ORIGINAL SUBMISSION

VENTRIA BIOSCIENCE

January 20, 2006

RECD JAN 2 3 2006

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

Re: GRAS Exemption Claim for Lysozyme (human) derived from rice as an ingredient for infant and pre-term formula and pediatric medical food, oral rehydration solution.

Dear Dr. Martin,

Ventria Bioscience is submitting data and information that establish that lysozyme (human) derived from rice is GRAS as an ingredient for addition to term infant formulas at levels up to 0.6 g/L, to pre-term formulas and fortifiers at levels up to 0.3 g/L and to pediatric oral rehydration solution at levels up to 2 mg/mL. Lysozyme is an important component of breast milk and the proposed levels are comparable to levels found in breast milk.

Ventria has convened an Expert Panel to review the published and unpublished data and information on lysozyme. The following individuals comprise the Expert Panel: Steven Abrams, MD; Roger Clemens, DrPH; Bo Lonnerdal, Ph.D. and Steve Taylor, Ph.D. After reviewing the available data, the Expert Panel has concluded in its January 2006 report that lysozyme (human) derived from rice is GRAS as an ingredient for addition to infant and pre-term formula and pediatric medical foods.

In accordance with the criteria set forth in the GRAS notification proposed regulation 21 CFR 170.36(c) (62 Federal Register 18937 (April 17, 1997)), Ventria is submitting the following information as part of its GRAS exemption claim.

Name and Address of Notifier:

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

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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 CAS Registry Number is 12671-19-1
The published literature may also refer to lysozyme as muramidase, mucopeptide N-acetylmuramylhydrolase, and muocopeptide glucohydrolase (Osserman, Canfield et al. 1974).

Conditions of Use:

Ventria Bioscience intends to make lysozyme (human) derived from rice available for addition to infant/pre-term infant formulas for nutritional support. This use will be for a target population of infants to 1 year of age at levels consistent with the level of lysozyme found naturally in human milk and the exposure of breast fed infants. Under 21 U.S.C. 321(z), infant formula is defined as a food that purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk. It is considered the sole source, or the major source, of nutrition during infancy, particularly during the first four to six months of life.

Ventria also intends to make lysozyme (human) derived from rice available for addition to pediatric medical foods (oral rehydration solutions, ORS) for additional nutritional support during episodes of dehydration associated with diarrhea. This target population will be infants up to 3 years of age at the levels consistent with the level of lysozyme found naturally in human milk.

1. Full-term infant formulas: Ventria proposes to make lysozyme (human) derived from rice (rhLZ) available for fortification of infant formulas at a level of 0.30 g rhLZ/L of formula up to 0.6 g rhLZ/L of formula.

2. Pre-term infant formulas or formula fortifiers: Ventria proposes make rhLZ available for fortification pre-term infant formulas at a level up to of 0.30 g rhLZ/L of formula.

3. Medical foods (under supervision of a physician only): 0.2 mg/mL rhLZ in oral rehydration solution (ORS).

Basis for GRAS Determination:

Ventria Bioscience has determined that lysozyme (human) derived from rice is a GRAS ingredient on the basis of scientific procedures. This document establishes the safety and the consensus by qualified experts that lysozyme (human) derived from rice is a GRAS ingredient.

Availability of Data:

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

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reasonable times at the offices Ventria Bioscience, 4110 N. Freeway Blvd., Sacramento, CA 95834.

GRAS Exemption Claim:

The use of lysozyme (human) derived from rice in term infant formulas, at a maximum use level of 0.6 g/L, pre-term infant formulas at a maximum use level of 0.3 g/L and oral rehydration solutions at a maximum use level of 0.2 mg/mL, are exempt from premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Ventria Bioscience has determined that such uses are GRAS.

Enclosed please find an original, two copies and one electronic copy on disk of this notification for review. If you have any questions, please contact me at the phone number and address above.

Sincerely,

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

Enclosures

cc: Scott Deeter
President & CEO

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GENERALLY RECOGNIZED AS SAFE (GRAS) NOTIFICATION FOR LYSOZYME (HUMAN) FROM RICE FOR USE AS AN INGREDIENT IN INFANT AND PRE-TERM FORMULAS AND IN PEDIATRIC MEDICAL FOODS

Prepared by

Delia R. Bethell, Ph.D.
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January 20, 2006

Contains No Confidential Business Information

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DESCRIPTION OF SUBSTANCE

I. Physical and Chemical Composition

Human lysozyme (hLZ) is present in human biological fluids and secretions (milk, tears, saliva, genital, nasal and bronchial secretions) as well plasma and circulating neutrophils. Human lysozyme is an enzyme protein (EC 3.2.1.17, CAS No. 12671-19-1) 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)n found in most bacterial cell walls. Lysozyme catalyzes the hydrolysis of β(-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).

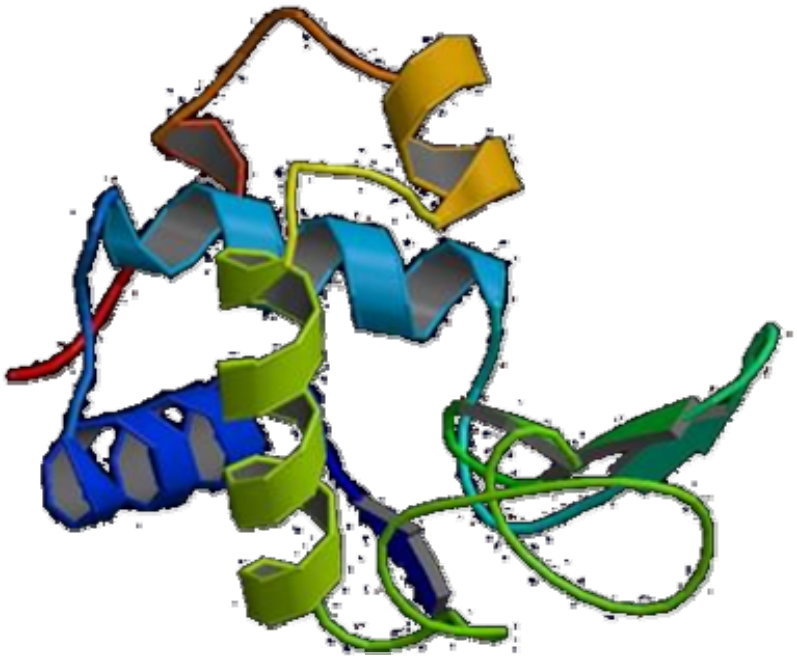


Figure 1. Human Lysozyme
Human lysozyme is a single polypeptide chain. The cDNA was isolated from a human placenta library. There is only one human lysozyme gene and it has been assigned to

chromosome 12 (Peters, Kruse et al. 1989). There are no N-glycosylation sites on lysozyme and there is no reported glycosylation of the protein. The complete amino acid sequence has been determined for lysozymes from many species: human, bovine, hen, quail, pheasant, guinea fowl, turkey, duck, chachalaca, baboon, rat, tortoise (Jolles and Jolles 1984). The human lysozyme gene sequence used by Ventria Bioscience was based on the DNA sequence from GenBank (GenBank accession number J03801).

Table 1 provides published levels of hLZ measured in human exocrine secretions.

Table 1. Lysozyme Levels in Human Exocrine Secretions

Fluid	Lysozyme (µg/mL)	Reference
Breast milk	9-800	(Peitersen, Bohn et al. 1975; Montagne, Cuilliere et al. 1998)
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)
Neutrophils	0.095	(Moreira-Ludewig and Healy 1992)

II. Molecular Biology of Production

Recombinant human lysozyme (rhLZ) 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 rhLZ in the endosperm of the seed. Ventria used a seed-specific promoter and signal peptide sequence to direct the rhLZ molecule to the protein bodies in the endosperm cell. While the genes for human 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 require expression of 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 rhLZ 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 rhLZ in rice grain was accomplished through the use of a seed-specific promoter and a signal peptide directing the resulting rhLZ 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 human 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 the rice glutelin gene. The signal peptide is used to guide the rhLZ through the endoplasmic reticulum and target it to the protein bodies of the endosperm cells in maturing rice grain. It is cleaved from the rhLZ 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). The selectable marker gene (hpt) is under tight control of the 940 bp rice β-glucanase 9 (Gns9) gene promoter that is specific for expression in rice callus tissue. Although the hpt protein is expressed and present in the calli of the cultured non-differentiated plant cells after transformation, it is not expressed or present in tissues of the mature plant including the grain. 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 rhLZ 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.

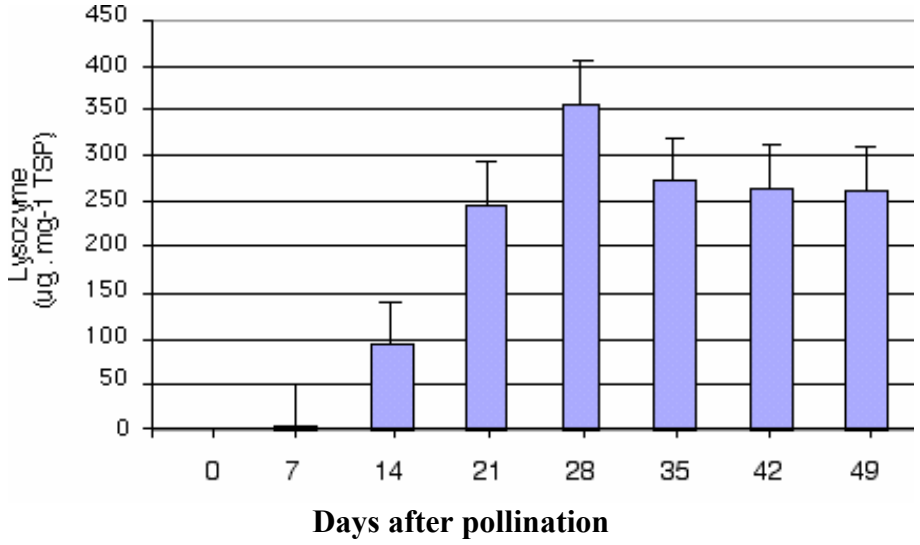


Figure 2. Recombinant Human Lysozyme Protein by ELISA in the Maturing Grain

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 20,800 rfc at 4° C for 10 min. Recombinant human 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).

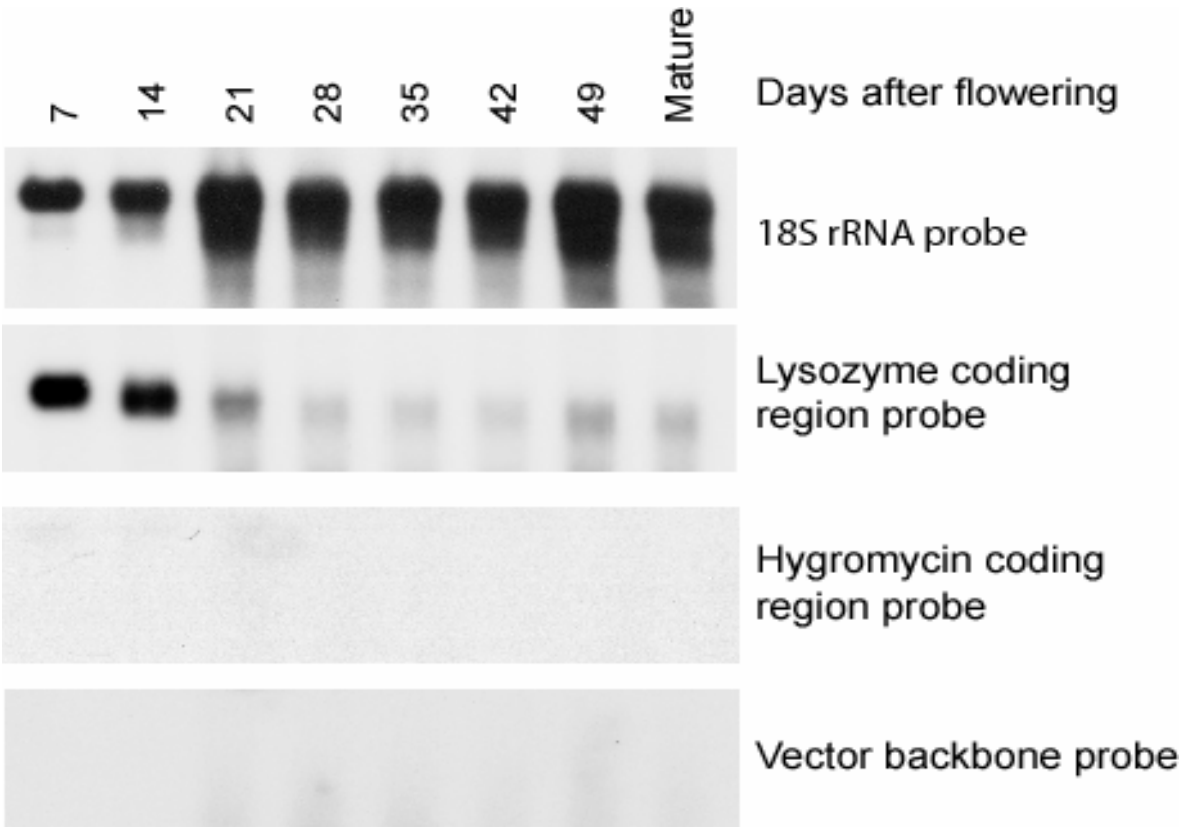


Figure 3. Northern Blot Analysis of Maturing Seed in LZ159 Rice

The human 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 rhLZ 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 phophotransferase 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 rhLZ protein by Western blot. Recombinant human 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 rhLZ protein is only expressed in the grain of the rice plant.

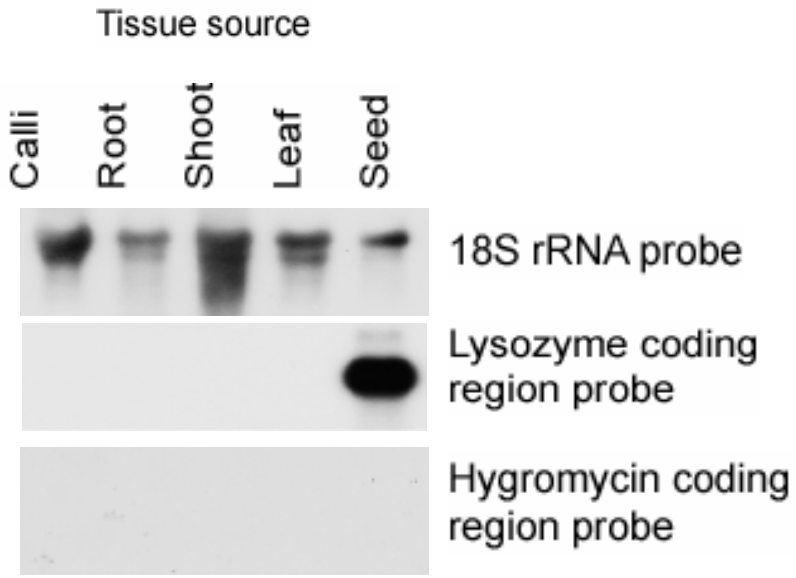


Figure 4. Northern Blot Analysis of Various LZ159 Plant Tissues

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.

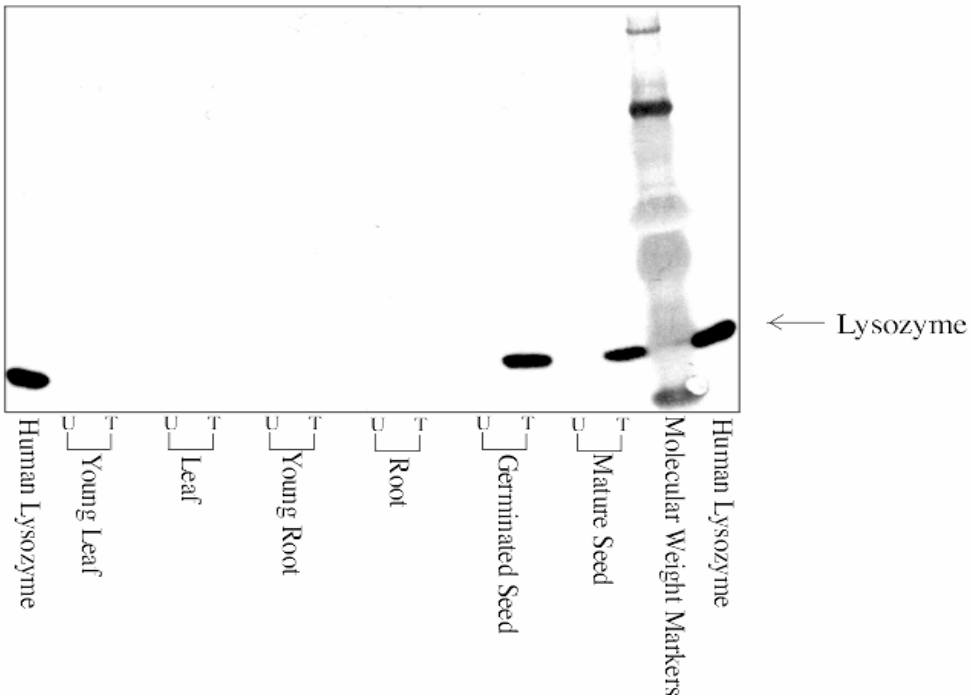


Figure 5. Tissue Specificity Studies of rhLZ

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. Recombinant human lysozyme expression was determined by Western blot analysis using polyclonal antiserum against human lysozyme. Native human lysozyme from milk was used as positive control.

Inheritance of the introduced genetic locus meets the single locus model of Mendelian inheritance in the R1 generation based on Chi square analysis. A transgenic plant was considered homozygous for rhLZ expression if all grains from the plant were positive for rhLZ in both the *Micrococcus luteus* cell lysis assay and Western blot analysis.

The transgenic rice expressing rhLZ 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 rhLZ expression level over nine succeeding generations (R2 – R10).

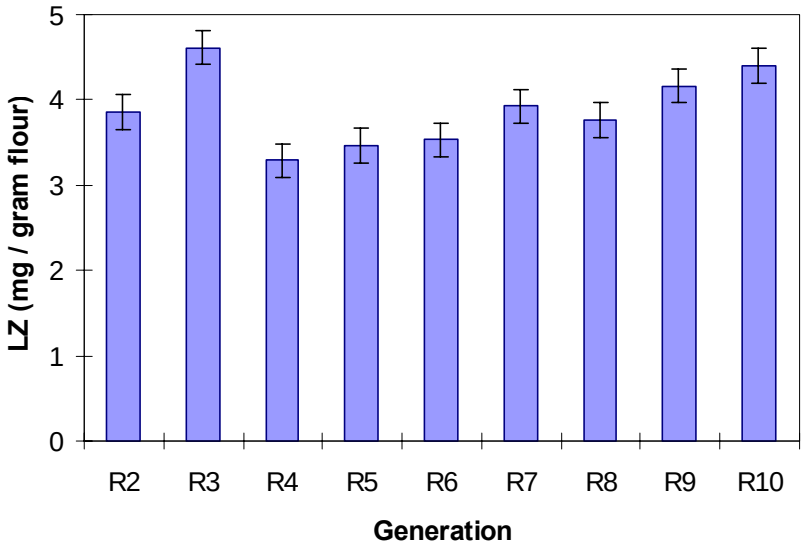


Figure 6. Recombinant Human Lysozyme Expression in LZ159 Rice

Brown rice flour corresponding to R2 to R10 generations of the LZ159 line was extracted with 0.3 M NaCl in 20 mM sodium acetate pH 4.0 at 37º C for 1.5 hr. The supernatant was collected by centrifuging the suspension at 20,800 rfc 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.

III. Rice grain

Extensive comparisons of the transformed rice grain and the parental rice grain against standard values for rice from the literature have been sponsored by Ventria Bioscience. Material from three harvest years has been analyzed and there are no significant variations in proximate analysis values, amino acid content, fatty acids, vitamins or minerals. The three anti-nutrients found in rice – phytic acid, trypsin inhibitor and lectin – are also present at the same levels in the parental and transformed grain.

IV. 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 rhLZ. 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.

V. 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).

A. General description of the production process

To date Ventria Bioscience has purified 1.4 kg of rhLZ from rice powder. 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, milled, and recombinant human lysozyme is extracted from the ground rice powder. The liquid phase, containing the soluble protein is a protein concentrate. The concentrate is further purified to an isolate using ion exchange chromatography.

All chemicals and processing aids are of food grade or higher quality and processing meets current good manufacturing practice for food. Process chemicals comply with 21 CFR § 182.1 (a) and 184.1721. Process aids are FDA compliant with 21 CFR § 170.30(b), 177.2910 (a) and 177.2510. Figure 7 gives a schematic of the purification process.

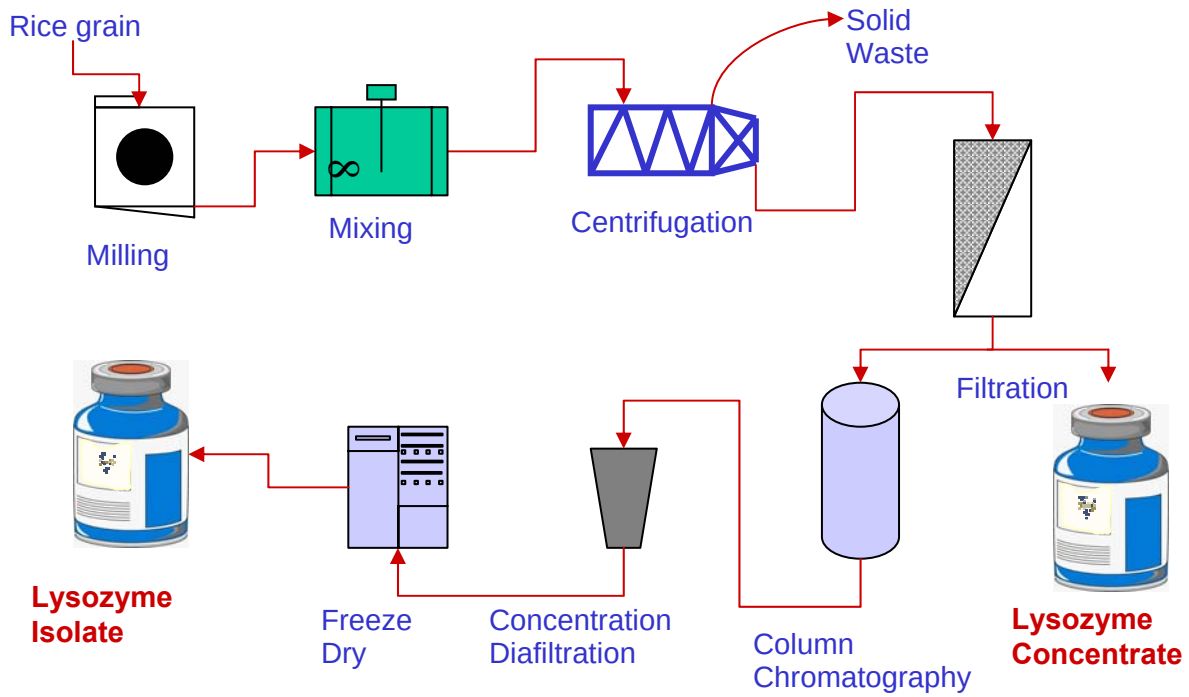


Figure 7. Purification of Recombinant Human Lysozyme

The process has been consistent and reproducible. A minimum of three separate batches tested and meet the appropriate food grade specifications

B. Stability of recombinant human lysozyme

Stability of the purified lysozyme dry powder stored at 37º C, ambient, and 4º C has been monitored by *Micrococcus luteus* activity assay for 59 weeks with no loss of activity.

C. Finished product specifications

Specifications for the two forms of rhLZ, concentrate and isolate, are similar to those for other ingredients found in infant formulas.

VI. Conclusion

Lysozyme (human) has been purified from transgenic rice using standard food industry procedures and materials in accordance with current good manufacturing practice for food. The process is reproducible and scalable and the material is stable in the dry powder form.

CONSUMER EXPOSURE AND SAFETY

I. Consumer Exposure

Ventria Bioscience intends to make lysozyme (human) derived from rice available for addition to infant/pre-term infant formulas and pediatric medical foods (oral rehydration solutions, ORS). The target population is infants 1 year of age for infant formulas and up to 3 years of age for ORS. The proposed use levels are consistent with the level of lysozyme found naturally in human milk and the exposure of breast fed infants (preterm formula, 0.3g rhLZ/L for preterm infants; full-term formula, 0.3-0.6g rhLZ/L; and medical foods [ORS, under supervision of a physician only]: 0.2 mg/mL rhLZ.)

A. Background exposure to lysozyme

Lysozyme is a natural constituent of the human body, found in exocrine secretions including saliva and milk, and in neutrophils and plasma.

1. Saliva

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 ± 0.61 mL/min. This flow rate would produce 2.8 liters of saliva each day. Lysozyme was measured at 11.76 ± 2.25 µg/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 µg/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 30 – 40 mg/day.

2. Human milk

Infants who are breast-fed consume human lysozyme, a natural constituent of human milk. A recent search of the scientific literature on the concentration of lysozyme in human milk was conducted. This literature search resulted in a range of reported lysozyme levels seen in various studies using different methods for measurement. Lysozyme levels in human milk have been reported from as low as 0.03 g/L to as high as

3.0 g/L. The variation is potentially attributable to methodological differences including study populations (e.g., Africa, Western Europe, US), sample treatment and analytical methods. The analytical methods used are antimicrobial activity assays (e.g., *Micrococcus lysodeikticus* assay), immunoassays that detect lysozyme protein (e.g., ELISA, immunoelectrophoresis/diffusion) or electrophoresis (e.g., SDS-PAGE). The stability of lysozyme is affected by sample storage conditions. In mature milk, lysozyme was stable at 37 º C and -20 º C, but lost activity after 24 hours at 4 º C (Goldman, Garza et al. 1982). Chandan, Shahani et al. 1964, reported that lysozyme activity was most effectively preserved frozen, but warned against repeated freeze thawing. They also indicated human milk could be held at room temperature or 30 º C for 3-4 days, until the milk spoiled, without significant loss of lysozyme activity. Accordingly, studies in the literature were deemed acceptable for use in this safety assessment if (1 there was sufficient information provided to ascertain that the samples were adequately stored prior to analysis; and (2 the study population was either from the US or a Western European nation with roughly comparable nutrition and diet. The mean and range of lysozyme values from studies meeting these criteria are presented in Table 2, converted where necessary to g/L.

Table 2. Concentration of Lysozyme in Human Milk

Lactation Stage	N	Range (g/L)	Mean (g/L)	Method	Reference
Colostrum	12	0.09 – 1.02	0.46	*Micrococcus lysodeikticus* assay	(Chandan, Shahani et al. 1964)
Milk	105	0.03 – 3.0	0.39		
Milk	3		0.4	*Micrococcus lysodeikticus* assay	(Chandan, Parry et al. 1968)
Colostrum (2d)	10		0.09	Electroimmunodiffusion	(Goldman, Garza et al. 1982)
1 m	11		0.03		
3 m	7		0.09		
6 m	11		0.25		
9 m	4		0.2		
12 m	5		0.2		
0-3 m	636	0.09 – 1.83	0.32	Microparticle-enhanced nephelometric immunoassay	(Montagne, Cuilliere et al. 1998)
0 – 140 d term	8	0.16 – 0.46	0.16	SDS-PAGE	(Velona, Abbiati et al. 1999)
0-140 d preterm	8	0.17 – 1.10	0.17		
1 – 14 d term	215		0.29	Microparticle-enhanced nephelometric immunoassay	(Montagne, Cuilliere et al. 1999)
1-14 d preterm delivery	153		0.35		
Colostrums (1-5d)	142		0.37	Microparticle-enhanced nephelometric immunoassay	(Montagne, Cuilliere et al. 2001)
6 – 14 d	106		0.27		
15 – 28 d	34		0.24		

Lactation Stage	N	Range (g/L)	Mean (g/L)	Method	Reference
29 – 56 d	50		0.33		
57 – 84 d	28		0.89		
Colostrums (<32 w gestational age)	36		0.00071 (0.71)	*Micrococcus lysodeikticus* assay	(Koenig, Diniz et al. 2005)
Colostrums (32-36 6/7 w gestational age)	32		0.00079 (0.79)		
Colostrums (≥37 w gestational age)	33		0.00055 (0.55)		

The overall average of the mean values presented is ~0.3g/L. Because of differences in methodologies and lactation stages studied no attempt was made to derive an overall distribution of the values. The values for milk and colostrum in Table 2 are reasonably consistent across different analytical methods, even considering that more recent methods have offered improved sensitivities.

The values for colostrums in Koenig, Diniz et al. 2005 are significantly below the other studies, and there is reason to believe there is an error in their units. The authors reported lysozyme values as 0.00051-79 µg/L, but noted that their findings were comparable to those of previous researchers, specifically mentioning Velona and Montagne (Montagne, Cuilliere et al. 1999; Velona, Abbiati et al. 1999). Velona reported 0.17-1.10 g/L, and Montagne, Cuillierre et al. 1999, 0.33 g/L. We therefore believe the Koenig values should have been reported as mg/L rather than µg/L. Due to this discrepancy, the Koenig data were included in the Table, but not used in determining the overall average value of lysozyme.

Based on the literature summarized above and discussions with experts in the field, we have used 0.3 g/L in estimating the intake of naturally-occurring lysozyme by pre-term and full-term infants who are breast-fed (personal communication, GRAS Expert Panel). However, it is important to note that the level of lysozyme seen in human milk has been reported to be as high as 3.0 g/L.

B. Exposure to naturally-occurring lysozyme in human milk

Natural exposure to lysozyme through the consumption of human milk was estimated for full-term and pre-term infants. The daily consumption of human milk was determined from intake studies conducted with results reported in scientific journals. The naturally occurring level of lysozyme in human milk was also estimated following a thorough review of the scientific literature. These data allowed for the calculation of the typical (mean) exposure to naturally occurring lysozyme among pre-term and full-term infants.

1. Human milk and lysozyme intake

a. Full-term infants

The American Academy of Pediatrics (AAP) currently recommends that healthy full-term infants be exclusively breast-fed from 0 to 6 months. Breastfeeding should be continued for the first year or longer if mutually desired by the mother and the infant. After 6 months, foods rich in iron should compliment breastfeeding ((AAP) 2005).

US data indicate that breastfeeding has increased in the US in the 1990s through 2003 following a slight decrease in the 1980s. Published data from the "Ross Mothers Survey" show that in 2003, the percent of women breastfeeding their infants in the hospital was 66.0% (national average), up 10.1% from 1993. At 6 months of age, the percent of women still breastfeeding their infants was 32.8% (Ross 2003). This data is based on a nationally representative sample of new mothers.

A review of the current scientific literature yielded several reliable sources for data on intake of human milk among full-term infants. A recent article, (Arcus-Arth, Krowech et al. 2005) reviewed the current literature and intake studies from 1975 through 2000 and derived population distributions of intake. Two datasets were constructed based on 1) those studies that had data consistent with AAP advice (AAP dataset) and 2) a 0-12 month exclusively breast fed dataset using the 0-6 month data in the AAP dataset combined with data from infants exclusively breast-fed from 6-12 months. As part of the DARLING study, Heinig, Nommsen et al. 1993 compared energy intake and growth between breast-fed and formula-fed infants at 3, 6, 9, and 12 months. In this study, infants in the breast-fed group received breast milk as the sole source of milk through the first 12 months. Infants in the formula-fed group were either not breast-fed or were completely weaned by 3 months of age. At 3 months of age, 100% of the infants' energy intake was from milk. At 6, 9, and 12 months, the percent of energy intake from milk was reported and therefore the intake of milk at these time points could be calculated as well.

Additionally, The Harriet Lane Handbook provides standard estimates of daily energy recommendations for 0-1 yr olds (Robertson and Shilkofski 2005).

Based on the standard energy content of human milk (0.67 kcal/mL) (Robertson and Shilkofski 2005), the daily volume of human milk intake can be calculated from energy intake estimates using the following formula:

$$\frac{\text{[Energy intake (kcal/kg bw/day)] x [\% energy intake from milk]}}{\text{0.67 kcal/mL milk}} = \text{mL milk/kg bw/day}$$

A summary of human milk intake estimates for full term infants is provided in Table 3.

Intake of lysozyme by full-term infants through the consumption of human milk was estimated by multiplying the concentration of lysozyme in human milk (0.30 g/L) with

human milk intakes. The estimated natural intakes of lysozyme by full-term infants are presented in Table 3. Mean natural lysozyme intake ranges from 0.015 g/kg/day to 0.054 g/kg/day.

The intakes presented in Table 3 are based on an estimate of the average concentration of naturally occurring lysozyme in human milk (0.3 g/L). It is important to note that lysozyme levels in milk have been reported as high as 3.0 g/L and therefore background intake could potentially be as high as ~0.5 g/kg/day (0.180 L milk/kg/day x 3 g LZ/L milk).

Table 3. Human Milk, Energy, and Natural Lysozyme Intakes in Full Term Infants

	Mean Intake		
Infant Age	**Human milk (ml/kg/day)**	**Energy[2] from milk (kcal/kg/day)**	**Natural LZ intake[1] (g/kg bw/day)**
0-12 months AAP Method 1[3,6]	100.7	67.5	0.030
0-12 months AAP Method 2[4,6]	101.6	68.1	0.030
0-12 months EBF[6]	113	75.7	0.034
3 months[7]	128	85.9	0.038
6 months[7]	102	68.1	0.030
9 months[7]	80	53.5	0.024
12 months[7]	51	34.1	0.015
0-12 months[5,8]	120-180	80-120	0.036 - 0.054

AAP = The American Academy of Pediatrics

EBF = Exclusive breast-feeding

[1] 0.3 g LZ/L milk

[2] Energy density of human milk = 0.67 kcal/mL.

[3] Method 1 = The average population daily intake at each age is described by a regression line, assuming normality.

[4] Method 2 = Probablistic model with distribution of intake based on empirical mean, SD, and assumption of normality.

[5] Assuming that 100% of energy requirements are met by milk consumption.

[6] (Arcus-Arth, Krowech et al. 2005)

[7] (Heinig, Nommsen et al. 1993)

[8] (Robertson and Shilkofski 2005)

b. Pre-term infants

Intake recommendations and/or target intakes for pre-term infants from relevant data in the available scientific literature are presented in Table 4. The Tsang, Uauy et al. 2005 guidelines are presented as "reasonable intakes" of energy per day and then converted to milk intake using the formula presented above. From the majority of the sources

reviewed, it is the general consensus that low birth weight, pre-term infants typically require 150-160 mL milk/kg bw/day.

Intake of lysozyme by pre-term infants through the natural consumption of human milk was estimated (Table 4). Mean natural lysozyme intake ranges from 0.018 g/kg/day to 0.054 g/kg/day.

The intakes presented in Table 4 are based on an estimate of the average concentration of naturally occurring lysozyme in human milk (0.3 g/L). It is important to note that lysozyme levels in milk have been reported as high as 3.0 g/L and therefore background intake could potentially be as high as ~0.5 g/kg/day (0.180 L milk/kg/day x 3 g LZ/L milk).

Table 4. Human Milk, Energy, and Natural LZ Intake in Pre-Term Infant

Infant Age/weight classification	Human milk[1] (ml/kg bw/day)	Energy[2] (kcal/kg/day)	Natural LZ Intake[3] (g/kg bw/day)	Human milk consumption Reference
Day 0				(Tsang, Uauy et al. 2005)
ELBW	60-75	40-50	0.018 - 0.023	
VLBW	60-75	40-50	0.018 - 0.023	
Transition (≤7 days)				(Tsang, Uauy et al. 2005)
ELBW	112-127	75-85	0.034 - 0.038	
VLBW	90-104	60-70	0.027 - 0.031	
Growing				(Tsang, Uauy et al. 2005)
ELBW	157-172	105-115	0.047 - 0.052	
VLBW	134-149	90-100	0.040 - 0.045	
VLBW	150-180	100-120	0.045 - 0.054	(Hawthorne, Griffin et al. 2004)
Pre-term	135-180	90-120	0.041 - 0.054	(Kleinman 2004)

[1] Assuming that 100% of energy requirements are met through milk consumption.

[2] Energy density of human milk = 0.67 kcal/mL.

[3] 0.3 g LZ/L human milk

C. EDI of rhLZ from uses proposed by Ventria

Ventria proposes the following uses for recombinant human lysozyme enrichment. The target population is infant formulas to 1 year of age and medical foods for children up to 3 years of age.

1. Full-term infant formulas: Ventria proposes to fortify term infant formulas at a level of 0.3 g rhLZ/L of formula up to 0.6 g rhLZ/L of formula. The estimated daily intake would therefore be 0.23 to 0.45 g/day, 0.022 g/kg/day to a maximum of 0.09 g/kg/day based on a 5 kg infant.

2. Pre-term infant formulas or formula fortifiers: Ventria proposes to fortify pre-term infant formulas up to 0.3 g rhLZ/L of formula. The estimated daily intake would therefore be 0.05 g/day, 0.018 g/kg/day to a maximum of 0.054 g/kg/day based on a 1 kg infant.

3. Medical foods (under supervision of a physician only): 0.2 mg/mL rhLZ in oral rehydration solution (ORS). The estimated intake during the critical four hour rehydration period would range from 40 to 240 mg.

1. Formulas for full-term infants

Formula consumption in full-term infants was determined using data from the Life Sciences Research Office (LSRO) term report (1998), Heinig, Nommsen et al. 1993 (Table 5). The LSRO report provides recommendations for the composition of formulas for full term infants through 1 year of age. Nutrient intake recommendations throughout the report are based on a 5 kg infant consuming 750 mL milk per day (i.e., 150 mL/kg/day).

As part of the DARLING study, Heinig, Nommsen et al. 1993 compared energy intake and growth between breast-fed and formula-fed infants at 3, 6, 9, and 12 months. Infants in the formula-fed group were either not breast-fed or were completely weaned by 3 months of age. Energy intakes were converted to formula intake using the formula presented earlier. For these calculations, it was assumed that infant formula has the same energy density as human milk (i.e. 0.67 kcal/mL).

In addition to the intake studies reported in the scientific literature, formula consumption was also determined using data from the U.S. Department of Agriculture's (USDA's) Continuing Survey of Food Intakes by Individuals, 1994-1998 (CSFII) USDA, 2000 and Exponent's proprietary Food and Residue Evaluation software program (FARE™). Data from both survey days was used to estimate a 2-day average, generally considered to represent consumption on a chronic (long-term) basis. In this analysis, mean intakes of infant formula were estimated for 2 subpopulations. The subpopulations were divided according to age (0-6 months and 0-12 months). The values we report are for "users," i.e., persons who reported eating the foods of interest (see Appendix A for a list of these foods) on either or both of the survey days.

When infant formula is fortified at 0.3 g/L, the mean estimated intake of lysozyme ranges from 0.022 g/kg/day to 0.045 g/kg/day (Table 5). When infant formula is fortified at 0.6 g/L, the mean estimated intake of lysozyme ranges from 0.045 g/kg/day to 0.09 g/kg/day.

Table 5. Daily Formula Intake and LZ Intake in Full-Term Infants at a Fortification Level of 0.3 g rhLZ/L and 0.6 g rhLZ/L

Infant Age	Formula intake (ml/kg/day)	Energy intake[1] (kcal/kg/day)	Fortified LZ intakes		Human milk consumption reference
			LZ intake[2] (g/kg bw/day)	LZ intake[3] (g/kg bw/day)	
3 months	147	98.7	0.044	0.088	(Heinig, Nommsen et al. 1993)
6 months	119	79.5	0.036	0.071	(Heinig, Nommsen et al. 1993)
9 months	89	59.9	0.027	0.054	(Heinig, Nommsen et al. 1993)
12 months	75	50.0	0.022	0.045	(Heinig, Nommsen et al. 1993)
0-6 months	150	100	0.045	0.090	LSRO (1998)
0-6 months	150.6	100.9	0.045	0.090	((USDA) April 2000)
0-12 months	116.5	78.1	0.035	0.070	((USDA) April 2000)

[1] Energy density of human milk = 0.67 kcal/mL

[2] 0.3 g LZ/L formula.

[3] 0.6 g LZ/L formula.

2. Formulas for preterm infants

Formula consumption in pre-term infants was determined using data from the Life Sciences Research Office (LSRO) term report (Klein 2002). The LSRO report provides recommendations for the composition of formulas for pre-term, low birth weight infants. Nutrient intake recommendations throughout the report are based on a 1 kg infant consuming 150 mL milk per day (i.e. 150 mL/kg/day). In addition, the sources used to estimate human milk intake in pre-term infants was based on energy requirements and the assumption that 100% of the infants' energy requirements were being met by human milk intake. Therefore, we can make the same conservative assumption that 100% of the energy requirements of a pre-term infant could also be obtained from formula consumption. A summary of formula intake estimates in pre-term infants is provided in Table 6.

When pre-term infant formula is fortified at 0.3 g/L (Table 6), the estimated intake of lysozyme ranges from 0.018 g/kg/day to 0.054 g/kg/day.

Table 6. Daily Formula Intake and Fortified LZ Intake in Pre-Term Infants

Infant Age/weight classification	Formula (ml/kg bw/day)	Energy[1] (kcal/kg/day)	LZ intake[2] (kcal/kg/day)	Reference
<36 weeks gestational age; <2500g	150	100	0.045	(Klein 2002)
Day 0				(Tsang, Uauy et al. 2005)
ELBW	60-75	40-50	0.018 - 0.023	
VLBW	60-75	40-50	0.018 - 0.023	
Transition (up to 7 days)				(Tsang, Uauy et al. 2005)
ELBW	112-127	75-85	0.034 - 0.038	
VLBW	90-104	60-70	0.027 - 0.031	
Growing				(Tsang, Uauy et al. 2005)
ELBW	157-172	105-115	0.047 - 0.052	
VLBW	134-149	90-100	0.040 - 0.045	
VLBW	150-160	100-107	0.045 - 0.048	(Hawthorne, Griffin et al. 2004)
Pre-term	135-180	90-120	0.041 - 0.054	(Kleinman 2004)

[1] Energy density of formula = 0.67 kcal/mL.

[2] Formula fortified at.0.3 g LZ/L formula.

3. Oral rehydration solutions (ORS)

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). 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 7.

Table 7. Estimated rhLZ Intake from ORS Enriched at 0.2 mg/mL

Weight	Age	Amount of ORS in first 4 hours	rhLZ intake
<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

5I. Safety Studies

Because of the cost of human lysozyme, most studies have used hen egg white lysozyme. 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* (FR FDA 03/13/98 PR 63 FR 12421). 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. The Joint FAO/WHO Expert Committee on Food Additives has also concluded that lysozyme added to cheese is not a hazard to consumer health (FAO/WHO 1992). 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)).

A. ADME Studies

Hen egg white lysozyme and human lysozyme have the same enzymatic substrate specificity, although there is a difference in the specific activity of the two enzymes, human lysozyme having a high specific activity. They are also very similar in amino acid sequence (60% homology) and secondary structure; however, there is no apparent immunological cross reactivity as discussed in the section on substantial equivalence, page 45.

Several studies have investigated the systemic appearance of lysozyme following oral intake. 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®, 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.

Lysozyme has been detected in the stool of both adults and infants. Lysozyme was detected in the stool of healthy adults at a production rate of 0.42 mg/day, a value that exceeds the predicted amount based on plasma seepage, indicating local production in the gastrointestinal tract (Bard, Laibe et al. 2003). Studies on fecal lysozyme in breast fed infants reported 0 to 131 mg/L of lysozyme (Eschenburg, Heine et al. 1990).

B. Animal studies

Ventria Bioscience's recombinant human lysozyme 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 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) of recombinant human lysozyme isolate (85% lysozyme) is considered to be greater than 0.36 g/kg/day.

C. 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.

D. 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 from rice, lactoferrin, at 1 mg/mL (Zavaleta, Figueroa et al. 2005). 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 dehydration and 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 were no material related adverse events.

III. Other Studies

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 and defense. The key role of the innate immune system is to provide not only 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 has both enzymatic and non-enzymatic functions that support its role in protection of the organism.

The use of lysozyme in pediatric medical foods and as an ingredient for pre-term and infant formulas can lend nutritional support to management of conditions associated with compromised host defense systems, impaired gastrointestinal or immune function, gastrointestinal disease and stress. The improved health status of breast fed children has been demonstrated in numerous epidemiological studies. Recently, PUFA and nucleotides have been accepted as conditional essential nutrients for addition to infant formulas. These changes are associated with attempts to provide an infant formula as similar to breast milk as possible. Lysozyme, lactoferrin and IgA are proteins found in breast milk which contribute to the innate defense system. Lysozyme can plays a natural defense 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.

B. Bacteria-Controlling Effects

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. It is important that the these studies were done using physiological levels of lysozyme. 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 allowing 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).

Lysozyme is considered one of the biologically active proteins involved in the development and healthfulness of the neonate. It is resistant to proteolysis in the infant intestine and is found in stools of breast fed infants (Hanson and Winberg 1972). Epidemiological studies have established that human milk is beneficial for infants, resulting in fewer incidences of infectious diseases of both the digestive and respiratory tracts. This is due to the passive protection conferred by antimicrobial substances present in human milk, including lysozyme (Hennart, Brasseur et al. 1991).

C. Immunomodulatory Studies

The immunomodulatory 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). 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. These activities support the use of lysozyme (human) derived from rice in pediatric medical foods and as an ingredient in pre-term and infant formulas.

D. Enzymes in Milk

Human milk contains numerous enzymes which can be arbitrarily divided into those that function in the mammary glands, in the milk and in the infant. Lysozyme is in the category of those that function in the infant (Hamosh 1988) and is the most abundant enzyme in human milk (Bezkorovainy 1977). Lysozyme is one of three whey proteins resistant to digestion and thus not considered nutritionally available (Raiha 1988). It is generally categorized as having a protective effect due to bactericidal activity. *In vitro* studies of the ability of lysozyme to degrade the beneficial *Bifidobacteria* and Lactobacilli of the intestine demonstrated with 5 species of *Bifidobacteria* and *Lactobacillus acidophilus* that they were not susceptible to lysis by lysozyme at concentrations as high as 0.500 mg/mL (Heine, Braun et al. 1995). Lysozyme may also have a role in proteoglycan and glycoprotein metabolism (Osserman, Canfield et al. 1974).

IV. 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 based on its substantial equivalence to lysozyme in human milk.

SAFETY ASSESSMENT

I. Substantial equivalence of lysozyme (human) derived from rice to native human lysozyme

A. Biochemical equivalence

Lysozyme (human) derived from rice has been purified from LZ159 rice and compared to native human lysozyme from 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 8 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 hLZ and rhLZ.

Table 8. Comparison of Physical, Chemical and Biochemical Properties of hLZ and rhLZ

Properties	hLZ	rhLZ (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	85o C, 5 min in PBS	85o 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

1. 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 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 composition data are not available for this residue using this method. There are five tryptophans in lysozyme and all were

identified in the complete sequence analysis. In the composition analysis Cys and Arg are known to generate larger variation.

Table 9 indicates that based on protein hydrolysis and amino acid analysis, recombinant human lysozyme and native human lysozyme have the same amino acid composition.

Table 9. Comparison of Amino Acid Composition of rhLZ And hLZ

Amino Acid	rhLZ	hLZ	Measurement variation
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%
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 hLZ sequence. The measured amino acid composition of rhLZ was also compared to the theoretical value for hLZ based on the nucleic acid sequence. Values for all amino acids in rhLZ were within acceptable analytical variation of the theoretical values based on the DNA sequence.

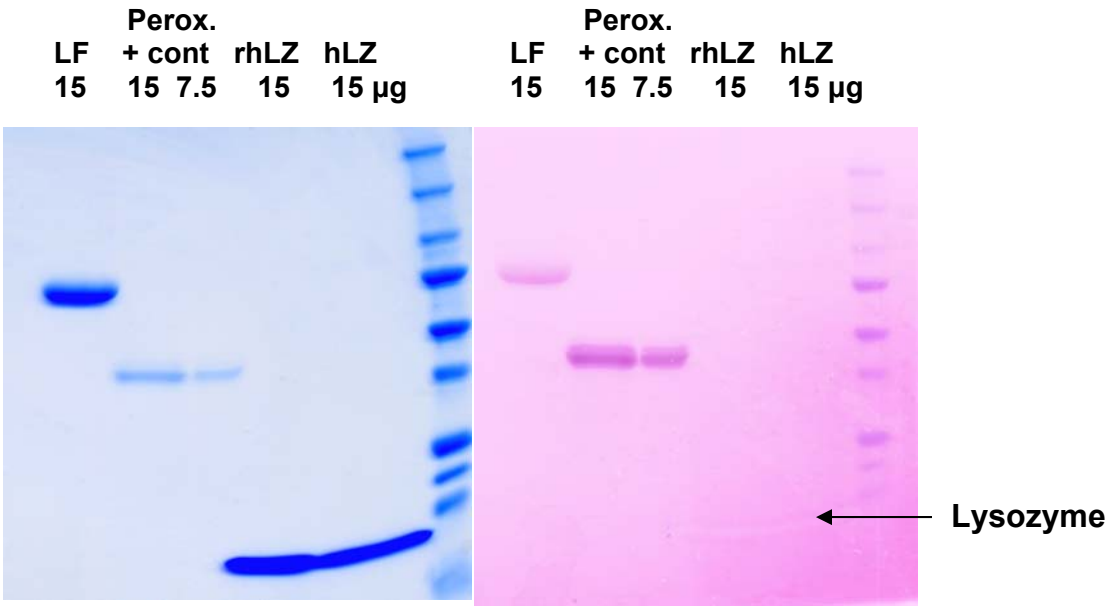
In addition to analysis of amino acid composition, the complete sequence of recombinant human lysozyme was determined. Purified lysozyme was digested by the endoproteinase Lys-c protocol to yield 5 fragments. Four of the fragments were analyzed for amino acid sequence by Edman sequence analysis. One 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.

2. N-terminal sequence

N-terminal sequence analysis of purified rhLZ gave the same sequence, KVFER(C)ELART, as hLZ, 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.

3. Glycosylation

There are no reports in the literature of glycosylation of native human lysozyme; therefore recombinant human lysozyme should not be glycosylated. There are no N-glycosylation sites on lysozyme. Although there is one potential O-glycosylation site, there is currently no consensus amino acid sequence related to O-glycosylation. 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 hLZ or rhLZ shows evidence of glycosylation at the concentrations loaded.



A. Coomassie Blue **B. Carbohydrate Stain - PAS**

Figure 8. Glycan Analysis of rhLZ and hLZ

4. Molecular weight

The molecular weight of rhLZ 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 µg). Native hLZ (5µg) was used as control. Both rhLZ and hLZ 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.

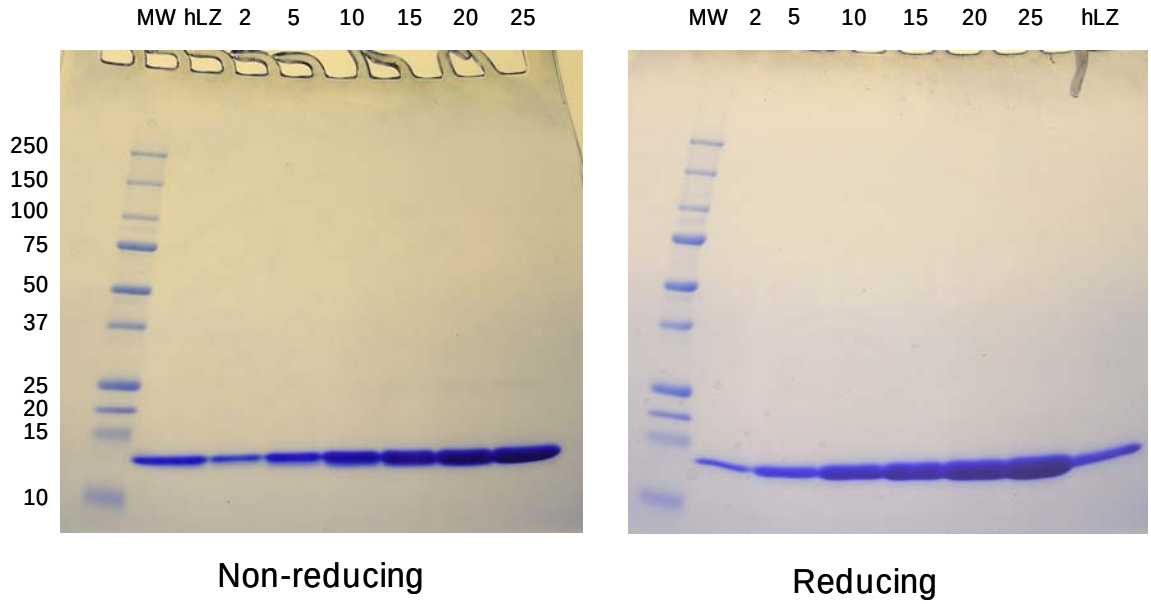


Figure 9. SDS-PAGE of rhLZ and hLZ

5. Molecular mass (MALDI)

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

Human lysozyme **Recombinant human lysozyme**

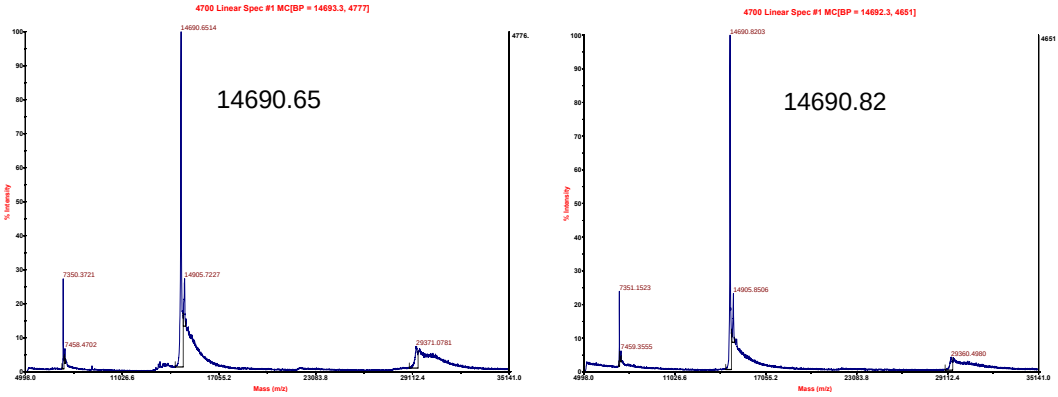


Figure 10. MALDI, Mass Spectrum of hLZ and rhLZ

6. Isoelectric focusing

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

7. pH stability

The pH stability of rhLZ was compared to that of hLZ 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 rhLZ and hLZ have the same pH stability profile and maintain their activity across a broad pH range (pH 2 to 10).

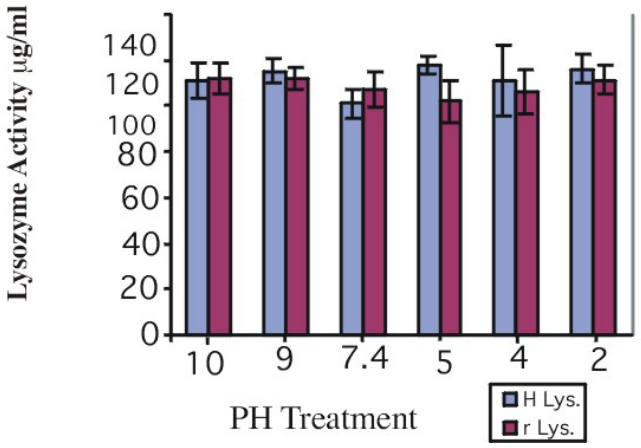


Figure 11. pH Stability of rhLZ and hLZ Activity

8. Thermal stability

Recombinant human lysozyme and hLZ 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 100º 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 12). At 60º C and 72º C, enzymatic activity survives 15 min of incubation time. Both proteins have the same thermal stability profile, losing their activity after 5 min at 85º C and almost immediately at 100º C.

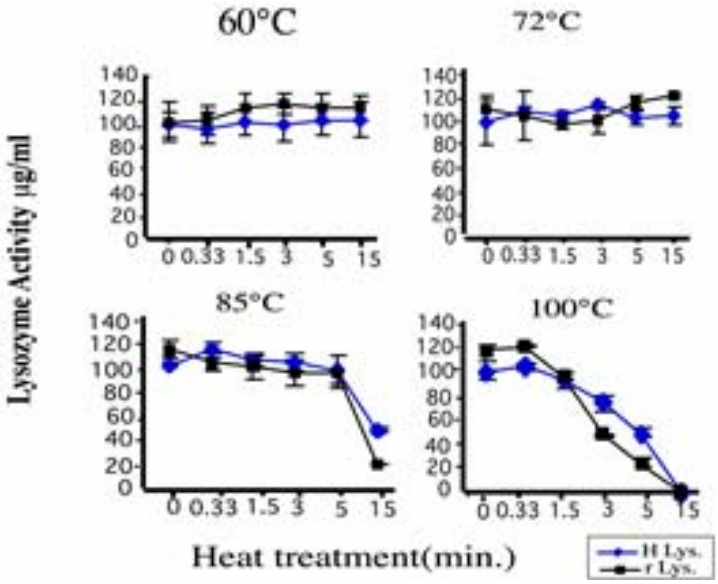


Figure 12. Thermal Stability of rhLZ and hLZ

9. Solubility

Both rhLZ and hLZ are soluble at > 50 mg/mL in phosphate buffered saline at room temperature.

10. Pepsin digestion

Ventria's rhLZ was compared with hLZ from breast milk and HEL 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 min 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 min and completely in 15 min when analyzed according to the standard protocol. Based on the results from the individual gels, the digestion was repeated on the hLZ and rhLZ using time points 0, 2, 5, 10 and 15 min. Both proteins were completely digested in < 10 min. Figure 13 shows the complete time course of digestion for hLZ (A), rhLZ (B) and hen egg white lysozyme (C) and the time course including 10 min for hLZ and rhLZ on the same gel (D).

In addition a theoretical digestion was performed using the computer program on ExPASy Proteomics tools, (http://kr.expasy.org/tools/peptidecutter/), Peptide Cutter. This program predicts the potential enzyme cleavage sites in a given protein sequence. The program was run using pepsin digestion of the rhLZ sequence at pH 1.3 and pH >2. Cleavage at pH 1.3 resulted in 32 peptides and amino acids, with only 3 peptides larger than 12 amino acids. From epitope mapping studies the theoretical peptide length for binding is 8 – 12 amino acids for a T-cell epitope and longer for B-cell epitopes (Rothbard and Gefter 1991; Metcalfe, Astwood et al. 1996). The three fragments were 23, 15 and 15 amino acids in length. Cleavage with pepsin at pH > 2 resulted in 14 peptides with the three largest fragments being 25, 21 and 13 amino acids in length. Results from the computer generated digestions and the actual digestion indicate there is no difference in the pepsin-resistance between hLZ and rhLZ.

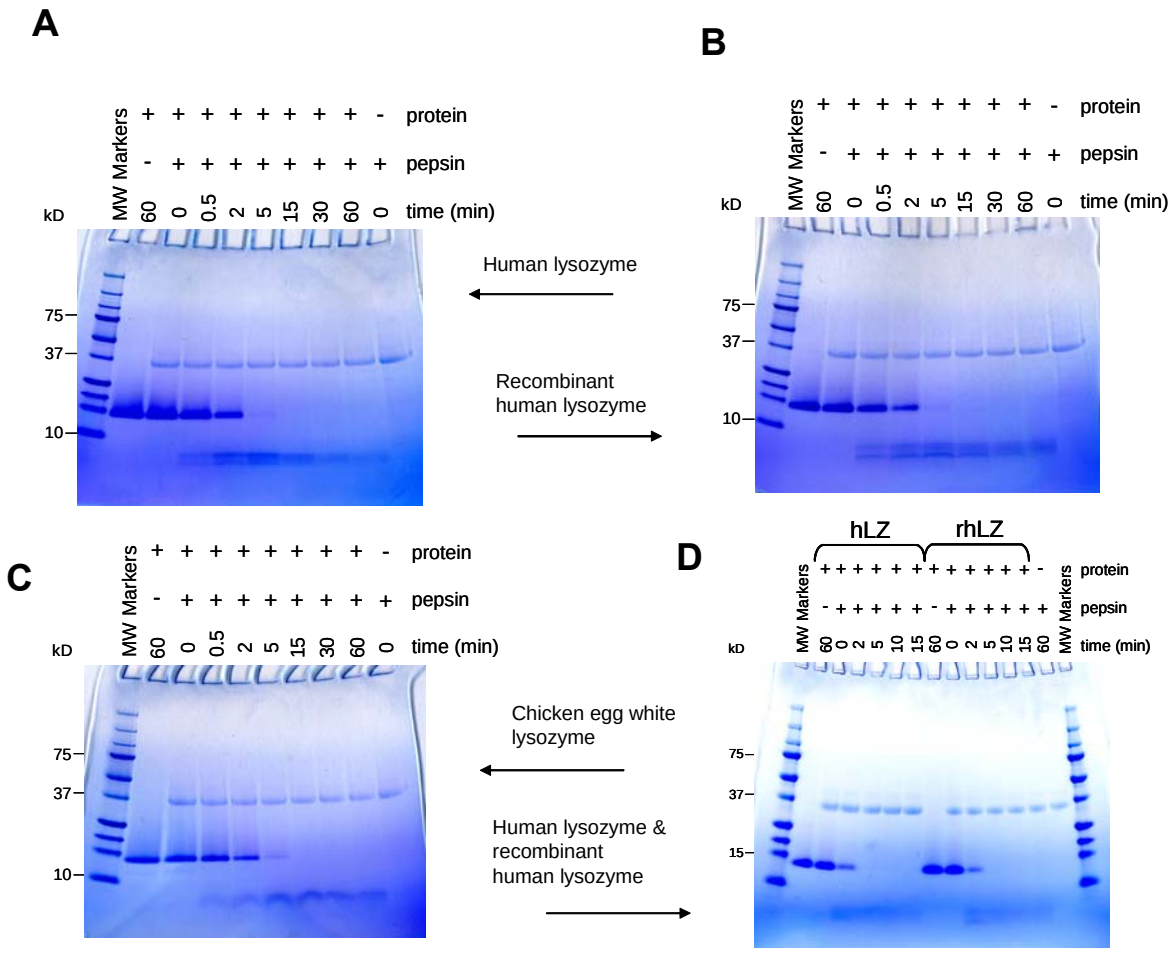


Figure 13. Digestion of hLZ from Breast Milk, rhLZ and HEL Using the ILSI Protocol.

B. Activity equivalence

In addition to comparing the physical and chemical properties of hLZ from milk to rhLZ from rice, activity comparisons were done. Table 10 summarizes the studies and the results. The data from the individual studies follow the table.

Table 10. Activity of rhLZ Compared with hLZ

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

1. Specific activity

The specific activity of rhLZ and hLZ was 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 hLZ has a specific activity of 134,206 units/mg protein, indicating substantial equivalence and activity of ~135,000 units/mg protein. As a comparison, HEL (cLZ) was measured and its specific activity is 32,393 units/mg protein.

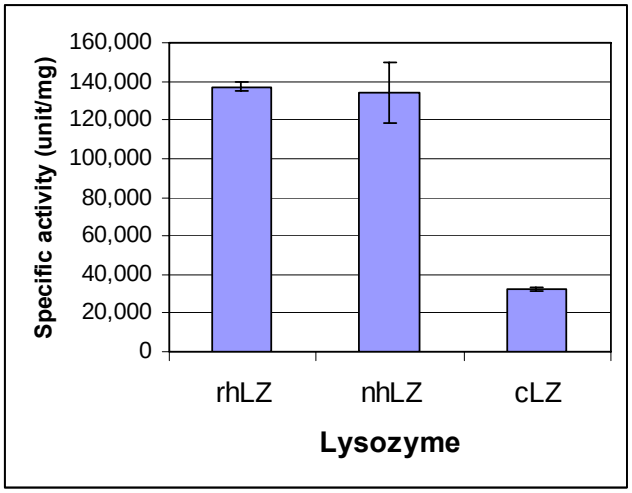


Figure 14. Specific Activity of rhLZ, hLZ and HEL (cLZ)

2. Bactericidal activity

Bactericidal activity of hLZ and rhLZ was compared using *E. coli* strain JM109. Bacteria were incubated in buffer, non-transformed rice extract, and hLZ or rhLZ 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 1 h incubation time, colony forming units were reduced to 100 and approached zero at 2 h.

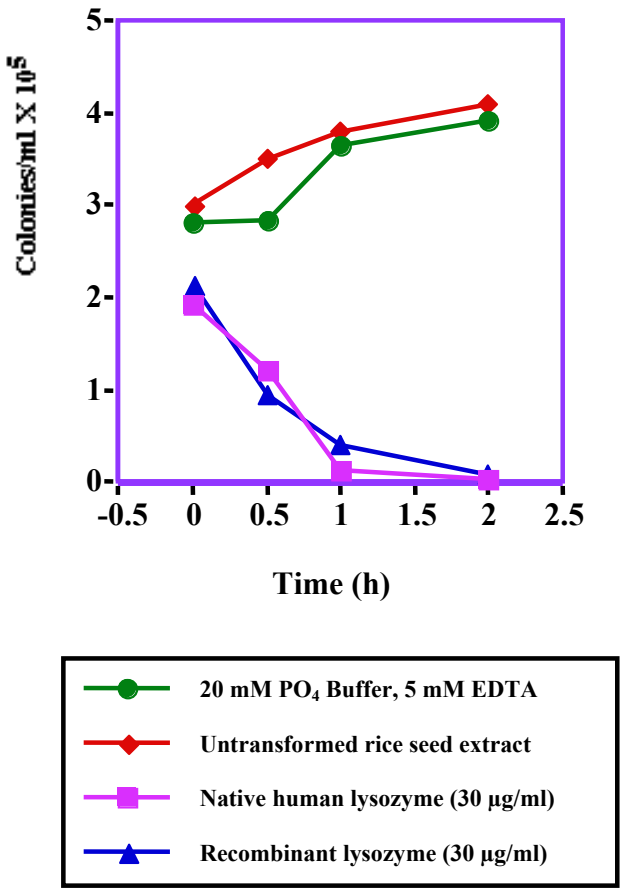
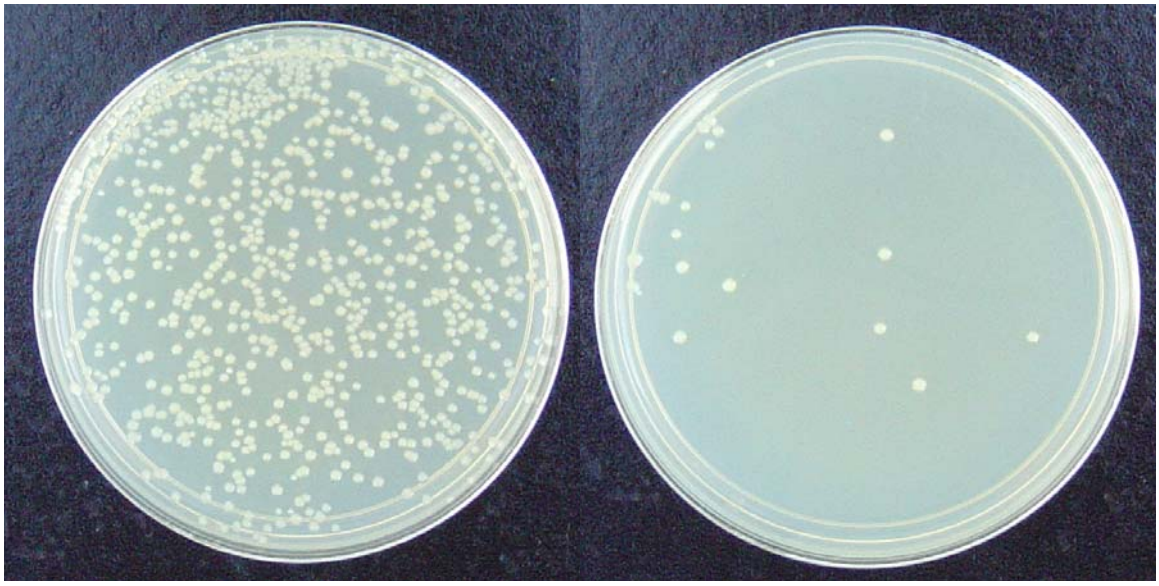


Figure 15. Bactericidal Activity of rhLZ and hLZ

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 µg of rhLZ; the control contained no lysozyme. The mixture was incubated at 37º C for 120 min, plated and colony forming units were determined.



Buffer only 20 µg/mL rhLZ

Figure 16. Bactericidal Activity of rhLZ

C. Safety equivalence

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

1. Gene source

The gene for rhLZ is synthetically produced based on the DNA sequence of hLZ. Therefore the gene is not from an allergenic source.

2. 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 can contain 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 hLZ 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 hLZ and HEL, 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. 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. HEL and a 24 amino acid lysozyme fragment from HEL 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 and that program was run as well. Chicken

lysozyme C (hen egg white lysozyme) and bovine alpha-lactalbumin were the only hits with this program.

The cross-reactivity of human IgE antibodies to HEL with rhLZ was investigated and the report from those studies, described below, concluded there is no cross-reactivity with IgE antibodies against HEL.

The alpha-lactalbumin increased from 40% to 46% homology using the 80mer sliding window. Several studies have been done on immunochemical cross-reactivity between bovine alpha-lactalbumin and lysozymes from several species, including human, bovine, chicken and duck. In studies with the native proteins, no cross-reactivity was detected in direct binding or inhibition studies using antibodies to both proteins (Arnon and Maron 1970; Atassi, Habeeb et al. 1970; Faure and Jolles 1970; Faure and Jollès 1970; Hopp and Woods 1982). Studies using peptides to inhibit suggest lysozyme and alpha-lactalbumin share a similar distribution of antigenic determinants in their surface, but enough sequence divergence to prevent cross-reactivity (Hopp and Woods 1982). One investigator has shown RAST inhibition of lysozyme binding by alpha-lactalbumin using sera from egg allergic subjects (Walsh, Elliott et al. 1987). It is also possible to develop cross-reacting antibodies to the reduced, carboxymethylated derivatives of lysozyme and alpha-lactalbumin, but the investigators felt this was indicative of structural resemblance of the unfolded proteins, they were not able to show cross-reactivity with the native proteins (Arnon and Maron 1971). Lysozyme is very stable in the intestine, appearing intact in fecal samples. In comparison to alpha-lactalbumin, lysozyme has no peptide bonds accessible to hydrolysis by trypsin, the disulfide bonds are less susceptible to reduction, and models of the two proteins reveal significant differences in shape (Atassi, Habeeb et al. 1970; Warme, Momany et al. 1974). Since the recombinant lysozyme is a human protein and there is strong evidence presented below that the IgE to hen egg white lysozyme, at 60% homology, is not cross reactive, it is highly unlikely that the alpha-lactalbumin at 46% homology would be cross reactive (Metcalfe, Astwood et al. 1996).

3. 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 hLZ from milk and rhLZ. Gels are shown on page 36.

D. Allergenicity

It is unlikely that hLZ 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 high concentrations found in tears and saliva. It has never been identified as an allergen. Due to the high cost of hLZ, however, HEL is the only commercially available lysozyme and most studies have used HEL, which is a minor allergen (Sava 1996).

For rhLZ 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

1. Human lysozyme as an auto-allergen

There is a small chance that hLZ could be an auto-allergen, but it is highly unlikely. 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 (Budde, de Heer et al. 2002). IgE recognition of rhLZ on the basis of sensitization to e.g. HEL 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 on a regular basis. In summary, although relevant for discussion, this possible allergenic property of rhLZ is is very remote.

2. 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 rhLZ from rice contain at least 20% residual rice protein, but possibly up to 50%. This residual rice material could include rice-derived allergens. As indicated, this product will be labeled as lysozyme (human) from rice. 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 α-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 grass pollens and allergens in rice.

Despite these studies, rice continues to be a component of recommended hypoallergenic diets and one of the first foods introduced to infants. 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 rhLZ but feel that this is unlikely given the rarity of rice allergy and the ubiquitous exposure to this foodstuff. In addition the product will be labeled as lysozyme (human) from rice.

3. Human lysozyme as a cross reacting allergen

Currently, the commercial source of lysozyme is HEL. 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 HEL and concluded the low additional intake of HEL 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, HEL is a minor allergen and there are reports of allergic reactions related to exposure to HEL 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 intake 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 hLZ and HEL. There are examples of polyclonal antibodies to HEL that are weakly cross reacting and non-cross reacting to hLZ. It is known that the major IgG binding epitopes of HEL are conformational, not linear epitopes (Bentley 1996; Smith-Gill 1996). There are three major IgG epitope regions on HEL (Bentley 1996). In 1984, Allen et al. identified amino acid region 46-61 of HEL as the region recognized by T-cells to initiate an immune response. Human lysozyme differs from HEL 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 HEL. These same cell lines had no detectable response to hLZ, 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 hLZ could cross-react with IgE antibodies to HEL.

To address this issue *in vitro* ELISA inhibition studies were done on banked and fresh sera from egg allergic, hen egg white lysozyme sensitized individuals. Patients ranged in age from 2 to 25; greater than 60% were between the ages of 2 and 5. Sera from 41 egg allergic patients with specific IgE to egg lysozyme were screened by ELISA inhibition using egg lysozyme and rhLZ. Sera were 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. 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.

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.

Of the 41 sera tested, none showed any cross-reactivity between rhLZ and HEL. The combined ELISA inhibition data are in Figure 17. Data are the mean and standard deviation of the percent inhibition of the individual sera at each concentration tested.

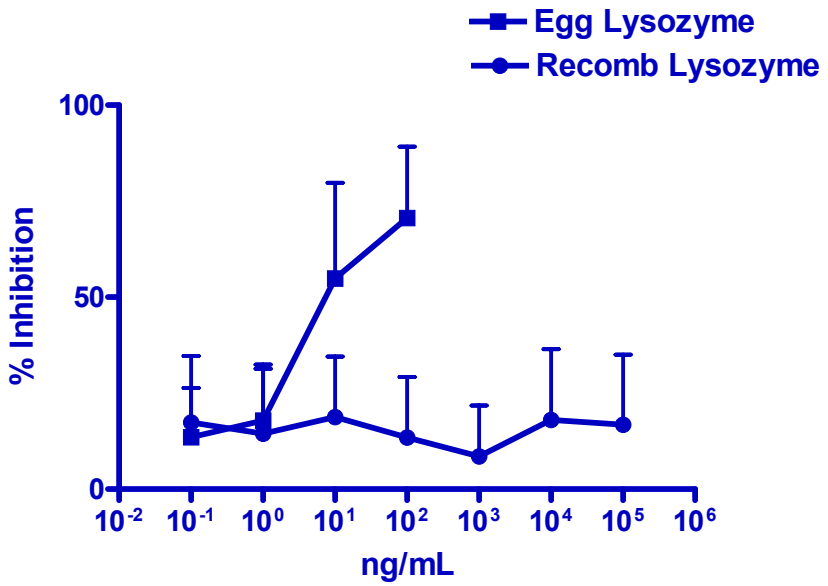


Figure 17. Percent Inhibition ELISA (mean ± SD) for 41 Egg Lysozyme Positive Sera

A key issue for allergenicity assessment is not only IgE binding but biological activity of IgE antibodies. Unfortunately, this notion has not really crystallized in the present guidelines for allergenicity assessment. There is much emphasis on IgE binding and very little attention to biological activity, i.e. clinical relevance. This is a dangerous pitfall for evaluation of a novel food or a plant-derived pharma product. It is easy to find some serum samples from food allergic patients that will have serum IgE antibodies recognizing the novel food or plant-derived recombinant protein.

Biological activity and clinical relevance of IgE antibodies can be assessed using functional *in vitro* tests which mimic the contact between the allergens and the cells responsible for release of histamine. Functional *in vitro* tests for allergic reactions focus on the circulating basophil: the basophil activation test (BAT). Flow cytometry can be used as a tool to identify basophil activation through identification of CD63. CD63 is

normally found in the basophilic granule membrane (the site of histamine storage). When CD63 is detected on the external surface of the basophil it indicates cell degranulation and histamine release as a result of fusion between the granules and the plasma membrane. CD63 expression is considered a reliable method for monitoring basophil activation (Boumiza, Debard et al. 2005).

A positive result indicates that membrane IgE are actually bridged by the allergen so that the activated cell expresses CD63 on the surface. It means that antibody sites of IgE recognize the antigen and that the affinity of linkage is sufficient to induce bridging. The purpose of the study was to compare the results obtained with hen lysozyme and recombinant human lysozyme. If cross reactivity between two molecules does exist, the activation is observed for both, but at a lower extent for the cross-reacting allergen: the intensity of activation is lower, and the concentrations needed for activation are higher. Twenty-eight children, age 1 to 11, allergic to egg and sensitized to hen egg lysozyme were recruited and fresh heparinized blood samples obtained for analysis using a fluorescence activated cell sorter and FITC labelled monoclonal antibody directed against CD63. Samples were tested with three preparations of rhLZ at 85%, 95% and 99% purity, human milk lysozyme, rice protein and egg lysozyme. Samples were added to whole blood at 3 different concentrations 0.1, 1 and 100 µg/mL and incubated for 20 min at 37°C. The basophil activation reaction was stopped and CD63 presence on the cell surface determined using a Beckman cytometer. For each sample n-formyl-Met-Leu-Phe was used as a positive control of basophil activation. The basophil activation test was considered positive if the following criteria were met:

positive control - 25 to 59 % positive
negative control - 1.9 to 9.5 % positive
egg lysozyme or recombinant human lysozyme - > 15 % positive.

Of the 28 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 rice proteins. The percentage of basophil activation decreased with the increased purity of the rhLZ.

These data support the conclusion that rhLZ does not cross react with human IgE directed against hen egg lysozyme.

4. 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.

5. 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 hLZ, 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 hLZ 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 apoAI 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 hLZ 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 intake (de Felice, Vieira et al. 2004).

Ventria Bioscience has done full sequence analysis of the rhLZ and there is 100% agreement with the sequence of wild-type hLZ as defined by the DNA sequence. The amino acid sequence from position 51-68 was confirmed identical to hLZ based on nucleic acid sequence using mass spectrometry of endoproteinase Lys-c and trypsin digested fragments (see page 30). None of the amyloid related amino acid substitutions occur in the rhLZ from rice.

6. Studies in animals and humans with oral intake of human lysozyme (human) derived from rice

Ventria Bioscience's rhLZ 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 rhLZ from an extract of the LZ159 rice flour. The formula also included recombinant human lactoferrin. 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 rhLZ 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 rhLZ 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% rhLZ.

Testing is completed in children with acute watery diarrhea using rhLZ 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 completed the study, one third received the rhLZ supplemented ORS and there were no material related adverse events.

II. 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 rhLZ produced in rice.

- The proteins are structurally identical and biochemically equivalent
- Recombinant lysozyme from rice is safe for egg white lysozyme sensitized patients.
- The gene source in non-allergenic
- The protein is not in the allergen databases
- Risk is remote of allergic sensitization to human lysozyme
- Residual rice proteins are not likely trigger an allergic response
- It is highly unlikely that ingestion of human lysozyme as a food ingredient, 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. As evidenced by the Expert Panel consensus statement in Appendix B.

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Appendix A

Infant formulas included in intake analysis

CSFII Food Code	Food Description
11710000	INFANT FORMULA, NFS
11710050	ALIMENTUM, W/ IRON, INFANT FORMULA, NS AS TO FORM
11710051	ALIMENTUM, W/ IRON, INFANT FORMULA, READY-TO-FEED
11710100	ENFAMIL, LOW IRON, INFANT FORMULA, NS AS TO FORM
11710101	ENFAMIL, LOW IRON, INFANT FORMULA, READY-TO-FEED
11710102	ENFAMIL, LOW IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11710103	ENFAMIL, LOW IRON, INFANT FORMULA, PREPARED FROM POWDER
11710110	GERBER, W/ IRON, INFANT FORMULA, NS AS TO FORM
11710111	GERBER, WITH IRON, INFANT FORMULA, READY-TO-FEED
11710112	GERBER, WITH IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11710113	GERBER, WITH IRON, INFANT FORMULA, PREPARED FROM POWDER
11710120	GERBER, LOW IRON, INFANT FORMULA, NS AS TO FORM
11710121	GERBER, LOW IRON, INFANT FORMULA, READY-TO-FEED
11710122	GERBER, LOW IRON, INFANT FORMULA, FR LIQ, CONCENTRATE
11710123	GERBER, LOW IRON, INFANT FORMULA, PREPARED FROM POWDER
11710200	SIMILAC, LOW IRON, INFANT FORMULA, NS AS TO FORM
11710201	SIMILAC, LOW IRON, INFANT FORMULA, READY-TO-FEED
11710202	SIMILAC, LOW IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11710203	SIMILAC, LOW IRON, INFANT FORMULA, PREPARED FROM POWDER
11710250	SIMILAC NATURAL CARE,LO IRON,INFANT FORMULA,NS FORM
11710251	SIMILAC NATURAL CARE,LO IRON,INFANT FORMULA,READY-TO-FEED
11710400	SIMILAC W/ IRON, INFANT FORMULA, NS AS TO FORM
11710401	SIMILAC W/ IRON, INFANT FORMULA, READY-TO-FEED
11710402	SIMILAC W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11710403	SIMILAC W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11710450	SIMILAC SPECIAL CARE 24, IRON, INFANT FORMULA, NS FORM
11710451	SIMILAC SPECIAL CARE 24, IRON, INFANT FORMULA, READY-TO-FEED
11710460	SIMILAC, TODDLER'S BEST, IRON, INFANT FORMULA, NS FORM
11710461	SIMILAC, TODDLER'S BEST, IRON, INFANT FORMULA, READY-TO-FEED
11710500	SMA, W/ IRON, INFANT FORMULA, NS AS TO FORM

CSFII Food Code	Food Description
11710501	SMA, W/ IRON, INFANT FORMULA, READY-TO-FEED
11710502	SMA, W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11710503	SMA, W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11710550	SMA, LOW IRON, INFANT FORMULA, NS AS TO FORM
11710551	SMA, LOW IRON, INFANT FORMULA, READY-TO-FEED
11710552	SMA, LOW IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11710553	SMA, LOW IRON, INFANT FORMULA, PREPARED FROM POWDER
11710600	ENFAMIL W/ IRON, INFANT FORMULA, NS AS TO FORM
11710601	ENFAMIL W/ IRON, INFANT FORMULA, READY-TO-FEED
11710602	ENFAMIL W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11710603	ENFAMIL W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11710710	LACTOFREE, W/ IRON, INFANT FORMULA, NS FORM
11710711	LACTOFREE, W/ IRON, INFANT FORMULA, READY-TO-FEED
11710712	LACTOFREE, W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11710713	LACTOFREE, W/ IRON, INFANT FORMULA, FROM POWDER
11710800	PEDIASURE, W/ IRON, INFANT FORMULA, NS AS TO FORM
11710801	PEDIASURE, W/ IRON, INFANT FORMULA, READY-TO-FEED
11710900	CARNATION GOOD START, IRON, INFANT FORMULA, NS FORM
11710901	CARNATION GOOD START, W/ IRON, INFANT FORMULA, READY-TO-FEED
11710902	CARNATION GOOD START,W/IRON,INFANT FORMULA,LIQUID CONCENTRAT
11710903	CARNATION GOOD START, W/ IRON, INFANT FORMULA, FROM POWDER
11710950	CARNATION FOLLOW-UP,W/ IRON,INFANT FORMULA,NS FORM
11710951	CARNATION FOLLOW-UP,W/ IRON,INFANT FORMULA,READY-TO-FEED
11710952	CARNATION FOLLOW-UP,W/IRON,INFANT FORMULA,LIQUID CONCENTRATE
11710953	CARNATION FOLLOW-UP, W/ IRON, INFANT FORMULA, FR POWDER
11720050	GERBER SOY, W/ IRON, INFANT FORMULA, NS FORM
11720051	GERBER SOY, W/ IRON, INFANT FORMULA, READY-TO-FEED
11720052	GERBER SOY, W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11720053	GERBER SOY, W/ IRON, INFANT FORMULA, FROM POWDER
11720200	NURSOY, W/ IRON, INFANT FORMULA, NS AS TO FORM
11720201	NURSOY, W/ IRON, INFANT FORMULA, READY-TO-FEED
11720202	NURSOY, W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11720203	NURSOY, W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11720300	PROSOBEE, W/ IRON, INFANT FORMULA, NS AS TO FORM

CSFII Food Code	Food Description
11720301	PROSOBEE, W/ IRON, INFANT FORMULA, READY-TO-FEED
11720302	PROSOBEE, W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11720303	PROSOBEE, W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11720400	ISOMIL, W/ IRON, INFANT FORMULA, NS AS TO FORM
11720401	ISOMIL, W/ IRON, INFANT FORMULA, READY-TO-FEED
11720402	ISOMIL, W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11720403	ISOMIL, W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11720450	ISOMIL SF, W/ IRON, INFANT FORMULA, NS AS TO FORM
11720451	ISOMIL SF, W/ IRON, INFANT FORMULA, READY-TO-FEED
11720452	ISOMIL SF, W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11720500	SOYALAC, W/ IRON, INFANT FORMULA, NS AS TO FORM
11720501	SOYALAC, W/ IRON, INFANT FORMULA, READY-TO-FEED
11720502	SOYALAC, W/ IRON, INFANT FORMULA, FROM LIQUID CONCENTRATE
11720503	SOYALAC, W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11720600	CARNATION ALSOY, W/ IRON, INFANT FORMULA, NS AS TO FORM
11720601	CARNATION ALSOY, W/ IRON, INFANT FORMULA, READY-TO-FEED
11720602	CARNATION ALSOY, W/ IRON, INFANT FORMULA, FROM LIQUID CONCEN
11720603	CARNATION ALSOY, W/ IRON, INFANT FORMULA, FROM POWDER
11720700	NEXT STEP SOY TODDLER FORMULA,W/ IRON,INFANT FORMULA,NS FORM
11720702	NEXT STEP SOY TODDLER FORMULA,W/ IRON,INFANT, LIQUID CONCEN
11720703	NEXT STEP SOY TODDLER FORMULA,W/ IRON,INFANT,FROM POWDER
11740100	LOFENALAC, W/ IRON, INFANT FORMULA, NS AS TO FORM
11740103	LOFENALAC, W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11740300	NUTRAMIGEN, W/ IRON, INFANT FORMULA, NS AS TO FORM
11740301	NUTRAMIGEN, W/ IRON, INFANT FORMULA, READY-TO-FEED
11740302	NUTRAMIGEN, W/ IRON, INFANT FORMULA, FR LIQUID CONCENTRATE
11740303	NUTRAMIGEN, W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11740400	PREGESTIMIL, W/ IRON, INFANT FORMULA, NS AS TO FORM
11740403	PREGESTIMIL, W/ IRON, INFANT FORMULA, PREPARED FROM POWDER
11740500	SIMILAC PM 60/40, LOW IRON, INFANT FORMULA, NS FORM
11740501	SIMILAC PM 60/40, LOW IRON, INFANT FORMULA, READY-TO-FEED
11740503	SIMILAC PM 60/40, LOW IRON, INFANT FORMULA, FROM POWDER
11740600	PORTAGEN, W/ IRON, INFANT FORMULA, NS AS TO FORM
11740603	PORTAGEN, W/ IRON, INFANT FORMULA, PREPARED FROM POWDER

Appendix B

Expert Panel Consensus Statement

In conclusion, there is no indication that adverse effects would result from consumption of foods containing lysozyme (human) from rice at the levels proposed in the intended uses as an ingredient in pre-term and infant formula and in pediatric oral rehydration solution. The literature and the experimental data provided by Ventria Bioscience support the substantial equivalence of human lysozyme and recombinant human lysozyme. There is minimal risk of allergenic or immunogenic responses in individuals consuming recombinant human lysozyme produced in rice.

- The proteins are structurally identical and biochemically equivalent
- Recombinant lysozyme from rice is safe for egg white lysozyme sensitized patients.
- The gene source in non-allergenic
- The protein is not in the allergen databases
- Risk is remote of allergic sensitization to human lysozyme
- Residual rice proteins are not likely trigger an allergic response
- It is highly unlikely that ingestion of human lysozyme as a food ingredient, 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 milk lysozyme.

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 medical foods and as a ingredient in infant formula.

Based on a critical evaluation of the currently available published and unpublished 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 in the target population of infants and children up to 3 years of age at the levels described below:

1. Full-term infant formulas: Ventria proposes to fortify infant formulas at a level of 0.30 g rhLZ/L of formula up to 0.6 g rhLZ/L of formula.

2. Pre-term infant formulas or formula fortifiers: Ventria proposes to fortify pre-term infant formulas up to a level of 0.30 g rhLZ/L of formula.

3. Medical foods (under supervision of a physician only): 0.2 mg/mL rhLZ in oral rehydration solution (ORS).

Steven Abrams, M.D.
Roger Clemens, DPH.
Bo Lonnerdal, Ph.D.
Stephen L. Taylor, Ph.D., Chairman

ingredient in pre-term and infant formula and in pediatric oral rehydration solution. The literature and the experimental data provided by Ventria Bioscience support the substantial equivalence of human lysozyme and recombinant human lysozyme. There is minimal risk of allergenic or immunogenic responses in individuals consuming recombinant human lysozyme produced in rice.

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3. Medical foods (under supervision of a physician only): 0.2 mg/mL in oral rehydration solution (ORS).

Steven Abrams, MD
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Signature: _____

Date: 1/8/2006

In conclusion, there is no indication that adverse effects would result from consumption of foods containing lysozyme (human) from rice at the levels proposed in the intended uses as an ingredient in pre-term and infant formula and in pediatric oral rehydration solution. The literature and the experimental data provided by Ventria Bioscience support the substantial equivalence of human lysozyme and recombinant human lysozyme. There is minimal risk of allergenic or immunogenic responses in individuals consuming recombinant human lysozyme produced in rice.

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Roger Clemens, DrPH, CNS, FACN, FIFT
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Date: January 10, 2006

In conclusion, there is no indication that adverse effects would result from consumption of foods containing lysozyme (human) from rice at the levels proposed in the intended uses as an ingredient in pre-term and infant formula and in pediatric oral rehydration solution. The literature and the experimental data provided by Ventria Bioscience support the substantial equivalence of human lysozyme and recombinant human lysozyme. There is minimal risk of allergenic or immunogenic responses in individuals consuming recombinant human lysozyme produced in rice.

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SUBMISSION END