters, Kruse et al. 1989). There are no N-glycosylation sites on lysozyme and there is no glycosylation of the protein. The complete amino acid sequence has been determined for lysozyme from many species: human, bovine, hen, quail, pheasant, guinea fowl, turkey, duck, chachalaca, baboon, rat, tortoise (Jolles and Jolles 1984). The recombinant human lysozyme gene sequence used by Ventria Bioscience was based on the DNA sequence from GenBank (GenBank accession number J03801).

Human lysozyme is present in biological fluids and mucous secretions (milk, tears, saliva, genital, and nasal secretions) as well as in neutrophils. Table 1 provides published levels of lysozyme measured in human exocrine secretions.

**Table 1. Lysozyme Levels in Human Exocrine Secretions**

Fluid	Lysozyme ( $\mu\text{g/mL}$ )	Reference
Breast milk	65-400	(Jolles and Jolles 1984)
Tears	1267	(Jolles and Jolles 1984)
Urine	0.5	(Jolles and Jolles 1984)
Nasal secretions	10-60	(Raphael, Jeney et al. 1989)
Amniotic fluid	9.3	(Jolles and Jolles 1984)
Gastric juice	74.2	(Jolles and Jolles 1984)
Cerebrospinal fluid	0.5	(Jolles and Jolles 1984)
Saliva	8.8	(Jolles and Jolles 1984)
Serum	10.8	(Jolles and Jolles 1984)
Neutrophil	0.095	(Moreira-Ludewig and Healy 1992)

## ***B. Molecular Biology of Production***

Recombinant human lysozyme was produced through the genetic modification of *Oryza sativa*, *Japonica*, Taipei 309. The parent rice variety, Taipei 309 (TP309), is a commercially cultivated Japonica variety that has ancestral origins in China. This variety is not grown commercially in the United States. The strain of Taipei 309 used by Ventria Bioscience was provided by a collaborator at the University of California in 1993. The variety has been maintained in the greenhouse to produce material for transformation as required. The varietal multiplication continues at Ventria Bioscience on an as-needed basis.

Ventria Bioscience has produced transgenic rice that expresses human lysozyme in the endosperm of the seed. Ventria used a seed-specific promoter and signal peptide sequence to direct the lysozyme molecule to the protein bodies in the endosperm cell. While the genes for the lysozyme and selectable markers are present in every transgenic plant cell, the expression is limited to the seed endosperm and does not occur in other parts of the rice plant. The promoters, signal peptide sequences, and selectable markers used in the transformation process were either isolated from the rice genome or are those used in other genetically modified grains currently in the food supply. As described below, Ventria Bioscience has used a selection technology which does not express the antibiotic resistance proteins in the plant or grain.

Ventria Bioscience uses a two-vector system in the transformation process and DNA from these vectors was introduced into plant cells via particle bombardment. One vector contains the chimeric target gene and the other contains the chimeric selectable marker gene. The frequency with which both vectors are integrated into the same cell in the plant transformation process is approximately 90%. The details of the transformation process and the characterization of the resulting lysozyme protein have been published (Huang, Nandi et al. 2002; Huang, Wu et al. 2002).

The human milk lysozyme gene sequence was based on the DNA sequence from GenBank (GenBank accession number J03801). The production of the recombinant human lysozyme in rice grain was accomplished through the use of a seed-specific promoter and a signal peptide directing the resulting lysozyme to the protein body of the seed endosperm cell. The Gt1 promoter was derived from the rice glutelin gene family (Okita, Hwang et al. 1989) and controls the specificity and level of expression of the lysozyme gene in rice endosperm. The Gt1 promoter was isolated from the rice genome and used in chimeric gene construction. The corresponding Gt1 signal peptide was also derived from this rice glutelin gene. The signal peptide is used to guide the lysozyme through the endoplasmic reticulum and target it to the protein bodies of the endosperm cells in maturing rice grain. It is cleaved from the lysozyme as the protein is translocated into the endoplasmic reticulum.

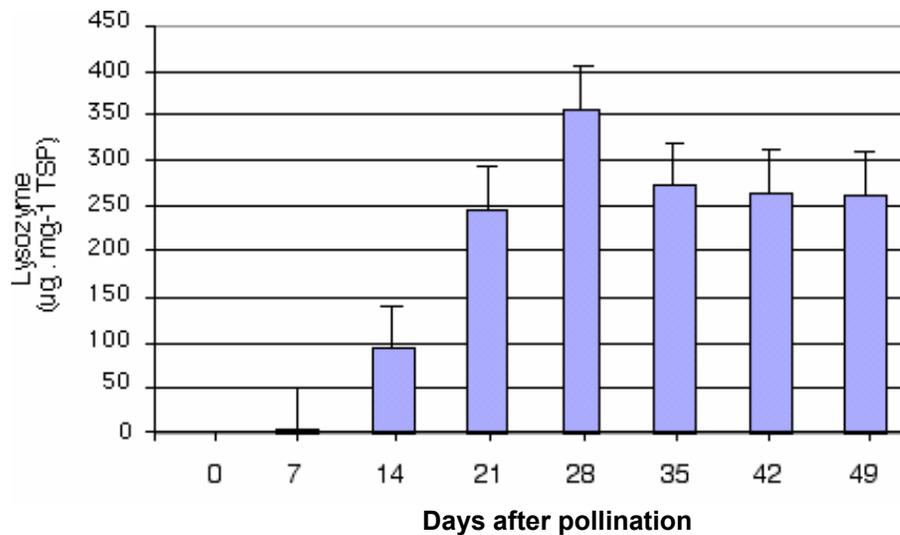
Transformed rice calli were selected by the expression of hygromycin B phosphotransferase (hpt), an enzyme that deactivates the antibiotic hygromycin B (Waldron, Murphy et al. 1985). Hygromycin B phosphotransferase (hpt) has been used in transformation of crop plants intended for human consumption. It has been the subject of an FDA consultation on the use of antibiotic resistant marker genes (FDA 1998). Although the hpt protein is present in the calli of the cultured non-differentiated plant cells after transformation, it is not present in tissues of the mature plant including the grain. The selectable marker gene (hpt) is under tight control of the 940 bp rice  $\beta$ -glucanase 9 (Gns9) gene promoter that is specific for expression in rice callus tissue. Therefore, hygromycin

phosphotransferase is expressed and detected in rice calli, but not in the rice plant (Huang, Wu et al. 2001).

During chimeric gene construction, the gene encoding kanamycin phosphotransferase (npt) was utilized as a selectable marker for plasmid maintenance in *E. coli*. The npt gene is under the control of a bacterial promoter. This promoter and gene pair were used in the propagation of the plasmid in bacteria prior to plant transformation by bombardment. The npt gene sequences are not active in plants. The npt promoter and gene pair have been used in the transformation of crop plants intended for human consumption and the safety addressed in the FDA consultation on antibiotic resistant markers (FDA 1998).

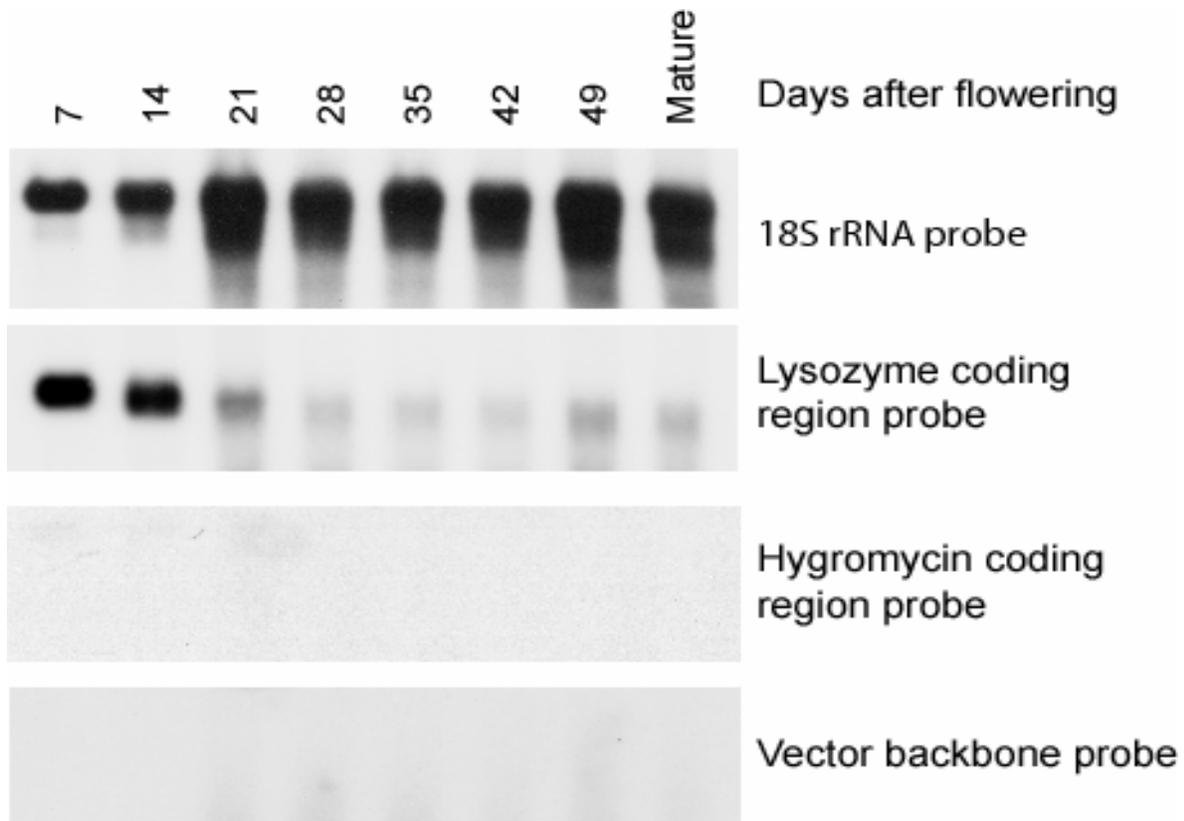
In summary, of the three open reading frames for the new genetic material introduced into Taipei 309 rice, only one is functional in the mature rice plant, the human lysozyme gene. The hygromycin B phosphotransferase gene is under control of a promoter that is not functional in the vegetative portions of the rice plant or the grain and the kanamycin phosphotransferase gene is under control of a bacterial promoter and is not functional in plants.

The correct functioning of the Gt1 promoter and signal peptide control sequences has been documented through the use of Northern blot analysis of RNA expression, Western blot analysis and ELISA measurement for levels of protein expression. Figures 2 and 3 present ELISA and Northern blot analysis of the rice seed (grain) during the maturation process, respectively. Figure 2 demonstrates the increasing accumulation of lysozyme protein in the rice grain during the maturation process. Figure 3 documents the presence of lysozyme RNA in the seed and the absence of RNAs from the antibiotic resistance genes and other vector backbone sequences.



**Figure 2. Figure Lysozyme Protein by ELISA in the Maturing Grain**

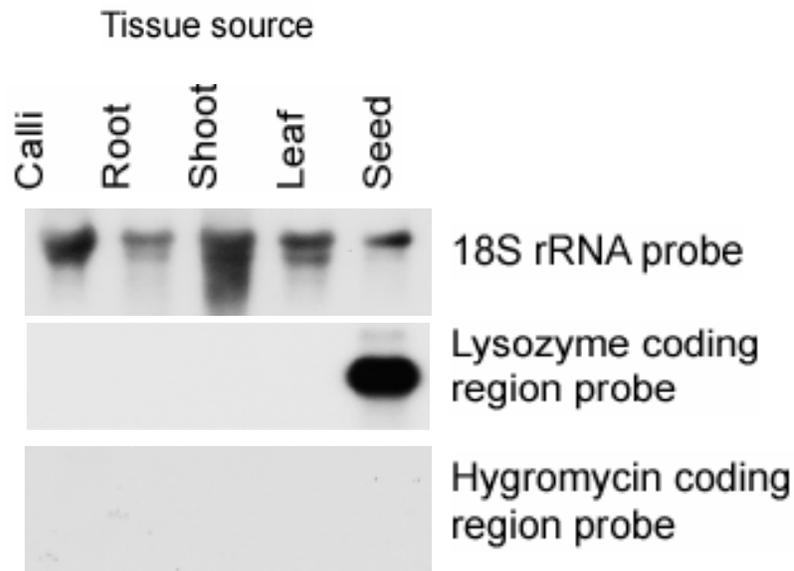
Ten spikelets were harvested from rice plants corresponding to the grain maturation stage shown. Soluble proteins were extracted with 0.35 M NaCl in PBS, and clear supernatant was obtained by centrifuging at 14,000 rpm at 4° C for 10 min. Lysozyme and total protein content were determined by ELISA and Bradford assays, respectively. The ELISA plates were coated with rabbit anti-human lysozyme IgG antibody (Dako). Flour extract was added followed by sheep anti-human lysozyme (Cal Biochem) and peroxidase conjugated donkey anti-sheep IgG (H&L) (Jackson Immunoresearch).



**Figure 3. Northern Blot Analysis of Maturing Seed in LZ159 Rice**

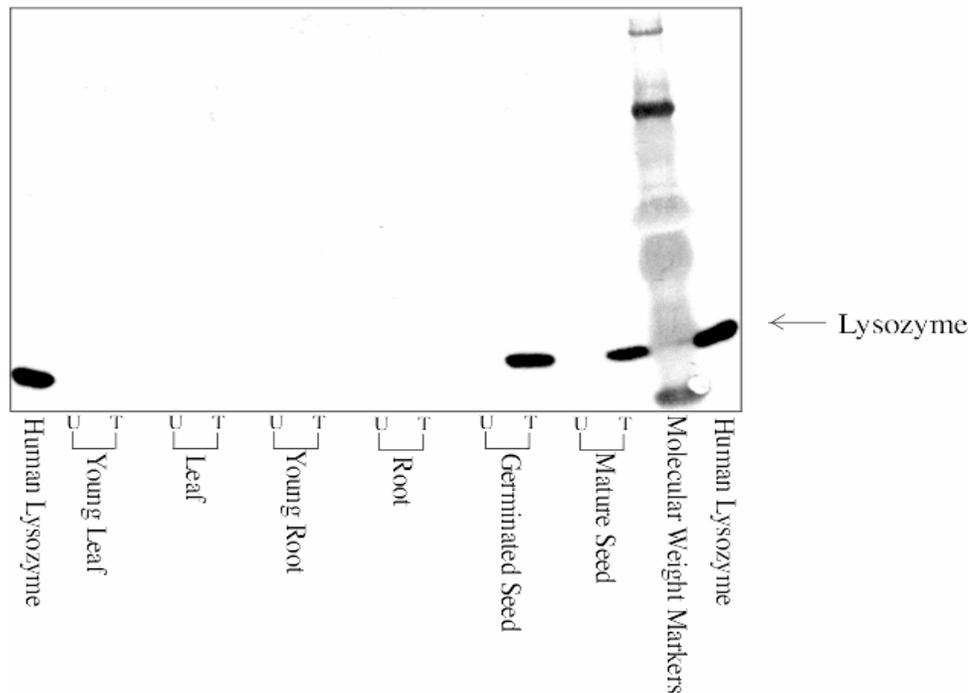
The lysozyme gene expression profile in grain of LZ159 was analyzed by Northern blotting. The total RNAs were isolated from the developing spikelets of LZ159 at 7, 14, 21, 28, 35, 42, 49 and 56 (mature) days after flowering. The RNAs were blotted onto a nylon membrane after separation in 1% agarose gel and probed with lysozyme, hygromycin phosphotransferase, vector backbone DNA sequences. The 18S rRNA was used as internal control.

Figures 4 and 5 illustrate the tissue specificity of the lysozyme protein using Northern and Western blot analyses, respectively. In Figure 4, plant tissues were analyzed for lysozyme and the antibiotic resistance protein RNAs. RNA of lysozyme is seen only in the seed material. Likewise, the hygromycin B phosphotransferase RNA is not seen in any plant tissues. In Figure 5 the various plant tissues from the parent non-transformed line and the LZ159 transformed line were analyzed for the presence of lysozyme protein by Western blot. Lysozyme protein is not detected in any parts of the non-transformed plants and only in the seed structures of the transformed plant. These data indicate that the promoters and signal peptides are functioning as expected and that the human lysozyme protein is only expressed in the grain of the rice plant.



**Figure 4. Northern Blot Analysis of Various LZ159 Plant Tissues**

Tissue-specific expression of the lysozyme gene in line LZ159 was determined by Northern analysis. Total RNA was extracted from roots, shoots, leaves, and seed of mature plants as well as calli derived from LZ159 mature embryo. The RNAs were blotted onto nylon membrane after separation by 1% agarose gel and probed with lysozyme and hpt genes. The 18S rRNA was used as an internal control.



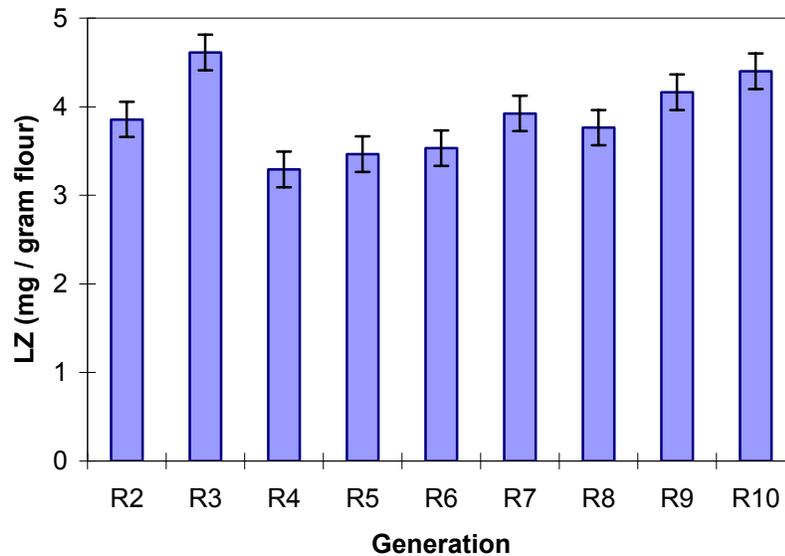
**Figure 5. Tissue Specificity Studies of rhLZ**

Total soluble protein was extracted from roots, leaves, germinated grain and mature grain of untransformed (U) and transgenic plants (T) with 0.35 M NaCl in PBS at room temperature for 2 h. Proteins were separated by PAGE and transferred to nitrocellulose membrane. Lysozyme expression was determined by Western blot analysis using polyclonal antiserum against human lysozyme. Native human lysozyme was used as positive control.

Inheritance of the introduced genetic locus meets the single locus model of Mendelian inheritance in the  $R_1$  generation based on Chi square analysis. A transgenic plant was considered homozygous for lysozyme expression if all grains from the plant were positive for lysozyme in both the *Micrococcus luteus* cell lysis assay and Western blot analysis.

The transgenic rice expressing recombinant human lysozyme has been grown in the greenhouse and field for 10 generations. The transformed line LZ159 displayed stable inheritance of the newly introduced genetic material. At each generation, the expression of rhLZ by the transgenic plants was confirmed by *Micrococcus luteus* cell lysis assay and Western blot analysis using rabbit anti-lysozyme antiserum. The stability of lysozyme expression has been monitored over 10 succeeding generations and is relatively consistent and reproducible.

Figure 6 illustrates stable and reproducible lysozyme expression level over nine succeeding generations (R<sub>2</sub> – R<sub>10</sub>).



**Figure 6. Lysozyme Expression in LZ159 Rice**

Brown rice flour corresponding to R<sub>2</sub> to R<sub>10</sub> generations of the LZ159 line was extracted with 0.3 M NaCl in 20 mM sodium acetate pH 4.0 at 37° C for 1.5 hr. The supernatant was collected by centrifuging the suspension at 14,000 rpm for 15 min. at room temperature. Lysozyme enzymatic activity was measured in the *Micrococcus luteus* turbidimetric assay. One unit of activity produced a 1 milliOD change in absorbance (450nm) in a 2.6 mL reaction volume (pH 6.24) and 1 cm path length.

## Conclusion

Recombinant human lysozyme is produced through standard methods of genetic modification and plant transformation. The rice grain in which the protein accumulates is stable and well suited as a production system. There is no evidence of any new material being expressed in the transformed rice grain other than the human lysozyme. The genetic elements used in the transformation process are currently found in the food supply, either in native rice or genetically modified foods designated as safe for food or feed by the FDA.

## C. Manufacturing Process

The production of recombinant human proteins in rice grain has several advantages.

- Rice grain proteins can accumulate to 9-19% of grain weight (Huang, Wu et al. 2001).
- The endosperm proteins are synthesized during grain maturation and stored in protein bodies for use in the germination and seedling growth of the next plant generation.
- Grain can be stored for years without loss of functionality, and therefore the downstream processing can be conducted independent of growing seasons.
- Specific promoters for expression of recombinant proteins are available from the genes of the major rice storage proteins, the glutelin, *Gt-1*, *Gt-2* and *Gt-3* (Okita, Hwang et al. 1989).
- Rice is a self-pollinating crop, which significantly reduces the possibility of gene flow in the environment.
- There are standard extraction methods available in the food industry for the extraction of rice protein fractions.
- Any material, other than the target protein, remaining after purification is from rice and thus considered to have low allergenicity. The plant matrix can actually serve as an excipient or inert carrier (Peterson and Arntzen 2004).

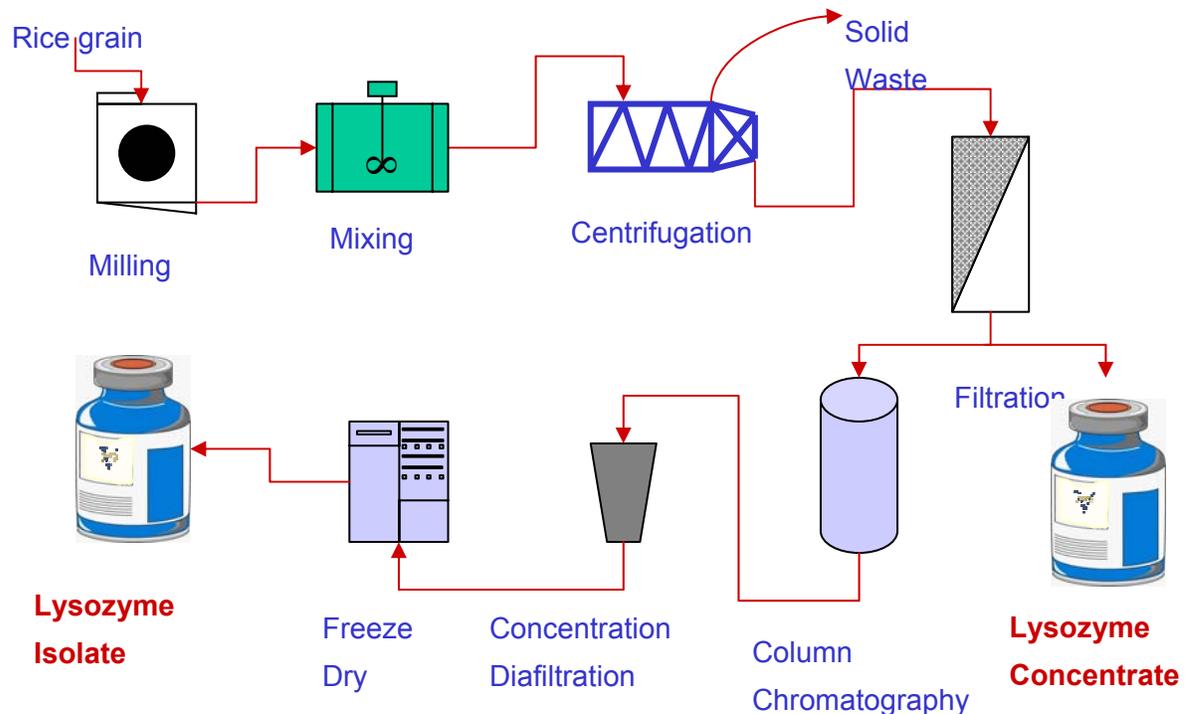
### 1. General Description of the Production Process

To date Ventria Bioscience has purified 1.4 kg of recombinant human lysozyme from rice flour. The protocols developed at pilot scale for 10 kg of rice flour have been scaled to 90 kg of rice flour in a linear fashion. Current efforts are underway to scale to 700 kg of rice flour.

Recombinant human lysozyme is produced in the LZ159 rice variety derived from *Oryza sativa*, *Japonica*, Taipei 309. The rice is dehusked and milled to an average of 100 mesh flour using standard food industry procedures.

Recombinant human lysozyme is extracted from ground rice flour using 0.02 M acetate buffer with 0.3 M NaCl pH 4.5. After 1.0 – 2.0 hours of extraction, the solid rice flour is separated from the liquid phase by centrifugation. The liquid phase, containing the soluble protein, is filtered and concentrated. At this point the product is a protein concentrate that is >10% lysozyme protein. The concentrate is further purified to an isolate using ion exchange chromatography. The concentrate is loaded on a column of SP Sepharose Big Bead Media, the column is washed and the bound lysozyme eluted with 0.8 M sodium chloride. The isolate is ultrafiltered to remove excess salt and concentrated prior to lyophilization. The isolate form of lysozyme is > 80% pure lysozyme protein as measured by HPLC analysis.

All chemicals and processing aids are of food grade or higher quality. The chemicals would include but not be limited to food grade sodium chloride, sodium acetate, and demineralized water. Processing aids would include but not be limited to SP Sepharose (or other cation exchange resin with an inert solid support), regenerated cellulose and/or polyvinylidene fluoride membranes. Figure 7 gives a schematic of the purification process. Safety and information sheets for the processing chemicals are located in Appendix B.



**Figure 7. Purification of recombinant human lysozyme**

The process has been consistent and reproducible. During the recent production of a 1.4 kg lot of recombinant human lysozyme, three batches were purified and blended to produce the lot. Table 2 gives the yield and purity of the three batches prior to blending. Each batch was purified from 90 kg of rice flour.

**Table 2. Batch results from recombinant human lysozyme purification**

Batch No.	Yield weight (gm)	LZ as % of Protein
032804	497.5	82.9
032904	502.5	83.8
033004	501.3	82.7

Rice is a production crop species that is well understood and characterized as to the presence of toxins and anti-nutrients. There are only three antinutrient factors in rice, all concentrated in the bran fraction. They are phytic acid, hemagglutinin-lectin and trypsin inhibitor. Ventria Bioscience has sponsored analysis of these factors and shown that there is no increase in the levels of these factors in the transformed rice grain. Phytic acid and lectin are removed during the extraction process based on solubility. Phytic acid has a molecular weight 880 and is removed during the ultrafiltration steps (Chen 2004). Analysis indicates 99% is removed in the first ultrafiltration step. The lectins of rice are only extracted with an alkaline based buffer (Tabary, Font et al. 1987). The trypsin inhibitor is in the albumin family of rice proteins and is extracted with water or saline. This protein could be present in the concentrate.

## 2. Finished Product Specifications

Specifications for the two forms of lysozyme are in Table 3. The sodium concentration is variable depending on the final product specifications.

**Table 3. Manufacturing specifications for recombinant human lysozyme**

Specification	Lysozyme Concentrate	Lysozyme Isolate
Appearance	Off white powder	Off white powder
Purity of lysozyme	> 10%	> 80% (w/w)
Total protein	> 50%	> 90%
Moisture	< 10%	< 10%
Solubility (1%)	na	A <sub>600</sub> <2.5
pH (1% in H <sub>2</sub> O)	5.0 – 7.0	5.0 – 7.0
<i>S. aureus</i>	Neg in 1 g	Neg in 1 g
<i>Pseudomonas spp</i>	Neg in 25 g	Neg in 25 g
<i>E. coli</i>	Neg in 1 g	Neg in 1 g
Salmonella	Neg in 25 g	Neg in 25 g
Total Plate Count	< 500 cfu/g	< 500 cfu/g
Yeast	< 10 cfu/g	< 10 cfu/g
Mold	< 10 cfu/g	< 10 cfu/g
Sodium	Product specific	Product specific
Heavy metals (as Pb)	< 10 ppm	< 10 ppm
Lead	< 0.5 ppm	< 0.5 ppm

## ***Conclusion***

Recombinant human lysozyme has been purified from transgenic rice using standard food industry procedures and current good manufacturing practices. The process is reproducible and scalable.

### III. USE AND CONSUMER EXPOSURE

#### A. Consumer Safety

##### 1. Use of lysozyme in animals and humans

An extensive library of literature is available to support the safety of lysozyme from human and avian origin. Human and hen egg white lysozymes have been consumed by humans through consumption of breast milk in infancy and eggs throughout life. Infants who are breast-fed consume human lysozyme, a natural constituent of human milk. Human milk contains from  $0.37 \pm 0.27$  g/L in colostrum to  $0.89 \pm 0.21$  g/L in mature milk (Montagne, Cuilliere et al. 2001).

Hen egg white lysozyme has been consumed by humans as a naturally occurring protein found in eggs. Egg white lysozyme has been affirmed as Generally Recognized as Safe (GRAS) as a direct food substance for use in preventing late blowing of cheese caused by the bacterium *Clostridium tyrobutyricum* (63 FR 12421, 3/13/98). The Joint FAO/WHO Expert Committee on Food Additives has concluded that hen egg lysozyme added to cheese is not a hazard to consumer health (FAO/WHO 1992), and its presence in ripened cheese at *quantum satis* (levels necessary to achieve the intended effect) is approved in the EU (Directive 95/2/EC, Food Additives, Annex III ([http://europa.eu.int/comm/food/fs/sfp/addit\\_flavor/flav11\\_en.pdf](http://europa.eu.int/comm/food/fs/sfp/addit_flavor/flav11_en.pdf))). Lysozyme has also been granted GRAS status for use as an antimicrobial agent in casings for frankfurters and on cooked meat and poultry products, GRN 000064. Hen egg lysozyme is also approved in the EU as a processing aid in winemaking to reduce lactic bacteria (Regulation No. 1493/1999, On the Common Organization of the Market in Wine, Annex IV [http://europa.eu.int/eur-lex/pri/en/oj/dat/1999/l\\_179/l\\_17919990714\\_en000100\\_84.pdf](http://europa.eu.int/eur-lex/pri/en/oj/dat/1999/l_179/l_17919990714_en000100_84.pdf))). This site provides a list of authorized oenological practices and processes which may be applied to fresh grapes, grape must, grape must in fermentation, grape must in fermentation extracted from raisined grapes, concentrated grape must and new wine still in fermentation. Hen egg white lysozyme has been used in the pharmaceutical industry in tablets, eye drops and vaginal suppositories (reviewed in (Proctor and Cunningham 1988; Sava 1996)).

##### 2. Animal Studies

Data have been published to support safety of rice derived recombinant human lysozyme. The recombinant protein was used in combination with a second recombinant human protein produced in rice, lactoferrin. The study included two trials using 660 3-day old broiler chicks (Humphrey, Huang et al. 2002). Chicks were fed corn-soy-rice diets containing conventional or recombinant rice extract

at levels from 0% to 10% lysozyme. There were no adverse events and feed efficiency based on grams body weight gained/gram feed consumed was significantly higher for chicks receiving lysozyme containing rice extract as compared to commercial rice extract supplemented in the feed.

**Table 4. Data on feed efficiency of LZ 159 rice fed to broiler chicks**

<b>Dietary Treatment</b>	<b>Feed Efficiency (g wt gain/g feed)</b>
<b>Experiment 1</b>	
Commercial rice	0.79
5% LF + 10% LZ	0.84 (p<0.05)
<b>Experiment 2</b>	
Commercial rice	0.72
10% LZ	0.77 (p<0.05)
5% LF + 10% LZ	0.77 (p<0.05)

Ventria Bioscience sponsored a 28-day feeding trial in rats with recombinant human lysozyme. The maximum dose of 0.36 g/kg/day of lysozyme resulted in a No Observed Adverse Effect Level (NOAEL). No adverse events were reported in the study.

#### **4. Infant Formula Supplementation Studies**

Lysozyme has been used in Japan in dried milk for pediatric use. It has also been added to infant formulas for neonates and premature infants (as reviewed (Proctor and Cunningham 1988)). Studies included addition to infant formula with the reporting of increased *Bifidus bacillus* in infants receiving lysozyme (Nishihara, Isoda et al. 1967). A study of neonates with opportunistic intestinal bacterial infections demonstrated reduction in disease duration when dosed with 0.025 g/kg/day for 7-10 days (Amirova, Lebenzon et al. 1990). Feeding of 29 premature infants with 50 mg/L gave results comparable to breast feeding (Bol'shakova, Shcherbakova et al. 1984). It is not clear what species lysozyme was used in these studies, but it is likely that the source is hen egg white.

#### **5. Pediatric Dosing of Lysozyme**

Ventria Bioscience has sponsored a study supplementing a rice-based oral rehydration solution (ORS) with lysozyme at 0.2 mg/mL and a second recombinant human milk protein, lactoferrin, at 1 mg/mL. The study conducted in Lima, Peru, enrolled 140 children with acute watery diarrhea in a blinded study. Children received the ORS based on a standard dosing protocol related to the amount of diarrhea. Children in the study received from 0.018 to 0.2 g of lysozyme in the first four hours based on level of dehydration. Dosing continued until diarrhea was resolved or dosing had continued for 14 days. There have

been no material related adverse events.

## 6. Adult Dosing of Lysozyme

Several studies have investigated the systemic appearance of lysozyme following oral delivery. All studies were performed with hen egg white lysozyme. Serum hen egg white lysozyme levels were monitored in four healthy volunteers for 24 hours following oral administration of 900 mg of hen egg white lysozyme (Yuzuriha, Katayama et al. 1978). The serum levels of hen egg white lysozyme peaked at 0.35 to 4.55 ng/mL in 30 minutes to 1 hour and were back to baseline in 24 hours. The investigators developed a hen polyclonal antibody based radioimmunoassay for hen egg white lysozyme that had very low cross-reactivity with human lysozyme.

In a similar study, a group of investigators used a hen egg white lysozyme, Neuzym<sup>®</sup>, available in Japan by prescription for the treatment of edema associated with chronic sinusitis and expectoration in respiratory disease (Hashida, Ishikawa et al. 2002). In this study serum levels of hen egg white lysozyme were measured following clinically relevant doses of 30 and 90 mg of lysozyme. Serum levels within 1 hour ranged from 0.04 to 1.7 ng/mL in 29 volunteers and were undetectable at 48 hours.

There has been extensive use of lysozyme for a range of applications from control of susceptible bacteria, modulation of host immunity and depression of the immune response. Dosing has been as high as a gram/day in antiviral applications. There have been minimal reports of adverse events, although there are instances of anti-lysozyme responses (see reviews (Proctor and Cunningham 1988; Sava 1996)). However, all studies have been done using the only commercially available source of lysozyme, hen egg white.

## B. Consumer Exposure

### 1. Background exposure to lysozyme in saliva

Lysozyme is a natural constituent of the human body, found in exocrine secretions including saliva and breast milk, and in neutrophils and plasma. Humans produce about 1.5 liters of saliva each day. Under basal conditions, the salivary glands produce saliva at the rate of approximately 0.5 mL/min, with a much slower flow rate during sleep. After stimulation, flow increases 10-fold over the basal rate (Marino and Gorelick 2003).

Several researchers have measured lysozyme levels in stimulated saliva. Tsang (Tsang and Samaranayake 1999) reported data on stimulated saliva from thirty-two healthy individuals. The mean flow rate of the saliva was  $1.92 \pm 0.61$  mL/min. This flow rate would produce 2.8 liters of saliva each day. Lysozyme

was measured at  $11.76 \pm 2.25$   $\mu\text{g/mL}$  saliva. Assuming 2.8 L saliva production/day, this would result in an exposure to lysozyme from saliva of 32.9 mg per day. Lin et al. (Lin, Johnson et al. 2001) reported stimulated saliva flow rates from 34 healthy control subjects. Flow rates ranged from 1.5 to 2.4 mL/min with a mean of 2.0 mL/min. This agrees with the data obtained by Tsang. In the Lin study, lysozyme levels ranged from 10.9 to 18.0  $\mu\text{g/mL}$  saliva and an average of 39.5 mg/day. The saliva lysozyme levels measured in these studies are comparable to that stated in a standard Medical Physiology text book (Marino and Gorelick 2003). The data from this research support an estimated mean exposure to lysozyme from saliva of 0.03 – 0.04 g/day.

Although hen egg white and human lysozyme share some amino acid sequence homology, the frequency of IgE cross-reactivity in hen egg white lysozyme sensitized subjects is rare and of questionable biological significance. This supports the safety of recombinant human lysozyme with respect to consumer safety (IgE from egg lysozyme sensitized patients do not cross-react with rhLZ at equimolar concentrations; see discussion in Section V).

## 2. EDI of rhLF from uses proposed by Ventria

Ventria proposes 4 categories of uses for recombinant human lysozyme enrichment:

1. General population: 500 mg/serving in yogurts, meal replacement and performance beverages and bars, including granola bars and “Ensure”-type drinks.
2. Medical foods (under supervision of a physician only): 0.2 mg/mL in oral rehydration solution (ORS)
3. Replacement of hen egg white lysozyme, a GRAS ingredient used to prevent late-blowing in cheese caused by the bacterium *Clostridium tyrobutyricum* (63 FR 12421, 13 March 1998) and to prevent spoilage in processed meats, GRN 000064.
4. Replacement of hen egg white lysozyme in the wine industry as a processing aid.

Dietary exposure to lysozyme from the winemaking use is expected to be negligible. Lysozyme may be used at 100-200mg/L in certain specific kinds of wines to control lactic bacteria (malolactic fermentation) in the grape must under certain specific conditions. When used in a red wine, lysozyme is inactivated by reacting with tannins; in white wines, 50%-80% of the added lysozyme may remain (Gerland).

a. Replacement of Egg White Lysozyme in Cheese and Meats

In the Proposed Rule (63 FR 12421, 3/13/98), FDA estimated that the mean long-term intake of chicken egg lysozyme by cheese consumers is 3.8mg/person/day, and at the 90<sup>th</sup> percentile, 8.1mg/person/day. The intake associated with use of lysozyme as an antimicrobial agent by consumers of frankfurters and cooked ready-to-eat meat and poultry products was estimated as 0.21mg/person/day at the mean and 0.40mg/person/day at the 90<sup>th</sup> percentile (Lysozyme GRAS Notice Exemption Claim, p. 39). Because the antimicrobial activity of rhLZ is 4 times that of egg white lysozyme (see Fig. 4, Section 5, above), the use level of rhLZ will be only 25% that of egg white lysozyme in the existing cheese and meat applications. A comparison of per user intakes of egg white lysozyme and the corresponding anticipated intakes of rhLZ, assuming 100% market penetration, is presented in Table 5.

**Table 5. Comparative intakes of egg white and rhLZ from cheese and meat uses (consumers).**

Replacement Use	Egg White Lysozyme		rhLZ	
	Mean	90 <sup>th</sup>	Mean	90 <sup>th</sup>
	(mg/p/day)	(mg/p/day)	(mg/p/day)	(mg/p/day)
Cheese	3.8	8.1	0.95	2.02
Frankfurters and cooked ready-to-eat meats	0.21	0.40	0.05	0.10

b. New uses - General population

Exponent estimated the EDI associated with adding rhLZ to the proposed foods at 500 mg/serving, using food consumption data from the 1994-96, 98 CSFII and their FARE™ software. The comprehensive list of codes included in the analysis is included in Appendix A.

Results of the analyses are in Table 6 (Total rhLZ intake from all enriched foods) and Table 7 (rhLZ intake by food category). “Per user” values are based on persons who reported consuming the foods of interest during the survey. Intake of rhLZ by the highly exposed consumer (represented by the 90<sup>th</sup> percentile per user values) ranges from 723 mg/day (teens) to about 1 g/day (teens and adults).

**Table 6. Average daily intake of lysozyme from proposed uses (500 mg/serving)**

Population	Per capita, mg/day		Per user, mg/day	
	Mean	90 <sup>th</sup> percentile	Mean	90 <sup>th</sup> percentile
Children, 1-12 yr	96	333	361	723
Teens, 13-19 yr	105	325	566	1021
Adults, 20+ yr	88	272	517	1000

**Table 7. Category contribution: Average daily intake of lysozyme from proposed uses (500 mg/serving)**

Food Group	Children, 1-12 yr		Teens, 13-19 yr		Adults, 20+ yr	
	mg/day		mg/day		mg/day	
	Per Capita	Per User	Per Capita	Per User	Per Capita	Per User
Dairy Containing Popsicles*	7	304	4	288	5	376
Dry Meal Replacements*	1	389	3	514	6	518
Fluid Meal Replacements*	15	429	43	861	19	700
Solid Meal Replacements*	19	303	17	314	13	400
Sports Beverages*	NA	NA	NA	NA	0	12
Yogurts Including Frozen	25	237	24	451	40	426

\* Population size insufficient for reliable estimate of the 90<sup>th</sup> percentiles

NA: No CSFII survey respondents in this age grouping

c. Medical food uses

The World Health Organization (WHO) has issued guidance for administration of ORS in the first 4 hours to children with some dehydration ([http://www.who.int/child-adolescent-health/publications/referral\\_care/chap4/chap41.htm](http://www.who.int/child-adolescent-health/publications/referral_care/chap4/chap41.htm)). Using these recommended dosages, an oral rehydration solution containing 0.2 mg/mL rhLZ (the proposed enrichment level) would correspond to the lysozyme intakes in Table 8.

**Table 8. Estimated rhLZ intake from ORS enriched at 0.2 mg/mL**

<b>Weight</b>	<b>Age</b>	<b>Amount of ORS in first 4 hours</b>	<b>rhLZ intake</b>
<5 kg	<4 months	200-400 mL	40-80 mg
5-<8 kg	4-<12 months	400-600 mL	80-120 mg
8-<11 kg	12 months to <2 years	600-800 mL	120-160 mg
11-<16 kg	2-<5 years	800-1200 mL	160-240 mg
16-50 kg	5-15 years	1200-2200 mL	240-440 mg

**Conclusion**

Recombinant human lysozyme from rice has been shown to be safe and free from adverse reactions. RhLZ is well tolerated and not related to any serious adverse events or allergic reactions. The dietary exposure to rhLZ associated with the proposed uses at the specified levels is safe.

## IV. INTENDED EFFECTS

### A. INTRODUCTION

Lysozyme is an enzyme found in the protective fluids (tears, saliva, mucous) of most animals. The natural substrate for the enzyme is a peptidoglycan polymer found in most bacterial cell walls. Although the primary target is the gram positive bacteria, lysozyme has demonstrated other activities important for its role in innate immunity. The key role of the innate immune system is to provide an immediate non-specific response against invading microorganisms, but also function as a bridge to the adaptive immune system (Qian and Walker 2004)

Since the discovery of lysozyme by Alexander Fleming in 1922, studies have been performed *in vitro*, in experimental animals, and in humans to analyze the functions of lysozyme (Sava 1996). It is clear that lysozyme also has non-enzymatic functions that support its role in protection of the organism.

The use of lysozyme as a preservative to prevent spoilage in foods has already been established through the use of hen egg white lysozyme in the cheese and processed meat industry. It is also used as an antimicrobial processing aide in the wine and beer industry. The use of lysozyme in functional foods and beverages and medical foods can lend nutritional support to management of conditions associated with gastrointestinal disease and stress. Lysozyme can play a role not only against pathogenic microbial organisms, but also in modulating leukocytes function during inflammation. A summary of the *in vitro* and *in vivo* studies on the various functions of lysozyme reported in the literature are below:

- Antimicrobial
- Anti-inflammatory

### B. ANTIMICROBIAL STUDIES

Lysozyme exhibits antibacterial activity against not only the gram positive bacteria, but also some gram negative organisms. In addition, it also has antiviral, antifungal and antiparasitic activities. Although most of the studies were conducted *in vitro* (for review, see (Sava 1996)), there are *in vivo* studies as well (Sava 1996).

The primary mechanism of antibacterial action is the enzymatic cleavage of the gram positive bacterial cell wall. Lysozyme, however, also demonstrates activity against some gram negative organisms as well. The resistance of gram negative

organisms to lysozyme is due to the lipopolysaccharide (LPS) outer layer that protects the cell wall. Another protein of the innate immune system, lactoferrin, can enhance lysozyme activity by binding to the LPS and allow lysozyme to penetrate the outer layer and enzymatically cleave the cell wall (Ellison and Giehl 1991). In combination, these two proteins have demonstrated bactericidal effects on the gram negative pathogens, *V.cholerae*, *S. typhimurium* and *E. coli*. However, other research has indicated lysozyme alone can have antimicrobial action, in a dose dependent fashion, against *E. coli*, *Salmonella enteritidis*, *P. aeruginosa*, *S. aureus* and *B. subtilis* (Ibrahim 1998). This activity was independent of lysozyme's enzymatic activity and appeared related to hydrophobic binding interaction with lipopolysaccharides (LPS). Direct binding of lysozyme to purified bacterial LPS was shown to inhibit the enzymatic activity of lysozyme and alter the activity of the LPS (Ohno and Morrison 1989).

Lysozyme can cause the precipitation of viral particles by forming an insoluble complex that results in rapid resolution of skin infections by herpes zoster and simplex (Jolles and Jolles 1984; Sava 1996). The anti-HIV viral activity in human chorionic gonadotropin preparations has been linked to lysozyme (Lee-Huang, Huang et al. 1999).

Lysozyme has been studied in oral health by a number of investigators. It showed activity against the bacteria of the oral cavity associated with caries and gingivitis, *S. mutans* (Iacono, MacKay et al. 1980). It also has an anti-fungal effect on oral isolates of *Candida krusei* and *Candida albicans* (Samaranayake, Samaranayake et al. 1997).

### **C. ANTI-INFLAMMATORY STUDIES**

The anti-inflammatory action of lysozyme was demonstrated in studies using physiologically relevant concentrations of human lysozyme to inhibit inflammatory chemotaxis of polymorphonuclear leukocytes (PMN) in agarose and *in vivo* in skin windows (Gordon, Douglas et al. 1979). It is of particular note that hen egg white lysozyme had no effect in these experiments. Human lysozyme was also able to inhibit oxidative metabolism and production of superoxide anions by PMN.

Lysozyme has been associated with anti-tumor effects *in vitro* and in mice. In studies with two tumor cell lines, a melanoma and transitional cell carcinoma of the bladder, human lysozyme isolated from the urine of patients with monocytic or myelomonocytic leukemia enhanced monocyte cytotoxicity towards the tumor cells (LeMarbre, Rinehart et al. 1981). Oral administration of hen egg white lysozyme to mice prior to inoculation with mammary carcinoma cells resulted in a reduction in the formation of lung tumors (Sava, Ceschia et al. 1988).

Lysozyme (human) derived from rice exhibits the same activities as human lysozyme and has greater enzymatic specific activity than hen egg white lysozyme. These activities support the use of lysozyme (human) derived from rice in functional and medical foods and as a replacement for hen egg white lysozyme in the food industry.

## V. SAFETY ASSESSMENT

### A. *Substantial equivalence of lysozyme (human) derived from rice to native human lysozyme*

#### 1. Biochemical Equivalence

Lysozyme (human) derived from rice has been purified from LF159 rice and compared to native human lysozyme from breast milk. Lysozyme found in breast milk, saliva, tears and other secretions is produced by the same gene sequence. The results of the physical, chemical and biochemical comparisons are summarized in Table 9 and have been published (Huang, Nandi et al. 2002; Huang, Wu et al. 2002); the data from each comparison follow. The data support substantial equivalence between human lysozyme and lysozyme (human) derived from rice.

**Table 9. Comparison of physical, chemical and biochemical properties of native human lysozyme and lysozyme (human) derived from rice**

Properties	Human LZ (hLZ)	Recombinant hLZ (rice-derived)
Amino acid composition and sequence	130 amino acids based on DNA sequence	Homologous composition and 100 % sequence agreement
N-terminal sequence	KVFERCELART Based on DNA sequence	KVFER(C)ELART Direct measurement
Glycosylation	None	None
MW by SDS-PAGE	14.5 kD	14.5 kD
MW by MALDI	14690.65	14,690.82
Isoelectric focusing point	pI 10.2	pI 10.2
pH stability	pH 2 to 10	pH 2 to 10
Temperature stability	85° C, 5 min in PBS	85° C, 5 min in PBS
Solubility	>50 mg/mL in PBS at RT	>50 mg/mL in PBS at RT
Resistance to pepsin digestion	Digested in < 10 min	Digested in < 10 min

#### a. Amino Acid Composition and Sequence

Amino acid analysis was done by cation exchange chromatography on 3 hydrolyzed samples of purified recombinant human lysozyme and native human lysozyme derived from human milk. Asn is deamidated to Asp during hydrolysis

and thus both Asn and Asp are reported as Asx. Similarly, Gln is deamidated to Glu and is reported as Glx. Recovery of Cys is typically incomplete unless oxidized procedures are performed. Tryptophan is destroyed upon hydrolysis and not recovered therefore data is not available for this residue. In the analysis Cys and Arg are known to generate larger variation.

Table 10 indicates that based on protein hydrolysis and amino acid analysis, recombinant human lysozyme and native human lysozyme have the same amino acid composition.

**Table 10. Comparison of amino acid composition of recombinant human lysozyme (rhLZ) comparison to native human lysozyme (hLZ)**

<b>Amino Acid</b>	<b>rhLZ</b>	<b>hLZ</b>	<b>Measurement variation</b>
Asx	14.33%	14.32%	0.008%
Thr	4.00%	3.96%	0.041%
Ser	4.47%	4.54%	-0.073%
Glx	7.47%	7.46%	0.004%
Gly	9.19%	9.09%	0.102%
Ala	11.66%	11.54%	0.114%
Cys	4.09%	4.72%	-0.632%
Val	7.19%	7.16%	0.031%
Met	1.66%	1.59%	0.065%
Ile	4.08%	4.02%	0.064%
Leu	6.83%	6.78%	0.053%
Tyr	4.93%	4.81%	0.130%
Phe	1.58%	1.70%	-0.123%
Lys	4.15%	4.17%	-0.025%
His	0.89%	0.90%	-0.003%
Trp	0.00%	0.00%	0.000%
Arg	11.65%	11.38%	0.269%
Pro	1.84%	1.86%	-0.023%

The amino acid composition of rhLZ, as determined by the nucleic acid sequence, is identical to the native human lysozyme sequence. The measured amino acid composition of recombinant human lysozyme was also compared to the theoretical value for human lysozyme based on the nucleic acid sequence. Values for all amino acids in recombinant human lysozyme were within acceptable analytical variation of the theoretical values based on the DNA sequence.

In addition to analysis of amino acid composition, the complete sequence of recombinant human lysozyme was determined. Purified lysozyme was digested by the Lys-c protocol to yield 5 fragments. Four of the fragments were analyzed for amino acid sequence by Edman sequence analysis. One fragment (amino acids 34-69) was further digested by trypsin into four fragments and analyzed by

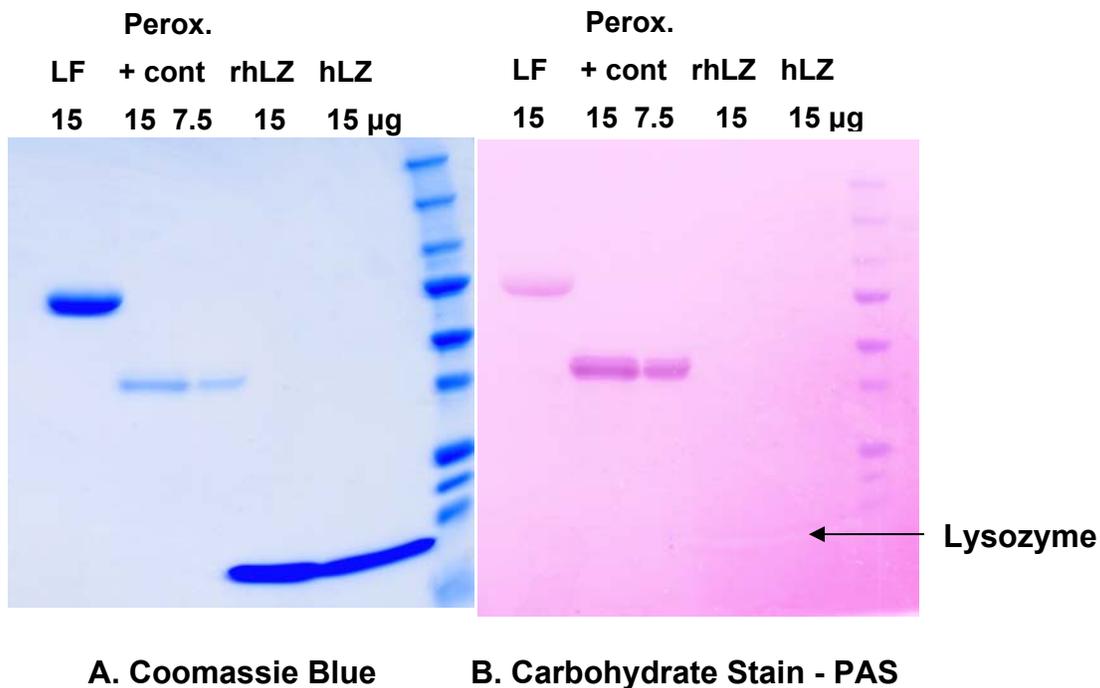
Edman sequence analysis and MS/MS (mass spectrometry) sequencing. There is 100% agreement of the 130 amino acid sequence of recombinant human lysozyme compared to that predicted based on the human lysozyme DNA sequence.

b. N-terminal sequence

N-terminal sequence analysis of purified rhLZ gave the same sequence, KVFER(C)ELART, as native human lysozyme, indicating that the signal peptide was removed correctly by rice signal peptidase. In expressing rhLZ in rice grain, a signal peptide obtained from rice storage protein glutelin 1 was fused with mature peptide of human lysozyme to direct the rhLZ into the endoplasmic reticulum and target it into protein bodies.

c. Glycosylation

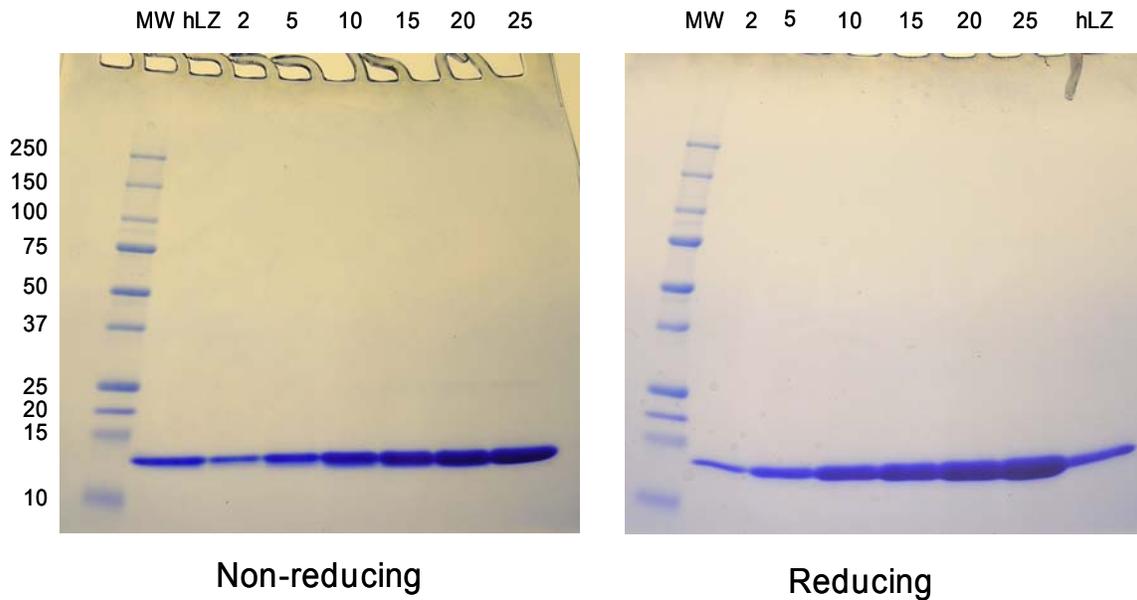
Native human lysozyme is not glycosylated; recombinant human lysozyme should not be glycosylated. To confirm the absence of glycosylation, a Glycoprotein Detection Kit using a periodic acid-Schiff (PAS) reaction was used to detect carbohydrates. This kit method has a stated limit of detection of 25 – 100 ng of carbohydrate, depending on the nature of protein glycosylation. Figure 8 shows the gels comparing protein staining with Coomassie Blue (A) and carbohydrate staining with PAS (B). There are two positive controls used on the gels. The peroxidase control was included in the kit. It contains two glycan chains equivalent to 16% of the weight and the protein was loaded at 15 and 7.5 µg. As an additional control, 15 µg of recombinant human lactoferrin from rice was run on the gel. Lactoferrin has two glycan chains equivalent to 5% of weight. Neither native nor recombinant lysozyme shows evidence of glycosylation at the concentrations loaded.



**Figure 8. Glycan analysis of rhLZ and hLZ**

d. Molecular weight

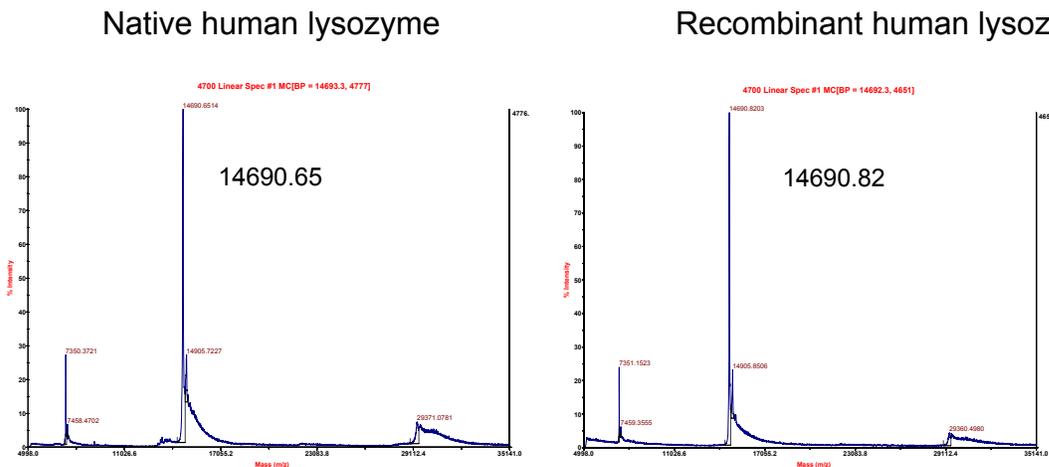
The molecular weight of recombinant human lysozyme was determined by SDS-PAGE using both non-reducing and reducing conditions. Flour from LZ159 rice grain was extracted, purified and analyzed by SDS-PAGE (2 – 25  $\mu\text{g}$ ). Native hLZ (5 $\mu\text{g}$ ) was used as control. Both recombinant and native human lysozyme were loaded on a 4-20% Tris glycine gradient gel. Results show that native hLZ and rhLZ migrate at the same speed and distance indicating that both enzymes have similar molecular weight. By referring to molecular weight markers, it is estimated that the molecular weight of the enzyme is 14.5 kD.



**Figure 9. SDS-PAGE of recombinant human lysozyme and control human lysozyme**

e. Molecular Mass (MALDI)

To accurately determine the molecular weight of recombinant human lysozyme, the latest technology, matrix assisted laser desorption/ionization (MALDI) combined with time of flight (TOF) mass spectrometry was used. Molecular weights for both recombinant and native human lysozyme were determined. The human lysozyme has a molecular weight of 14,690.65 and recombinant human lysozyme is 14,690.82. The mass of recombinant human lysozyme is identical to that of native human lysozyme and both are in good agreement with the predicted theoretical mass of 14699.75. Experimental error is 0.1% or 14.7 Daltons.



**Figure 10. MALDI mass spectrum of native and recombinant human lysozyme**

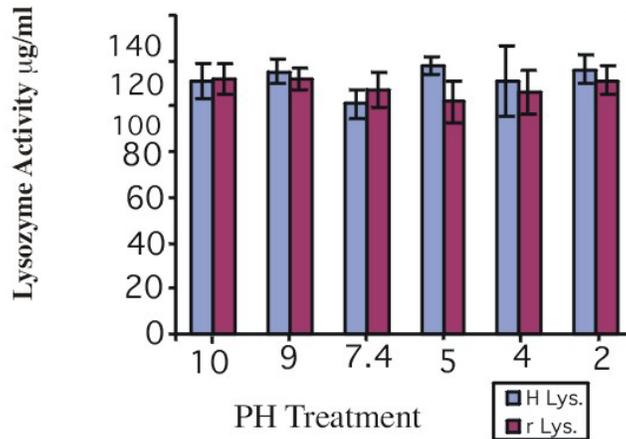
f. Isoelectric focusing

Lysozyme is a very basic protein with a calculated pI of 10.2. In order to compare the pI of native and recombinant lysozyme, reverse IEF gel electrophoresis was done. Results (not shown) indicated no difference in the pI of the two forms of lysozyme.

g. pH Stability

The pH stability of recombinant human lysozyme was compared to that of native human lysozyme using the *Micrococcus luteus* turbidimetric assay. For acidic and neutral pH (2, 4, 5 and 7.4), lysozyme was dissolved in PBS at 100 µg/mL and adjusted to the desired pH with HCl. For basic pH (9 and 10), lysozyme was dissolved in TBS adjusted to the desired pH with sodium bicarbonate. The lysozyme was incubated at 37° C for 30 minutes and assayed for enzymatic activity. One unit of enzyme activity produced a 1 milliOD change in absorbance (450nm) in a 2.6 mL reaction volume (pH 6.24) and 1 cm path length. The

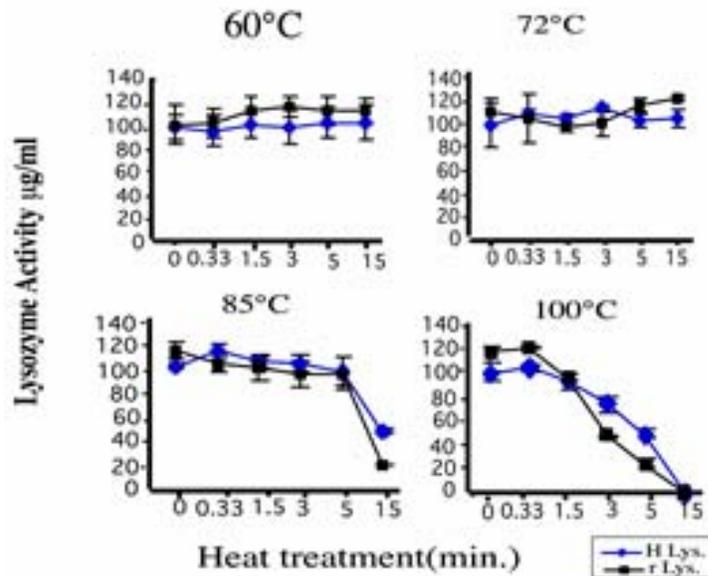
recombinant and native human enzymes have the same pH stability profile and maintain their activity across a broad pH range (pH 2 to 10).



**Figure 11. pH stability of recombinant human lysozyme and native human lysozyme activity**

h. Thermal stability

Recombinant human lysozyme and native human lysozyme were compared for thermal stability. Approximately 50 µL of hLZ and rhLZ in PBS (pH 7.4) at 100 µg/mL were subjected to heat treatment. Four different temperatures of 60, 72, 85 and 100° C were tested. For each temperature, samples were removed at 0 min, 0.33 min, 1.5 min, 3 min, 5 min and 15 min for analysis of lysozyme enzymatic activity by *Micrococcus luteus* turbidimetric assay (see page 35). At 60° C and 72° C, enzymatic activity survives 15 minutes of incubation time. Both proteins have the same thermal stability profile, losing their activity after 5 min at 85° C and almost immediately at 100° C.



**Figure 12. Thermal stability of recombinant human lysozyme and native human lysozyme**

i. Solubility

Both native and recombinant lysozyme are soluble at > 50 mg/mL in phosphate buffered saline at room temperature.

j. Pepsin digestion

Ventria recombinant lysozyme was compared with native human lysozyme from breast milk and hen egg white lysozyme for resistance to pepsin digestion. The studies were done using the digestion protocol evaluated by nine different laboratories working with the ILSI Health and Environmental Sciences Institute (Thomas, Alalbers et al. 2004). Lysozyme samples were digested in simulated gastric fluid, pH 1.2. Samples were removed for analysis at time 0, 0.5, 2, 5, 15, 30, and 60 minutes and neutralized with 0.2 M sodium carbonate, pH 11. Digests were run on 10-20% Tris/Tricine gels from Invitrogen under reducing conditions. All forms and sources of lysozyme were digested to the same extent in 5 minutes and completely in 15 minutes when analyzed according to the standard protocol. Based on the results from the individual gels, the digestion was repeated on the human lysozyme and recombinant human lysozyme using time points 0, 2, 5, 10 and 15 minutes. Both proteins were completely digested in < 10 minutes. Figure 13 shows the complete time course of digestion for native human lysozyme (A), recombinant human lysozyme (B) and hen egg white lysozyme (C) and the time course including 10 minutes for hLZ and rhLZ on the same gel (D).



## 2. Activity Equivalence

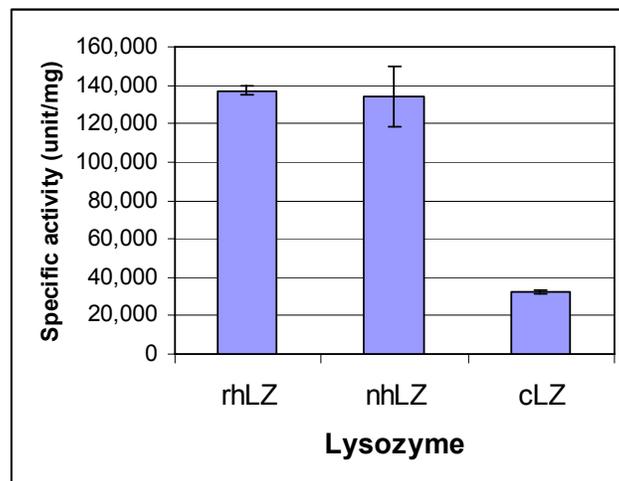
In addition to comparing the physical and chemical properties of hLZ from milk to rhLZ from rice, activity comparisons were done. Table 11 summarizes the studies and the results. The data from the individual studies follow the table.

**Table 11. Activity of recombinant human lysozyme compared with human lysozyme**

Property	Human LZ (hLZ)	Recombinant hLZ (rice derived)
Specific activity (U/mg)	134,206 ± 15,600	137,122 ± 2,468
Bactericidal (10 <sup>5</sup> to 0 CFU at 20 µg/mL)	2 hrs	2 hrs

### a. Specific Activity

The specific activity of recombinant human lysozyme and native human lysozyme were compared (Figure 14). Lysozyme specific activity was measured in the *Micrococcus luteus* turbidimetric assay. One unit of activity produced a 1 milliOD change in absorbance (450nm) in a 2.6 mL reaction volume (pH 6.24) and 1 cm path length. Recombinant human lysozyme has a specific activity of 137,122 unit/mg protein and native human lysozyme has a specific activity of 134,206 units/mg protein, indicating substantial equivalence and activity of ~135,000 units/mg protein. As a comparison, hen egg white lysozyme (cLZ) was measured and its specific activity is 32,393 units/mg protein.



**Figure 14. Specific activity of recombinant human lysozyme (rhLZ), human lysozyme (hLZ) and hen egg white lysozyme (cLZ)**

b. Bactericidal Activity

Bactericidal activity of hLZ and rhLZ were compared using *E. coli* strain JM109. Bacteria were incubated in buffer, non-transformed rice extract, and human or recombinant human lysozyme at 30 µg/mL. After incubation for the time indicated, an aliquot of the mixture was plated and colony forming units determined. There was no reduction in colony forming units in the negative controls while there was a significant decrease of *E. coli* with lysozyme treatment. At 60 min incubation time, colony forming units were reduced to 100 and approached zero at 120 min.

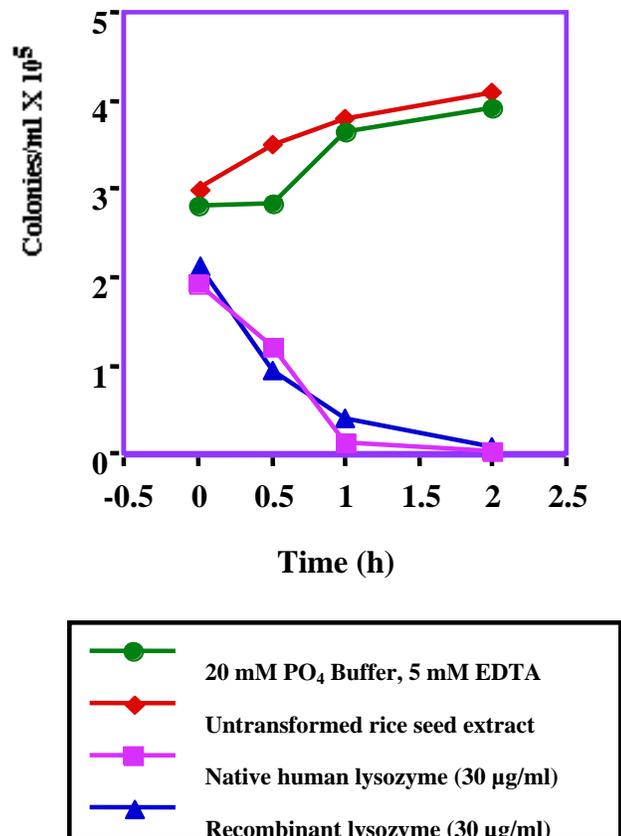
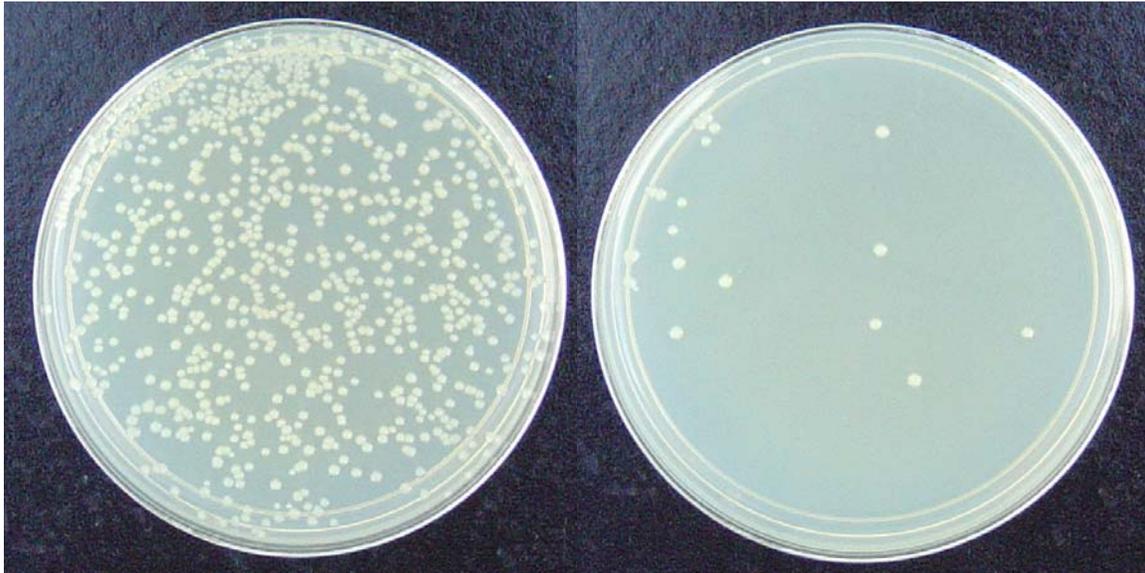


Figure 15. Bactericidal activity of rhLZ and hLZ

The activity of rhLZ was also tested against the K12 strain of *E. coli*.  $10^5$  CFU of *E. coli* in one mL were mixed with 20  $\mu$ g of rhLZ; the control contained no lysozyme. The mixture was incubated at 37° C for 120 minutes, plated and colony forming units were determined.



Buffer only

20  $\mu$ g/mL rhLZ

**Figure 16. Bactericidal activity of recombinant human lysozyme (rhLZ)**

### 3. Safety Equivalence

Immunogenicity and allergenicity are important factors for consideration when determining the safety of human lysozyme purified from rice. The goal is to demonstrate that the lysozyme (human) derived from rice is substantially equivalent to, and thus as safe as, human lysozyme found in breast milk, saliva and other secretions.

#### a. Gene Source

The gene for recombinant human lysozyme is synthetically produced based on the DNA sequence of lysozyme from human secretions. Therefore the gene is not from an allergenic source.

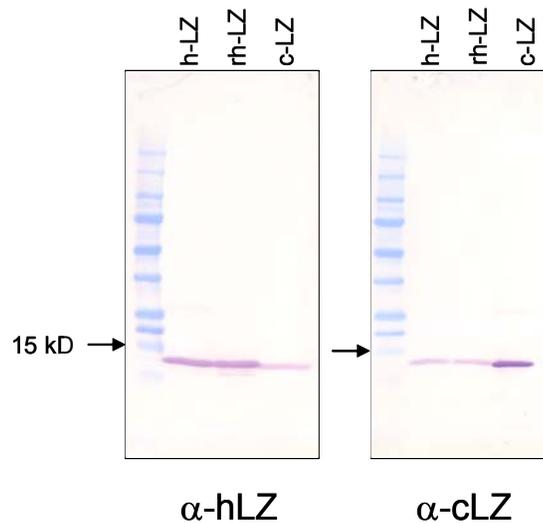
#### b. Sequence similarity, toxin and allergen database search

Lysozyme is present at significant concentrations in the milk and other biological fluids of most mammals – including humans. Human milk contains up to 250 µg/mL of lysozyme (Lönnerdal 1985). Due to its ubiquitous presence in human secretions, it is highly unlikely that this molecule is either a toxin or allergen. Recombinant human lysozyme from rice and human lysozyme have 100% sequence homology. Human lysozyme is not listed as a toxin in the SWISS-PROT and TrEMBL databases; nor does it share any amino acid sequence homology with known toxins, as determined by FASTA search against known toxins listed in the SWISS-PROT and TrEMBL databases. The criteria for the FASTA search are described by Gendel (Gendel 1998). The wordsize used initially was set to two, and the regions of amino acid sequence identity greater than 6 were scored; none were found.

Human lysozyme is not listed as an allergen. A search against a database of known allergens, identified in the SWISS-PROT and TrEMBL databases, also using techniques described by Gendel, found 60% amino acid sequence homology between human lysozyme and hen egg lysozyme, which is a known minor allergen, Gal d4. The lysozyme sequence was also searched using the Food Allergy Research and Resource Program (FARRP) allergen database, [www.allergenonline.com](http://www.allergenonline.com). This site uses the FASTA comparison routine of Pearson and Lipman to compare protein sequences in an allergen database (Pearson and Lipman 1988). Using this data base, historical data indicate cross-reactivity is not likely for proteins with less than 50% homology over the entire protein sequence. Hen egg white lysozyme and a 24 amino acid lysozyme fragment from hen egg white lysozyme were the only proteins with an identity greater than 50% homology (60.9% and 52.2% respectively). At this level of homology, the possibility of cross-reactivity exists and additional testing was performed (see below).

There were some matches in the 40% range. A new 80mer sliding window search is available on [www.allergenonline.com](http://www.allergenonline.com) and that program was run as well. Chicken lysozyme C (hen egg white lysozyme) and bovine alpha-lactalbumin were the only hits using this program. The alpha-lactalbumin increased from 40% to 46% homology using the 80mer sliding window. Since lysozyme is a human protein and there is strong evidence presented that the IgE to hen egg white lysozyme (at 66.% homology by sliding scale) is not cross reactive (page 46), it is highly unlikely that the alpha-lactalbumin at 46% homology would be cross reactive (Metcalf, Astwood et al. 1996).

*In vitro* cross reactivity studies were done using Ventria's recombinant human lysozyme, native human lysozyme and hen egg white lysozyme with polyclonal antibodies. Equal amounts of rhLZ and hLZ and cLZ (5µg) were used in Western blot analyses. Two identical blots were prepared: one probed with rabbit anti-hLZ IgG (Dako) and the other probed with rabbit anti-cLZ IgG (BioDesign Int). Both antibodies were purified polyclonal IgG allowing recognition of a wide range of epitopes. Five µg each of native human lysozyme (hLZ), recombinant human lysozyme (rhLZ) and hen egg white lysozyme (cLZ) were separated on a 4-20% SDS polyacrylamide gel and then transferred to nitrocellulose paper. The blot on the left was developed with rabbit anti-human lysozyme antibody while the blot on the right was developed with rabbit anti-hen lysozyme antibody. In the blot probed with anti-hLZ antibody, the same band intensity was observed in lanes for rhLZ and hLZ indicating that the two proteins have the same epitopes. Less intensity was observed in lane with cLZ. The same relationship was observed when the blots were probed with a polyclonal IgG antibody to hen egg white lysozyme. Although these commercially available antibodies showed cross-reactivity, there are also examples in the literature with reports on the preparation of rabbit anti-human lysozyme antibodies that do not cross-react with hen egg white lysozyme (Osserman and Lawlor 1966).



**Figure 17. Cross-reactivity of anti-hLZ antibody and anti-cLZ antibody to recombinant human lysozyme (rhLZ), native human lysozyme (hLZ) and hen egg white lysozyme (cLZ)**

In the Western blot developed with antibody to human lysozyme, there is a faint band observed just below the primary lysozyme band. Ventria Bioscience has determined that this band is an experimental artifact (e.g. non-specific binding of antibody) and not a degradation product. The MALDI data (see page 35) was obtained using the same lot of recombinant human lysozyme and there is no indication of a lower molecular weight fragment. In addition, N-terminal sequencing and complete protein sequencing gave no evidence of a lower molecular weight specie. Ventria Bioscience has 12 weeks of real time stability at 37° C and ambient temperature of recombinant human lysozyme. Stability samples were analyzed by HPLC and enzymatic activity. After 12 weeks there was no loss of enzymatic activity and a single peak by HPLC.

The cross-reactivity in the blots is similar to data observed in the development of an radioimmunoassay for hen egg white lysozyme (Yuzuriha, Katayama et al. 1978). The radioimmunoassay measured competitive binding of <sup>125</sup>I-hen egg white lysozyme with a rabbit anti-hen egg white lysozyme polyclonal antibody. Unlabeled hen egg white lysozyme was detectable at 0.3 – 5.0 ng/mL while human lysozyme required concentrations greater than 2 µg/mL. Based on the results, the study determined the cross-reactivity between hen egg white and human lysozyme to be 1:10,000. This suggests there is no clinically significant cross-reactivity between the lysozymes.

The cross-reactivity of human IgE antibodies to hen egg white lysozyme with recombinant human lysozyme was investigated and the report from those studies, described below, came to a similar conclusion.

c. Pepsin digestion

There is no difference in the susceptibility of hLZ and rhLZ to digestion with pepsin at pH 1.2. Although there is some resistance to digestion, there is no difference in human lysozyme from breast milk, human lysozyme from rice and hen egg white lysozyme. Gels are shown on page 38.

#### 4. Allergenicity

It is unlikely that human lysozyme will cause a clinical allergic response. It is a protein component of breast milk, and as such is one of the earliest proteins infants are exposed to in their diet. There are also a high concentrations found in tears and saliva. It has never been identified as an allergen. Due to the high cost of human lysozyme, however, hen egg white lysozyme is the only commercially available lysozyme and most studies have used hen egg white lysozyme, which is a minor allergen (Sava 1996).

For human recombinant lysozyme to have allergic potential there must be IgE binding structures in the product. If these structures occur, it must be determined whether they can induce mediator release in a clinically relevant manner. There are three potential allergenic structures:

- Human lysozyme as an auto-allergen
- Residual rice proteins being known allergens
- Cross-reactivity with IgE to hen egg white lysozyme allergenic epitopes

a. Human lysozyme as an auto-allergen

The likelihood of human lysozyme being an auto-allergen is small, but it can not be excluded completely. Auto-allergens are sometimes also referred to as IgE-dependent histamine-releasing factors (HRF) (Kleine Budde, de Heer et al. 2002; Kleine Budde and Aalberse 2003). IgE-binding to human proteins on the basis of cross-reactivity with a non-self sensitizing allergen has been reported. An example is the rare report of human profilin that was shown to be a cross-reactive (auto-) allergen in some patients sensitized to birch pollen profilin (Valenta, Duchene et al. 1991). IgE recognition of recombinant human lysozyme on the basis of sensitization to e.g. hen egg white lysozyme is remote. Even if such a patient would be identified, we can argue that this is not a property that is a result of its expression in rice. The potential role of a human protein as an auto-allergen holds for any human protein, including many blood-derived products e.g. albumin and immunoglobulin that are administered to patients on a regular basis. In summary, although relevant for discussion, this possible allergenic property of a recombinant human lysozyme product is not a reason to come to a negative verdict.

b. Residual rice proteins being known allergens

Although rice is considered to have low allergenicity, there are reported instances of rice-allergy/sensitivity. The products with recombinant human lysozyme from rice contain at least 20% residual rice protein, but possibly up to 50%. This residual rice material could include rice-derived allergens. Cereal grains have been reported to be a cause of food allergies; however rice is not considered a major food allergen. *In vitro* studies on the IgE-binding reaction to rice proteins have suggested that the major IgE binding protein in rice is a 14-16 kD protein. More recent studies have suggested this protein is a salt soluble albumin belonging to the  $\alpha$ -amylase/trypsin inhibitor family (Izumi, Sugiyama et al. 1999), however, the subjects with these antibodies have not been tested by food challenge and it is questionable whether this is a true allergy or perhaps the result of a grass pollen allergy.

In Europe and the USA, rice is not considered an issue for allergy. In Japan, IgE-mediated rice sensitivity is reported at 10% in atopic individuals (Besler, Tanabe et al. 2001), however, there is no indication that these individuals have an allergic response following the ingestion of rice. Studies of rice allergies and rice allergens are complicated by the fact that allergens exist in rice pollens; there is evidence that rice pollen allergens cross react with allergens in rice and grass pollens.

Despite these studies, rice continues to be a component of recommended hypoallergenic diets. It remains one of the major components of the elimination diets used in the evaluation and diagnosis of food allergies (Bock 2003; Mofidi 2003).

Recent studies have suggested that certain organisms, tumors or whole allergens (e.g. peanut) contain glycans that serve as natural adjuvants directing a Th2 skewed response (Atochina, Daly-Engel et al. 2001; Okano, Satoskar et al. 2001; Thomas and Harn Jr 2004). Many of these glycans bind to ligands on dendritic cells, promoting DC2 differentiation (promoting a Th2 response). While there is limited evidence to support the existence of rice allergy, glycans present in rice may, in combination with a potent antigen, alter the immune response to that antigen. We are cognizant of this possibility with recombinant human lysozyme but feel that this is unlikely given the rarity of rice allergy and the ubiquitous exposure to this foodstuff.

c. Human lysozyme as a cross reacting allergen

Currently, the commercial source of lysozyme is hen egg white. Hen egg white lysozyme is used extensively in the food industry to protect certain foods from bacterial contamination, especially meat and cheese (Hughey and Johnson 1987; Hughey, Wilger et al. 1989). Egg white lysozyme was affirmed as GRAS for use as an antimicrobial in cheese manufacture in the tentative rule published

March 13, 1998 (63 FR 12421 et.seq). The Joint Food and Agricultural Organization/World Health Organization Expert Committee on Food Additives evaluated lysozyme and concluded the low additional intake of lysozyme via cheese was not a hazard to consumer health (FAO/WHO 1992). Hen egg white lysozyme received GRAS designation (GRN 64) for use as an antimicrobial agent for use on cooked meat and poultry products.

However, hen egg white lysozyme is a minor allergen and there are reports of allergic reactions related to exposure to egg white lysozyme in cheese and drugs (Pichler and Campi 1992; Fremont, Kanny et al. 1997). Sensitization to egg has also been identified in exclusively breast fed children. Investigators have attributed this reaction to ovalbumin present in breast milk due to egg ingestion by the mother. In a controlled egg intake study, ovalbumin in human breast milk was present for up to eight hours following egg intake and had a dose response correlation (Palmer, Gold et al. 2005).

The egg white protein allergen designations and their proportion by weight are as follows:

ovomucoid	Gal d1	(11%)
ovalbumin	Gal d2	(54%)
ovotransferrin (conalbumin)	Gal d3	(12%)
lysozyme	Gal d4	(3.5%)

(Anet, Back et al. 1985; Poulsen, Hansen et al. 2001) .

One report tested serum from 34 adults with a positive history of egg allergy using a battery of *in vitro* tests. That group reported the order of allergenicity frequency as ovotransferrin (53%) > ovomucoid (38%) > ovalbumin (32%) > lysozyme (15%) (Aabin, Poulsen et al. 1996). In a study using repurified egg white proteins, Mine and Zhang determined the order of dominance in IgE binding to be ovomucoid > ovotransferrin (conalbumin) > ovalbumin > lysozyme (Mine and Zhang 2002). In contrast, the IgG response was lysozyme > ovomucoid > ovotransferrin > ovalbumin.

The most common food allergies are mediated by IgE reactions, type I immediate hypersensitivity and anaphylaxis reactions. Type II, antibody dependent cytotoxic hypersensitivity and Type III hypersensitivity reactions are mediated by IgG antibodies and not generally associated with oral delivery of the antigens. In a study comparing IgG and IgE antibodies in milk allergic and control subjects, IgG, but not IgE, antibodies to milk were found in the controls who were tolerant of cow's milk (Beyer and Teuber 2005).

Some of the reported differences in the dominance of allergens may be related to the study populations. For example, adults and children may process the egg proteins differently due to differences in their digestive systems. However, cross contamination of the protein preparations may be another source of variation. It may require very rigorous purification of the egg white proteins to obtain truly

pure preparations (Ebbehoj, Dahl et al. 1995). A commercial preparation of egg white lysozyme (Sigma) contained ovalbumin and ovotransferrin as well as some smaller non-identified proteins prior to repurification with a two-step ion-exchange and gel filtration method.

There is 60% sequence homology between human lysozyme and hen egg white lysozyme. There are examples of polyclonal antibodies to hen egg white lysozyme that are weakly cross reacting and non-cross reacting to human lysozyme. It is known that the major IgG binding epitopes of lysozyme are conformational, not linear epitopes (Bentley 1996; Smith-Gill 1996). There are three major IgG epitope regions on hen egg white lysozyme (Bentley 1996). In 1984, Allen et al. identified amino acid region 46-61 of hen egg white lysozyme as the region recognized by T-cells to initiate an immune response. Human lysozyme differs from chick lysozyme by four residues in this region, three of which are located in the amino terminus. Additionally, they isolated two T-cell lines that recognized and responded to a challenge from hen egg white lysozyme. These same cell lines had no detectable response to human lysozyme, even when a 10 to 100-fold excess of antigen was used (Allen, Strydom et al. 1984; Allen and Unanue 1984). These studies have looked at IgG antibodies. Although it is unlikely, the possibility exists that human lysozyme could cross-react with IgE antibodies to hen egg white lysozyme.

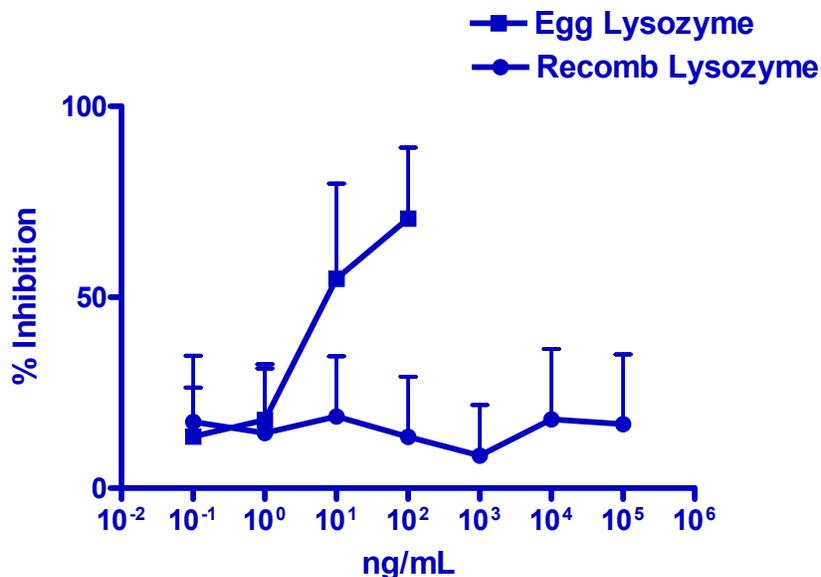
To address this issue *in vitro* ELISA inhibition studies were done on sera from hen egg white lysozyme sensitized individuals. Sera from 60 patients with egg allergy based on Double Blind Placebo Controlled Food Challenge to egg were screened for lysozyme specific IgE using the CAP System. Forty (67%) of the patient's sera were positive for hen egg white lysozyme IgE. This number appears high based on estimates in the literature that 15% to 35% of the egg allergic population are allergic to lysozyme (Aabin, Poulsen et al. 1996; Fremont, Kanny et al. 1997; Martorell Aragones, Bone Calvo et al. 2001; Poulsen, Hansen et al. 2001). There are two explanations of this high rate of positives. The hen egg white lysozyme did not undergo additional purification. Ebbehoj demonstrated that commercial lysozyme was contaminated with both ovotransferrin and ovalbumin (Ebbehoj, Dahl et al. 1995). Second, the investigator suggested that the frequency of hen egg white lysozyme allergy is increasing in France due to hen egg white lysozyme inclusion in cheese. This observation has been published (Fremont, Kanny et al. 1997).

Four of the subjects with IgE to egg white lysozyme participated in a DBPCFC with hen egg white lysozyme. Two of the four with reactive IgE were negative on oral challenge and two were reactive in the challenge. Of the two subjects with a positive DBPCFC to hen egg white lysozyme, serum IgE from one subject was not bound by recombinant human lysozyme in the IgE ELISA inhibition at concentrations up to 100 µg/mL. Serum from the second reactive subject (number 19) did show IgE inhibition at 10 µg/mL, but not 100 µg/mL using recombinant human lysozyme. Inhibition was 86% with hen egg white lysozyme

at 100 ng/mL. The results for this subject are presented and discussed in Figure 19.

Sera from 39 subjects, including the four described above, were tested by IgE ELISA binding of hen egg white lysozyme using non-repurified Sigma lysozyme. Sera were then tested in an IgE ELISA inhibition with hen egg white lysozyme (Sigma) and recombinant human lysozyme isolate in a concentration range from 0.1 ng/mL to 100 ng/mL. For the ELISA tests, a direct binding optical density of >0.10 was considered positive for egg lysozyme IgE reactivity. A 30% decrease in optical density of specific binding IgE was considered clear inhibition and reported based on the lysozyme concentration resulting in 30% or greater inhibition. The inhibition was confirmed by shape of the curve with increasing inhibition by increasing concentrations of lysozyme. The recombinant human lysozyme was also tested in ELISA inhibition at concentrations from 0.1 ng/mL to 100,000 ng/mL, a 1000-fold excess over the hen egg white lysozyme.

Of the 39 sera tested, nine showed no inhibition of IgE binding by hen egg white lysozyme and were eliminated from the study. The combined ELISA inhibition data for the remaining 30 sera are in Figure 18. Data are the mean and standard deviation of the percent inhibition of the 30 individual sera at each concentration tested. There is little suggestion of inhibition of IgE binding to hen egg white lysozyme by recombinant human lysozyme.

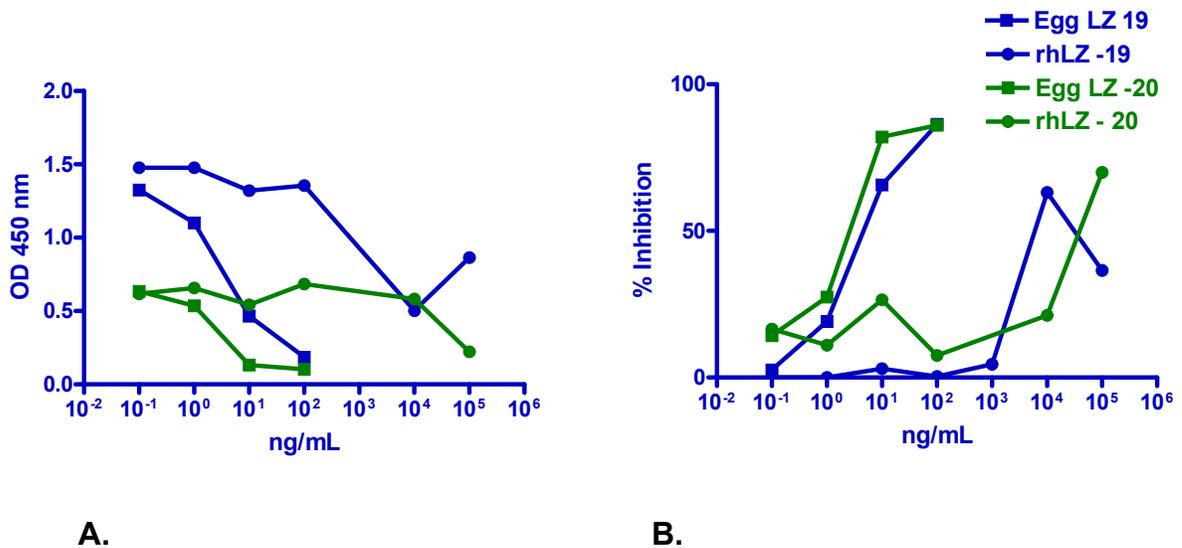


**Figure 18. Percent inhibition ELISA (mean  $\pm$  SD) for 30 egg lysozyme positive sera**

When recombinant human lysozyme was tested at an equimolar concentration range to the hen egg white lysozyme, there was no inhibition of IgE binding to egg white lysozyme by human recombinant lysozyme with any serum. When recombinant human lysozyme was tested up to a 1000-fold excess over the hen egg white lysozyme, three samples showed some inhibition.

Inhibition curves for two of the three sera that were inhibited by high levels of recombinant human lysozyme are in Figure 19. The third serum, from subject 04 (not shown), had a direct binding egg lysozyme ELISA value of 0.15, near the lower level cut off, and a hen egg white lysozyme ELISA inhibition of 36% at 100 ng. Using a concentration of rhLZ of 100 µg/mL, a 1000-fold increase, recombinant human lysozyme caused a 31% inhibition (OD<sub>450</sub> 0.10). The low direct binding value makes this patient difficult to evaluate for significance of the inhibition.

Sera from subjects 19 and 20, Figure 19, showed no inhibition with recombinant human lysozyme at 100 ng/mL. Data are shown as ELISA result of OD<sub>450</sub> in Figure 19A and percent inhibition in 191B. At 100 ng/mL hen egg white lysozyme inhibited serum IgE binding to egg white lysozyme by 86% for both subjects. Subject 19, with documented extreme sensitivity to egg lysozyme by DBPCFC, showed inhibition of 63% at 100-fold excess (10 µg/mL), but marginal inhibition of 36% at 1000-fold excess (100 µg/mL). Subject 20 had 70% inhibition at 1000-fold excess (100 µg/mL). In both cases it is difficult to interpret the shape of the recombinant human lysozyme inhibition curve. Subject 19's egg allergy was identified as abnormally high in a screen of 250 egg allergic patients. It is considered by the investigator an extremely rare occurrence and not applicable to the general egg allergic population. Additional testing is being done on both sera, but it is unlikely that either represents a clinically significant response to recombinant human lysozyme. There is no evidence of human lysozyme allergy in either of these patients, thus these two subjects are not likely to react to recombinant human lysozyme under normal circumstances of ingestion.



**Figure 19. ELISA inhibition for 2 egg lysozyme positive sera with inhibition by human recombinant lysozyme at high concentration.**

**A is data based on optical density at 450 nm**

**B is % inhibition.**

Binding of antigen to IgE bound to circulating basophils triggers a specific cellular response that causes expression on the cell surface of CD 63 (gp 53). The occurrence of this response can be measured using a fluorescence activated cell sorter and FITC labelled monoclonal antibody directed against CD 63. Heparinized blood samples were obtained from eighteen patients diagnosed with egg allergy/sensitivity. Egg white lysozyme or human lysozyme was added to whole blood at 3 different concentrations 0.1, 1 and 100 µg/mL and after 20 min incubation at 37°C the basophil activation reaction was stopped. CD 63 presence on the cell surface was determined using a Beckman cytometer. For all samples n-formyl-Met-Leu6phe was used as a positive control of basophil activation. The basophil activation test was considered positive if the positive control yield 25 to 59 % positive, negative control yield 1.9 to 9.5 % positive and either egg or human lysozyme yielded >15 % positive cells.

Of the eighteen samples tested, two showed basophil activation at high concentrations of recombinant human lysozyme at 80% purity. When the rhLZ was further purified by HPLC to 95% purity and the activation test repeated, the samples were negative. This indicates that the activation observed was due to cross reacting rice proteins or carbohydrate cross reacting determinants. The basophil data provides additional confirmation that cross reactivity of human recombinant lysozyme with IgE to hen egg white lysozyme is not clinically significant. These data from A.D. Moneret-Vautrin will be published.

## 5. Autoimmune disease and lysozyme

Anti-lysozyme autoantibodies may be present in patients with autoimmune diseases, forming a component of anti-neutrophil cytoplasmic antibodies (ANCA; for a review see (Schulz and Tozman 1995)). However, anti-lysozyme autoantibodies appear to be a minor component of the ANCA antibody response. In one study of a cohort of 566 patients with systemic lupus erythematosus only 4.6% of the ANCA antibodies were lysozyme specific (Galeazzi, Morozzi et al. 1998). In another study of 57 patients with inflammatory bowel disease, none had antibodies to lysozyme (Ooi, Lim et al. 2000). The clinical literature on anti-lysozyme autoantibodies as a component of ANCA provides no evidence anti-lysozyme autoantibodies have a causative role, but rather that they are a result of the autoimmune disease and have a minor effect. It thus seems highly unlikely that oral ingestion of human lysozyme, even by individuals with an ongoing autoimmune disease, would have any adverse effect.

## 6. Amyloid formation and lysozyme

Amyloidosis is a disorder in which normally soluble proteins are deposited as insoluble fibrils as a result of abnormal protein folding. There are at least 20 different unrelated proteins which can form amyloid fibrils *in vivo* (Hawkins 2003). The first association between lysozyme and hereditary systemic amyloidosis was reported in two families in 1993 (Pepys, Hawkins et al. 1993). In this report, affected individuals had a mutant form of lysozyme, although each family had a different mutation. In all cases a single amino acid mutation occurred in a highly conserved region of the lysozyme protein and structural disruption was predicted from the change. This was the first report of a natural mutation in the human lysozyme gene or a variation in the amino acid sequence. Currently four families have been identified with lysozyme amyloidosis. All have different clinical presentations and they represent three different single amino acid substitutions (Valleix, Drunat et al. 2002).

- Isoleucine            56     to Threonine
- Tryptophan           64     to Arginine
- Aspartic acid        67     to Histidine

As a result of this finding, amyloidotic tissues from patients with other forms of amyloidosis (transthyretin and apoA1 variants) were analyzed using anti-lysozyme antibodies. The investigators specifically tested senile amyloid of articular cartilage and localized ocular amyloid deposits because articular cartilage and tears have relatively high concentrations of lysozyme. No lysozyme was detected in these amyloid deposits.

Wild-type lysozyme is not amyloidogenic. The individuals affected by hereditary amyloidosis are heterozygous for the mutation, but the amyloid deposits in different tissues contain only the mutant variant and not wild-type lysozyme (Hawkins 2003). In addition, the serum lysozyme in these patients is wild-type, not mutant.

Lysozyme has been very well characterized to the level of atomic resolution. Lysozyme was the first protein to be characterized by x-ray crystallography and as such has been the model protein for structural and functional characterization of proteins in general. Amyloid diseases are of great clinical importance due to their association with Alzheimer's and prion induced diseases. Lysozyme has provided an excellent model for *in vitro* study to help understand the formation of amyloidogenic conformations of proteins and the mechanisms of aggregation. It was one of the earliest systems for the development of NMR techniques for studying folded and unfolded proteins. It is utilized extensively today in protein experimentation (Dyson and Wright 2004). Wild-type human lysozyme can be induced *in vitro* to form amyloid fibrils (Morozova-Roche, Zurdo et al. 2000). However, the conditions required are those which typically destabilize native proteins and favor protein aggregation: acidic pH (pH 2.0 – 4.0), elevated temperature (37 - 65° C), extended time (2 – 10 days), ethanol or trifluoroethanol (Frare, Polverino de Laureto et al. 2004). The conditions used are generally not reproducible *in vivo* and therefore should not be a factor in the use of lysozyme with oral delivery (de Felice, Vieira et al. 2004).

Ventria Bioscience has done full sequence analysis of the recombinant human lysozyme and there is 100% agreement with the sequence of wild-type lysozyme as defined by the DNA sequence. The amino acid sequence from position 51-68 was confirmed identical to human lysozyme based on nucleic acid sequence using mass spectrometry of Lys-c and trypsin digested fragments (see page 31). None of the amyloid related amino acid substitutions occur in the recombinant human lysozyme from rice.

## **7. Studies in animals and humans with oral delivery of human lysozyme (human) derived from rice**

Ventria Bioscience's lysozyme (human) derived from rice has been fed to animals and humans with no reports of adverse events or indication of IgE allergic reactions. The rhesus monkey animal model is often chosen to test the safety of products prior to human trials. In collaboration with a team at UC Davis, infant rhesus monkeys were fed with a modified infant formula containing 0.1 mg/mL of rhLZ and 1.0 mg/mL rhLF from day one after birth to four months. Animals were fed a whey-based formula containing recombinant human lysozyme from an extract of the LZ159 rice flour. The formula also included recombinant human lactoferrin. There were no infant deaths and no adverse

effects were observed at a dosage ranging from 0.05 to 0.08 g/kg body weight for rhLZ and 0.5 to 0.8 g/kg body weight for rhLF.

To provide information on possible health hazards that might arise from repeated oral administration of recombinant human lysozyme produced and extracted from rice, a 28 day repeated dose oral toxicity study was done in rats. Fifteen healthy male and fifteen healthy female Wistar albino rats were randomly selected and assigned to one of three groups of five males and five females per group. All animals were dosed once daily, seven days a week by oral gavage technique. One group received vehicle (saline), two groups received recombinant human lysozyme from rice dosed at 0.36 and 0.036 g/kg/day. No animals exhibited any symptoms of allergic reaction during the feeding phase; the histopathology and serum chemistry indicated no evidence of allergic response.

Based on the hematological, clinical chemistry and microscopic data, the no observed adverse effect level (NOAEL) is considered to be greater than 0.36 g/kg/day of 85% recombinant human lysozyme.

Currently, testing is being done in children with acute watery diarrhea using lysozyme at 0.2 mg/mL in an oral rehydration solution. In a case of extreme dehydration, this would be a dose of 0.048 to 0.090 g/kg/day. One hundred forty children have completed the study, one third have received the lysozyme supplemented ORS and there have been no material related adverse events.

## ***Conclusion***

The literature and the experimental data provided by Ventria Bioscience support the conclusion that there is minimal risk of allergenic or immunogenic responses in individuals consuming recombinant human lysozyme produced in rice.

- The proteins are biochemically equivalent
- The gene source is non-allergenic
- The protein is not in the allergen databases
- Risk is remote of allergic sensitization to human lysozyme
- Recombinant lysozyme from rice appears to be biologically safe for egg white lysozyme sensitized patients.
- Residual rice proteins are not likely trigger an allergic response
- It is highly unlikely that ingestion of human lysozyme as a food supplement, even by individuals with an ongoing autoimmune disease, would have any adverse effect.

Recombinant human lysozyme is substantially equivalent to and therefore as safe as human lysozyme.

## **8. Expert Panel Consensus Statement**

In conclusion, there is no indication that adverse effects would result from consumption of foods containing lysozyme (human) derived from rice at the levels proposed in the intended uses.

- The amino acid sequence and structure of the protein are identical to human lysozyme, which has been studied extensively.
- There is no difference in the resistance to pepsin digestion of recombinant human lysozyme and human lysozyme.
- Based on ELISA inhibition studies, it is not likely that the presence of IgE antibodies to hen egg white lysozyme would cause a clinically significant reaction following consumption of recombinant human lysozyme
- For the rare individual with true rice allergy/sensitivity, the products will be labeled as lysozyme (human) derived from rice
- The clinical literature on autoantibodies to lysozyme provides no evidence that these antibodies play a causative role and it is unlikely that ingestion of recombinant human lysozyme, even by individuals with autoimmune disease, would have any adverse effect.
- Using the data generated for this application and other generally available and accepted scientific data, information, methods, and principles, there is reasonable certainty that recombinant human lysozyme from rice will be safe under the intended conditions of use in functional and medical foods and as a replacement for hen egg white lysozyme in the food industry.

Based on a critical evaluation of the currently available pertinent data and information summarized in this report, the Expert Panel concludes that the use of recombinant human lysozyme from rice, meeting the proposed specifications and produced by current good manufacturing practice is generally recognized as safe, based on scientific procedures, for use as an ingredient in functional foods in amounts not to exceed 500 mg/serving, in medical foods in amounts not to exceed 0.2mg/mL and as a replacement for hen egg white lysozyme in the food and beverage industries as specified by Ventria Bioscience.

Robert Bush, M.D.  
Bo Lonnerdal, Ph.D.  
Lloyd Mayer, M.D.  
Stephen L. Taylor, Ph.D., Chairman  
Ronald van Ree, Ph.D.

Steve Taylor, PhD, Chairman of Panel  
Professor and Head  
Department of Food Science and Technology  
University of Nebraska-Lincoln  
Institute of Agriculture and Natural Resources  
143 H.C. Filley Hall, East Campus  
Lincoln, NE 68583-0919  
402-472-5302  
[Staylor@unlnotes.unl.edu](mailto:Staylor@unlnotes.unl.edu)

Signature: \_\_\_\_\_

Date: 6 JUNE, 2005

Robert K. Bush, MD  
Department of Medicine, Allergy & Immunology  
University of Wisconsin – Madison  
K4/910 CSC #9988  
600 Highland Ave  
Madison, WI 53792  
608-263-6174  
[Robert.Bush@med.va.gov](mailto:Robert.Bush@med.va.gov)

Signature: \_\_\_\_\_

Date: 6 June 2005

Bo Lonnerdal, PhD  
Professor of Nutrition and Internal Medicine  
University of California  
Department of Nutrition  
One Shields Avenue  
Davis, CA 95616  
530-752-8347  
[bllonerdal@ucdavis.edu](mailto:bllonerdal@ucdavis.edu)

Signature: \_\_\_\_\_

Date: June 6, 2005

Lloyd F. Mayer MD  
Professor & Director, Center for Immunology  
Professor, Medicine/ Clinical Immunology, Gastroenterology  
Professor, Microbiology  
The Mount Sinai Hospital  
One Gustave L. Levy Place  
Box 1089  
New York, NY 10029  
212-659-9266  
[lloyd.mayer@mssm.edu](mailto:lloyd.mayer@mssm.edu)

Signature: \_\_\_\_\_

Date: 6/6/05

Ronald van Ree, PhD  
Sanquin Research  
Department of Immunopathology  
Plesmanlaan 125  
1066 CX Amsterdam  
The Netherlands  
(020) 612 33 33  
[r.vanree@sanquin.nl](mailto:r.vanree@sanquin.nl)

Signature: \_\_\_\_\_

Date: 6/6/05

## VI. REFERENCES

- Aabin, B., L. K. Poulsen, et al. (1996). "Identification of IgE-binding egg white proteins: Comparison of results obtained by different methods." Int Arch Allergy Appl Immunol **109**: 50-57.
- Allen, P. M., D. J. Strydom, et al. (1984). "Processing of lysozyme by macrophages: Identification of the determinant recognized by two T-cell hybridomas." Proc Natl Acad Sci U S A **81**: 2489-2493.
- Allen, P. M. and E. R. Unanue (1984). "Processing and presentation of hen egg-white lysozyme by macrophages." Immunobiology **168**(3-5): 182-8.
- Amirova, T. D., S. S. Lebenzon, et al. (1990). "[Characteristics of cellular immunity in newborn infants with intestinal bacterial infection after administration of lysozyme]." Pediatrriia(1): 21-4.
- Anet, J., J. F. Back, et al. (1985). "Allergens in the white and yolk of hen's egg. A study of IgE binding by egg proteins." Int Arch Allergy Appl Immunol **77**(3): 364-71.
- Atochina, O., T. Daly-Engel, et al. (2001). "A Schistosome-expressed immunomodulatory glycoconjugate expands peritoneal Gr1+ macrophages that suppress naive CD4+ T cell proliferation via an IFN-g and nitric oxide-dependent mechanism." J Immunol **167**: 4293-4302.
- Bentley, G. (1996). The crystal structures of complexes formed between lysozyme and antibody fragments. Lysozymes: Model Enzymes in Biochemistry and Biology. P. Jolles. Basel Switzerland, Birkhauser Verlag: 301 - 319.
- Besler, M., S. Tanabe, et al. (2001). "Allergen Data Collection - Update: Rice (*Oryza sativa*)." Internet Symposium on Food Allergens <http://www.food-allergens.de> **3**(Suppl 2): 1 - 17.
- Beyer, K. and S. S. Teuber (2005). "Food allergy diagnostic: scientific and unproven procedures." Curr Opin Allergy Clin Immunol **5**: 261-266.
- Bock, S. A. (2003). "Diagnostic Evaluation." Pediatrics **111**(6): 1638-1644.
- Bol'shakova, A. M., E. G. Shcherbakova, et al. (1984). "[Lysozyme in the feeding of premature infants with mixed pathology]." Antibiotiki **29**(10): 784-90.
- Canfield, R. E., S. Kammerman, et al. (1971). "Primary structure of lysozymes from man and goose." Nat New Biol **232**(27): 16-7.
- Chen, Q. (2004). "Determination of phytic acid and inositol pentakisphosphates in foods by high-performance ion chromatography." J Agric Food Chem **52**(15): 4604-4613.
- de Felice, F. G., M. N. N. Vieira, et al. (2004). "Formation of amyloid aggregates from human lysozyme and its disease-associated variants using hydrostatic pressure." FASEB J **18**: 1099-1101.
- Dyson, H. J. and P. E. Wright (2004). "Unfolded proteins and protein folding studied by NMR." Chem Rev **104**: 3607-3622.
- Ebbehoj, K., A. M. Dahl, et al. (1995). "Purification of egg-white allergens." Allergy **50**: 133-141.

- Ellison, R. T., 3rd and T. J. Giehl (1991). "Killing of gram-negative bacteria by lactoferrin and lysozyme." J Clin Invest **88**(4): 1080-91.
- FAO/WHO (1992). Lysozyme, <http://www.inchem.org/documents/jecfa/jecmono/v30je04.htm>. **2005**.
- FDA (1998). Guidance for Industry: Use of antibiotic resistance marker genes in transgenic plants. Washington, D.C., US Food and Drug Administration, Center for Food Safety and Nutrition.
- Frare, E., P. Polverino de Laureto, et al. (2004). "A highly amyloidogenic region of hen lysozyme." J Mol Biol **340**: 1153-1165.
- Fremont, S., G. Kanny, et al. (1997). "Prevalence of lysozyme sensitization in an egg-allergic population." Allergy **52**(2): 224-8.
- Galeazzi, M., G. Morozzi, et al. (1998). "Anti-neutrophil cytoplasmic antibodies in 566 European patients with systemic lupus erythematosus: prevalence, clinical associations and correlations with oather autoantibodies. European Concerted Action on the Immunogenetics of SLE." Clin Exp Rheumatol **16**(5): 541-546.
- Gendel, S. (1998). "The use of amino acid sequence alignments to assess potential allergenicity of proteins used in genetically modified foods." Adv Food Nutr Res **42**: 45-62.
- Gerland, C. Use of lysozyme in enology: Applications and limits, <http://dspace.library.cornell.edu/bitstream/1813/401/1/13.+Christophe+Gerland.doc>.
- Gordon, L. I., S. D. Douglas, et al. (1979). "Modulation of neutrophil function by lysozyme." J Clin Invest **64**: 226-232.
- Hashida, S., E. Ishikawa, et al. (2002). "Concentration of egg white lysozyme in the serum of healthy subjects after oral administration." Clin Exp Pharmacol Physiol **29**(1-2): 79-83.
- Hawkins, P. N. (2003). "Hereditary systemic amyloidosis with renal involvement." J Nephrol **16**: 443-448.
- Huang, J., S. Nandi, et al. (2002). "Expression of natural antimicrobial human lysozyme in rice grains." Molecular Breeding **10**: 83-94.
- Huang, J., L. Wu, et al. (2002). "Expression of functional recombinant human lysozyme in transgenic rice cell culture." Transgenic Res **11**: 229-239.
- Huang, N., L. Y. Wu, et al. (2001). "The tissue-specific activity of a rice beta-glucanase promoter (*Gns9*) is used to select rice transformants." Plant Science **161**: 589-595.
- Hughey, V. L. and E. A. Johnson (1987). "Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease." Appl Environ Microbiol **53**(9): 2165-70.
- Hughey, V. L., P. A. Wilger, et al. (1989). "Antibacterial activity of hen egg white lysozyme against *Listeria monocytogenes* Scott A in foods." Appl Environ Microbiol **55**(3): 631-8.
- Humphrey, B. D., N. Huang, et al. (2002). "Rice expressing lactoferrin and lysozyme has antibiotic-like properties when fed to chicks." J Nutr **132**: 1214-1218.
- Iacono, V. J., B. J. MacKay, et al. (1980). "Selective antibacterial properties of lysozyme for oral microorganisms." Infect Immunity **29**(2): 623-632.

- Ibrahim, H. R. (1998). "On the novel catalytically-independent antimicrobial function of hen egg-white lysozyme: a conformation-dependent activity." Nahrung **42**(3-4): 187-93.
- Izumi, H., M. Sugiyama, et al. (1999). "Structural characterization of the 16-kDa allergen, RA17, in rice seeds." Biosci Biotechnol Biochem **63**(12): 2059-2063.
- Jolles, J. and P. Jolles (1971). "Human milk lysozyme: unpublished data concerning the establishment of the complete primary structure; comparison with lysozymes of various origins." Helv Chim Acta **54**(8): 2668-75.
- Jolles, P. and J. Jolles (1984). "What's new in lysozyme research? Always a model system, today as yesterday." Mol Cell Biochem **63**(2): 165-89.
- Kleine Budde, I. and R. Aalberse (2003). "Histamine-releasing factors, a heterogeneous group of different activities." Clin Exp Allergy **33**(9): 1175-1182.
- Kleine Budde, I., P. G. de Heer, et al. (2002). "Studies on the association between immunoglobulin E autoreactivity and immunoglobulin E-dependent histamine-releasing factors." Immunology **107**(2): 243-251.
- Lee-Huang, S., P. L. Huang, et al. (1999). "Lysozyme and RNases as anti-HIV components in beta-core preparations of human chorionic gonadotropin." Proc. Natl. Acad. Sci. USA **96**(6): 2678-81.
- LeMarbre, P., J. J. Rinehart, et al. (1981). "Lysozyme enhances monocyte-mediated tumoricidal activity: a potential amplifying mechanism of tumor killing." Blood **58**(5): 994-9.
- Lin, A. L., D. A. Johnson, et al. (2001). "Salivary anticandidal activity and saliva composition in an HIV-infected cohort." Oral Microbiol Immunol **16**(5): 270-8.
- Lönnerdal, B. (1985). "Biochemistry and physiological function of human milk proteins." Am. J. Clin. Nutr. **42**(6): 1299-317.
- Marino, C. R. and F. S. Gorelick (2003). Pancreatic and Salivary Glands. Medical Physiology. W. F. Boron and E. L. Boulpaep. Philadelphia, Saunders: 929.
- Martorell Aragones, A., J. Bone Calvo, et al. (2001). "Allergy to egg proteins." Allergol Immunopathol **29**: 72-83.
- Metcalfe, D. D., J. D. Astwood, et al. (1996). "Assessment of the allergenic potential of foods derived from genetically engineered crop plants." Critical Reviews Food Science Nutrition **36**: S165-S186.
- Mine, Y. and J. W. Zhang (2002). "Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins." J Agric Food Chem **50**: 2679-2683.
- Mofidi, S. (2003). "Nutritional management of pediatric food hypersensitivity." Pediatrics **111**(6): 1645-1653.
- Montagne, P., M. Cuilliere, et al. (2001). Changes in lactoferrin and lysozyme levels in human milk during the first twelve weeks of lactation. Bioactive Components of Human Milk. Newburg. New York, Kluwer: 241-247.

- Moreira-Ludewig, R. and C. T. Healy (1992). "A rapid microtiter plate method for the detection of lysozyme release from human neutrophils." J Pharmacol Toxicol Methods **27**(2): 95-100.
- Morozova-Roche, L. A., J. Zurdo, et al. (2000). "Amyloid fibril formation and seeding by wild-type human lysozyme and its disease-related mutational variants." J Structural Biology **130**: 339-351.
- Nishihara, K., K. Isoda, et al. (1967). "[Fecal bacterial flora in the use of dry milk treated with lysozyme]." Nippon Shonika Gakkai Zasshi **71**: 95-102.
- Ohno, N. and D. C. Morrison (1989). "Lipopolysaccharide interaction with lysozyme." J Biol Chem **264**: 4434-4441.
- Okano, M., A. R. Satoskar, et al. (2001). "Lacto-N-fucopentatose III found on *Schistosoma mansoni* egg antigen functions as an adjuvant for proteins by inducing Th2-type response." J Immunol **167**: 442-450.
- Okita, T. W., Y. S. Hwang, et al. (1989). "Structure and Expression of the Rice Glutelin Multigene Family." J. Biol. Chem. **264**(21): 12573-12581.
- Ooi, C. J., B. L. Lim, et al. (2000). "Antineutrophil cytoplasmic antibodies (ANCA) in patients with inflammatory bowel disease show no correlation with proteinase 3, lactoferrin, myeloperoxidase, elastase, cathepsin G and lysozyme: a Singapore study." Ann Acad Med Singapore **29**(6): 704-707.
- Osserman, E. F., R. E. Canfield, et al., Eds. (1974). Lysozyme. New York, Academic Press.
- Osserman, E. F. and D. P. Lawlor (1966). "Serum and urinary lysozyme (muramidase) in monocytic and myelomonocytic leukemia." J Exp Med **124**(921 - 951).
- Palmer, D. F., M. S. Gold, et al. (2005). "effect of cooked and raw egg consumption on ovalbumin content of human milk: a randomized, double-blind, cross-over trial." Clin Exp Allergy **35**(2): 173-178.
- Pearson, W. R. and D. J. Lipman (1988). "Improved tools for biological sequence comparison." PNAS **85**(8): 2444-2448.
- Pepys, M. B., P. N. Hawkins, et al. (1993). "Human lysozyme gene mutations cause hereditary systemic amyloidosis." Nature **362**: 553-557.
- Peters, C. W., U. Kruse, et al. (1989). "The human lysozyme gene. Sequence organization and chromosome location." Eur J Biochem **182**(3): 507-516.
- Peterson, R. K. D. and C. J. Arntzen (2004). "On risk and plant-based biopharmaceuticals." Trends Biotech **22**(2): 64-66.
- Pichler, W. J. and P. Campi (1992). "Allergy to lysozyme/egg white-containing vaginal suppositories." Ann Allergy **69**: 521-525.
- Poulsen, L. K., T. K. Hansen, et al. (2001). "Allergens from fish and egg." Allergy **56**(Suppl. 67): 39-42.
- Proctor, V. A. and F. E. Cunningham (1988). "The chemistry of lysozyme and its use as a food preservative and a pharmaceutical." Crit Rev Food Sci Nutr **26**(4): 359-95.
- Qian, Y. and W. A. Walker (2004). "Innate immunity of the gut: Mucosal defense in health and disease." J Pediatr Gastroenterol Nutr **38**: 463-473.
- Raphael, G. D., E. V. Jeney, et al. (1989). "Pathophysiology of rhinitis. Lactoferrin and lysozyme in nasal secretions." J Clin Invest **84**(5): 1528-35.

- Rothbard, J. B. and M. L. Gefter (1991). "Interactions between immunogenic peptides and MHC proteins." Annu Rev Immunol **9**: 527-565.
- Samaranayake, Y. H., L. P. Samaranayake, et al. (1997). "The antifungal effect of lactoferrin and lysozyme on *Candida krusei* and *Candida albicans*." Apmis **105**(11): 875-83.
- Sava, G. (1996). Pharmacological aspects and therapeutic applications of lysozyme. Lysozyme: model enzyme in biochemistry and biology. P. Jolles: 433-449.
- Sava, G., V. Ceschia, et al. (1988). "Evidence for host-mediated antitumor effects of lysozyme in mice bearing the MCa mammary carcinoma." Eur J Cancer Clin Oncol **24**(11): 1737-43.
- Schulz, D. R. and E. C. Tozman (1995). "Antineutrophil cytoplasmic antibodies: major autoantigens, pathophysiology, and disease associations." Aemin Arthritis Rheum **25**: 143-159.
- Smith-Gill, S. (1996). Molecular recognition of lysozyme by monoclonal antibodies. Lysozymes: Model Enzymes in Biochemistry and Biology. P. Jolles. Basel Switzerland, Birkhauser Verlag: 277-300.
- Tabary, F., J. Font, et al. (1987). "Isolation, molecular and biological properties of a lectin from rice embryo: relationship with wheat germ agglutination properties." Arch Biochem Biophys **259**(1): 79-88.
- Thomas, K., M. Alalbers, et al. (2004). "A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins." Regulatory Toxicology Pharmacology **39**: 87-98.
- Thomas, P. G. and D. A. Harn Jr (2004). "Immune biasing by helminth glycans." Cell Microbiol **6**(1): 13-22.
- Tsang, C. S. P. and L. P. Samaranayake (1999). "Salivary lysozyme and related parameters of a predominantly Chinese, HIV-infected cohort in Hong Kong." Oral Dis **5**: 241-246.
- Valenta, R., M. Duchene, et al. (1991). "Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals." Science **253**: 557-560.
- Valleix, S., S. Drunat, Philit, Jean-Paptiste, et al. (2002). "Hereditary renal amyloidosis caused by a new variant lysozyme W64R in a French family." Kid Internat **61**: 907-912.
- Waldron, C., E. Murphy, et al. (1985). "Resistance to hygromycin B: A new marker for plant transformation studies." Plant Mol. Biol. **5**: 103-108.
- Witholt, B., H. V. Heerikhuizen, et al. (1976). "How does lysozyme penetrate through the bacterial outer membrane?" Biochim Biophys Acta **443**(3): 534-544.
- Yuzuriha, T., K. Katayama, et al. (1978). "Studies on biotransformation of lysozyme. IV. Radioimmunoassay of lysozyme and its evaluation." Chem Pharm Bull (Tokyo) **26**(3): 908-14.

## APPENDIX A

### ***CSFII Food Codes Included in EDI Analysis for Ventria Proposed Uses***

#### POPSICLES (DAIRY CONTAINING ONLY)

13120050	ICE CREAM BAR OR STICK, NOT CHOC- OR CAKE-COVERED
13120100	ICE CREAM BAR/STICK, CHOCOLATE COVERED
13120130	ICE CREAM BAR/STICK,RICH ICE CREAM,CHOC COVER,W/NUT
13120140	ICE CREAM BAR/STICK, CHOC ICE CREAM, CHOC COVER
13120400	ICE CREAM BAR/STICK W/ FRUIT
13161630	LIGHT ICE CREAM,BAR/STICK, W/ LOW-CAL SWEETENER, CHOC COAT
13235000	PUDDING, POPS, CHOCOLATE
13235100	PUDDING, POPS, NOT CHOCOLATE
91611050	ICE POP FILLED W/ ICE CREAM, ALL FLAVOR VARIETIES

#### MEAL REPLACEMENTS (DRY, FLUID AND SOLID)

11830800	INSTANT BREAKFAST POWDER, NOT RECONSTITUTED
11830810	INSTANT BFAST,PWDR,SWT W/ LO CAL SWT,NOT RECONSTUT
11830850	HIGH CALORIE MILK BEVERAGE, POWDER, NOT RECONST
11830900	PROTEIN SUPPLEMENT, MILK BASED, DRY POWDER
11830940	MEAL REPLACEMENT,PROTEIN,MILK BASED,FRUIT JUICE MIX
11830950	NUTRIENT SUPP,MILK-BASED,POWDERED,NOT RECONSTITUTED
11830960	PROTEIN SUPP, MILK BASE, SODIUM CONTROLLED, POWDER
11830970	MEAL REPLACEMENT, PROTEIN TYPE, MILK-BASE, POWDER
11830980	PROTEIN SUPP, MILK-BASE, POWDER (INCL SUSTACAL)
11830990	NUTRIENT SUPP, MILK-BASE, POWDER (INCL SUSTAGEN)
11831500	NUTRIENT SUPPLEMENT,MILK-BASE,HIGH PROT,NOT RECONST
11832000	MEAL REPLACEMENT,MILK-&SOY-BASE,POWDER,NOT RECONST
11832500	MEAL REPLACEMENT,PROTEIN TYPE,MILK-BASE,W/SUGAR&ART
11835000	MEAL REPLACEMENT, CAMBRIDGE, POWDER, NOT RECONST
11835100	MEAL REPLACEMENT, POSITRIM DRINK MIX, DRY POWDER
11835150	DYNATRIM, MEAL REPLACEMENT, POWDER
11835200	LOSE-IT (NANCI), MEAL REPLACEMENT, POWDER
41430000	PROTEIN POWDER, NFS
41430010	PROTEIN SUPPLEMENT, POWDERED
41430310	PROTEIN DIET POWDER W/ SOY & CASEIN
41440000	TEXTURED VEGETABLE PROTEIN, DRY
11611000	INSTANT BREAKFAST, FLUID, CANNED
11612000	INSTANT BREAKFAST, POWDER, MILK ADDED
11613000	INSTANT BFAST,PWDR,SWT W/ LO CAL SWT, MILK ADDED
11621000	DIET BEVERAGE, LIQUID, CANNED
11623000	MEAL SUPPLEMENT / REPLACEMENT,PREPARED,RTD
11631000	HIGH CALORIE BEV, CANNED OR POWDERED, RECONSTITUTED
11641000	MEAL REPLACEMENT, MILK BASED, HIGH PROTEIN, LIQUID
11651010	MEAL REPLACEMENT, CAMBRIDGE, RECONST, ALL FLAVORS
41430200	MEAL REPLACE / SUPP, SOY-MILK-BASE,POWD,WATER ADDED
41440010	MEAL REPLACEMENT/SUPPLEMENT, LIQUID, HI PROTEIN
41440020	ENSURE W/ FIBER, LIQUID

41440050	ENSURE PLUS LIQUID NUTRITION
----------	------------------------------

## MEAL REPLACEMENTS (DRY, FLUID AND SOLID)-Cont'd

41440100	MEAL REPLACEMENT, LIQUID, SOY-BASE (ISOCAL, OSMOLITE)
92553000	FRUIT-FLAVORED THIRST QUENCHER BEVERAGE, LOW CAL
92560000	FRUIT-FLAVORED THIRST QUENCHER BEVERAGE
41435110	HIGH PROTEIN BAR, CANDY-LIKE, SOY & MILK BASE
53540000	BREAKFAST BAR, NFS
53540100	BREAKFAST BAR, CAKE-LIKE
53540200	BREAKFAST BAR, CEREAL CRUST W/ FRUIT FILLING, LOWFAT
53540250	BREAKFAST BAR, CEREAL CRUST W/ FRUIT FILLING, FAT FREE
53540500	BREAKFAST BAR, DATE, W/ YOGURT COATING
53541100	BREAKFAST BAR, DIET MEAL TYPE
53541200	MEAL REPLACEMENT BAR (INCL SLIM FAST BAR)
53542100	GRANOLA BAR W/ OATS, SUGAR, RAISINS, COCONUT
53542200	GRANOLA BAR, OATS, FRUIT, NUTS, LOWFAT
53542210	GRANOLA BAR, NONFAT
53543100	GRANOLA BAR W/ PEANUTS, OATS, SUGAR, WHEAT GERM
53544100	GRANOLA BAR, W/ NOUGAT
53544200	GRANOLA BAR, CHOCOLATE-COATED
53544210	GRANOLA BAR, W/ COCONUT, CHOCOLATE-COATED
53544220	GRANOLA BAR W/ NUTS, CHOCOLATE-COATED
53544250	GRANOLA BAR, COATED W/ NONCHOCOLATE COATING
53544300	GRANOLA BAR, HIGH FIBER, YOGURT COATING, NOT CHOC
53544400	GRANOLA BARS, W/ RICE CEREAL
53544450	POWERBAR (FORTIFIED HIGH ENERGY BAR)

## YOGURTS, INCLUDING FROZEN

11410000	YOGURT, NS AS TO TYPE OF MILK/FLAVOR
11411010	YOGURT, PLAIN, NS AS TO TYPE OF MILK
11411100	YOGURT, PLAIN, WHOLE MILK
11411200	YOGURT, PLAIN, LOWFAT MILK
11411300	YOGURT, PLAIN, NONFAT MILK
11420000	YOGURT, VANILLA, LEMON, COFFEE, NS AS TO MILK TYPE
11421000	YOGURT, VANILLA, LEMON, COFFEE, WHOLE MILK
11422000	YOGURT, VANILLA, LEMON, COFFEE, LOWFAT MILK
11423000	YOGURT, VANILLA, LEMON, COFFEE, NONFAT MILK
11424000	YOGURT, VANILLA, LEMON, COFFEE, NONFAT MILK, LOW CAL SWEET
11425000	YOGURT, CHOCOLATE, NS AS TO TYPE OF MILK
11426000	YOGURT, CHOCOLATE, WHOLE MILK
11427000	YOGURT, CHOCOLATE, NONFAT MILK
11430000	YOGURT, FRUIT VARIETY, NS AS TO MILK TYPE
11431000	YOGURT, FRUIT VARIETY, WHOLE MILK
11432000	YOGURT, FRUIT VARIETY, LOWFAT MILK
11433000	YOGURT, FRUIT VARIETY, NONFAT MILK
11433500	YOGURT, FRUITED, NONFAT MILK, LOW CAL SWEETENER

## YOGURTS, INCLUDING FROZEN-CONT'D

11444000	YOGURT, FRUIT & NUTS, NS AS TO TYPE OF MILK
11445000	YOGURT, FRUIT & NUTS, LOWFAT MILK
11459990	YOGURT, FROZEN, NS AS TO FLAVOR, NS TO TYPE OF MILK
11460000	YOGURT, FROZEN, NOT CHOCOLATE, TYPE OF MILK NS
11460100	YOGURT, FROZEN, CHOCOLATE, TYPE OF MILK NS
11460150	YOGURT, FROZEN, NS AS TO FLAVOR, LOWFAT MILK
11460160	YOGURT, FROZEN, CHOCOLATE, LOWFAT MILK
11460170	YOGURT, FROZEN, NOT CHOCOLATE, LOWFAT MILK
11460190	YOGURT, FROZEN, NS AS TO FLAVOR, NONFAT MILK
11460200	YOGURT, FROZEN, CHOCOLATE, NONFAT MILK
11460250	YOGURT, FROZEN, NOT CHOCOLATE, W/ SORBET/SORBET-COATED
11460300	YOGURT, FROZEN, NOT CHOCOLATE, NONFAT MILK
11460400	YOGURT, FRZ, CHOCOLATE, NONFAT MILK, W/ LOW-CAL SWEET
11460410	YOGURT, FRZ, NOT CHOC, NONFAT MILK, W/ LOW-CAL SWEET
11460420	YOGURT, FROZEN, NS AS TO FLAVOR, WHOLE MILK
11460430	YOGURT, FROZEN, CHOCOLATE, WHOLE MILK
11460440	YOGURT, FROZEN, NOT CHOCOLATE, WHOLE MILK
11461000	YOGURT, FROZEN, CHOCOLATE-COATED
11461100	YOGURT, FROZEN, CAROB-COATED
11461200	YOGURT, FROZEN, SANDWICH
11461250	YOGURT, FROZEN, CONE, CHOCOLATE
11461260	YOGURT, FROZEN, CONE, NOT CHOCOLATE
11461270	YOGURT, FROZEN, CONE, NOT CHOCOLATE, LOWFAT MILK
11461280	YOGURT, FROZ, CONE, CHOCOLATE, LOWFAT MILK

## SPORTS BEVERAGES

92900300	FRUIT-FLAV THIRST QUENCH BEV, DRY CONC (GATORADE)
----------	---

## **APPENDIX B**

### ***Processing Chemicals and Materials***

**SIoux**  
**Biochemical, Inc.**  
140 19th St. S.W., Sioux Center, IA 51250-1868

October 7, 2003

Confidential

Dr. Ning Huang  
Vice President of R & D  
Ventria Biosciences  
4110 North Freeway  
Sacramento, CA 95834

Dear Ning,

Attached are the following requested documents:

- Pest control report from P & K Pest & Control for August, 2003;
- Testing reports from the City of Sioux Center;
- Spreadsheet of chemicals and reagent used for Ventria's production; and
- Certificates of Analysis or technical bulletins for chemicals and reagents used for Ventria's production.

Please review all documents.

At Sioux Biochemical, Inc., we continue to put together additional documents requested by Ventria Biosciences.

Sincerely,

Helen Vandermeer

cc. Dr. Allan Kramer



Pest Control  
Fumigation

# P & K PEST & CONTROL

Dennis Bosma

Phone 712-324-4656  
1134 10th Street  
Sheldon, IA 51201



Pest Control  
Fumigation

## P & K PEST CONTROL REPORT

CUSTOMER: Sioux Biochemical

### INFESTATION INVENTORY:

TYPE: crickets

LOCATION: Front door + loading room

TREATMENT: Spot spray

- A. Location free of dead rodents
- B. Location free of dead insects
- C. Exterior of premises inspected / serviced
- D. Interior of premises inspected / serviced
- E. Rodent bait stations inspected / serviced
- F. Storage area clean, free of spillage
- G. Storage lift, or more from walls
- H. Doors rodent proof, windows screened
- I. Building free of holes, harborages, etc.
- J. Lot clean, free of harborages / burrows

	Yes	No
A	<input checked="" type="checkbox"/>	<input type="checkbox"/>
B	<input checked="" type="checkbox"/>	<input type="checkbox"/>
C	<input checked="" type="checkbox"/>	<input type="checkbox"/>
D	<input checked="" type="checkbox"/>	<input type="checkbox"/>
E	<input checked="" type="checkbox"/>	<input type="checkbox"/>
F	<input checked="" type="checkbox"/>	<input type="checkbox"/>
G	<input checked="" type="checkbox"/>	<input type="checkbox"/>
H	<input checked="" type="checkbox"/>	<input type="checkbox"/>
I	<input checked="" type="checkbox"/>	<input type="checkbox"/>
J	<input checked="" type="checkbox"/>	<input type="checkbox"/>

No infestation found. Preventive treatment rendered.  
 Infestation found. Treatment rendered.

### RECOMMENDATIONS:

### APPROVED CHEMICALS USED:

#### Non-residual:

	Concentrate	Qty.	Areas Treated		Concentrate	Qty.	Areas Treated
A. Pyrenone Food Plant Fogging Insecticide (Fairfield Am. Corp.)				C. PT 555 (Whitman)			
B. Pyrenone Industrial Spray E.C. (Fairfield Am. Corp.)				D. BP 100 (Microgen)			
				E. OTHER:			

#### Residual:

A. Dursban LO (Dow Chemical Co.)				B. Dursban 4E Emulifiable Concentrate (Dow Chemical Co.)			
C. OTHER							

(Used only in inedible areas with no open windows or open vents) (to be used after approval by inspector)

#### Rodenticides:

	Qty.	Areas Treated		Qty.	Areas Treated
A. Talon G Rodenticide Pellets / Weatherblok bait (I.C.I. Americas Inc.)			C. Final Rat and Mouse Bait (Bell Laboratories, Inc.)		
B. P.C.Q Rat and Mouse Bait (Bell Laboratories, Inc.)			D. OTHER		

### Trap / Station Inventory:

No. in place	<u>10</u>
No. added	<u>0</u>
TOTAL:	<u>10</u>
No. replaced	<u>1</u>

Discussed & Accepted: \_\_\_\_\_  
 P & K Representative: \_\_\_\_\_  
 Date: 8.21.63

310



MANGOLD ENVIRONMENTAL TESTING, INC.
2004 EXPANSION BLVD
712-732-7786
STORM LAKE IA 50588
FAX 712-732-3925

03/12/2003
FRED CORBIN
SIOUX CENTER WATER DEPT
335 1ST AVENUE NW
SIOUX CENTER, IA 51250

Table with 2 columns: Field Name and Value. Fields include Account Number (C283), Order Number (2003030025), Sample Number (8), and Lab ID Number (267447).

Sampling Information
Date: 03/10/2003 10:50
Collector: Kruid Haran
Matrix Type: Water Nitrate Routine
Location: 921 9th St SE
RE

Receiving Information
Date Received: 03/11/2003 09:55
Received By: SYS
Sample Notes: 10.5 degrees

Table with 7 columns: Analyte, Results, Test Units, Analyst, Date of Analysis, Method Code. Rows include Nitrate as NO3 (14.6 mg/L, DLF, 03/12/2003, SM 2540E) and Nitrate-Nitrogen (as N) (3.30 mg/L, MG, 03/11/2003, EPA 300.0).

Maximum Contaminant Limit for your Nitrate-Nitrogen (as N) is 10.0 mg/L.
Any Nitrate-Nitrogen (as N) result exceeding this value is UNSATISFACTORY.

Prepared and Submitted by:
MANGOLD ENVIRONMENTAL TESTING, INC.

Brent J. Mangold, Manager

# CERTIFICATE OF ANALYSIS

Date: 03/26/03

Page 1

PRODUCT: SODIUM PHOSPHATE, DIBASIC, ANHYDROUS FCC

CATALOG NO:	S1402	**	CUSTOMER P.O.:	3306
LOT NO:	PT2441			

DESCRIPTION	LIMIT		RESULT
	MIN.	MAX.	
ASSAY (DRIED BASIS)	98.0 %		100.33 %
LOSS ON DRYING	- 5.0 %		3.06 %
ARSENIC (As)	- 3 mg/kg		0.3 mg/kg
FLUORIDE	- 0.005 %		< 0.005 %
HEAVY METALS (as Pb)	- 10 mg/kg		< 10 mg/kg
INSOLUBLE SUBSTANCES	- 0.2 %		< 0.2 %
IDENTIFICATION	TO PASS TEST		PASSES TEST

APPROVED BY:

encl 1023752

CHRIS TERPAK  
QUALITY CONTROL CHEMIST  
New Brunswick, N.J. Plant

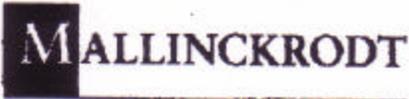
**SPECTRUM**<sup>®</sup>  
www.spectrumchemical.com

**IQ**<sup>™</sup>  
INTEGRATED QUALITY  
PROGRAM



### Spectrum Chemicals & Laboratory Products

Corporate Headquarters: 14422 S. San Pedro St. Gardena, CA 90248 (310) 516-8000 • Fax (310) 516-9843 • (732) 214-1300	East Coast Plant: 755 Jersey Ave. New Brunswick, NJ 08901
--	---



Mallinckrodt Inc.  
P.O. Box 5439  
St. Louis, MO 63147

Technical Services:  
Telephone (314) 539-1421  
Facsimile (314) 539-1128

**CERTIFICATE OF ANALYSIS**

CUSTOMER PRODUCT: 566570

MSCC ORDER NO.: 402453  
CUSTOMER ORDER NO.: 097890  
SHIPPED TO: BRENTAG GREAT LAKES L

-----  
Item: SODIUM PHOSPHATE DIBASIC USP POWDER (DRIED)  
Code: 7920 Specification 23 JUN 2000  
Lot#: D11313 Released 16 SEP 2002  
Date of Manufacture: 9 SEP 2002  
-----

COMMENTS:  
Retest every 2 years  
-----

TESTS	LIMITS	RESULTS
Identification (Sodium) (USP)	dense precipitate	dense precipitate
Identification (Phosphate) (USP)	yellow precipitate	yellow precipitate
Loss on Drying (USP)	5.0% max	0.0%
Insoluble Substances (USP)	0.38% max	0.00%
Chloride (USP)	0.05% max	<0.05%
Sulfate (USP)	0.19% max	<0.19%
Arsenic (USP)	15 ppm max	<15ppm
Heavy Metals (USP)	0.0019% max	<0.0010%
Assay (USP)	98.0-100.5%	99.8%

It is certified that the above is a true copy of the analysis.

Department of Quality Assurance



**CARGILL FOODS**  
Salt Products

## Certificate of Analysis

Identification:		Shipped to:	
Product	TopFlo	Customer: Johnson Feed	
Lot	H2193	Order # 500153	
Date of Manufacture	8/7/2003	OrderDate: 8/6/2003	ShipDate: 8/7/2003
		PO #	Todd e-mail
		Trailer #	33
			Code #

Comments:

		Retained on JSS Mesh	
Chemical Analysis:		USS 8	
NaCl %	99.92	USS 10	
Ca & Mg as Ca ppm	254	USS 12	
		USS 14	
Additives:		USS 16	
YPS ppm	10.0	USS 20	
TCP %	.00	USS 30	.6
KI %	.000	USS 40	23.0
Zeolex %	.000	USS 50	61.3
PG %	.000	USS 60	12.1
		USS 70	2.7
Physical:		USS 80	
surface moisture (%)	.01	USS 100	
bulk density (#/cu ft)	79	USS 140	
pH (20% solution)	8.50	USS 150	
water insolubles (ppm)	12	USS 200	
		USS 325	

Pan .3

*This salt product is a white, crystalline solid with a characteristic saline taste. Its low water activity and astringent nature inhibit pathogen growth. Periodic testing for microbiological activity confirms this assertion.*

*This ingredient is tested regularly by the manufacturer and meets all USDA and FDA requirements as specified in the Food Chemical Codex at time of shipment from the manufacturer.*

*All statements and data above are based on our laboratory results and we believe same to be reliable.*

**Manufacturer:**

Hutchinson Evap. Plant  
609 East Avenue G  
Hutchinson KS 67501-7574

Quality Assurance (620) 669-2171  
Fax (620) 663-8006

Customer Service (800) 282-6777

Submitted by:

QA Chemist

4023

ProdNo: 679375

LotNo:

JUN. 16. 2003 8:41AM ROWELL CHEMICAL

NO. 776 P 2/2

JUN 30 2003



**REAGENT CHEMICAL & RESEARCH, INC.**  
 20 SOUTHRIDGE DRIVE  
 GLENDALE, MO

Product = 679375  
 Lot # = OM073821127

**CERTIFICATE OF ANALYSIS  
 HYDROCHLORIC ACID**

CUSTOMER NAME & ADDRESS ROWELL CHEMICAL  SHIP TO: UNIVAR OMAHA, NE  ATTN: JOHN WARD/ROWELL	DATE: 05-29-03	CONTROL NUMBER: 102610
	LOT NUMBER: RCRX:351053003	QUANTITY 190.163 #
	RCR IC RCRX 1351	CUSTOMER ORDER #: OM46739 REL 307802
		PS45225
		PRIORITY: 2185/22188/45225

338

THIS IS TO CERTIFY THAT TYPICAL SAMPLES OF HYDROCHLORIC ACID HAVE BEEN WITHDRAWN USING STATISTICAL QUALITY CONTROL SAMPLING PROCEDURES AND UPON ANALYSIS IS FOUND TO CONTAIN THE FOLLOWING:

IDENTIFICATION	SAMPLE
STRENGTH IN BAUME'	20.73 Be'
% HCL CONTENT	32.80 %
SPECIFIC GRAVITY	1.1665 @ 60°F
COLOR (A.P.H.A)	10
EXTRACTABLE ORGANIC COMPOUNDS	0.4 PPM
IRON (Fe)	0.4 PPM

**QUALITY ASSURANCE**  
 (800) 972-3047

Quality First  
 To Do It Right - The First Time and Every Time

FCRM # 221174  
 RVSD 05/03

2302  
 QUAY

ARMETTA

JUL 23 2003



P.O. Box 385015  
Birmingham, Alabama 35238-5015  
(205) 298-3000

A DIVISION OF  
VULCAN MATERIALS COMPANY  
ATTENTION :

CERTIFICATE OF ANALYSIS

Product = 705849  
Lot # = OM0738:1266

UNIVAR USA INC  
KAY WILSON  
TRACK 782 ZONE C2  
OMAHA NE

50% CAUSTIC, DIAPHRAGM

ORDER NUMBER: 3034890  
DATE SHIPPED: JUL 07 03  
DESTINATION: OMAHA NE  
SOURCE TANK: C17  
SEAL NBR: 133085 86 87

CUSTOMER ORDER NO.: OM468125  
TANK CAR NO.: GATX 49533  
SHIPPED FROM: WICHITA KS  
THIRD PARTY P.O.:

COMPONENT	ANALYSIS
NaOH, Wt. %	50.12
Na2CO3, Wt. %	0.031
Total Alkalinity, as Na2O, Wt. %	38.85
NaCl, Wt. %	1.00
Iron, as Fe, ppm	2.6

Denotes Storage Tank Analysis (\*\*)

MEETS FOOD CHEMICALS CODEX SPECIFICATION

JESSICA RHOADES

SUPPLIER'S REPRESENTATIVE

DISTRIBUTION: FAX  
FAX NUMBER: 14027333152 KAY WILSON

REMARKS:

ORM NUMBER: 211

Univar USA Inc  
3002 F Street  
Omaha, NE 68107  
Ph: (402) 733-3266  
Fax: (402) 733-3152  
Website: [www.univarusa.com](http://www.univarusa.com)



Product = 678442  
Lot # = OM07:821113

### Certificate of Analysis

#### Acetic Acid Glacial, FCC

Date 07/01/2003 Product Code # 678442

Univar Lot # OM073821113

	Results	Limits
Assay	101 %	≥99.5 %
Readily Oxidizable	Pass	Pass
Appearance	Pass	Clear liquid free of matter
Solidification Point	16.5°C	≥ 15.6°C
Specific Gravity @ 20°C	1.0455	1.030 to 1.055
Identification (IR)	Pass	Pass

Signed:   
Jean Busboom-Piercy  
Omaha QC

Date: July 1, 2003

FLORIST.

From: TAB, Chemicals

10-02-01

6359

BRENNTAG  
PC: 03624  
10/02/01



TAB CHEMICAL COMPANY  
4801 S AUSTIN AVE  
FAX: 773-586-9882  
CHICAGO, IL 60638  
Attn: DENISE

Purchase Order No.: A150662  
Date: 01/24/01

Certificate of Analysis for SODIUM ACETATE, ANHYDROUS, FCC

Lot Number: 03624  
Date of Analysis: 12/28/00

PURITY (DRY BASIS)	-	99.95 %
pH OF 10% SOLUTION	-	8.41
POTASSIUM COMPOUNDS	-	PASS
WATER	-	NIL %
HALIDES (AS NaCl)	-	<0.05 %
HEAVY METALS (AS Pb)	-	< 5 PPM
IRON	-	3.0 PPM
ARSENIC (AS As)	-	< 1 PPM
ALKALINITY (AS NaOH)	-	0.0 %
LOSS ON DRYING	-	NIL %
APPEARANCE	-	FINE, WHITE CRYSTAL
SULFATE	-	< 25 PPM

S. J. D'Angelo  
Manager Quality Assurance

**TECHNICAL INFORMATION**

# Oakite Sanitizer 1

Quaternary ammonium germicide disinfectant for all purpose sanitizing, control of mold and algae.

EPA Reg. No. 1020-1

### PRIMARY APPLICATION

Oakite Sanitizer 1 is a liquid germicide disinfectant for sanitizing and deodorizing food processing and pharmaceutical plants. A quaternary ammonium compound, it combines fast killing action with excellent residual bacteriostatic protection. Oakite Sanitizer 1 is effective on a wide range of bacteria, fungi, mold, and other disease causing pathogens such as staphylococcus, streptococcus and escherichiacoli. Sanitizer 1 does not require a potable water rinse when used at concentrations up to 200 ppm.

Oakite Sanitizer 1 contains ingredients acceptable to the FDA and is authorized by the USDA for use in federally inspected meat and poultry plants.

### CHEMICAL CHARACTERISTICS

chemical composition	alkyl dimethyl benzyl ammonium chloride
physical form	concentrate: light yellow as used: clear solution
specific gravity (approx.)	0.987 at 20°C (68°F)
bulk density	987 g/l (8.2 lb/gal)
viscosity	4 cps, Brookfield Spindle 1, 60 rpm at 27°C (80°F)
flash point	none
foaming tendency	moderate when circulated; high when sprayed
recommended diluent	water
maximum solubility	completely miscible in cold or hot water
behavior in hard water	unaffected
rinsability	good
biodegradable surfactants	yes
phosphate-free	yes
normal working concentrations	1 fl. oz/8 gals of water
normal working temperatures	21° to 71° (70° to 160°F)
pH at working concentrations	7-9 depending on alkalinity of water
effect of prolonged boiling	some loss of sanitizing properties
effect of working solutions on metals	rate of metal loss from 24-hour immersion in a 200 ppm solution of Oakite Sanitizer 1 at 38°C (100°F), projected for one year, is as follows:

metal (alloy)	mm/yr	in/yr
stainless steel (316)	0.00	0.000
steel (1010)	0.07	0.003
aluminum (2024)	0.06	0.002
aluminum (3003)	0.07	0.003
brass	0.01	0.000
copper	0.00	0.000

**Oakite.**

## APPLICATION PROCEDURE

**Directions:** Thoroughly clean and rinse all surfaces before applying this solution for sanitizing or germicidal treatment. Prepare a fresh solution daily or when use solution becomes visibly dirty.

**General Disinfection:** For walls, floors, equipment, other hard, nonporous inanimate surfaces: After proper cleaning, apply Oakite Sanitizer 1 by mop, brush or by flooding areas to be treated. Treated surfaces must remain wet for 10 minutes. Prepare solution by adding 1 fluid ounce (about 2 tablespoons) of Oakite Sanitizer 1 to 4 gallons of water. May be used in cold, warm and hot water. Disinfection more rapid with warm or hot water.

**Dairy Farms, Milk Plants:** For sanitizing milking machines and rubber parts, pails, strainers, separators, sanitary piping, coolers, pasteurizing equipment, etc. After proper cleaning of equipment, sanitize with solution of Oakite Sanitizer 1. Apply by circulating, flooding or spraying 1/4 fluid oz. to 2 gallons of water which approximates 200 ppm (1 to 5000).

**Note:** Do not use with soap or anionic detergents.

**Sanitizing, Mold Control:** For general sanitizing in bakeries, food and bottling plants a solution containing 1/2 fluid oz. to 4 gallons of water is applied by spraying or wiping previously cleaned surfaces until they are moist. Solution must have at least one minute contact time. Residual solution will control mold and yeast growth on hard, smooth surfaces such as metal or tile, as well as on painted walls and ceilings. Application should be repeated every 7 days or as necessary depending on the severity of the mold problem.

**Algae:** To control algae in previously cleaned circulating water systems, apply an initial slug addition of Oakite Sanitizer 1, at the rate of 13-26 fluid oz/per 1000 gal of water in the system (20-40 ppm active) ingredients. Subsequent slug additions of 4-8 oz of Oakite Sanitizer 1 per 1000 gal of water (8-12 ppm) should be applied every 2 to 5 days or as needed depending on the volume of bleed-off and the severity of the algae problem. Additions should be introduced gradually into the sump of the water cooling tower.

**Sanitizing food eggs:** To sanitize previously cleaned food grade eggs in shell egg and egg product processing plants, spray with 1-1/4 oz Oakite Sanitizer 1 in 10 gallons of water (200 ppm active). The solution should be warmer than the eggs, but not to exceed 130°F. Wet eggs thoroughly and allow to drain. Eggs sanitized with this product shall be subjected to a potable water rinse only if they are to be immediately broken for use in the manufacture of egg products. Eggs should be reasonably dry before casing or breaking. The solution should not be reused for sanitizing eggs.

**Restaurants, Luncheon Counters and Taverns:** For sanitizing glasses, dishes, utensils, destroys many disease-producing bacteria. After proper cleaning, apply solution of Oakite Sanitizer 1 (1/2 fluid ounce per 4 gallons of water). Allow to drain thoroughly before reuse.

This product fulfills the criteria of Appendix F of the Grade "A" Pasteurized Milk Ordinance 1965 Recommendations of the U.S. Public Health Service in waters up to 550 ppm of hardness calculated as CaCO<sub>3</sub> when tested by the A.O.A.C. Germicidal and Detergent Sanitizers-Official Method.

**SOLUTION CONTROL:** To determine concentration of active ingredient (quat), use Procedure 93.

## NOTES ON USE

(See Material Safety Data Sheet)

**Safety and Handling Precautions:** Direct contact causes eye damage and skin irritation. Avoid contact with skin, eyes and clothing. When handling, wear safety goggles or face shield and rubber gloves. Harmful or fatal if swallowed. Avoid contamination of food.

**First Aid In Case of Contact:** Immediately flush eyes or skin with plenty of water for at least 15 minutes. For eyes, get medical attention; for skin, remove and wash clothing before reuse. If swallowed, promptly drink a large quantity of water. Avoid alcohol. Call a physician immediately.

**Note to Physician:** Probable mucosal damage may contraindicate the use of gastric lavage. Measures against circulatory shock, respiratory depression and convulsion may be needed.

**KEEP OUT OF REACT OF CHILDREN**

**DISPOSAL**

Dispose of according to all federal, state and local regulations.

**SHIPMENT**

May be shipped by any common carrier. Freight classification is "Cleaning Compound, NOI, Liquid", Product Code No. 5080.

**STORAGE**

Suitable for general indoor storage. Keep container closed when not in use. Keep from freezing

effect of high temperature storage .....	slight pressure may build up
effect of low temperature storage .....	will freeze at 0°C (32°F), contents may be restored by thawing
effect of aging .....	none

Oakite Products, Inc. warrants that the product or products described herein will conform with its published specifications. The products supplied by Oakite and information related to them are intended for use by buyers having necessary industrial skill and knowledge. Buyers should use and make sufficient verification and testing to determine the suitability of the Oakite materials for their own particular purpose. Since buyer's conditions of use of products are beyond Oakite's control, Oakite does not warrant any recommendations and information for the use of such products. OAKITE DISCLAIMS ALL OTHER WARRANTIES INCLUDING THE IMPLIED WARRANTY OF MERCHANTABILITY AND FITNESS FOR ANY PARTICULAR PURPOSE IN CONNECTION WITH THE USE OF ITS PRODUCTS.



Oakite Products, Inc. P.O. Box 602, 50 Valley Road, Berkeley Heights, NJ 07922  
Oakite Canada Limited 115 East Drive, Bramalea, Ontario L6T1B7  
Tel. (905) 526-4473 (905) 464-6900 FAX (905) 464-4658  
Distributors and Licensees Worldwide

F13300R7807  
Printed in the USA

# Q & SP Sepharose<sup>®</sup> Big Beads

## BioProcess<sup>™</sup> Media

## Data File

### Ion Exchange Media

Q and SP Sepharose Big Beads are strong ion exchangers designed for industrial applications. The large particle size (100–300  $\mu\text{m}$ ) and excellent physical stability of the base matrix ensure maintained speed even with viscous samples. Q and SP Sepharose Big Beads are therefore the ultimate ion exchange media for initial purifications when high viscosity precludes the use of ion exchangers with smaller bead size, such as Sepharose Fast Flow ion exchangers. The unique flow characteristics are also invaluable when adsorption needs to be done quickly, e.g. in order to minimize proteolytic breakdown.

- Easy to scale-up
- High flow rates
- High chemical resistance for effective cleaning-in-place (CIP)
- Easy maintenance

### Media characteristics

The ion exchange groups are coupled to the highly cross-linked agarose matrix through chemically stable ether bonds. The strong ion exchange groups maintain full protein binding capacity over the whole operating pH range. Q Sepharose Big Beads and SP Sepharose Big Beads have the same selectivities as the corresponding Sepharose Fast Flow and Sepharose High Performance ion exchangers.

### General maintenance

Sepharose Big Beads ion exchange media are easy to pack and handle. The very high flow rates that can be used save valuable time in equilibration and during regeneration. Even with viscosities as high as 2.5 times water a high flow rate (500 cm/h) is maintained in industrial column operation. Packed columns can be cleaned- and sanitized-in-place to minimize production losses. The media can also be autoclaved.

### Column packing

Q and SP Sepharose Big Beads are easy to pack in small and large scale columns. Narrow peaks with high symmetry are reproducible whether you pack



Fig. 1. Q and SP Sepharose Big Beads ion exchange media.

the ion exchanger bed with a constant pressure of between 1 to 3 bar, or let the slurry sediment and then compress it with the adaptor. Suction packing can easily be performed as well.

Table 2. Characteristics of Q and SP Sepharose Big Beads.

Property	Q	SP
<i>Ion exchange type</i>	Strong anion	Strong cation
<i>Ionic capacity</i>	180–250 pmole/ml gel	
<i>Exclusion limit</i>	4x10 <sup>6</sup> daltons (globular proteins)	
<i>Matrix</i>	Macroporous, cross-linked agarose, 6%	
<i>Bead form</i>	Spherical, 100–300 $\mu\text{m}$	
<i>Flow rate</i>	1200–1800 cm/h*	
<i>Working temperature</i>	4–40°C	
<i>pH stability</i>	2–12 (working range) 2–14 (cleaning-in-place)	4–12 (working range) 3–14 (cleaning-in-place)
<i>Chemical stability</i>	All commonly used aqueous buffers – 1 M NaOH – 70% ethanol – Organic solvents	
<i>The following should be avoided</i>	– Oxidizing agents – Long exposure (1 week, 20°C) to pH <4	

\* 2.5 cm bed height, 1 bar, 20°C distilled water in Amersham Biosciences XK 50 column.

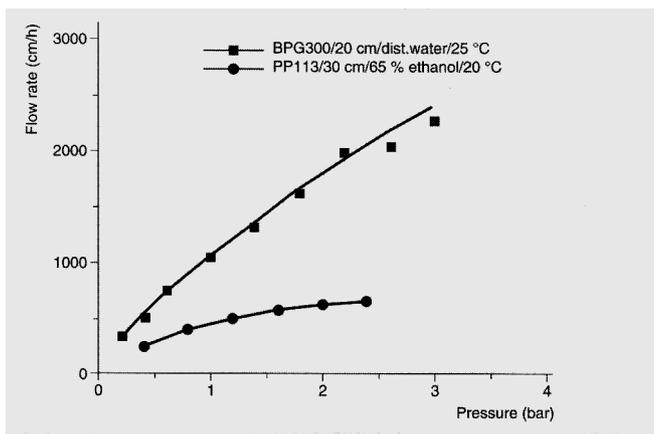


Fig. 2. The excellent flow characteristics of Sepharose Big Beads allow high flow rates even with high viscosity feed.

- Bed height 20 cm, diameter 30 cm in distilled water at 25°C.
- Bed height 30 cm, diameter 11 cm in 65% ethanol at 20°C. i.e. viscosity 2.5 times water.

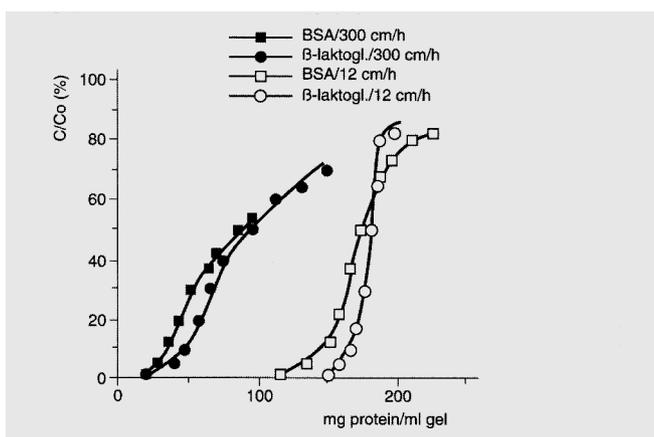


Fig. 3. Typical binding capacities of Sepharose Big Beads media. The above shows the binding capacity of SP Sepharose Big Beads measured with frontal analysis in acetate pH 5 Bovine serum albumin (BSA) and formate pH 4.1 ( $\beta$ -lactoglobulin) at linear flow rates of 12 (corresponding to total available capacity) and 300 cm/h.

## Cleaning, sanitization, regeneration and storage

Due to the high chemical resistance of these media, severe conditions can be used to clean and sanitize the column.

### Cleaning-in-place

- **Ionically bound proteins:**  
Wash with filtered 2M NaCl at approximately 100 cm/h. Contact time: 10–15 min.
- **Hydrophobically bound proteins or lipoproteins:**  
Wash with 1 M NaOH at 40 cm/h. Contact time: 1–2 h.
- **Lipids and very hydrophobic proteins:**  
Wash with 70% ethanol at 40 cm/h, reversed flow, or with saw-tooth gradient 0–30–0% isopropanol. Contact time: 1–2 h.

## Sanitization

A reduction of microbial contamination in the ion exchanger bed is obtained by washing the column with 0.5–1 M NaOH, allowing a contact time of 30–60 min.

## Regeneration

Regeneration is performed by passing one bed volume of 1 M NaCl through the column. After regeneration, equilibrate the column with five column volumes of buffer.

## Storage

Q and SP Sepharose Big Beads can be stored either at neutral pH in buffer containing 20% ethanol or in 0.01 M NaOH.

## Operation

### Equipment

Any standard chromatographic system from Amersham Biosciences can be used. Make sure the capacity of the pump is high enough to handle the very high flow rates used during column packing.

### Process optimixation

Normal optimization procedures for choosing buffer, ionic strength, pH, gradient shape and elution volume should be followed. The use of a higher bed height can give a better result due to the increased residence time.

## Ordering information

Product	Pack size	Code No.
SP Sepharose Big Beads	1 l	17-0657-03
	10 l	17-0657-05
Q Sepharose Big Beads	1 l	17-0989-03
	10 l	17-0989-05

### Copyright

1994 Amersham Biosciences AB, S-751 82 Uppsala, Sweden. Please request the written permission before reproducing any of this information.

### Trademarks

The following designations are trademarks owned by Amersham Biosciences: BioProcess, Sepharose.

Submission End

AM

**Gaynor, Paulette M**

**From:** delia bethell [dbethell@ventria.com]  
**Sent:** Thursday, July 14, 2005 3:20 PM  
**To:** Gaynor, Paulette M; Merker, Robert I  
**Subject:** Lysozyme GRAS

Dear Drs. Gaynor and Merker,

As per our phone conversation, there is no significance to the order of the pages in Appendix B of the GRN 000162 or GRN 000174. The paper copies can be reorder to so the electronic and paper copies are identical.

In GRN 000174, the letter in Appendix B marked Confidential is not considered confidential business information. It should be in both the paper and electronic copy,

If there are any questions, please don't hesitate to contact me.

Regards,

Delia Bethell

Delia R. Bethell, Ph.D.  
Vice President for Clinical Development  
Ventria Bioscience  
4110 N. Freeway Blvd.  
Sacramento, CA 95834  
PH: 916-921-6148 x 21  
FX: 916-921-5611  
[dbethell@ventria.com](mailto:dbethell@ventria.com)  
[www.Ventria.com](http://www.Ventria.com)

This message (including any attachments) contains confidential information intended for a specific individual and purpose, and is protected by law. If you are not the intended recipient, you should delete this message. Any disclosure, copying, or distribution of this message, or the taking of any action based on it, is strictly prohibited.

7/21/2005

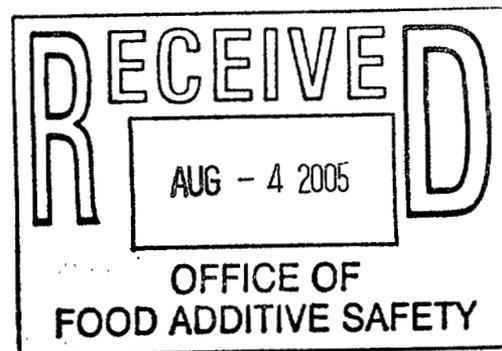


AM



# VENTRIA BIOSCIENCE

August 2, 2005



Paulette M. Gaynor, Ph.D.  
Division of Biotechnology and GRAS Notice Review  
Center for Food Safety and Applied Nutrition  
HFS-255  
Harvey W. Wiley Federal Building  
5100 Paint Branch Parkway  
College Park, MD 20740-3835

**Re: GRN 000174**

Dear Dr. Gaynor:

As a clarification in Section III, the exposure levels associated with medical foods in solid form would be similar to the exposure levels proposed for use in functional foods, 500 mg per serving. This would result in exposures similar to that from liquid medical foods for diarrhea, Table 8, and fluid or dry meal replacements. The average intakes for the fluid and dry meal replacements are found in Tables 6 and 7.

If you have any questions, please do not hesitate to contact me.

Sincerely,

(b)(6)

Delia R. Bethell, Ph.D.  
Vice President for Clinical Development

cc: Scott Deeter  
President & CEO

BEST ORIGINAL COPY



# VENTRIA BIOSCIENCE



REC'D SEP 13 2005

September 6, 2005

Paulette M. Gaynor, Ph.D.  
Division of Biotechnology and GRAS Notice Review  
Center for Food Safety and Applied Nutrition  
HFS-255  
Harvey W. Wiley Federal Building  
5100 Paint Branch Parkway  
College Park, MD 20740-3835

**Re: GRN 000174**

Dear Dr. Gaynor,

In light of our discussions, Ventria is withdrawing GRN No. 000174 on lysozyme (human) enzyme preparation from rice.

Thank you for your time.

Regards,

(b)(6)

Delia Bethell  
Vice President for Clinical Development

**BEST ORIGINAL COPY**