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ORIGINAL SUBMISSION

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**GRAS
EXEMPTION CLAIM
FOR
MILK BASIC PROTEIN (MBP®)**

Prepared for
Snow Brand Milk Products Co., Ltd.
Tokyo, Japan
and
IAS Co., Ltd.
Tokyo, Japan

Prepared by
ENVIRON International Corporation
Arlington, Virginia

March 3, 2006

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ENVIRON

March 3, 2006

REC'D MAR - 6 2006

Robert Martin
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Exemption Claim for the Use of Milk Basic Protein as a Dietary Ingredient in Selected Foods and Beverages

Dear Mr. Martin:

On behalf of Snow Brand Milk Products Company, Ltd., and IAS Company, Ltd., we are submitting this Generally Recognized As Safe (GRAS) notification for the use of Milk Basic Protein (MBP[®]) as a dietary ingredient in selected foods and beverages. As documented in this notification, MBP is GRAS for use as a dietary ingredient in selected foods and beverages at maximum concentrations ranging from 10 to 40 mg MBP per serving.

We have enclosed three copies of this exemption claim, the signed statement of the Expert Panel, and supporting documentation for your review. Should you have any questions, please feel free to contact me.

Sincerely,

Claire L. Kruger, Ph.D., D.A.B.T.
Principal
ENVIRON International Corporation

Enclosures

cc: Antonia Mattia, Ph.D.

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GRAS Exemption Claim

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GRAS Exemption Claim for Milk Basic Protein (MBP[®])

A. NAME AND ADDRESS OF NOTIFIER

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* IAS Co., Ltd. is a joint venture company established by Snow Brand Milk Products Co., Ltd. and Itochu Corporation to market MBP in the United States.

B. IDENTITY OF GRAS SUBSTANCE

The product that is the subject of this Generally Recognized as Safe (GRAS) determination is a specific basic protein fraction derived from pasteurized New Zealand skim milk. This protein is named Milk Basic Protein (MBP[®]) by Snow Brand Milk Products Co., Ltd.

C. INTENDED USE

MBP is intended for use as a dietary ingredient in selected foods and beverages at maximum concentrations ranging from 10 to 40 mg MBP per serving. The intended maximum use of MBP is 10 mg per serving of cottage cheese and salad dressing (excluding mayonnaise and mayonnaise-type dressings), 30 mg per serving of processed cheese, and 40 mg per serving of imitation milk, juice, including 100% citrus juices and citrus blends, prune juice and vegetable

juices, meal replacement bars, meal replacement/supplement drinks, milk, including skim, 1%, kefir, and yogurt (excluding frozen yogurt).

D. BASIS FOR DETERMINATION OF GRAS

This GRAS determination for the use of MBP as an ingredient in cottage cheese, salad dressing, processed cheese, imitation milk, juice, meal replacement bars, meal replacement/supplement drinks, milk, and yogurt at the maximum use levels described in Section C of this chapter, is based upon scientific procedures as described under 21 CFR §170.30(b). Using scientific procedures, the intake of MBP from the intended uses specified above, estimated by ENVIRON International Corporation (ENVIRON), has been shown to be safe, and GRAS, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that MBP is safe, and GRAS, under its intended conditions of use, the safety of the intake of MBP resulting from its consumption in cottage cheese, salad dressing, processed cheese, imitation milk, juice, meal replacement bars, meal replacement/supplement drinks, milk, and yogurt has been established. Subsequently, this intake of MBP has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts, qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of MBP as an ingredient in cottage cheese, salad dressing, processed cheese, imitation milk, juice, meal replacement bars, meal replacement/supplement drinks, milk, and yogurt has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b), based on the following:

- 1) MBP is a well-characterized, basic protein fraction derived from New Zealand skim milk, which reproducibly meets compositional standards and complies with limits on contaminants appropriate for food-grade ingredients. Product specifications are set to assure MBP is suitable for use in food.
- 2) The estimated daily intakes of MBP, based on the maximum proposed uses are 47 and 100 mg/day/user for the population 2+ years at the mean and 90th percentiles of MBP consumption. For very young child (infants < 1 year, infants 1 year, and children 2-5 years), the mean and 90th percentile consumptions of MBP are lower. For these age groups, respectively, mean intakes are 20, 32, and 40 mg/day/user and 90th percentile intakes are 51, 69, and 89 mg/day/user.

- 3) Protein components of basic whey proteins have been determined to contribute to bone health. Lactoferrin has been identified as a basic whey protein that may have a role in bone physiology. Additional studies conducted that using the Whey Protein Fraction (WPC) of milk and the basic fraction of WPC, known as MBP, have shown that whey proteins, and more specifically, other components found in the basic proteins of the whey fraction, strongly stimulate bone formation and suppress bone resorption *in vitro* and *in vivo*. MBP has been shown to increase the number of osteoblastic cells and the amount of bone proteins such as collagen. Two components of MBP that have this growth-promoting activity have been purified and sequenced. One component was identified as a high-mobility group (HMG)-like protein, the other as a small fragment of high molecular weight (HMW) kininogen fragment 1·2. MBP also suppresses bone resorption. One of the components identified as having this activity is cystatin C.

- 4) Lactoferrin and lactoperoxidase comprise approximately 98% of total protein of the final MBP product. Both lactoferrin and lactoperoxidase are proteins found in milk and products derived from milk, such as whey, and are thus widely consumed in the diet by Americans through dairy sources. Both milk-derived lactoferrin and lactoperoxidase are found in numerous dietary supplements marketed in the U.S. Milk-derived lactoferrin is also a GRAS ingredient for use in foods in the U.S., and has been the subject of three GRAS Notifications to FDA (GRN Nos. 67, 77, and 130). Lactoferrin has been determined to be GRAS (with source labeling) for use as an ingredient in sports and functional foods at an estimated daily intake (EDI) of up to 196 mg/person/day for the 90th percentile consumer.

- 5) The estimated intakes of lactoferrin for the mean and 90th percentile consumers of MBP (population 2+ years), respectively, are 26 and 54 mg/person/day. Thus, intakes are below levels already determined to be GRAS for use in functional foods. In addition, these levels of lactoferrin consumption from MBP are respectively estimated to be equivalent to that consumed from approximately 1 and 2 cups of milk for the mean and 90th percentile MBP consumers.

- 6) Little scientific and clinical information exists to document whether bovine milk-derived lactoferrin is a cows' milk allergen. An Expert Panel convened to consider lactoferrin ingestion for GRN 000077, concluded that increased exposure to milk-derived lactoferrin at the levels of exposure specified therein would be highly unlikely to induce allergy or autoimmune disease. Since lactoferrin is not a known milk allergen, the only remaining question would concern the possibility that exposure might lead to increased sensitization

to this milk protein. This possibility seems unlikely, given the rather low level of predicted exposure to bovine milk lactoferrin.

- 7) Data and analysis of lactoferrin do not indicate any elevated risk of potential food allergy for this protein at levels of ingestion expected from MBP. It is expected that consumers of MBP containing lactoferrin will be protected with a labeling statement indicating that the product has been derived from a milk source. The United States has permitted the use of lactoferrin in certain food products with the provision that the presence of lactoferrin be declared on the label as arising from a milk source.
- 8) The estimated intake of lactoperoxidase for the mean and 90th percentile consumers of MBP (population 2+ years) is respectively estimated to be 19 and 41 mg/person/day. This is respectively equivalent to the amount of lactoperoxidase consumed from approximately 2.7 and 5.7 cups of milk for the mean and 90th percentile MBP consumers. The per capita milk intake in the U.S. for the population 2+ years is about 1 cup per day (USDA 2000a). Therefore, the intake of lactoperoxidase for the average consumer of MBP is approximately two to three times the background from milk consumption.
- 9) For children, consumption of milk is greater than for adults. For example, children ages 2 to 5 have a per capita milk intake of 1.5 cups of milk per day (USDA 2000a). Lactoperoxidase consumed by this population of children from MBP intake is respectively estimated to be equivalent to 2.3 and 5.1 cups of milk per day for mean and 90th percentile MBP consumers. Therefore, intake of lactoperoxidase for the average child consumer of MBP is estimated to be only 1.5 times the background from milk consumption. Thus, exaggeration of lactoperoxidase exposure from MBP ingestion compared to background milk consumption is less in children than adults. This is important to note, since children are a sensitive subpopulation, with respect to milk allergy.
- 10) Little scientific and clinical information exists to document whether bovine milk-derived lactoperoxidase is a cows' milk allergen. If lactoperoxidase were not a known milk allergen, the only remaining question would concern the possibility that exposure might lead to increased sensitization to this milk protein. This possibility seems unlikely, given the rather low level of predicted exposures to bovine milk lactoperoxidase.
- 11) Lactoperoxidase is not stable to pepsin hydrolysis, using the ILSI-HESI recommended method. However, stable fragments were produced. Stable peptide fragments have been a concern for novel proteins such as cry9c; however, lactoperoxidase and its fragments are

natural components of milk, so consumers are already exposed. The sequence homology research shows that these proteins have no biologically significant sequence homology with known allergens, and would therefore not be expected to show cross-reactivity.

- 12) Data and analysis of lactoperoxidase do not indicate any elevated risk of potential food allergy for this protein at levels of ingested expected from MBP. In addition, it is expected that consumers of MBP containing lactoperoxidase will be protected with a labeling statement indicating that the product has been derived from a milk source.
- 13) Cystatin C is present naturally in conventional bovine milk. Thus, exposure to cystatin C will occur from ingestion of milk products such as milk, skim milk, non-fat dry milk, whey protein powder, etc. Because MBP is simply isolated from bovine milk, ingestion of MBP also results in exposure to cystatin C. The amount of cystatin C consumed per day from MBP is equivalent to the amount of cystatin C, found in no more than ¼ cup of fluid milk for all population groups. These volumes of milk equate to approximately 20-25% of the background daily fluid milk intake by the U.S. population (USDA 2000a).
- 14) Cystatin C is not stable to pepsin hydrolysis using the ILSI-HESI recommended method; however, stable (digestion-resistant) fragments are produced. These fragments also are expected to be generated from the native cystatin C in conventional milk products; thus, ingestion of MBP does not result in exposure to unique fragments.
- 15) The bioinformatics analysis of cystatin C resulted in no amino acid sequence matches that met the analytical criteria for potential allergenic cross-reactivity. These results suggest that ingestion of cystatin C from consumption of MBP does not create an elevated risk of potential allergenic cross-reactivity. Thus, the consumption of products containing MBP does not appear to elevate allergenic risk. Importantly, products containing MBP will have content labels indicating milk-derived ingredients.
- 16) Kininogen fragment 1-2 is present naturally in conventional bovine milk products; thus, exposure to it will occur from ingestion of milk products such as milk, skim milk, non-fat dry milk, whey protein powder, etc. Because MBP is simply isolated from bovine milk, ingestion of MBP also results in exposure to kininogen fragment 1-2. LF, LPO and cystatin C can be used as markers for predicting the relative concentrations of various protein components in MBP relative to milk. These markers can then be used as surrogates to predict a reasonable expectation of exposure to other basic proteins identified in MBP (kininogen fragment 1-2). These marker proteins suggest that ingestion

of MBP will not result in an exaggerated exposure to kininogen fragment 1·2 relative to background ingestion from dairy. Importantly, MBP-containing products will be labeled as containing milk.

- 17) Kininogen fragment 1·2 is enzymatically cleaved from high molecular weight kininogen by kallikrein in bovine plasma. Kininogen fragment 1·2 protein was digested in a standardized *in vitro* pepsin digestion assay. The test material for pepsin digestion was a mixture of peptides representing both de-glycosylated and glycosylated forms of fragment 1·2, as would be expected from natural milk. Fragment 1·2 digested relatively quickly in pepsin, with no bands detectable between 15 and 25 kDa after approximately 20 minutes of digestion. However the presence of lower molecular weight bands of approximately seven and 13 kDa begin to appear following 30 seconds of digestion with pepsin, and the continued accumulation of these bands for at least 20 minutes of digestion indicates that moderately stable significant fragments are formed during digestion. This stability is consistent with that found for a number of food allergens. In evaluating the safety of these fragments or products, it is important to consider that the kininogen fragment 1·2 is present in blood and milk from bovine naturally. It should be similarly stable in meat and dairy foods. Based on that knowledge, and the low level of presence in the final MBP-containing products, the stability of these fragments should not raise any special concerns relative to potentially new allergenic foods. Importantly, the MBP-containing products will be labeled as milk; this should mitigate any reasonable question regarding allergenicity. It is unlikely there would be any marked increase in allergenic risk (as long as products containing these are labeled as milk) from the products with MBP.
- 18) Bioinformatics analyses of amino acid sequence homologies with bovine HMW kininogen I sequence resulted in no matches that met the analytical criteria for potential allergenic cross-reactivity. These results support the conclusion that there is not an elevated risk of potential allergenic cross-reactivity from ingestion of the bovine HMW kininogen fragment 1·2.
- 19) HMG-like protein is present in MBP. Because MBP is simply isolated from bovine milk, the bovine HMG-like protein is also expected to be present naturally in conventional milk and whey products. LF, LPO and cystatin C can be used as markers for predicting the relative concentrations of various protein components in MBP relative to milk. These markers can then be used as a surrogate to predict a reasonable expectation of exposure to other basic proteins identified in MBP (HMG-like protein). These marker proteins

suggest that ingestion of MBP will not result in an exaggerated exposure to HMG-like protein relative to background ingestion from dairy.

- 20) A standard pepsin digestion assay of HMG protein using the ILSI-HESI recommended method resulted in rapid digestion of the HMG protein, but resistant fragments were produced. These fragments are also expected to be generated by digestion of conventional milk. Thus, the stability of these fragments should not increase allergenic risks, as long as products containing MBP are labeled as milk.
- 21) The allergenic potential of HMG protein was evaluated using bioinformatics analyses of sequence homologies. The results of the bioinformatics analyses found two sequence matches with the bovine HMG protein 1. The only exact contiguous eight amino acid sequence match was to a low complexity region of the protein Ara h 1, a peanut allergen. This does not seem to be an added risk, however, because the sequence is in a low complexity region of the protein. This same sequence is present in many innocuous proteins. A second amino acid sequence match, and an 80-mer-sequence match, was also in a low complexity region. It matched a parasite allergen. This match does not seem significant for potential allergenic cross-reactivity. These results, therefore, suggest that there is no significant increase in risk of potential allergenic cross-reactivity from ingestion of the HMG-like protein in MBP-containing products.
- 22) MBP has been tested for potential toxicity and mutagenicity in a number of *in vitro* and *in vivo* tests. MBP was tested for the potential to induce point mutations in a reverse mutation assay using *Salmonella typhimurium*; results demonstrated that MBP was not mutagenic under the conditions of the assay. In an acute oral toxicity study, the lethal dose of MBP for 50% of the Crj:CD (SD) IGS rats (LD₅₀) was estimated to be greater than 2,000 mg/kg body weight. Teratogenicity was evaluated in pregnant Crj:CD (SD) IGS rats following administration of MBP via gavage at doses of 0 and 2,000 mg/kg body weight/day between Day 7 and Day 17 of gestation. Under these test conditions, MBP had no adverse effects on reproduction or development. Repeated-dose toxicity was evaluated in a 90-day gavage study of MBP at doses of 0, 200, and 2,000 mg/kg body weight/day in male and female Crj:CD (SD) IGS rats. Based on this study, the no-observed adverse effect level (NOAEL) of MBP in rats was 2,000 mg/kg body weight/day.
- 23) In a study evaluating the human health effects of MBP consumption, 30 healthy adult men consumed daily 5 bottles of an MBP-containing beverage (60 mg MBP per bottle, 300 mg

MBP per day) for 17 days. No significant adverse changes in group mean body weights, fat percentages, blood pressures, hematology, clinical chemistry, serum immunology, or urinalysis parameters were observed. In addition, results suggested that 300 mg MBP per day for 17 days promoted bone formation and suppressed bone resorption in healthy adult men.

- 24) In a randomized, placebo-controlled, double blind study, 34 healthy adult women consumed daily a beverage containing either 40 mg MBP or a placebo for 6 months. Based on the results, no adverse health effects (based on subjective symptoms, hematology, clinical chemistry, and urinalysis) were associated with the consumption of 40 mg per day of MBP by healthy adult women for 6 months. An increase in bone mineral density in the MBP-treated group was observed that was independent of dietary intake of vitamins and minerals. Under the conditions of this long-term clinical trial in women, MBP was safe and well tolerated at a dose of 40 mg/day.
- 25) The EDI of MBP is 47 and 100 mg/day/user for the population 2 years and older at the mean and 90th percentile of MBP consumption based on the maximum proposed use level in foods. The safety of ingestion of this level is supported by estimation of the resulting intake of the major proteins compared with background exposure. The ingestion of MBP results in an exaggeration of exposure, for the typical consumer, to lactoferrin and lactoperoxidase, which is not expected to be above three times the background exposure from milk consumption for adults and twice the background for children. In addition, active protein components presented in MBP are also ingested through the consumption of milk, and the products arising from the whey fraction of milk. Studies evaluating the mechanism of action of active protein components provide data on the potential beneficial effect of MBP on bone health. Animal toxicology studies support the safety of acute and repeated-dose exposure to MBP as well as a lack of developmental toxicity or mutagenic potential. A long term human clinical trial in women at a dose of 40 mg/day for 6 months corroborates the safety of MBP and shows that it is well tolerated at these levels of ingestion.

Determination of the GRAS status of MBP under its intended conditions of use has been made through the deliberations of Joseph F. Borzelleca, Ph.D., Steve Taylor, Ph.D., and E. Allen Foegeding, Ph.D. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of MBP and the potential human exposure to MBP resulting from its intended use as an

ingredient in cottage cheese, salad dressing, processed cheese, imitation milk, juice, meal replacement bars, meal replacement/supplement drinks, milk, and yogurt and have concluded:

There is no evidence in the available information on MBP that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when MBP is used at levels that might reasonably be expected from the proposed applications. MBP is GRAS for use in products as proposed by Snow Brand Milk Products Co. and IAS Co. Ltd.

E. AVAILABILITY OF INFORMATION

The detailed data and information that serve as the basis for this GRAS determination will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., DABT, Principal, ENVIRON International Corporation, 4350 North Fairfax Drive, Suite 300, Arlington, Virginia 22203, telephone: (703) 516-2309, facsimile: (703) 516-2393, or e-mail: ckruger@environcorp.com.

F. SIGNATURE

This notice of a GRAS Exemption Claim for Milk Basic Protein (MBP®) under its intended conditions of use is submitted by ENVIRON International Corporation on behalf of Snow Brand Milk Products, Ltd. and IAS Co., Ltd.

Claire L. Kruger, Ph.D., D.A.B.T.
Principal
ENVIRON International Corporation

3/3/06
Date

Expert Panel Statement

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ENVIRON

THE GENERALLY RECOGNIZED AS SAFE STATUS OF MILK BASIC PROTEIN (MBP)

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of food and food ingredients, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized as Safe (GRAS) status of Milk Basic Protein (MBP) summarized in the GRAS determination document, Generally Recognized As Safe Determination For The Use of Milk Basic Protein (MBP), June 30, 2005.

MBP is intended for use as a dietary ingredient in selected foods and beverages at maximum concentrations ranging from 10 to 40 mg MBP per serving. The GRAS determination for the use of MBP as an ingredient in cottage cheese, salad dressing, processed cheese, imitation milk, juice, meal replacement bars, meal replacement/supplement drinks, milk, and yogurt at the maximum use levels described in the GRAS determination, is based upon scientific procedures as described under 21 CFR §170.30(b), and corroborated by a history of safe exposure and unpublished data. The intake of MBP has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

Therefore, MBP is GRAS for its intended uses as proposed by Snow Brand Milk Products Co. Ltd. and IAS Co. Ltd. Because MBP is GRAS for its intended use, it is excluded from the definition of a food additive, and thus may be marketed for this use without the need to promulgate a specific food additive regulation under 21 CFR.

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Based on independent and collective critical evaluation of the available information, the Expert Panel concludes that MBP, produced in accordance with current Good Manufacturing Practice (cGMP), and meeting the specifications referenced in the GRAS determination document, Generally Recognized As Safe Determination For The Use of Milk Basic Protein (MBP), is safe for the intended uses. The Expert Panel further concludes that this use is GRAS based on scientific procedures and corroborated by history of safe exposure and unpublished data. The Expert Panel believes that other experts qualified by training and/or experience to evaluate the safety of food ingredients would concur with this conclusion.

Joseph F. Borzelleca, Ph.D.
Chairman of the Expert Panel
Professor Emeritus
Pharmacology & Toxicology
Medical College of Virginia
Richmond, Virginia

Signature: _____
Date: 18 July 2005

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Date: _____

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Signature: _____
Date: _____

Scientific Advisor to the Expert Panel
Claire L. Kruger, Ph.D.
Principal
ENVIRON Health Sciences
Arlington, Virginia

Signature _____
Date: Aug 1, 2005

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Based on independent and collective critical evaluation of the available information, the Expert Panel concludes that MBP, produced in accordance with current Good Manufacturing Practice (cGMP), and meeting the specifications referenced in the GRAS determination document, Generally Recognized As Safe Determination For The Use of Milk Basic Protein (MBP), is safe for the intended uses. The Expert Panel further concludes that this use is GRAS based on scientific procedures and corroborated by history of safe exposure and unpublished data. The Expert Panel believes that other experts qualified by training and/or experience to evaluate the safety of food ingredients would concur with this conclusion.

Joseph F. Borzelleca, Ph.D.
Chairman of the Expert Panel
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Steve Taylor, Ph.D.
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Date: 7 July 2005

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Date: _____

Scientific Advisor to the Expert Panel
Claire L. Kruger, Ph.D.
Principal
ENVIRON Health Sciences
Arlington, Virginia

Signature: _____

Date: _____

000017

Based on independent and collective critical evaluation of the available information, the Expert Panel concludes that MBP, produced in accordance with current Good Manufacturing Practice (cGMP), and meeting the specifications referenced in the GRAS determination document, Generally Recognized As Safe Determination For The Use of Milk Basic Protein (MBP), is safe for the intended uses. The Expert Panel further concludes that this use is GRAS based on scientific procedures and corroborated by history of safe exposure and unpublished data. The Expert Panel believes that other experts qualified by training and/or experience to evaluate the safety of food ingredients would concur with this conclusion.

Joseph F. Borzelleca, Ph.D.
Chairman of the Expert Panel
Professor Emeritus
Pharmacology & Toxicology
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Richmond, Virginia

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Signature: _____
Date: 7/8/05

Scientific Advisor to the Expert Panel
Claire L. Kruger, Ph.D.
Principal
ENVIRON Health Sciences
Arlington, Virginia

Signature: _____
Date: _____

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GRAS Determination

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**GENERALLY RECOGNIZED
AS SAFE DETERMINATION
FOR THE USE OF
MILK BASIC PROTEIN (MBP)**

Prepared for
Snow Brand Milk Products Co., Ltd.
Tokyo, Japan
and
IAS Co., Ltd.
Tokyo, Japan

Prepared by
ENVIRON International Corporation
Arlington, Virginia

March 3, 2006

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March 3, 2006

I. Introduction

This dossier provides supporting documentation for the GRAS exemption claim for Milk Basic Protein (MBP[®]) under its intended conditions of use. It presents a critical review of the published literature, data, and other information regarding MBP.

II. Description of Substance

A. *IDENTITY*

1. **Chemical Identity**

The substance that is the subject of this Generally Recognized as Safe (GRAS) determination is Milk Basic Protein (MBP), a specific basic protein fraction derived from pasteurized skim milk.

The final MBP product is added in liquid form or powder form to the proposed foods at the proposed use levels (see Section IV).

2. **Chemical Name**

Milk Basic Protein (MBP), the substance that is the subject of this GRAS determination does not have a systematic chemical name; however, the individual protein components have standard names based on dairy industry identities. These names are provided in Section II.C., Batch data and composition.

3. **Common or Trade Name**

MBP will be marketed and sold by Snow Brand Milk Products/IAS under the trade name S-PROTEIN-T2.

4. **CAS Registry Number**

This substance does not have a Chemical Abstracts Service Registry Number.

5. **Standards of Identity**

The milk product, MBP, described herein is derived from pasteurized skim milk. No identity requirements have been established for this type of product. The Food and Drug Administration (FDA) does not specifically define MBP identity.

B. *PRODUCTION PROCESS*

1. **Source Material**

The MBP product is made from pasteurized skim milk. The components of this skim milk are presented in Table 1.

Table 1. Components of Pasteurized Skim Milk		
Principal Components	From New Zealand ^(a)	From United States ^(b)
Milk fat (%)	0.06	0.18
Carbohydrate (lactose) (%)	5.19	4.85
Protein (%)	3.98	3.41
Casein (%)	3.18	2.73
Whey proteins (%)	0.80	0.68
Moisture (%)	90.04	90.80
Ash (%)	0.73	0.45
TOTAL (%)	100.00	99.69
NOTES: (a) MBP starting material; data provided by Snow Brand Milk Products (November 2004). (b) U.S. Department of Agriculture, Agricultural Research Service, 2004. USDA National Nutrient Database for Standard Reference, Release 17. Nutrient Data Laboratory Home Page, http://www.nal.usda.gov/fnic/foodcomp . Milk protein is approximately 80% casein and 20% whey.		

2. Production Process

MBP is produced from pasteurized skim milk. Skim milk is directly applied to a cationic exchange chromatographic column. Acidic milk proteins, including casein, and lactose are removed and the basic proteins are eluted from the resin using sodium chloride (NaCl). The resultant eluate is concentrated and dialyzed to produce MBP solids. These MBP solids are crushed and packaged. This MBP powder can then be added to proposed foods.

3. Byproducts and Side Reactions

No unconventional whey protein byproducts are generated. Due to the minimal processing, use of only food-grade materials, and the mild conditions of pH and temperature, no unconventional whey protein byproducts are expected.

4. Good Manufacturing Practice (GMP)

All materials used in the manufacture of MBP may be used in food.

C. BATCH DATA AND COMPOSITION

1. Batch Analysis Results

Batch results for analyses of the principal components, related substances, microbiological contaminants, and other contaminants of concern are summarized in Table 2. Each batch of MBP complies with the food-use specifications (Table 3) established by Snow Brand Milk Products Co., Ltd. for this product. The production process for MBP can consistently yield a product suitable for addition to food.

Table 2. Summary of MBP Batch Data				
Components, Residues, and Contaminants	Mean^(a)	Standard Deviation	Units	Number of Batches
Total Protein	97.2	2.5	g/100g	10
Principal Protein Components				
Lactoferrin	54.3	4.3	g/100g	10
Lactoperoxidase	40.6	7.3	g/100g	10
Cystatin C	0.1	0.03	g/100g	49
High mobility group (HMG)-like protein	present ^(b)			1
Kininogen fragment 1:2	present ^(b)			1
Residual Milk Proteins^(c)				
Total	2.4	6.5	g/100g	10
Non-Protein Components				
Fat	0.10	0.02	g/100g	3
Carbohydrate (lactose)	<0.01	NA	g/100g	3
Ash	0.75	0.40	g/100g	10
Moisture	1.67	0.8	g/100g	11
Heavy Metals, Inorganics				
Arsenic (As)	< 0.2	NA	ppm	5
Lead (Pb)	< 0.1	NA	ppm	6
Total heavy metals (as lead (Pb))	< 10	NA	ppm	5
Potential Contaminants of Concern				
Aldrin	<0.003	NA	ppm	5
Hexachlorocyclohexane (BHC)	<0.01	NA	ppm	5
Dichlorodiphenyl trichloroethane (DDT)	<0.01	NA	ppm	5
Dieldrin	<0.003	NA	ppm	5
Polychlorinated biphenyls (PCBs)	<0.01	NA	ppm	5
Inhibitory substances	<0.005	NA	IU/mL	8
Physical Properties				
Color	Dark brown	NA	NA	49
pH	6.0	0.2	NA	49
Sediment	Better than disk A	NA	/5 g	49
Foreign matter	ND	NA	NA	49
Biological Activity				
Cysteine protease inhibitor activity	33	6	mU/mg	49
Proliferative activity of osteoblastic cells	Positive	NA	NA	8
Microbiology				
Total aerobic plate count	ND	NA	CFU/g	49
Coliform bacteria count	ND	NA	/0.1g	49
Yeast count	ND	NA	CFU/g	49
Mold count	0.2	0.3	CFU/g	49
<i>E. coli</i>	ND	NA	/g	49
<i>Staphylococcus aureus</i>	ND	NA	/0.01g	49
<i>Salmonella</i>	ND	NA	/25g	49

Table 2. Summary of MBP Batch Data				
Components, Residues, and Contaminants	Mean^(a)	Standard Deviation	Units	Number of Batches
<i>Listeria</i>	ND	NA	/25g	49
<i>Staphylococcus enterotoxin</i>	<0.5	NA	ppb	49
Thermophile	ND	NA	CFU/g	49

NOTES:

(a) Average of measured values from batch data or for some physical properties, a qualitative determination. Methods of analysis are provided in Table 3.

(b) Present = minor, active protein components of MBP are confirmed by Western Blot analysis (Figure 1).

(c) Residual proteins may include casein, beta-lactoglobulin, alpha-lactalbumin, immunoglobulins, bovine serum albumin, and lysozyme.

ABBREVIATIONS:

NA: Not applicable.

ND: Analyzed for, but not detected in MBP based on standard method detection limits.

2. Principal Protein Components

a) Lactoferrin and Lactoperoxidase

MBP is approximately 97.2% total protein (Table 2). Most of this protein is lactoferrin (LF) and lactoperoxidase (LPO); other active, basic proteins and other milk proteins are approximately 2.4% of MBP.

LF and LPO are the major components of MBP and compose 54.3% and 40.6% of the total MBP protein, respectively (Table 2). Both LF and LPO are well known components of bovine milk, occurring in the whey protein fraction (Fox 2003). LPO is approximately 1.0% of the whey proteins of bovine milk (10-30 $\mu\text{g/mL}$) (Reiter 1985, as cited in Pruitt 2003); whereas LF has been reported at concentrations of up to 0.6% of total proteins (more than 20 to 200 $\mu\text{g/mL}$) (data compiled by Steijns and van Hooijdonk 2000).

b) Cystatin C

Cystatins are low Molecular Weight (MW) proteins produced by all nucleated cells (Abrahamson *et al.* 1990). Several research papers examining protein fractions of milk demonstrate the presence of cystatin C in conventional milk. Matsuoka and associates (2002) prepared MBP from fresh bovine milk and isolated approximately 9.3 mg of a cysteine protease inhibitor (CPI) (verified by inhibition activity in the protease reaction of papain) from 100 g of the prepared MBP. Sequencing of this CPI isolated from MBP shared 18 amino acid residues from the N-terminus with amino acid residues from cystatin C from bovine colostrum.

Cystatin C has also been isolated from human biological samples; Abrahamson and associates (1986) reported isolation of cystatin C from human milk at a concentration of 3.4 mg per L (27.2 mg per 100 g solids¹). Human cystatin C from alveolar macrophages had a MW of 14 kDa (Chapman *et al.* 1990), and cystatin C isolated from human serum had a MW of 13 kDa (Watanabe *et al.* 2003). Laterza and associates (2002) found a 122 amino acid, 13-kDa protein identified as a member of the CPIs in serum from humans.

Research indicates that cystatin C is present in fresh bovine milk. MBP is prepared from bovine milk, and is therefore expected to contain cystatin C. As shown by the batch data reported in Table 2, approximately 100 mg of cystatin C is present per 100 g of MBP as determined by ELISA.

c) Kininogen Fragment 1·2 (16-17 kDa)

The presence of kininogens of various sizes in the whey fraction of bovine milk has been documented (Wilson *et al.* 1989; Yamamura *et al.* 2000). Yamamura and associates (2000)

¹ Based on solid fraction of human milk of 12.5% (USDA 2004).

isolated a 17-kDa kininogen from a commercial New Zealand WPC using HPLC and SDS-PAGE analysis. Wilson and associates (1989) isolated a low MW 16-17 kDa kininogen fragment from the whey fraction of pasteurized skim bovine milk using HPLC. Abrahamson and associates (1986) confirmed the presence of kininogens in human milk. Using SDS-PAGE, these investigators identified a kininogen of approximately 60 kDa, found to occur at a concentration of 3.1 mg per L (24.8 mg per 100 g solids). Admundsen and Nustad (1965) identified kinin-forming activity in human milk by incubating samples in stable substrate plasma and testing for activity using isolated rat uterus. Snow Brand Milk Products has confirmed the presence of a 17-kDa kininogen (kininogen fragment 1-2) in the MBP product by Western Blot analysis (Figure 1). Although these analyses demonstrate the presence of a 17-kDa kininogen in MBP, the amount (concentration) of this protein in MBP is not known.

d) High Mobility Group (HMG)-Like Protein (10 kDa)

The MBP product that is the subject of this GRAS determination is prepared from the skim milk of New Zealand cows. The presence of this 10-kDa protein in milk is corroborated by Yamamura and associates (1999). These investigators isolated a 10-kDa high-mobility group (HMG)-like protein from a New Zealand WPC, based on SDS-PAGE analysis. Similarly, Snow Brand Milk Products demonstrated that MBP prepared by the manufacturer contains HMG-like protein using Western Blot analysis (Figure 1). Although these analyses demonstrate the presence of HMG-like protein in MBP, the quantity (concentration) of this protein is not known.

Figure 1. Western Blot Analysis of MBP to Determine Presence of Kininogen Fragment 1·2 and HMG-like Protein

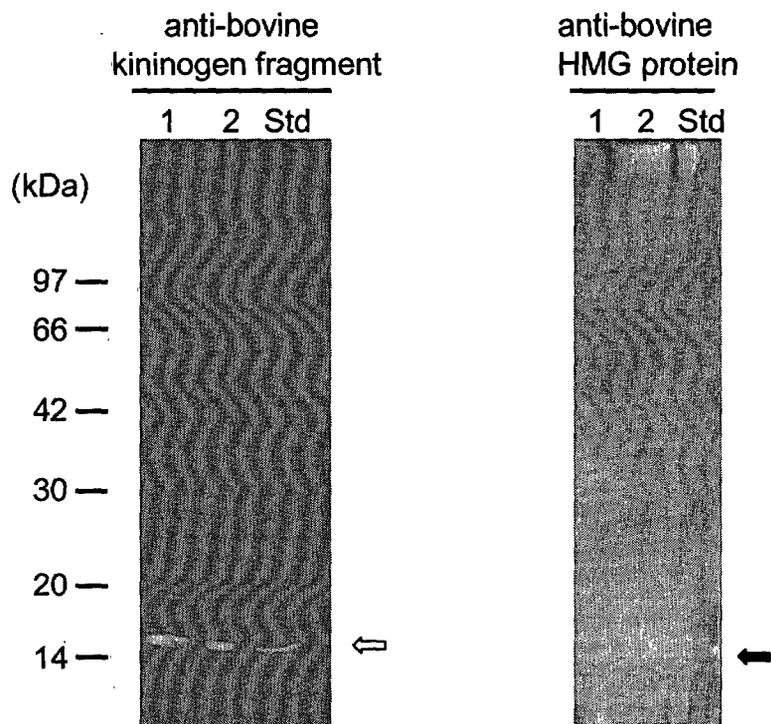


Figure Western blotting analysis of MBPs. MBPs (lanes 1 and 2) were electrophoresed under reducing condition using SDS-polyacrylamide gels (12%T). After the electrophoresis, the proteins were transferred onto PVDF membrane. The membrane was reacted with anti-bovine kininogen fragment 1.2 antibody (left) or anti-bovine HMG protein antibody (right), followed by chemiluminescence detection.

3. Other Milk Proteins

Unidentified milk proteins comprise approximately 2.4% of MBP (Table 2). Moreover, these components are residual acidic proteins from conventional fluid milk and whey. The major conventional proteins are listed below:

- **Casein:** A major milk protein; some residual may be present in MBP due to incomplete removal during processing. Chromatograms of MBP compared to standards suggest that alpha-S-casein is not present or, if present, is below 250 mg/100g MBP.
- **alpha-Lactalbumin (a-La):** A minor milk protein composing approximately 3.5% of total milk protein (Fox 2003); some residual may be present in MBP, due to incomplete removal during processing. Snow Brand Milk Products has conducted HPLC analysis of MBP to determine if a-La is present. Comparison of chromatograms of MBP to standards suggests that a-La is not present or, if present, is below 62.5 mg/100g MBP.
- **beta-Lactoglobulin (b-Lg):** A minor milk protein composing approximately 12% of total milk protein (Fox 2003); some residual may be present in MBP due to incomplete removal during processing. Snow Brand Milk Products has conducted HPLC analysis of MBP to determine if b-Lg is present. Comparison of chromatograms of MBP to standards suggests that b-Lg is not present or, if present, is below 125 mg/100g MBP.
- **Immunoglobulins (Ig):** Minor milk proteins composing approximately 3% of total milk nitrogen (Fox 2003); some residual may be present in MBP due to incomplete removal during processing. Snow Brand Milk Products has conducted HPLC analysis of MBP to determine if IgG is present. Comparison of chromatograms of MBP to standards suggests that IgG is not present or, if present, is below 125 mg/100g MBP.
- **Bovine Serum Albumin (BSA):** A minor milk protein composing 0.3 to 1.0% of total milk nitrogen (Fox 2003); some residual may be present in MBP due to incomplete removal during processing. Snow Brand Milk Products has conducted HPLC analysis of MBP to determine if BSA is present. Comparison of chromatograms of MBP to standards suggests that BSA is not present or, if present, is below 250 mg/100g MBP.
- **Lysozyme:** A minor milk protein composing less than 1% of total protein in the whey fraction of bovine milk (Farkye 2003); some residual may be present in MBP due to incomplete removal during processing. No data on presence or quantity in MBP is available.

4. Non-Protein Components, Impurities, Residues, and Contaminants of Concern

MBP components, other than protein, include fat, carbohydrate (e.g., lactose), ash, and moisture, all of which are components of conventional milk. Potential contaminants of MBP include pesticides, microbes potentially introduced during processing or present in raw milk, naturally occurring toxins, residual processing aids, heavy metals, and dioxins. Table 2 presents batch data for these components in MBP. These batch data show that non-protein components, impurities, residues, and contaminants of concern in MBP are consistently within established specification guidelines.

5. Stability of MBP

The MBP product is stored as a dry powder for eventual sale to food manufacturers for addition to various food products. This dry powder product is stable for at least 2 years at 5°C and 25 °C, and up to 1 year at 37°C.

D. SPECIFICATIONS AND ANALYTICAL METHODS

1. Food-Grade Specifications

Specifications for the MBP product established by Snow Brand Milk Products are listed in Table 3. Batches of MBP are tested periodically to comply with the specifications prior to packaging and sale.

Table 3. MBP Specifications				
Components, Residues, Contaminants	Min	Max	Units	Test Method
Protein Components				
Total Protein	90		g/100g	Kjeldahl method
Lactoferrin	40	70	g/100g	HPLC
Lactoperoxidase	15	60	g/100g	HPLC
Cystatin C ^(a)	0.02		g/100g	ELISA
Non-Protein Components				
Fat		2.0	g/100g	IDF method
Carbohydrate (lactose)		2.0	g/100g	UV method
Ash		2.5	g/100g	Ashing method
Moisture		6.0	g/100g	Oven drying method
Heavy Metals				
Arsenic (As)		1.0	mg/kg	Colorimetric
Lead (Pb)		5.0	mg/kg	FCC III

Table 3. MBP Specifications				
Components, Residues, Contaminants	Min	Max	Units	Test Method
Total heavy metals (as lead (Pb))		10.0	mg/kg	Sulfide method
Potential Contaminants of Concern				
Aldrin		0.005	mg/kg	GLC method
Hexachlorocyclohexane		0.2	mg/kg	GLC method
Dichlorodiphenyl trichloroethane (DDT)		0.05	mg/kg	GLC method
Dieldrin		0.005	mg/kg	GLC method
Polychlorinated biphenyls (PCBs)		0.1	mg/kg	GLC method
Physical Properties				
Color	Dark brown			Visual observation
pH	5.5	7.5		pH meter (5% solution)
Sediment	Better than Disk A		/5 g	ADMI (2.5% solution)
Foreign matter		ND		Visual observation
Microbiology				
Total aerobic plate count		10,000	CFU/g	JG
Coliform bacteria count		ND	/0.1g	JG
Yeasts count		50	CFU/g	JG
Molds count		50	CFU/g	JG
<i>Escherichia coli</i>		ND	/g	IDF method
<i>Listeria</i>		ND	/25g	USFDA method
<i>Salmonella</i>		ND	/25g	JG
<i>Staphylococcus aureus</i>		ND	/0.01g	JG
<i>Staphylococcus enterotoxin</i>		0.5	µg/kg	ELISA method
Thermophile		1,000	CFU/g	JG
<p>NOTES:</p> <p>(a) The minor protein components – HMG-like protein and kininogen fragment 1·2 – will be monitored using the surrogate, Cystatin C.</p> <p>ABBREVIATIONS:</p> <p>ADMI: American Dry Milk Institute.</p> <p>CFU: Colony forming units.</p> <p>ELISA: Enzyme-linked immunosorbent assay.</p> <p>FCC: Food Chemicals Codex.</p> <p>GLC: Gas liquid chromatography.</p> <p>HPLC: High performance liquid chromatography.</p> <p>IDF: International Dairy Federation.</p> <p>JG: Japanese Guidelines: Standard Methods of Analysis in Food Safety Regulation, edited by Japan Food Hygiene Association.</p> <p>LOD: Limit of detection.</p> <p>Max: Maximum.</p> <p>Min: Minimum.</p> <p>ND: Not Detected using standard methodology.</p> <p>USFDA: United States Food and Drug Administration, Bacteriological Analytical Manual.</p>				

2. Analytical Methods

All methods conform to Japan Food Hygiene Association approved methods, IDF method or FDA method, or specific methods reported in the literature and are listed in Table 3.

III. Intended Technical Effect

Milk is a core component of the American diet. U.S. government guidance documents recommend daily intake of milk or other dairy products (USDA 2005). The milk product that is the subject of this GRAS determination, MBP, is a derivative of milk proteins. Therefore, the intended effect of the MBP product is to provide a source of a specific milk protein fraction to the diet.

IV. Use and Consumer Exposure

A. *HISTORICAL USE: BACKGROUND EXPOSURE TO MBP CONSTITUENTS*

Milk and products derived from milk, such as whey, are widely consumed by Americans of all ages in the form of fluid milk, and as milk or milk-derived ingredients. Given that MBP is produced from milk, the U.S. population is continuously exposed to the constituents of MBP through consumption of milk and milk-containing foods. The MBP constituents are present in whey proteins, so Americans also are exposed to the constituents through consumption of whey protein-containing foods.

Federal guidance documentation recommends daily consumption of dairy products. The USDA Food Guide Pyramid currently recommends 2 to 3 servings of dairy products (milk, yogurt, or cheese) per day for individuals ages 2 years and older to promote adequate protein and calcium intake (USDA 2005). The U.S. population, ages two years and older, consumes an average of 1.0 serving of milk per day, though young children (2 to 11 years) and teenage males consume an average of 1.5 to 1.7 servings of milk per day (USDA 2000a). Each serving of milk is equivalent to one cup, or 8 fluid ounces.

Fluid milk contains approximately 0.1 and 0.03 g per liter of lactoferrin (LF) and lactoperoxidase (LPO), respectively (Korhonen and Pihlanto 2003). The U.S. population 2 years and older consumes an average of approximately one cup of fluid milk per day, which provides approximately 24 mg lactoferrin, and 7 mg lactoperoxidase daily for each American. Americans also ingest approximately 4.0 g of whey products per person per day, according to estimates from FDA (Rulis 2000). The consumption of whey products therefore provides small additional contributions to the total daily intake of the MBP constituents.

B. *POTENTIAL CONSUMER EXPOSURE TO MBP*

1. **Proposed Use**

Snow Brand Milk Products proposes to add MBP to nine categories of foods. These categories include: cottage cheese, imitation milk (including soy and rice milk), juice (including only 100% citrus, prune and vegetable juices), meal replacement bars, meal replacement/supplement drinks, milk (including only skim milk, 1% milk, and kefir [fluid and dry forms]), processed cheese, salad dressing (excluding mayonnaise and mayonnaise-type dressings), and yogurt (excluding frozen yogurt). The MBP product will be added as a specified amount of MBP per serving to each of the nine food categories. The Reference Amount Customarily Consumed (RACCs) of these foods, as defined in the Code of Federal Regulations

(CFR § 101.12) provide the reference amount, or standard serving size, of these products. The categories of foods to which MBP will be added, the serving size (RACC) per category, and the maximum use of MBP per food serving are presented in Table 4. As shown, the proposed MBP use is 40 mg MBP per serving of imitation milk, juice, meal replacement bars, meal replacement/supplement drinks, milk, and yogurt, 30 mg per serving of processed cheese, and 10 mg per serving of cottage cheese and salad dressing.

Table 4. Foods Proposed for MBP-Fortification		
Food Category	Serving Size ^(a)	Proposed Maximum MBP Fortification Level (mg/serving)
Cottage cheese	110 g	10
Imitation milk (including soy and rice milk)	8 fluid ounces, or 240 mL	40
Juice 100% Citrus and citrus blends, prune, and vegetable juices	8 fluid ounces, or 240 mL	40
Meal replacement bar	40 g	40
Meal replacement/supplement drink	8 fluid ounces, or 240 mL	40
Milk Skim milk, 1% milk, kefir (fluid and dry forms)	8 fluid ounces, or 240 mL	40
Processed cheese	30 g	30
Salad dressing ^(b)	30 g	10
Yogurt ^(c)	225 g	40
NOTES: (a) As defined by Reference Amounts Customarily Consumed (21 CFR § 101.12). (b) Excluding mayonnaise and mayonnaise-type dressings. (c) Excluding frozen yogurt.		

2. Estimated Intake of MBP from Proposed Uses

a) Method of Estimating MBP Intakes

The potential intake of a new food additive may be estimated from nationwide food consumption data. The 1994-1996 USDA Continuing Survey of Food Intakes by Individuals, and its 1998 Supplemental Children's Survey provide the most recent survey data detailing food intakes by Americans on multiple days; these surveys are collectively known as the CSFII 1994-96, 1998. The data collected in these surveys are publicly available on CD-ROM (USDA 2000b).

Using USDA recipe files for the foods reported in CSFII 1994-96, 1998, ENVIRON identified all food codes in the recipe files corresponding to one of the proposed use categories as defined in Table 4, including foods containing one or more of the MBP food categories as an ingredient. This list of food codes was then linked to the CSFII food consumption data reported for each survey respondent. For each reported intake of the MBP-containing food codes,

ENVIRON estimated potential food intake by food category. If the proposed MBP use represented an ingredient in a food mixture, the calculations were based on the portion of the food code intended for fortification with MBP (e.g., the milk portion of oatmeal cooked in skim milk).

b) Results of MBP Intake Estimates

The percentage of the U.S. population in CSFII reporting consumption of potential MBP-containing foods, the estimated intakes of foods (in servings per day) proposed for fortification with MBP, and estimated intakes of MBP by consumers of these foods are presented in Table 5.

It is important to note that all estimates of intake presented in Table 5 are likely overestimates. In the calculations of estimated intakes, all individuals consuming foods in the proposed use categories were assumed to consume foods fortified with MBP at the maximum proposed MBP use level.

Table 5. Estimated Daily Intake of MBP Based on Proposed Uses

Population n ^(a)	Food Category ^(b)	Users ^(c) (%)	Mean 2-Day Average Intake per User			
			Number of Servings (servings/day/user)		MBP ^(d) (mg/day/user)	
			Mean	90 th percentile	Mean	90 th percentile
Infants < 1 y n = 1,065	Cottage cheese	1	0.3	1.0	3	10
	Imitation milk	<0.5	1.7	3.7	69	147
	Juice (citrus, prune, vegetable)	8	0.5	1.0	19	40
	Meal replacement bar	<0.5	0.3	0.8	14	33
	Meal replacement/supplement drink	<0.5	4.0	4.0	160	160
	Milk	1	0.2	0.4	6	15
	Processed cheese	11	0.4	0.9	13	28
	Salad dressing	0	0	0	--	--
	Yogurt	5	0.4	0.8	15	33
	<i>All categories combined</i>	20	0.6	1.4	20	51
Infants 1 y n = 972	Cottage cheese	3	0.4	0.7	4	7
	Imitation milk	1	0.9	2.5	38	100
	Juice (citrus, prune, vegetable)	37	0.6	1.3	26	50
	Meal replacement bar	5	0.5	0.7	20	28
	Meal replacement/supplement drink	<0.5	0.6	1.1	24	45
	Milk	9	0.8	2.5	31	98
	Processed cheese	51	0.5	1.0	15	31
	Salad dressing	7	0.3	0.5	3	5
	Yogurt	13	0.4	0.7	17	27
	<i>All categories combined</i>	74	0.9	2.0	32	69
Children 2-5 y n = 5,437	Cottage cheese	3	0.4	0.8	4	8
	Imitation milk	1	1.4	2.5	54	100
	Juice (citrus, prune, vegetable)	42	0.7	1.3	28	52
	Meal replacement bar	5	0.5	0.9	21	37
	Meal replacement/supplement drink	<0.5	0.9	2.7	34	107
	Milk	19	1.0	2.2	42	90
	Processed cheese	57	0.6	1.3	19	39
	Salad dressing	13	0.3	0.6	3	6
	Yogurt	10	0.4	0.6	15	22
	<i>All categories combined</i>	83	1.2	2.4	40	89
Children 6-12 y n = 2,089	Cottage cheese	2	0.4	1.0	4	10
	Imitation milk	<0.5	0.8	1.5	32	60
	Juice (citrus, prune, vegetable)	37	0.7	1.4	29	55
	Meal replacement bar	7	0.6	1.1	25	43
	Meal replacement/supplement drink	1	0.6	1.0	24	40
	Milk	28	1.2	2.7	48	107
	Processed cheese	59	0.7	1.4	22	43
	Salad dressing	18	0.5	1.0	5	10
	Yogurt	7	0.4	0.5	15	22
	<i>All categories combined</i>	86	1.4	2.9	48	102
Teenagers 13-19 y n = 1,222	Cottage cheese	3	0.7	1.4	7	14
	Imitation milk	<0.5	0.7	1.0	28	39
	Juice (citrus, prune, vegetable)	32	1.0	2.0	41	80
	Meal replacement bar	5	0.6	1.1	25	42

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Table 5. Estimated Daily Intake of MBP Based on Proposed Uses						
Population n ^(a)	Food Category ^(b)	Users ^(c) (%)	Mean 2-Day Average Intake per User			
			Number of Servings (servings/day/user)		MBP ^(d) (mg/day/user)	
			Mean	90 th percentile	Mean	90 th percentile
	Meal replacement/supplement drink	1	1.1	1.5	43	60
	Milk	21	1.3	3.0	53	120
	Processed cheese	62	0.9	1.8	28	54
	Salad dressing	19	0.8	1.8	8	18
	Yogurt	3	0.5	0.9	21	35
	<i>All categories combined</i>	85	1.7	3.6	54	123
Adults 20+ y n = 9,323	Cottage cheese	5	0.5	1.0	5	10
	Imitation milk	<0.5	0.6	1.4	26	55
	Juice (citrus, prune, vegetable)	30	0.9	1.5	34	60
	Meal replacement bar	3	0.7	1.4	30	57
	Meal replacement/supplement drink	2	1.1	2.0	46	80
	Milk	25	0.9	2.0	37	80
	Processed cheese	51	0.8	1.7	24	50
	Salad dressing	31	0.8	1.6	8	16
	Yogurt	6	0.5	1.0	22	40
	<i>All categories combined</i>	83	1.5	3.1	47	98
Population 2+ y n = 18,071	Cottage cheese	5	0.5	1.0	5	10
	Imitation milk	<0.5	0.7	1.5	30	60
	Juice (citrus, prune, vegetable)	32	0.8	1.5	34	60
	Meal replacement bar	4	0.7	1.2	27	46
	Meal replacement/supplement drink	2	1.1	2.0	45	80
	Milk	25	1.0	2.2	40	87
	Processed cheese	54	0.8	1.6	24	49
	Salad dressing	27	0.8	1.5	8	15
	Yogurt	6	0.5	1.0	20	40
	<i>All categories combined</i>	83	1.5	3.1	47	100

NOTES:
 Data Source: U.S. Department of Agriculture, Agricultural Research Service. Continuing Survey of Food Intakes by Individuals 1994-96, 1998 (USDA 2000b).
 (a) Excludes breastfeeding infants and children. Includes only survey respondents with complete dietary recall information for both survey days.
 (b) Serving sizes and food categories are as defined in Table 4.
 (c) Percentage of survey respondents reporting intake of one or more products from the corresponding food category.
 (d) Proposed use levels of MBP corresponding to each food category are described in Table 4.

C. COMPARISON OF ESTIMATED INTAKES OF MBP TO EXPOSURE TO MBP CONSTITUENTS THROUGH CONSUMPTION OF MILK

As previously described, the U.S. population has a long history of exposure to MBP constituents through the consumption of fluid milk and milk-derived ingredients. Table 5 presents estimates of MBP intake based on the proposed uses of the product; it follows that

estimates of intake of each MBP component can be calculated from estimates of total MBP intake (Table 5) and data quantifying the concentration of each component in MBP (Table 2). Data in the published literature quantify the concentrations of select MBP constituents in fluid milk, namely LF and LPO (Korhonen and Pihlanto 2003).

ENVIRON calculated the number of servings of fluid milk (8 fluid ounces per serving) corresponding to mean MBP intakes of the primary constituents LF and LPO, using the MBP intake data and the LF and LPO concentration data. Results of the calculations are presented in Table 6. As shown in Table 5, the estimated mean and 90th percentile daily intakes of MBP by the U.S. population 2 years and older, resulting from all proposed MBP uses are 47 mg (26 mg LF and 19 mg LPO) and 100 mg (54 mg LF and 41 mg LPO), respectively. Based on LF and LPO concentrations in fluid milk and the quantities of these constituents in MBP, this amount of MBP is equivalent to the amount of MBP constituents naturally occurring in approximately 1.1 to 2.7 cups of fluid milk for the average MBP consumer. Per capita milk intake in the U.S. is approximately 1 serving (i.e., 1 cup) per day (USDA 2000a). The potential intake of MBP constituents that is estimated to result from the proposed uses of MBP therefore is not above approximately three times background from milk consumption for the average consumer. Additionally, given that young children typically consume more than 1 cup of milk per day (USDA 2000a), the estimated intake of MBP constituents from MBP products is likely smaller than current exposure to the MBP constituents from consumption of milk for this population. Thus, exaggeration of LPO exposure from MBP ingestion compared to background milk consumption is less in children than adults. This is important to note, since children are a sensitive subpopulation with respect to milk allergy.

Table 6. Milk Intake Equivalents Corresponding to Estimated Daily Intakes of LF and LPO from MBP

Population	Mean Daily MBP Intake ^(a) (mg/day/user)	Equivalent Cups of Fluid Milk (cups/day/user)		
		Based on LF ^(b)	Based on LPO ^(b)	Based on Cystatin C ^(c)
Infants <1 y	20	0.5	1.1	0.1
Infants 1 y	32	0.7	1.8	0.2
Children 2-5 y	40	0.9	2.3	0.2
Children 6-12 y	48	1.1	2.7	0.3
Teenagers 13-19 y	54	1.2	3.1	0.3
Adults 20+ y	47	1.1	2.7	0.3
Population 2+ y	47	1.1	2.7	0.3

NOTES:
 (a) Mean 2-day average MBP intakes as presented in Table 5.
 (b) Fluid milk equivalents are calculated based on known concentrations of lactoferrin and lactoperoxidase in fluid milk (0.1 and 0.03 g/L, respectively [Korhonen and Pihlanto 2003]) and known concentrations of these proteins in MBP (0.543 and 0.406 g/g solids, respectively [Table 2]).
 (c) Fluid milk equivalents are calculated based on known concentration of cystatin C in skim milk powder (8 µg/g [provided by Snow Brand February 16, 2005]) and known concentration in MBP (0.001 g/g solids [Table 2]). Approximately 23-g of skim milk powder is used to prepare one serving (8 fluid ounces) of skim milk (USDA 2004).

ABBREVIATIONS:
 LF: Lactoferrin
 LPO: Lactoperoxidase
 MBP: Milk Basic Protein

These calculations indicate that the MBP constituents LF and LPO are provided in quantities roughly comparable to LF and LPO from background fluid milk intake. A comparison of exposure to other MBP protein constituents (e.g. cystatin C, 10 kDa HMG-like protein, or 16-17 kDa kininogen fragment) with background intake of these constituents from fluid milk was also completed. In the absence of published fluid milk data for these proteins, Snow Brand provided analytical data quantifying the concentration of cystatin C in New Zealand skim milk powder. As shown in Table 7, the average concentration of cystatin C in the skim milk powder was 8 µg/g. Using the mean concentration of 8 µg cystatin C in 1 g of skim milk powder, we calculated how many cups of milk contain the same amount of cystatin C that is provided by MBP. Equivalent fluid milk servings were then calculated using the same methods described for LF or LPO data. Based on the concentration of cystatin C in MBP, the mean and 90th percentile intakes of cystatin C by the U.S. population 2 years and older are estimated to be <0.1 and 0.1 mg of cystatin C per day, respectively. Based upon the skim milk powder data, this amount of cystatin C consumed per day from MBP is equivalent to the amount of cystatin C found in no

more than ¼ cup of fluid milk for all population groups (Table 6). These volumes of milk equate to approximately 20-25% of the background daily fluid milk intake by the U.S. population (USDA 2000a).

Table 7. Cystatin C Concentration of New Zealand Skim Milk Powder ^(a)

	Sample Identification (Lot No.)						n	Mean	SD
	AN26 2208 M0090	CN09 2208 M0903	EN12 2200 A4440	GO07 2200 M4153	HO28 2204 M5668	JO02 2200 M6358			
Cystatin C (mcg/g)	5	6	11	9	9	8	6	8	2.1

NOTES:
 (a) Data provided by Snow Brand (February 16, 2005). Cystatin C concentration determined by ELISA.

To further illustrate the relationship between intake of specific protein constituents from MBP and fluid milk, data in Table 8 identify the amounts LF, LPO, and cystatin C consumed per day from each source by the average consumer of MBP and fluid milk. We have assumed a typical intake of 40 mg MBP, and an average daily intake of 1 cup of milk. The ratio presented in the final column directly compares the concentrations of each protein constituent in MBP to the concentration in fluid milk. These data indicate that the concentration of LF in a typical daily intake of MBP is similar to that provided by intake of fluid milk, of which LPO is approximately twice as concentrated in MBP compared to fluid milk, and the concentration of cystatin C in MBP is approximately 1/5 that of fluid milk. Overall, these data suggest that the estimated daily intake of MBP provides no more than approximately twice the quantity of any one of these protein constituents when compared to fluid milk. LF, LPO and cystatin C can be used as markers for potential exposure to other basic proteins identified in MBP (kininogen fragment 1·2; HMG-like protein). These marker proteins suggest that ingestion of MBP will not result in an exaggerated exposure to other protein components relative to background ingestion from dairy.

Table 8. Protein Component Concentrations in MBP Compared to Fluid Milk			
Protein Component	Amount (mg)^(a)		Concentration Ratio MBP:Milk^(d)
	Amount in 40 mg MBP^(b)	Amount in 1 Cup of Fluid Milk^(c)	
LF	21.7	23.6	0.9
LPO	16.2	7.1	2.3
Cystatin C	0.04	0.2	0.2

NOTES:
 Calculations are based on known the following data for each protein component:
 (a) Concentrations of lactoferrin and lactoperoxidase in fluid milk (0.1 and 0.03 g/L, respectively [Korhonen and Pihlanto 2003]), approximate concentration of cystatin C in skim milk powder (8 mcg/g [provided by Snow Brand February 16, 2005]), and approximate concentrations of these proteins in MBP (0.543, 0.406, and 0.001 g/g solids, respectively [Table 2]).
 (b) 40 mg of MBP is approximately the estimated intake by the average consumer based on proposed uses (Table 8).
 (c) Approximate per capita milk intake in the U.S. (USDA 2000a).
 (d) Calculated as (Amount of protein component in 40 mg MBP)/(Amount of protein component in 1 cup of fluid milk).
 ABBREVIATIONS:
 LF: Lactoferrin
 LPO: Lactoperoxidase
 MBP: Milk Basic Protein

D. CONCLUSIONS

Under the proposed uses of MBP, the estimated 2-day average intake of MBP by consumers of MBP-fortified products ages 2 years and older is approximately 47 mg per day. Based on concentrations of LF and LPO in fluid milk and concentrations of these constituents in MBP, the addition of MBP to the proposed foods is estimated to provide no more than the equivalent of 2.7 milk servings per day for mean consumers of MBP ages 2 years and older, and no more than the equivalent of 1.8 milk servings per day for mean consumers 1 year or younger. Based on cystatin C concentration data in New Zealand skim milk powder, MBP provides an amount of cystatin C per day equivalent to the amount of cystatin C found in no more than ¼ cup of fluid milk for all population groups. These estimates indicate that MBP intake from proposed uses at the proposed fortification levels would provide exposure to the MBP constituents in the range of current intake of these components from naturally occurring sources in the U.S. food supply. LF, LPO and cystatin C can be used as markers for predicting the relative concentrations of various protein components in MBP relative to milk. These marker proteins suggest that ingestion of MBP will not result in an exaggerated exposure to other protein components relative to background ingestion from dairy.

V. Safety Assessment

A. IDENTIFICATION AND ACTIVITY OF PRINCIPAL COMPONENTS IN MBP

Milk is recommended as an excellent calcium source for bone health. However, milk contains other components in addition to calcium that are efficacious for bone health. Milk whey proteins (WP), and in particular those from its basic fraction, are actively involved in bone metabolism. An active component of a WP fraction of bovine milk stimulated proliferation and differentiation of osteoblastic MC3T3-E1 cells, as represented by [³H]thymidine incorporation, increased DNA and hydroxyproline content, and increased protein synthesis (Takada *et al.* 1996). In other studies, an active component of a WP fraction of bovine milk had bone resorption inhibitory activity, as represented by inhibition of pre-existing and newly-formed osteoclast bone resorption and inhibition of osteoclast formation from splenic blast cells (Takada *et al.* 1997b).

Two studies were performed with ovariectomized (OVX) rats to examine the effects of WP in the diet on calcium and bone metabolism and on bone resorption and bone strength (Takada *et al.* 1997a,c). When WP (1% or 2% in diet) was fed to animals in the diet, bone protein metabolism, but not bone calcium metabolism, was increased, which resulted in higher levels of bone protein (amino acid content) and increased breaking energy of the bone. In addition, WP also appeared to counteract the effect of the removal of the ovaries (i.e., decreased female hormones) on bone resorption (e.g., osteoporosis). The breaking strength, adjusted for bone calcium, was significantly higher in all WP groups relative to controls, which suggests that a mechanism other than bone calcium content is involved in enhancing the breaking force.

The specific components of the milk WP that are responsible for bone health were identified from *in vitro* and *in vivo* studies of the milk WP source for MBP (Takada *et al.* 1996, 1997 a-c) and from the MBP fraction itself (Kato *et al.* 2000; Toba *et al.* 2000; Takada *et al.* 2000, 2001). Studies conducted using WPC and the fraction of WPC known as MBP have shown that whey proteins and, more specifically, components found in the basic proteins of the whey fraction strongly stimulate bone formation and inhibit bone resorption *in vitro* and *in vivo*. MBP has been shown to increase the number of osteoblastic cells and the amount of bone proteins such as collagen. Two components of the MBP that have this growth-promoting activity have been purified and sequenced. One component was identified as a high-mobility group (HMG)-like protein, the other as a component of high molecular weight kininogen fragment 1·2. High molecular weight kininogen I is a 621 amino acid protein that is processed *in vivo* by kallikrein (Han *et al.*, 1976; Kitamura *et al.*, 1983) to produce a mature protein of 110 amino acids, also known as fragment 1·2, representing amino acids 389-498 of the full length protein, which is secreted in milk (Wilson *et al.*, 1989). High mobility group protein-1 is

produced as a 215 amino acid translation product (Kaplan and Duncan 1988). The HMG protein 1 is known to be processed as a secreted protein and is found in milk (Walker et al., 1980; Yamamura et al., 1999). MBP also suppresses bone resorption. One of the components identified as having this activity is cystatin C. Bovine cystatin C is a 118 amino acid inhibitor of cysteine proteases (Olsson et al., 1997). Lactoferrin has also been identified as a basic whey protein that may have a role in bone physiology. In one study, it has been shown to promote osteoblast growth and inhibit osteoclastogenesis *in vitro* and increase local bone formation *in vivo* (Cornish et al. 2004).

The active components in MBP that play a role in bone formation by producing growth-promoting activity of osteoblastic cells were identified by [³H]thymidine incorporation into osteoblastic MC3T3-E1 cells (Yamamura et al. 1999). The active components were purified using cationic-exchange column chromatography of basic whey proteins prepared from WPC. Whey proteins were treated with acid and heated, and the supernatant was loaded onto an S-Sepharose column. The bound proteins were eluted with a linear gradient of 0 to 1.0 M NaCl in sodium phosphate buffer. The eluted protein fractions were collected, and the growth promoting activities of them were measured. The osteoblastic cell growth-promoting fractions were pooled and loaded onto a Mono S cation exchange column. The bound proteins were also eluted with a linear gradient of 0 to 1.0 M NaCl in sodium phosphate buffer. Osteoblast growth-promoting fractions were split into two peaks. The latter fraction was subjected to Mono Q anion exchange column, reversed phase HPLC purification, and then subjected to electrophoresis and amino acid sequence analysis. Electrophoretic analysis revealed that the purified protein migrated as a single band that corresponded to 10 kDa. To characterize this 10 kDa active protein, the amino-terminal 33 amino acids were identified. The identified sequence was analyzed by a homology search using SWISS PROT protein sequence database. The homologous sequence was the amino terminal sequence of bovine high mobility group (HMG) protein 1. HMG protein 1 is a well known nuclear non-histone chromosomal protein (Bustin et al. 1990, cited in Yamamura et al. 1999).

The purification and identity of the active component corresponding to the former fraction of the Mono S chromatography were reported in a subsequent paper by Yamamura's group (Yamamura et al. 2000). The fraction was subjected to Mono Q anion exchange column, gel filtration column (TSK-GEL G2000SW), reversed phase HPLC purification (phenyl column and C4 column) and then subjected to electrophoresis and then to amino acid sequence analysis. Molecular weight of the growth-promoting protein was estimated as 17 kDa by electrophoresis. The activity of the 17-kDa band was confirmed by eluting the proteins from the gel and assayed them for MC3T3-E1 proliferation activity. To characterize the active protein, the amino-terminal 19 amino acid residues were analyzed, and the homology was searched using SWISS PROT protein sequence database. The homologous sequence is an internal sequence of bovine

high molecular weight (HMW) kininogen (SWISS PROT accession number P01044). The HMW kininogen is well known as a plasma protein. The 17-kDa kininogen has been reported to occur in bovine milk (Wilson *et al.* 1989).

The milk basic protein fraction of whey has also been shown to suppress osteoclast-mediated bone resorption *in vitro*. Since osteoclasts on the bone surface secrete cathepsin K, a member of the cysteine protease family, to digest collagen in the bone matrix, it is believed that a cysteine protease inhibitor identified in MBP suppresses bone resorption by inhibiting this protease (Matsuoka *et al.* 2002). In a study reported by Matsuoka and colleagues (2002), an MBP solution was prepared from bovine milk and loaded into a CM-papain affinity column. The cysteine protease inhibitor that adsorbed to papain was eluted after fraction 23. Reduced SDS-PAGE showed the high-molecular-weight protein bands in fractions 23-40, and an approximately 12 kDa major band was silver stained after fraction 50. Fractions 25-55 were pooled, concentrated and purified. An 11.4 mg amount of purified cysteine protease inhibitor was obtained from 122 grams of MBP. Eighteen amino acid residues from the N-terminal of the 12 kDa protein were determined and the sequence corresponded to that reported for bovine cystatin C isolated from bovine colostrum and bovine parotid glands (Hirado *et al.* 1985; Cimerman *et al.* 1996). Cystatin C has also been identified in human biological fluids (Abrahamson *et al.* 1986). The inhibitory activity of 12 kDa cystatin C to osteoclast mediated bone resorption was determined *in vitro*.

B. SAFETY OF PRINCIPAL PROTEIN COMPONENTS OF MBP

1. Lactoferrin

a) Regulatory History

As discussed previously in this document, MBP is derived from the basic protein fraction of milk. Lactoferrin comprises approximately 56% of total protein of the final product. Lactoferrin is a protein found in milk and products derived from milk, such as whey, and thus is widely consumed in the diet by Americans through dairy sources. Fluid milk contains approximately 0.1 grams per liter lactoferrin (Korhonen and Pihlanto 2003). Milk-derived lactoferrin is found in numerous dietary supplements marketed in the U.S. Milk-derived lactoferrin is also a GRAS ingredient for use in foods in the U.S. and has been the subject of three GRAS Notifications to FDA: GRN No. 67, GRN No. 77, and GRN No. 130. In GRN No. 67, milk-derived lactoferrin was determined to be GRAS for use as a component (up to 2%) of an antimicrobial spray on beef carcasses, subprimals, and finished cuts. The cumulative EDI from these uses is up to 9.1 mg/person/day at the 90th percentile of consumption. Source

labeling is required. In GRN No. 77, milk-derived lactoferrin was determined to be GRAS for use as an ingredient in sports and functional foods at up to 100 mg/serving, resulting in an EDI of 196 mg/person/day at the 90th percentile of consumption. Source labeling is required. In GRN No. 130, milk-derived lactoferrin was determined to be GRAS for use as a component (up to 2%) of an antimicrobial spray on beef carcasses without the requirement of labeling for the source protein; resulting residue of milk-derived lactoferrin is less than 800 micrograms per kilogram edible beef. The FDA has no questions with regard to the conclusion that bovine milk-derived lactoferrin is GRAS for the uses and at the levels proposed in these three GRAS Notifications.

b) Potential for Allergenicity at Proposed Levels of Exposure

The issue of potential allergenicity for lactoferrin at the proposed levels of exposure resulting from MBP consumption should be addressed as part of the safety evaluation of MBP. Allergic reactions to cows' milk are quite common especially amongst young children where the prevalence approaches 2% of the population (Host and Halken 1990). Cows' milk allergy is caused by sensitization to certain milk proteins. The major allergens are casein, lactoglobulin, and lactalbumin (Bush and Hefle 1996).

Lactoferrin has been determined to be GRAS (with source labeling) for use as ingredient in sports and functional foods at an EDI of up to 196 mg/person/day for the 90th percentile consumer; the estimated intake of lactoferrin for the mean and 90th percentile consumers of MBP (population 2+ years) are 26 and 54 mg/person/day, respectively. Thus, intake is below levels already determined to be GRAS for use in functional foods. In addition, this level of lactoferrin consumption from MBP is equivalent to approximately 1 and 2 cups of milk for the mean and 90th percentile MBP consumers, respectively.

In the determination of GRAS status for lactoferrin for GRN 000077, FDA had identified two issues that were pivotal to their review of the Notification. The issues were (1) the potential that the increased level and overall exposure to milk-derived lactoferrin would sensitize individuals who are not already allergic, and (2) the potential that this immunologically active food component is correlated with autoimmune disorders. GRN 000077 presented a report of a specially convened panel of individuals to address these issues. The panel concluded that increased exposure to milk-derived lactoferrin at the levels of exposure specified would be highly unlikely to induce allergy or autoimmune disease.

The Expert Panel assembled to consider these issues for GRN 000077 indicated that little scientific and clinical information exists to document whether bovine milk-derived lactoferrin is a cows' milk allergen. While there is some evidence to suggest that bovine milk-derived lactoferrin is a minor cows' milk allergen, the existing information is difficult to interpret because of methodological problems in the manner in which much of the data were collected.

Certainly, no argument exists that the major cows' milk allergens in IgE-mediated cows' milk allergy are casein, β -lactoglobulin, and α -lactalbumin (Besler *et al.* 2001; Sharma *et al.* 2001; Wal 1998; Wal 2001). Several clinical investigators have demonstrated that some cows' milk-allergic individuals have IgE antibodies directed against lactoferrin (Baldo 1984; Businco *et al.* 2000; Host *et al.* 1992; Wal *et al.* 1995; Wal 1998; Wal 2001). However, the vast majority of these milk-allergic infants displayed IgE antibodies to one or more of the major cows' milk allergens in addition to lactoferrin. Wal (1998) appears to have identified two milk-allergic infants out of 92 studied (2.2%) who were mono-sensitized to lactoferrin only. However, the clinical significance of this observation is unclear, because the study does not indicate if these or other infants in the study had received blinded oral challenges to document their milk allergies. Without confirmation by oral challenge, the evaluation of specific IgE antibodies in patient serum can be clinically meaningless and misleading. Furthermore, in studies where quantitative estimates were obtained of the levels of specific IgE antibodies to lactoferrin (Wal *et al.* 1995; Wal 1998), the levels of anti-lactoferrin IgE antibodies were far lower than the levels of specific IgE antibodies to the major milk allergens in the vast majority of the patients.

Milk-allergic patients seem to occasionally have IgE antibodies directed at other minor milk proteins (Baldo 1984); the clinical significance of the presence of these antibodies is unproven. To demonstrate that bovine milk-derived lactoferrin is a clinically significant milk allergen, patients who were challenge-positive to milk ingestion would have to demonstrate specific IgE antibodies to bovine milk-derived lactoferrin (and possibly other milk proteins), positive skin prick tests to bovine milk-derived lactoferrin, and positive oral challenges to bovine milk-derived lactoferrin. Such information does not exist to unequivocally demonstrate that bovine milk-derived lactoferrin is a human allergen.

The Expert Panel assembled for GRN 000077 also concluded that the likelihood of increased consumer exposure to milk-derived lactoferrin, causing or exacerbating autoimmune disease is remote. Although there is extensive literature documenting the presence of anti-lactoferrin autoantibodies in various autoimmune diseases, there is no evidence that these antibodies play any role in the pathology of these diseases. The development of a harmful autoimmune response as a result of the ingredient use of lactoferrin would require a combination of circumstances, which the Panel considered to be unlikely.

In conclusion, data and analysis of lactoferrin do not indicate any elevated risk of potential food allergy for this protein at levels of ingestion expected from MBP. It is expected that consumers of MBP containing lactoferrin will be protected with a labeling statement indicating that the product has been derived from a milk source. The United States has permitted the use of lactoferrin in certain food products with the provision that the presence of lactoferrin be declared on the label as arising from a milk source.

2. Lactoperoxidase

a) Regulatory History

MBP, derived from the basic protein fraction of milk, also contains lactoperoxidase, which comprises approximately 42% of total protein of the final product. Lactoperoxidase is a protein found in milk and products derived from milk, such as whey, and thus is widely consumed in the diet by Americans through dairy sources. Fluid milk contains approximately 0.03 grams per liter lactoperoxidase (Korhonen and Pihlanto 2003). Milk-derived lactoperoxidase is found in numerous dietary supplements marketed in the U.S.

Lactoperoxidase has been considered for food use in Australia/New Zealand. The final assessment report for lactoperoxidase, manufactured by Tatua Cooperative Dairy Co Ltd, permitting its use as a component of a processing aid for meat with the function of inhibiting bacteria, has been approved by the Food Standards Australia New Zealand (FSANZ) (FSANZ 2002). Source labeling is required. Codex Standard (CAC/GL 13-1991) provides for the use of the lactoperoxidase system for the stabilization of milk.

b) Potential for Allergenicity at Proposed Levels of Exposure Occurrence in Milk

The issue of potential allergenicity for lactoperoxidase, at the proposed levels of exposure resulting from MBP consumption should be addressed as part of the safety evaluation of MBP. Allergic reactions to cows' milk are quite common especially amongst young children where the prevalence approaches 2% of the population (Host and Halcken 1990). Cows' milk allergy is caused by sensitization to certain milk proteins. The major allergens are casein, lactoglobulin, and lactalbumin (Bush and Hefle 1996).

Lactoperoxidase is a minor milk protein. No evidence exists to suggest that bovine milk lactoperoxidase is allergenic. Some weak evidence exists to suggest that bovine milk lactoperoxidase may be a weak sensitizer of the immune system in sensitive individuals (Baldo 1984). In a small group of 6 cows' milk-allergic infants, IgE antibodies were detected to lactoperoxidase in 4 of the 6 individuals. However, all of these infants also had IgE antibodies against one or more of the major cows' milk proteins – casein, lactoglobulin, and lactalbumin. These individuals also had IgE antibodies against other minor cows' milk proteins including lactoferrin, alkaline phosphatase, catalase, bovine serum albumin, and the bovine immunoglobulins. The presence of IgE antibodies alone does not infer that the cows' milk allergies suffered by these infants were caused in any way by lactoperoxidase. In fact, the techniques used to detect the presence of anti-lactoperoxidase IgE antibodies were fairly crude by today's standards. At most, the study of Baldo (1984) indicates that lactoperoxidase may be a weak sensitizer.

If lactoperoxidase is not a known milk allergen and if commercial lactoperoxidase contains insufficient amounts of other milk proteins to elicit allergic reactions in the vast

majority of milk-allergic individuals, the only remaining question would concern the possibility that increased exposure to lactoperoxidase might lead to increased sensitization to this milk protein. This possibility seems unlikely given the rather low level of predicted exposure to bovine milk lactoperoxidase. Any novel protein or novel food carries some risk of provoking occasional sensitization in consumers but this circumstance is most likely to occur in situations where exposure to the novel protein is comparatively high. The intake of lactoperoxidase for the mean and 90th percentile consumers of MBP (population 2+ years) is estimated to be 19 and 41 mg/person/day, respectively. This is equivalent to the amount of lactoperoxidase consumed in approximately 2.7 and 5.7 cups of milk for the mean and 90th percentile MBP consumers, respectively. Per capita milk intake in the U.S. is about 1 cup per day (USDA 2000a) for the population ages 2 years and older. Therefore, the intake of lactoperoxidase for the average consumer of MBP is approximately two to three times the background consumption from milk.

For children, consumption of milk is greater than for adults. For example, children ages 2-5 have a per capita milk intake of 1.5 cups of milk per day (USDA 2000a). Lactoperoxidase consumed by this population of children from MBP intake is estimated to be equivalent to 2.3 and 5.1 cups of milk per day for mean and 90th percentile MBP consumers, respectively. Therefore, intake of lactoperoxidase for the average child consumer of MBP is estimated to be only 1.5 times the background from milk consumption. Thus, exaggeration of lactoperoxidase exposure from MBP ingestion compared to background milk consumption is less in children than adults. This is important to note since children are a sensitive subpopulation with respect to milk allergy.

c) Pepsin Resistance

Pepsin digestion analysis (January 2005) results are shown in Figure 2A. Test protein was digested in a standardized in vitro pepsin digestion assay (Thomas *et al.* 2004). The gels were loaded in the same order with the same amount of digestion reaction mix or markers. Molecular weights (kDa) are indicated for lanes labeled "M". All other lanes contained 15 μ L of quenched reaction mix sampled at the times indicated. The reactions contained either test protein alone in SGF (simulated gastric fluid; 0,60 min); test protein in SGF with pepsin (0, 0.5, 2, 5, 10, 20, 30, and 60 min); or SGF with pepsin alone (0, 60 min). These 10-20% polyacrylamide Tricine gels were fixed and stained with CBB-G250.

A pattern of digestion results for lactoperoxidase was observed in this study. The undigested fraction yields a single component with a MW of 70 kDa or greater. Based on the protein sequencing data for lactoperoxidase, the protein has 612 amino acids; this would result in a molecular weight in the range of 62-65 kDa. The undigested MW component is consistent with data indicated that up to 10 percent of the weight of lactoperoxidase may be due to glycan (Watanabe *et al.* 2000). Therefore, glycosylation accounts for the starting MW of the

lactoperoxidase. In the digestion study, the lactoperoxidase was rapidly digested, but produced faint low molecular weight bands at 3.6 kDa (Figure 2A).

Although lactoperoxidase was rapidly digested and produced resistant, this protein is naturally occurring in milk and exposure to these fragments would also be expected to come from milk sources.

d) Evaluation of Sequence Homology

Lactoperoxidase was evaluated using bioinformatics approaches to identify any potential sequence matches with any allergenic proteins that might indicate an elevated risk of allergic cross reactivity if this protein were consumed at higher concentrations in milk or in other foods. At the request of Snow Brand, the bioinformatics analysis was conducted by Drs. Goodman, Wise, & Taylor at the Food Allergy Research and Resource Program (FARRP) laboratory of the University of Nebraska.

The full-length sequence of bovine lactoperoxidase is 712 amino acids (aa) long. The protein form secreted in milk is processed to remove the first 100 amino acids. Therefore, the bioinformatics analysis was performed on the mature sequence, represented by aa 101-712. Four bioinformatics searches with lactoperoxidase were conducted by FARRP. None of the results from these searches meet the analytical criteria for potential allergenic cross-reactivity. The results of the bioinformatics analysis, therefore, support the conclusion that there is not an elevated risk of potential allergenic cross-reactivity from ingestion of the milk-derived lactoperoxidase.

e) Conclusion

Lactoperoxidase is naturally occurring in milk. Therefore, exposure will occur through this source as well as other ingredient sources such as nonfat dry milk, whey, and whey protein concentrate. The intake of lactoperoxidase for the average consumer of MBP is approximately two to three times the background consumption from milk. For children, consumption of milk is greater than for adults and intake of lactoperoxidase for the average child consumer of MBP is estimated to be only 1.5 times the background from milk consumption. Thus, exaggeration of lactoperoxidase exposure from MBP ingestion compared to background milk consumption is less in children than adults. This is important to note since children are a sensitive subpopulation with respect to milk allergy.

Lactoperoxidase is not stable to pepsin hydrolysis using the ILSI-HESI recommended method. However, stable fragments were produced. Stable peptide fragments have been a concern for novel proteins such as cry9c; however, lactoperoxidase and its fragments are natural components of milk so consumers are already exposed. The sequence homology research shows that these proteins have no biologically significant sequence homology with known allergens and would therefore not be expected to show cross-reactivity.

Data and analysis of lactoperoxidase do not indicate any elevated risk of potential food allergy for this protein at levels of ingested expected from MBP. In addition, it is expected that consumers of MBP containing lactoperoxidase will be protected with a labeling statement indicating that the product has been derived from a milk source.

3. Cystatin C

As discussed previously in Chapter II, cystatin C is present in MBP at a very low level of 0.1 g/100g solids. Cystatin C is present naturally in conventional bovine milk and whey products. Because MBP is derived from bovine milk, it also contains cystatin C. Snow Brand provided analytical data quantifying the concentration of cystatin C in New Zealand skim milk powder. The average concentration of cystatin C in the skim milk powder was 8 µg/g. Based on the concentration of cystatin C in MBP, the mean and 90th percentile intakes of cystatin C by the U.S. population 2 years and older are estimated to be <0.1 and 0.1 mg of cystatin C per day, respectively. Based upon the skim milk powder data, this amount of cystatin C consumed per day from MBP is equivalent to the amount of cystatin C found in no more than ¼-cup of fluid milk for all population groups. These volumes of milk equate to approximately 20-25% of the background daily fluid milk intake by the U.S. population (USDA 2000a).

To evaluate the safety of consumption of MBP-containing products, several analyses of the characteristics of this protein were conducted. The resistance of cystatin C to digestion was evaluated using a standard pepsin digestion assay. The allergenic potential of this protein was evaluated using bioinformatics analyses of sequence homologies. The analyses, results and interpretations of these studies are presented below.

a) Pepsin Resistance

A standardized *in vitro* pepsin digestion assay was performed (Thomas *et al.* 2004). The gels were loaded in the same order with the same amount of digestion reaction mix or markers. The molecular weights (kDa) were calibrated and are indicated in the figures for the lanes labeled "M." All other lanes on the plates contained quenched reaction mix (15 µL) sampled at the times indicated. The reactions contained either test protein alone in simulated gastric fluid (SGF) at 0 and 60 minutes duration; test protein in SGF with pepsin at 0, 0.5, 2, 5, 10, 20, 30, and 60 minutes duration; or SGF with pepsin alone at 0 and 60 minutes duration. These 10-20% polyacrylamide-tricine gels were fixed and stained with CBB-G250.

The results for bovine cystatin C are shown in Figure 2B. A pattern of digestion results for cystatin C was observed in this study. The cystatin C was rapidly digested, but produced digestion-resistant fragments with molecular weights between 3 and 6 kDa. It is important to remember, however, that because cystatin C is naturally occurring in milk and whey products, these fragments will also be generated from the native protein in these conventional milk and whey products. Products containing MBP will have content labels indicating milk-derived

ingredients. Thus, the presence of these protein fragments does not raise a concern about allergenicity from novel proteins or proteins. It is unlikely there would be any marked increase in allergenic risk (as long as products containing these are labeled as milk) from the products with MBP.

b) Evaluation of Sequence Homology

Because of the presence of cystatin C, among other proteins, in MBP, the allergenic potential of these proteins was evaluated using bioinformatics analysis of sequence homologies, i.e., comparison of amino acid sequences of the MBP protein components to known allergenic proteins. The bioinformatics analysis attempted to identify any potential amino acid sequence matches with any allergenic proteins. Such matches might indicate an elevated risk of allergic cross reactivity, if these proteins were consumed at higher concentrations in milk or in other foods.

The sequence identity for bovine cystatin C (GI:27806675) was reported by Olsson *et al.* (1997). Four bioinformatics searches with the bovine cystatin C were conducted by FARRP. The results from the four different types of bioinformatics analyses (sequence searches) indicate that no matches meet the analytical criteria for potential allergenic cross-reactivity. The results of the bioinformatics analysis, therefore, support the conclusion that there is not an elevated risk of potential allergenic cross-reactivity from ingestion of the bovine cystatin C.

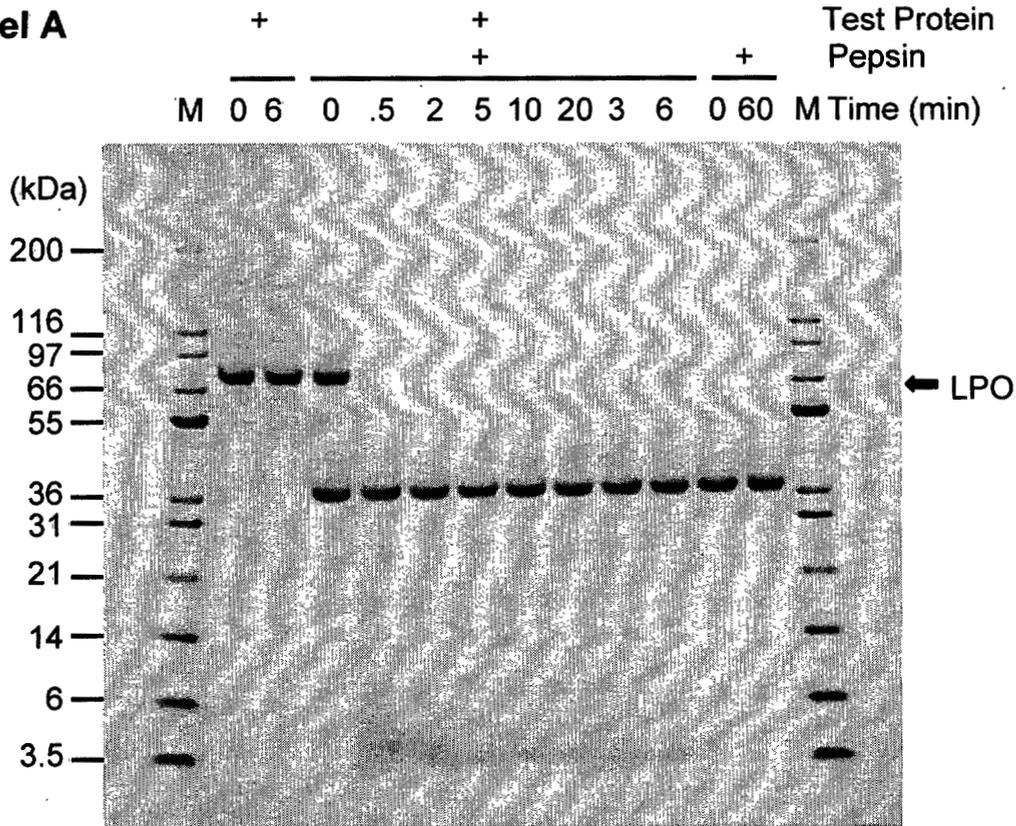
c) Conclusion

Pepsin digestion of cystatin C produces resistant fragments; however, the presence of these protein fragments does not raise allergenicity risk because these fragments would also be produced as a result of consumption of conventional bovine milk and whey products. Thus, it is unlikely there would be any marked increase in allergenic risk from products containing MBP so long as the product labels indicate that the presence of milk ingredients. Further, the sequence homology evaluation for cystatin C indicates that there is not an elevated risk of potential allergenic cross-reactivity from ingestion of the bovine cystatin C. Thus, the consumption of products containing MBP does not appear to elevate allergenic risk.

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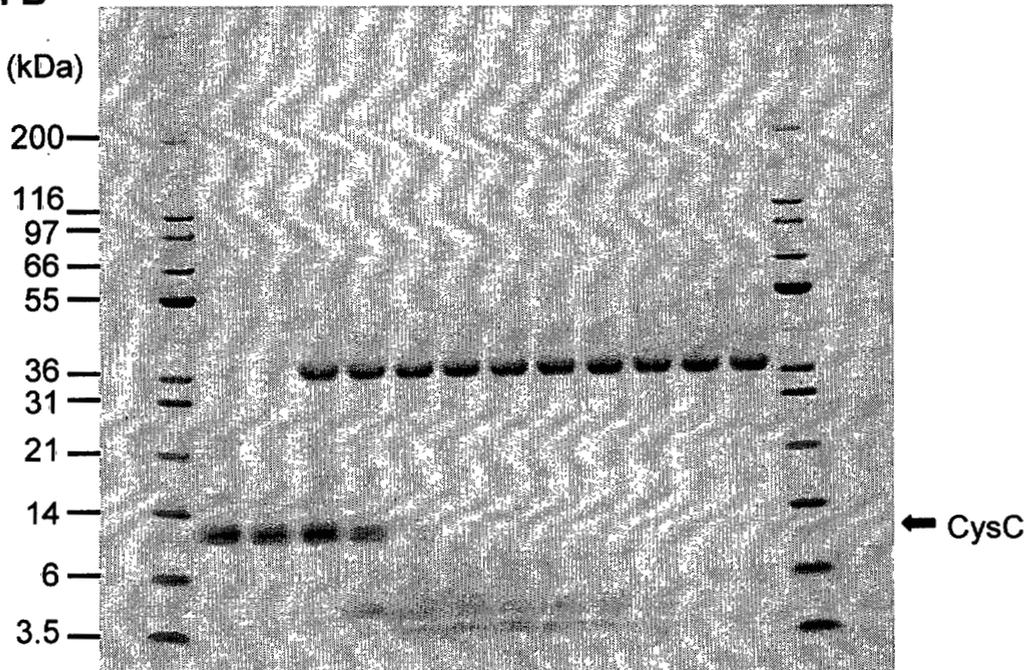
Figure 2. Pepsin Digestion Study: Lactoperoxidase and Cystatin C

Panel A



Test protein digested at different rates in a standardized in vitro pepsin digestion assay. A pattern of digestion results of lactoperoxidase (panel A) and cystatin C (panel B) were observed in this study. The LPO was rapidly digested, but produced resistant fragments. The cystatin C was rapidly digested, but produced resistant fragments. The gels were loaded in the same order with same amount of digestion reaction mix or markers. Molecular weights (kDa) are indicated for lanes labeled "M." All other lanes contained 15 µl of quenched reaction mix sampled at the times indicated. The reactions contained either test protein alone in SGF (simulated gastric fluid; 0, 60 min); test protein in SGF with pepsin (0, 0.5, 2, 5, 10, 20, 30, and 60 min); or SGF with pepsin alone (0, 60 min). These 10-20% polyacrylamide Tricine gels were fixed and stained with CBB-G250.

Panel B

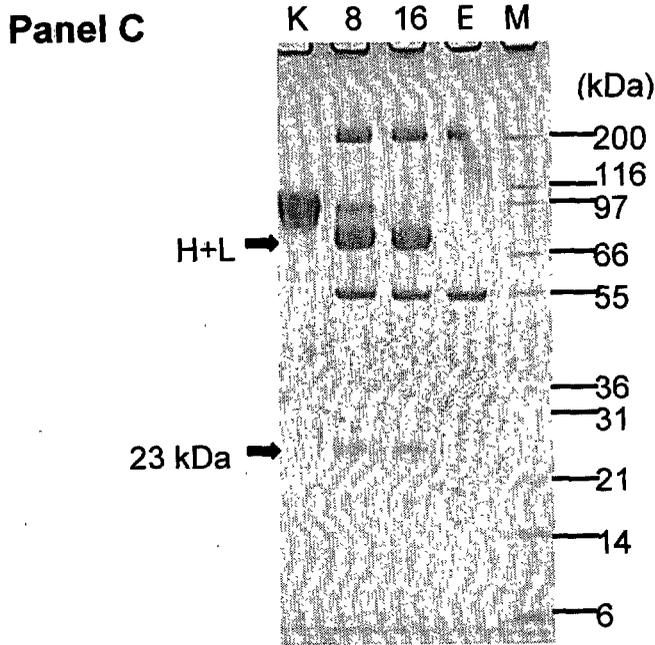


4. Kininogen Fragment 1·2

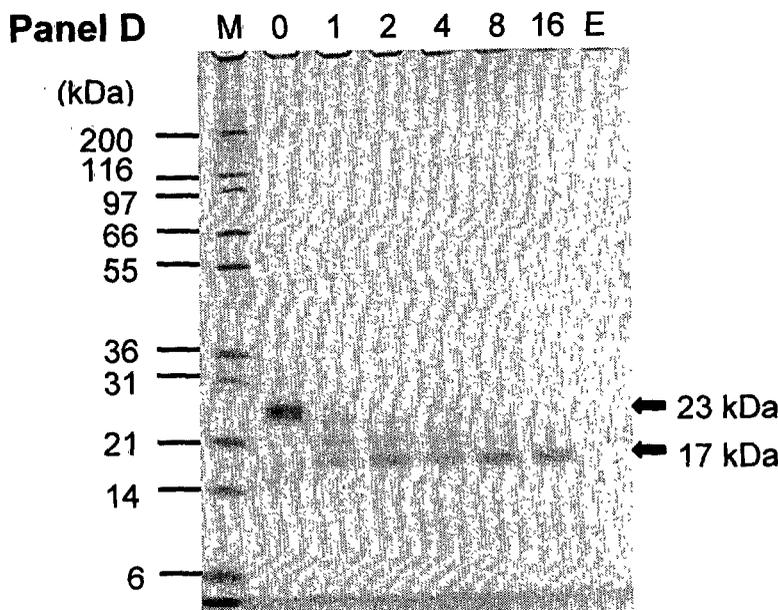
The presence of kininogen fragment 1·2 in MBP is confirmed as discussed in Section II. Because MBP is derived from bovine milk, the kininogen fragment 1·2 is also expected to be present naturally in conventional milk and whey products. To evaluate the safety of consumption of MBP-containing products (all of which will be labeled as containing milk ingredients), several analyses of the characteristics of this protein were conducted. The resistance of kininogen fragment 1·2 to digestion was evaluated using a standard pepsin digestion assay. The allergenic potential of this protein was evaluated using bioinformatics analyses of amino acid sequence homologies. The analyses, results and interpretations of these studies are presented below. Kininogen fragment 1·2 is enzymatically cleaved from high molecular weight kininogen by kallikrein in bovine plasma. Results of kallikrein digestion of high molecular weight kininogen are shown in Figure 3 panel C. Due to differential glycosylation, the peptide representing fragment 1·2 appear as multiple molecular weight bands from approximately 15 kDa to 25 kDa, which are reduced to a single band of approximately 15 kDa following digestion with sialidase and o-glycanase (Figure 3, panel D). The details of the procedures are discussed in Figure 3.

Han *et al.* (1976) discusses the amino acid sequence analysis and the amino acid composition of the kininogen fragments 1·2 and, individually, fragment 1 and fragment 2, as well as carbohydrate determination and electrophoretic mobility on SDS PAGE. Han *et al.* (1976) also support the observation that all protein forms described above have lower mobilities than expected based on amino acid content, and, thus, that not all of the low mobility relates to glycosylation. The results by Han *et al.* are comparable to the current results demonstrating that the sequence of the fragment is represented appropriately by the 110 amino acid sequence. (This is the sequence for which the bioinformatics analyses were performed as discussed below.) Further, Hayashi *et al.* (1985), demonstrates that there is no effect of reducing agent in mobility. Those results also are consistent in molecular weight with the 17 kDa and 23 to 25 kDa fragments for unglycosylated and glycosylated forms, respectively. Thus, the published research studies support the argument that the correct sequence for the kininogen fragment 1·2 has been evaluated.

Figure 3. Kallikrein Digestion Study: HMW Kininogen and Kininogen Fragment 1-2



Kallikrein digestion of bovine high molecular weight (HMW) kininogen. The HMW kininogen was purified from bovine plasma by chromatography according to the method of Shimada (Shimada et al. (1985) *J. Biochem. (Tokyo)*, 97, 429-39). The HMW kininogen (K) was incubated with bovine plasma kallikrein for the time indicated (8, 16 h). Each sample and blank contained enzyme only (E) were loaded to a SDS-polyacrylamide gels (10%T) under non-reducing condition. After the electrophoresis the gel was stained with Coomassie brilliant blue. The HMW kininogen liberated the fragment 1-2 and a nanopeptide, bradykinin, as Kato and Hayashi reported (Kato H et al. (1981) *Methods in Enzymology*, 80, 172-198; Hayashi I et al. (1985) *J. Biol. Chem.* 260, 6115-23). The position of the heavy and light chain complex (H+L, 70 kDa) and the 23 kDa protein were indicated.



Enzymatic deglycosylation of bovine high molecular weight kininogen fragment 1-2. The fragment 1-2 was purified from kallikrein-digested plasma kininogen (shown in Figure 1) by chromatography. The 23 kDa protein was incubated with sialidase and o-glycanase for the time indicated (0-16 h). Each sample and blank contained enzyme only (E) were loaded to a SDS-polyacrylamide gels (10%T) under non-reducing condition. After the electrophoresis the gel was stained with Coomassie brilliant blue. The position of 23 kDa and 17 kDa were indicated. The enzymatic deglycosylation reduced the mobility of fragment 1-2 by removing the o-glucoside sugar chains.

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a) Pepsin Resistance

Kininogen fragment 1·2 protein was digested in a standardized *in vitro* pepsin digestion assay (Thomas *et al.* 2004). The results of the pepsin digestion analysis are shown in Figure 4E. The test material for pepsin digestion was a mixture of peptides representing both de-glycosylated and glycosylated forms of fragment 1·2, as would be expected from natural milk. As evident in Figure 4E, fragment 1·2 digests relatively quickly in pepsin, with no bands detectable between 15 and 25 kDa after approximately 20 minutes of digestion. However, the presence of lower molecular weight bands of approximately seven and 13 kDa begin to appear following 30 seconds of digestion with pepsin, and the continued accumulation of these bands for at least 20 minutes of digestion indicates moderately stable significant fragments are formed during digestion. This stability is consistent with that found for a number of food allergens (Astwood *et al.* 1996).

In evaluating the safety of these fragments or products, it is important to consider that the kininogen fragment 1·2 is present in blood and milk from bovine naturally. It should be similarly stable in meat and dairy foods. Based on that knowledge, and the low level of presence in the final MBP-containing products, the stability of these fragments should not raise any special concerns relative to potentially new allergenic foods. Importantly, the MBP-containing products will be labeled as milk; this should mitigate any reasonable question regarding allergenicity. It is unlikely there would be any marked increase in allergenic risk (as long as products containing these are labeled as milk) from the products with MBP.

b) Evaluation of Sequence Homology

The sequence identity for high molecular weight (HMW) kininogen I (GI:125505) from the bovine (*Bos taurus*) was reported by Kitamura *et al.* (1983). Han *et al.* (1976) showed that the full-length sequence of bovine HMW kininogen I (GI:125505) is cleaved proteolytically *in vivo* by kallikrein to produce a mature peptide of 110 amino acids. Wilson *et al.* (1989) found that this 110 amino acid fragment – also known as kininogen fragment 1·2 – is secreted in milk. Therefore, Goodman, Wise & Taylor conducted a bioinformatics analysis of amino acid sequence homologies with the kininogen fragment 1·2. The results from the four different types of bioinformatics analyses indicate that no matches with the bovine HMW kininogen I sequence meet the analytical criteria for potential allergenic cross-reactivity. The results of the bioinformatics analyses, therefore, support the conclusion that there is not an elevated risk of potential allergenic cross-reactivity from ingestion of the bovine HMW kininogen I. Thus, ingestion of kininogen fragment 1·2 in products containing MBP is not expected to elevate the risk of potential allergenic cross-reactivity.

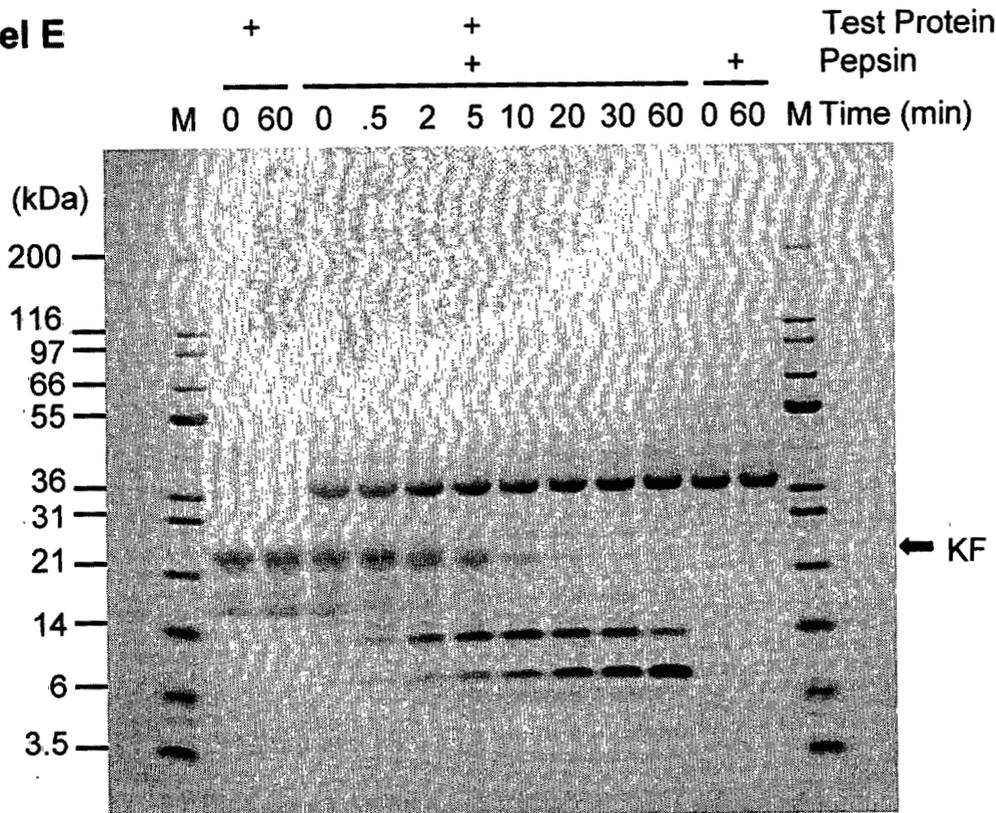
c) Conclusions

The finding that the pepsin digestion study showed some resistant proteins is tempered by the knowledge that these proteins would also be produced in digestion of conventional milk products. In addition, the results of the bioinformatics analyses support the conclusion that there is not expected to be an elevated risk of potential allergenic cross-reactivity from ingestion of the bovine HMW kininogen I. Thus, ingestion of kininogen fragment 1·2 in MBP-containing products is unlikely to create a marked increase in allergenic risk as long as products containing these are labeled as milk.

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Figure 4. Pepsin Digestion Study: Kininogen Fragment 1-2 and HMG Protein

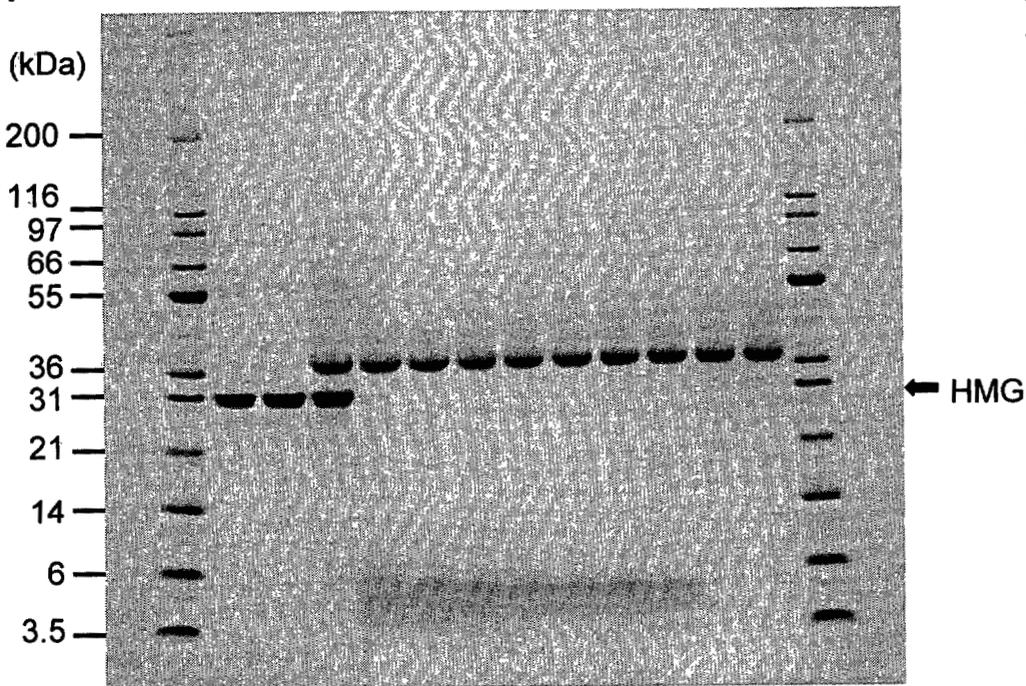
Panel E



← KF

Test protein digested at different rates in a standardized in vitro pepsin digestion assay. A pattern of digestion results of kininogen fragment 1-2 (panel E) and HMG protein (panel F) were observed in this study. The kininogen fragment 1-2 was relatively resistant and also produced resistant fragments. The HMG protein was rapidly digested, but produced resistant fragments. The gels were loaded in the same order with same amount of digestion reaction mix or markers. Molecular weights (kDa) are indicated for lanes labeled "M." All other lanes contained 15 µl of quenched reaction mix sampled at the times indicated. The reactions contained either test protein alone in SGF (simulated gastric fluid; 0, 60 min); test protein in SGF with pepsin (0, 0.5, 2, 5, 10, 20, 30 and 60 min); or SGF with pepsin alone (0, 60 min). These 10-20% polyacrylamide Tricine gels were fixed and stained with CBB-G250.

Panel F



← HMG

5. HMG-like Protein

The presence of high mobility group (HMG)-like protein in MBP is confirmed as discussed in Section II. Because MBP is derived from cow's milk, the HMG-like protein is also expected to be present naturally in conventional milk and whey products. Although all of the MBP-containing products will be labeled as containing milk ingredients, the safety of consumption of MBP-containing products was evaluated by employing several analyses of the characteristics of this protein. The resistance of HMG protein to digestion was evaluated using a standard pepsin digestion assay. The allergenic potential of this protein was evaluated using bioinformatics analyses of sequence homologies. The analyses, results and interpretations of these studies are presented below.

a) Pepsin Resistance

HMG protein was digested in a standardized in vitro pepsin digestion assay (Thomas *et al.* 2004). A pattern of digestion results for HMG protein was observed in this study (Figure 4F), which demonstrates that the HMG protein was rapidly digested (< 30 seconds), but that digestion produced pepsin-resistant fragments with molecular weights in the range 4 to 6 kDa. (The digestion and gel fixing and staining procedure is described above in section V.B.3.)

b) Evaluation of Sequence Homology

The sequence identity for bovine high mobility group (HMG) protein (GI:123367) was reported by Kaplan and Duncan (1988) to be 215 amino acids long and secreted intact in milk. The bioinformatics analysis conducted by FARRP was performed on this mature sequence. Four bioinformatics searches with the bovine HMG protein 1 sequence were conducted. On the bioinformatics analyses, there were two matches to allergenic sequences with the bovine HMG protein 1. One exact match of 8 amino acids was identified corresponding to a low complexity region of the peanut allergen Ara h 1 (GI:1168390). Based on other studies the sequence is considered to be unlikely to represent an IgE epitope. Although the match for a continuous 8 amino acid sequence was to Ara h 1, a peanut protein, it does not seem to be an added risk because the sequence is in a low complexity region of the protein and this same sequence is present in many innocuous proteins. In addition, a matching 80-mer-sequence was identified (of approximately 36% identity) in a low complexity region and matched a parasite allergen. These matches do not seem significant for potential allergenic cross-reactivity. The results of the bioinformatics analysis, therefore, support the conclusion that there is not an elevated risk of potential allergenic cross-reactivity from ingestion of the HMG-like protein.

c) Conclusions

The pepsin digestion study showed that some resistant protein fragments are formed during digestion by pepsin. This knowledge must be tempered by the knowledge that these protein fragments would also be produced in digestion of conventional milk products. The results of the bioinformatics analyses found two sequence matches. Neither match, however, was believed to significantly increase the allergenic risk. Thus, ingestion of bovine HMG-like protein in MBP-containing products is unlikely to create a marked increase in allergenic risk as long as products containing these are labeled as milk.

C. SAFETY STUDIES OF MBP

Toxicology studies were performed in accordance with "Ordinance on Standard of Conduct of Non-clinical Studies of Drug Safety," Ministry of Health and Welfare Ordinance No. 21, Japan, March 26, 1997 and "Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives," Notification No. 29 of the Environmental Health Bureau, Ministry of Health and Welfare, Japan, March 22, 1996.

1. Mutagenicity

MBP was tested for the potential to induce point mutations in a reverse mutation assay using *Salmonella typhimurium* strains TA98 and TA100 (Safety Research Institute for Chemical Compounds Co., Ltd. 1997; Kruger et al. 2005). All tests were conducted in the presence and absence of metabolic activation (i.e., rat liver homogenate, S9). MBP concentrations of 1.6, 8.0, 40, 200, 1,000, and 5,000 µg/plate (first test) and 156, 313, 625, 1,250, 2,500, 5,000 µg/plate (second test) were employed. Negative control samples (i.e., purified water) were run in triplicate and MBP and appropriate positive control samples were run in duplicate.

In both activated and non-activated MBP-treated plates, the average number of revertant colonies was less than two-times the number of revertant colonies observed in the negative control plates, up to 5,000 µg/plate. Results from the positive controls indicated that all plates showed increases in the average number of revertant colonies demonstrating appropriate sensitivity of the test system. There was no MBP concentration-dependent increase in revertants, and MBP-related inhibition of growth was not observed.

Based on these results, under these testing conditions MBP was not mutagenic.

2. Acute Oral Toxicity in Rats

Acute oral toxicity of MBP in rats was determined following a single gavage administration of 2,000 mg/kg body weight (Safety Research Institute for Chemical Compounds Co., Ltd. 2000a; Kruger et al. 2005). MBP was dissolved in purified water and was administered to 20 male and 20 female Crj:CD (SD) IGS rats (Charles River Japan, Inc.) at a dose volume of 10 mL/kg body weight. The rats each weighed approximately 158.1 g (males) and 129.5 g

(females). The animals were fasted 17 to 18 hours before and 4 hours after administration. All animals were observed daily from Day 1 to Day 14, on which day each animal was sacrificed in preparation for necropsy.

No mortality was observed during the 14-day study period. There were no abnormalities in general appearance in any animal, and no adverse changes in body weight occurred in any animal group during the study. Additionally, no organ pathology was observed at necropsy on Day 14.

Accordingly, the lethal dose of MBP for 50% of the Crj:CD (SD) IGS rats (LD₅₀) in this test system was estimated to be greater than 2,000 mg/kg body weight.

3. Teratogenicity in Rats

Teratogenicity was evaluated in pregnant Crj:CD (SD) IGS rats (n=20 per dose group) following administration of MBP via gavage at doses of 0 and 2,000 mg/kg body weight/day between Day 7 and Day 17 of gestation (Safety Research Institute for Chemical Compounds Co., Ltd. 2001; Kruger et al. 2005). MBP was dissolved in purified water (also used as control) and administered to each animal at a dose volume of 10 mL/kg body weight. All dams were observed for mortality and clinical conditions twice daily from Day 0 of gestation to necropsy during the administration period, and once daily before initiation and after completion of administration. Body weights were taken on Day 0, Day 3, and daily from Day 7 to Day 20 of gestation. Food consumption was measured on Days 0, 3, 7, 9, 11, 13, 15, 17, and 20. Necropsy was performed on Day 20 of gestation. At necropsy, selected organs and tissues were preserved. Ovaries and uteri were removed and the gravid uteri were weighed. After observation of intrauterine conditions and embryo-fetal and placental conditions, the uteri (after removal of live fetuses and their placentas) were weighed. Implantation index, viability index of fetuses, incidence of dead or resorbed embryos and fetuses, and sex ratio were calculated. The fetal examination consisted of external examination, visceral examination, and skeletal examination.

There were no MBP-related adverse clinical effects observed over the course of the study. There were no differences between treatment and control animals in body weight, body weight gain, food consumption, numbers of corpora lutea, numbers of implantation sites, numbers of live and dead fetuses, numbers of resorbed embryos, viability indices of fetuses, sex ratio, placental weight, and body weight of fetuses. In live fetuses, there were no MBP-related external, visceral, or skeletal anomalies. Based on this study, under these test conditions MBP had no adverse effects on reproduction or development in Crj:CD (SD) IGS rats up to 2,000 mg/kg body weight/day.

4. Four-Week Oral Toxicity in Rats

The toxicity of MBP was assessed in a four-week rat oral gavage study (10 rats/sex/dose group) given dose levels of 0, 1,000, and 2,000 mg MBP/kg/day (Safety Research Institute for Chemical Compounds Co., Ltd. 2000b; Kruger et al. 2005). All animals were observed for mortality, external appearance, and behavior. Body weights were obtained before test article administration, Days 1, 2, 7, 14, 21, and 28 of administration and on the necropsy day. Food consumption was measured for all animals on Days 1, 2, 7, 14, 21, and 28 of administration. In Week 4 of administration, ophthalmology examinations were conducted and urinalysis was performed on urine collected from non-fasted animals using metabolic cages for rats. Before necropsy, rats were fasted and blood was collected for hematology and clinical chemistry. Necropsy was conducted on all animals the day after Day 28 of test article administration. Animals were observed for external appearance and gross pathology was performed. Organs and tissues of all animals were weighed. The organs and tissues fixed and preserved at necropsy were sectioned and stained for histopathological analysis.

No adverse clinical observations were noted in males or females. There were no significant differences in body weights of males or females in the 1,000 or 2,000 mg/kg dose groups compared to control. No treatment related adverse effects were noted in ophthalmological examination of males and females from any dose group. Urinalysis revealed no statistically significant treatment related effects in males or females given the 1,000 mg/kg dose or in females given the 2,000 mg/kg dose. A statistically significant increase in protein excretion was noted in males in the 2,000 mg/kg/day dose group. Despite lack of statistical significance, there appeared to be a dose-related increase in protein excretion noted in the urinalysis for both males and females. No treatment-related adverse findings were noted in hematology or clinical chemistry. There were no statistically significant effects on organs weights, gross pathology or histopathology with the exception of statistically significantly increased absolute and relative liver weights in females from the 2,000 mg/kg/day dose group. This was not considered to be toxicologically significant or treatment related, because there were no corresponding adverse findings in clinical chemistry or histopathology.

5. Subchronic Oral Toxicity in Rats

a) Materials & Methods

A 4-week oral gavage range, finding study in rats, was conducted to set the MBP dose levels for a subsequent 13-week toxicity study (Safety Research Institute for Chemical Compounds Co., Ltd. 2000c; Kruger et al. 2005). Based upon results of this 4-week study (Safety Research Institute for Chemical Compounds Co., Ltd. 2000b), MBP doses of 0, 200, and 2,000 mg/kg body weight/day were administered for 91 days via gavage to groups of 10 male and 10 female Crj:CD (SD) IGS rats (Charles River Japan, Inc.) (Safety Research Institute for

Chemical Compounds Co., Ltd. 2000c; Kruger et al. 2005). MBP was dissolved in purified water (which also was used as control) and doses were administered at a dose-volume of 10 mL/kg body weight. Body weight and food consumption were measured and recorded on Days 1, 2, 7, and weekly thereafter. The general physical condition of each animal was observed once per day during the study. In Week 13, urinalysis (i.e., pH, protein, glucose, ketone body, urobilinogen, bilirubin, occult blood, urinary sediment, volume, specific gravity, sodium, potassium, and chloride) and ophthalmologic examinations were conducted. Before necropsy, a blood sample was collected from the abdominal aorta of each animal under anesthesia for hematological evaluations and clinical chemistry evaluations. Hematology parameters examined were red blood cells, hematocrit, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, platelet count, white blood cells, hemogram of white blood cells, prothrombin time, and activated partial thromboplastin time. Clinical chemistry parameters examined were aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ -GTP), alkaline phosphatase, glucose, total cholesterol, triglyceride, total bilirubin, urea nitrogen, creatinine, sodium, potassium, chloride, calcium, inorganic phosphorous, total protein, albumin, albumin to globulin ratio, and protein fractions.

Necropsies were performed on all animals at the termination of the study and weights were determined for the following organs: heart, liver, spleen, kidneys, adrenals, prostate, testes, seminal vesicles (including the coagulating glands), ovaries, uterus, brain, pituitary, salivary glands (submandibular and sublingual glands), thymus, lung, and thyroid (including parathyroid).

Histopathological examinations were performed for all animals in the control and high dose groups. Most tissues (i.e., skin, mammary gland, mandibular lymph nodes, mesenteric lymph node, thoracic aorta, submandibular glands, sublingual glands, parotid glands, sternum, femur, thymus, trachea, lung (including bronchus), heart, thyroids, parathyroids, tongue, larynx, esophagus, stomach (including forestomach and glandular stomach), duodenum, jejunum, ileum (including agmen peyerianum), cecum, colon, rectum, liver, pancreas, spleen, kidneys, adrenals, urinary bladder, seminal vesicles (including coagulating gland), prostate, ovaries, oviducts, uterus (uterine horns and cervix), vagina, brain (including cerebrum and cerebellum), pituitary gland, sciatic nerve, skeletal muscle, spinal cord, nasal cavity (turbinate), and Zymbal's gland) were fixed and preserved in 10% neutral buffered formalin; eyeballs and harderian gland were fixed and preserved in Davidson's fixative; testes and epididymides were fixed in Bouin's solution and preserved in 70% ethanol.

One-way parametric ANOVA with Dunnett's test was used to compare body weights, body weight gain, food consumption, feed efficiency, quantitative parameters of urinalysis (except specific gravity), hematological values, blood chemistry values, and organ weights. The

Kruskal-Wallis test with the Mann-Whitney U-test was used to analyze the qualitative parameters of urinalysis and specific gravity.

b) Results

(1) Clinical observations

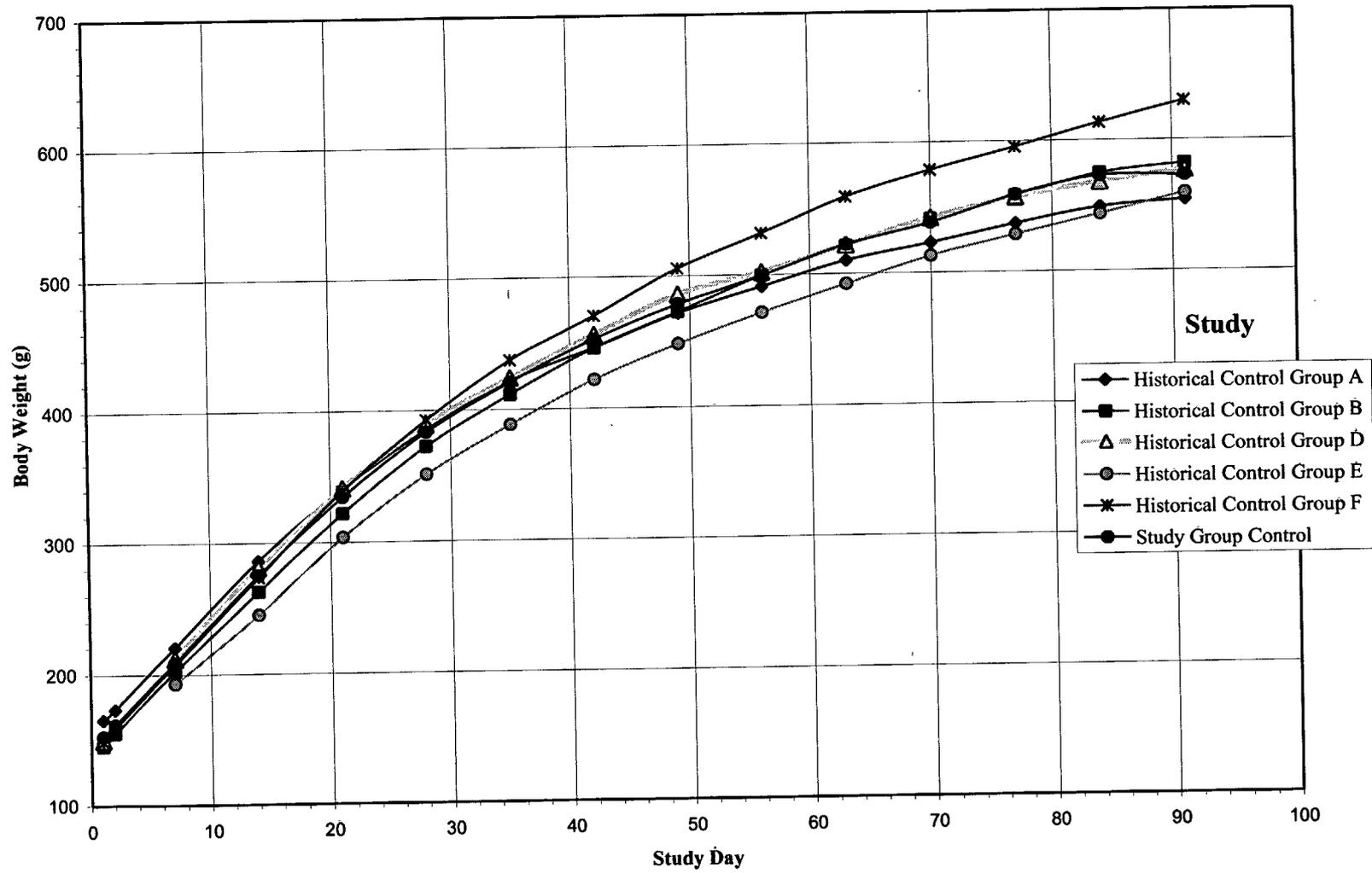
No MBP-related adverse clinical effects were observed in any animal. One female in the 2,000 mg/kg body weight/day dose group exhibited loss of the 4th digit of the right forelimb on Day 49, but this effect was not considered treatment-related.

(2) Body weight

A statistically significant decrease in group mean body weight was observed in female animals in the 200 mg/kg/day dose group on Day 28. This difference was considered anomalous and not treatment-related. A statistically significant decrease in group mean body weight was observed in female animals in the 2,000 mg/kg/day dose group between Days 14 through 56 compared to controls. The effect on body weight did not occur in males at this dose level and was not seen from day 63 to the end of the study. There was no statistically significant difference in body weight change over the duration of the study for males or females (i.e., between Days 1 to 91). A comparison of group mean body weights with historical control body weights does not show any treatment related decrease in body weights of the male or female animals from the 2,000 mg/kg/day dose group compared to historical ranges (Figures 5 and 6).

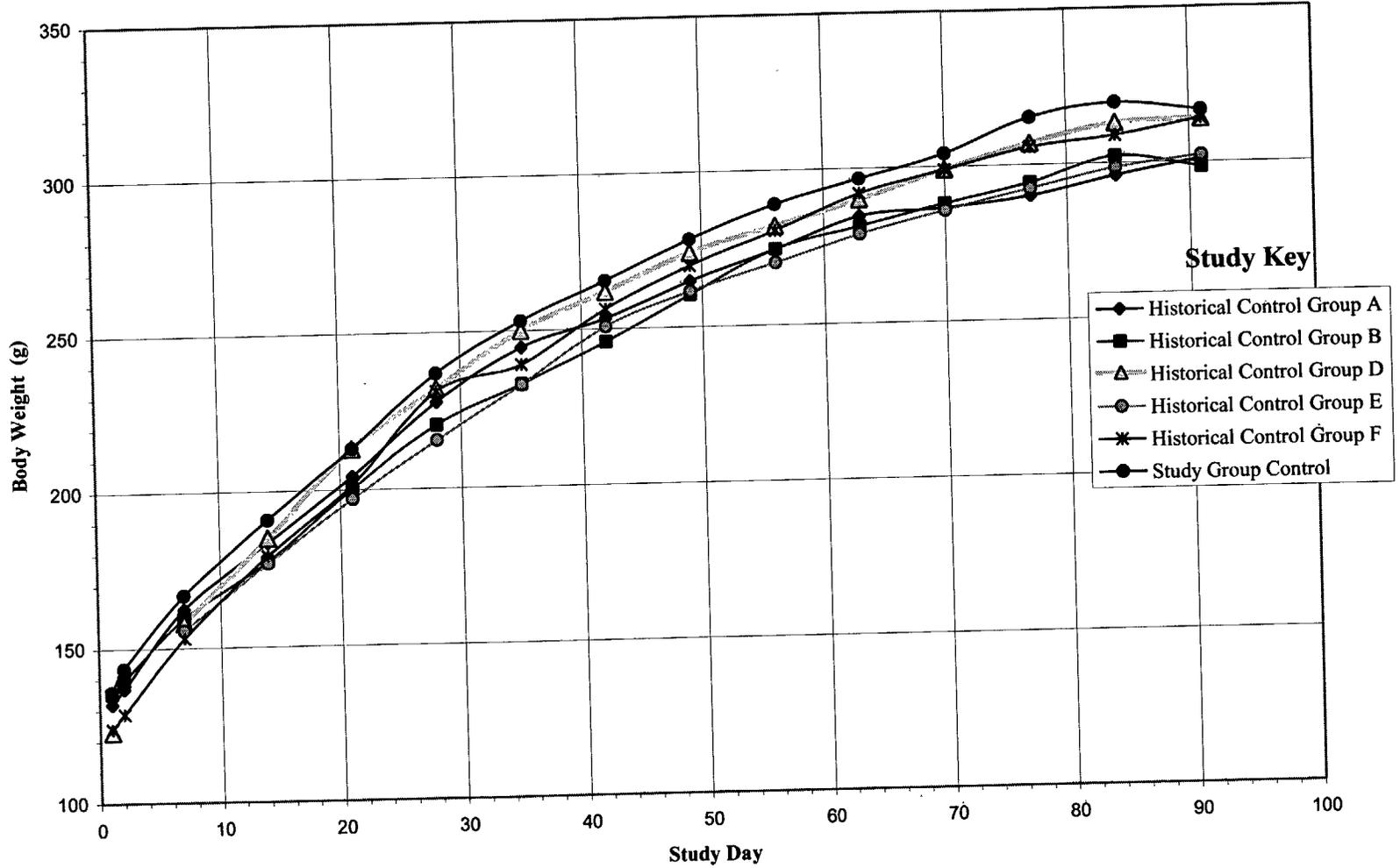
The following statistically significant differences in group mean body weight gain were observed between treated and control animals: in female animals of the 200 mg/kg/day dose group, an increase on Days 84 through 91; in female animals of the 2,000 mg/kg/day dose group, a decrease on Days 14 through 21; and in male animals of the 2,000 mg/kg/day dose group, an increase between Days 84 and 91. Differences observed in body weight gain between treated and control animals were not consistent over time or across sexes and the changes did not occur in a dose-dependent manner.

Figure 5. Body Weights in Male Rats (Study Group Control vs. Historical Controls)



690000

Figure 6. Body Weights in Female Rats (Study Group Control vs. Historical Controls)



000070

(3) *Food Consumption*

No statistically significant differences in group mean food consumption were observed in animals in the 200 mg/kg/day group compared with controls. In male animals from the 2,000 mg/kg/day dose group, a statistically significant decrease in group mean food consumption was observed on Day 77; however this finding was considered anomalous and not biologically significant. In female animals in the 2,000 mg/kg/day dose group, a statistically significant decrease in group mean food consumption was observed between Days 14, 35, and 49, which correlated with the period during which these animals had statistically significantly lower body weights. Food consumption was not statistically significantly different between controls and females in the 2,000 mg/kg/day dose group from Day 56 to the end of the study. A comparison of the food consumption from males and females of the 2,000 mg/kg/day dose group with historical controls does not show any adverse treatment related effect on food intake compared to historical ranges.

(4) *Feed Efficiency*

In the 200 mg/kg/day dose group, statistically significantly increased group mean feed efficiency was observed in female animals between Days 84 and 91, and statistically significantly decreased group mean feed efficiency was observed in male animals between Days 21 and 28. In the 2,000 mg/kg/day dose group, statistically significantly group mean increased feed efficiency was observed in male animals between Days 84 and 91; however, no differences were observed in female animals of this dose group. Because any differences in feed efficiency were inconsistent over time and between sexes, and they did not occur in a dose-dependent manner, these effects were not considered adverse or treatment-related.

(5) *Ophthalmic Effects:*

No adverse or treatment-related ophthalmic effects were observed in any animals at any dose group (data not shown).

(6) *Urinalysis*

Urinalysis results (Table 9) showed a dose-dependent increase in sodium and chloride excretion in male and female animals. The values for sodium excretion, however, were well within the range of historical controls (male, from 0.838 ± 0.561 to 1.918 ± 0.300 mEq/21 hours; female, from 0.681 ± 0.212 to 1.613 ± 0.417 mEq/21 hours) and the values for sodium excretion for the male and female control animals in this study were very low compared to background (0.513 ± 0.265 and 0.542 ± 0.213 mEq/21 hours for males and females, respectively). Similarly, values for chloride excretion were well within the range of historical controls (male, from 0.972 ± 0.677 to 2.549 ± 0.391 mEq/21 hours; female, from 0.665 ± 0.374 to 2.056 ± 0.445 mEq/21 hours) and the values for chloride excretion for the male and female control animals in this study

were very low compared to background (0.944 ± 0.426 and 0.796 ± 0.288 mEq/21 hours for males and females, respectively). Accordingly, the statistically significant differences of the sodium and chloride excretion values in the treated animals were attributed to unusually low control values for sodium and chloride excretion and were not considered to be a biologically adverse finding. No dose-dependent significant effect was seen on potassium excretion in female rats and no significant effects on potassium excretion were seen in male rats.

One (out of 10 total) male animal in the 200 mg/kg/day dose group exhibited a glucose-positive reaction in urine; no males in the 2,000 mg/kg/day dose group and females in either dose group exhibited a glucose-positive reaction in urine. A statistically significant increase in protein excretion was noted in males and females of the 2,000 mg/kg/day dose group. A statistically significant increase in urine specific gravity was observed in males of this dose group. Urinary protein was detected qualitatively using Multistix test paper. It is reasonable to suggest that the qualitative determination of increased protein excretion in the urine with increasing doses of the protein containing test article, MBP, may be due in part to the excretion of protein and not damage to the kidney. This is corroborated by a lack of change in organ weight or histopathology in the kidneys.

Table 9. Urinary Findings in Rats Administered MBP for 13 Weeks^(a)

Parameter	Males ^(b)			Females ^(b)		
	Control	200 mg/kg	2,000 mg/kg	Control	200 mg/kg	2,000 mg/kg
pH						
6.5	0	0	0	0	0	1
7.0	0	0	0	1	0	1
7.5	0	0	0	2	2	2
8.0	0	0	1	3	3	2
8.5	10	10	9	4	5	4
Protein						
-	0	0	0	5	2	1
±	7	4	1	5	7	6
+	3	6	6	0	0	3*
++	0	0	3**	0	1	0
Glucose						
-	10	9	10	10	10	10
+	0	1	0	0	0	0
Ketone bodies -	10	10	10	10	10	10
Urobilinogen (0.1 EU/dL)	10	10	10	10	10	10
Bilirubin -	10	10	10	10	10	10
Occult blood						
-	9	10	9	10	10	9
±	1	0	0	0	0	1
+	0	0	1	0	0	0
Specific gravity:						
1.011-1.020	0	1	0	0	0	0
1.021-1.030	4	2	1	1	1	0
1.031-1.040	5	3	3	3	0	4
1.041-1.050	1	4	3	4	8	4
>1.050	0	0	3**	2	1	2
Volume (mL/21 hr)	18.65 ± 2.71	18.30 ± 5.97	19.05 ± 6.54	10.10 ± 4.31	10.90 ± 2.02	11.40 ± 3.27
Na (mEq/21 hr)	0.513 ± 0.265	0.949 ± 0.464*	1.183 ± 0.333**	0.542 ± 0.213	0.870 ± 0.267*	0.882 ± 0.261**
K (mEq/21 hr)	2.525 ± 0.701	2.858 ± 0.722	3.078 ± 0.926	1.741 ± 0.502	2.355 ± 0.392*	1.984 ± 0.740
Cl (mEq/21 hr)	0.944 ± 0.426	1.355 ± 0.618	1.864 ± 0.583**	0.796 ± 0.288	1.321 ± 0.348**	1.335 ± 0.443**

NOTES:

Data source: Safety Research Institute for Chemical Compounds Co., Ltd. 2000c.

(a) Each value is the number of animals (i.e., pH, protein, glucose, ketone body, urobilinogen, bilirubin, occult blood, and specific gravity) or the group mean ± SD (i.e., volume, Na, K, and Cl). Na, sodium; K, potassium; Cl, chloride.

(b) n = 10 per group.

*p ≤ 0.05

**p ≤ 0.01

(7) Hematology:

There were no statistically significant differences in hematological values in any animals at any dose group compared with control animals. In one male animal in the 200 mg/kg/day

dose group, a high white blood cell count (i.e., 26,900/ μ L) and an increase in segmented cells (i.e., 27%) was observed; however this effect was not considered treatment-related.

(8) *Blood Chemistry:*

Compared with the control group, there were no statistically significant differences in blood chemistry values in male and female animals in the 200 mg/kg/day dose group or female animals in the 2,000 mg/kg/day dose group. In male animals in the 2,000 mg/kg/day dose group, there was a statistically significant differences in potassium concentration, compared with control animals; however, this effect was not considered biologically significant because it only occurred in males, there was no change in urine potassium excretion values, and there were no abnormalities observed in the kidneys or adrenals in these animals.

(9) *Necropsy & organ weights:*

There were no adverse treatment-related effects observed in any animal at necropsy. There were no statistically significant differences in absolute or relative organ weights in any animals at any dose compared with control animals.

(10) *Histopathology*

No treatment-related effects were recorded following the histological examination of organs with the exception of evidence of kidney damage in one male in the 2,000 mg/kg/day dose group. In the males of the 2,000 mg/kg/day dose group, the following effects were observed and noted: accumulation of foam cells in the lungs and cellular infiltration in the prostate. In the female animals of the 2,000 mg/kg/day dose group, fibrosis in the pleura and cysts in the pituitary gland were observed. Because these findings were also found in the controls and there was a low incidence of these effects, none were considered treatment-related.

With the exception of one male in the 2,000 mg/kg/day group, a low incidence of hyaline casts and cellular infiltration of lymphocytes were observed in both control and treated male and female animals. Therefore, these findings were not considered to be treatment-related (Table 10). In male number 310 from the 2,000 mg/kg/day group, there was evidence of a slight renal alteration reflected in the tubular epithelium indicative of an infection. This finding was not considered to be related to test article toxicity.

Table 10. Histopathological Findings^(a)

Female, Control										
Animal No.	151	152	153	154	155	156	157	158	159	160
Right kidney: Cast, hyaline	-	-	-	-	+	-	-	-	-	-
Left kidney	N	N	N	N	N	N	N	N	N	N
Female, MBP 2,000 mg/kg										
Animal No.	351	352	353	354	355	356	357	358	359	360
Right kidney	N	N	N	N	N	N	N	N	N	N
Left kidney	N	N	N	N	N	N	N	N	N	N
Male, Control										
Animal No.	101	102	103	104	105	106	107	108	109	110
Right kidney: Cast, hyaline	-	-	-	-	+	-	-	-	-	-
Left kidney: Cellular infiltration, lymphocyte	-	-	-	-	-	-	-	+	-	-
Male, MBP 2,000 mg/kg										
Animal No.	301	302	303	304	305	306	307	308	309	310
Right kidney: Cast, hyaline	-	-	-	-	-	-	+	-	-	+
Cellular infiltration, lymphocyte	-	+	-	-	-	-	-	-	-	-
Regeneration, tubular epithelium	-	-	-	-	-	-	-	-	-	+
Dilatation, tubule	-	-	-	-	-	-	-	-	-	+
Left kidney: Cast, hyaline	-	-	-	-	-	-	+	-	-	-
Cellular infiltration, lymphocyte	-	-	-	-	-	-	-	-	-	+
Regeneration, tubular epithelium	-	-	-	-	-	-	-	-	-	+
NOTE:										
(a) N = no abnormal findings, - = normal and + = slight change.										

c) Discussion and Conclusion

Female rats, having received an MBP dose of 2,000 mg/kg/day via gavage, exhibited decreases in body weight and food consumption during the study. However, the differences in body weight were not observed on or after Day 63 of administration, and the differences in food consumption were not observed on or after Day 56 of administration. This effect was

accompanied by a statistically significant decrease in food consumption between Day 14 and Day 49 (excluding Day 42). Feed efficiency during this period was not adversely affected. In addition, recovery was observed for both parameters, and body weights and food consumptions were comparable to ranges seen for historical controls. There were no similar differences in body weight and food consumption observed for male animals of the 2,000 mg/kg/day dose group. Therefore, the statistically significant effects noted for body weight and food consumption in female animals of the 2,000 mg/kg/day dose group were not considered to be biologically significant. Statistically significant increases in sodium and chloride excretion in the treated animals at the 2,000 mg/kg/day dose were considered to be attributed to unusually low control values and not considered to be a biologically adverse finding. A qualitative determination of significantly increased protein excretion in the urine with increasing dose is attributed in part to the excretion of protein after ingestion of the protein containing test article and not damage to the kidney.

One male in the 2,000 mg/kg/day dose group exhibited evidence of slight renal alteration reflected in the tubular epithelium. There was a finding of cellular infiltration of lymphocytes in this animal indicative of an infection. The conclusion that the protein and electrolyte data are not indicative of direct toxicity is corroborated by a lack of overall change in organ weight or histopathology of the kidney.

Accordingly, based on this study, the no-observed adverse effect level of MBP in rats was 2,000 mg/kg body weight/day.

D. HUMAN CLINICAL TRIALS

In a study evaluating the human health effects of MBP consumption, 30 healthy adult men consumed daily 5 bottles of an MBP-containing beverage (60 mg MBP per bottle, 300 mg MBP per day) for 17 days (Snow Brand Milk Products 2000a). Participants were examined 5 days before study commencement (prior to MBP ingestion; Exam 1), after 2 days of MBP ingestion (Exam 2), after 16 days of MBP ingestion (Exam 3), and 7 days after the termination of MBP ingestion (Exam 4). During each of the 4 examination periods, the following data were evaluated: body weight, body fat percentage, blood pressure, hematology, serum biochemistry, serum immunology, and urinalysis. Hematological parameters examined were white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), and platelet count. Serum biochemistry parameters examined were total protein, albumin/globulin ratio (A/G), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (γ -GTP), leucine amino peptidase (LAP), total bilirubin, creatinine, urea nitrogen, uric acid, creatinine phosphokinase (CPK), total cholesterol, free cholesterol, ester-type cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, apoprotein AI, apoprotein AII, apoprotein B, apoprotein E, sodium

(Na), potassium (K), chloride (Cl), calcium (Ca), inorganic phosphorus (IP), and magnesium (Mg). Serum immunology parameters examined were immunoglobulins G and E (IgG and IgE), and RAST-milk allergy screen. Urine analytical parameters examined were protein, glucose, urobilinogen, bilirubin, ketone bodies, occult blood, pH, Na, K, Cl, Ca, IP, and Mg.

Subjective symptoms (e.g., abdominal pain, bloating, dyspepsia, diarrhea, nausea, vomiting, anorexia, rash, itching, facial hot flushes, poor circulation, drowsiness, irritated feeling, fatigue, headache) were reported each day using a standardized questionnaire. The questionnaire was also used to record use of medicines (e.g., medicine for cold or constipation, antibiotics), consumption of food that might induce diarrhea (e.g., beverage containing dietary fiber or oligosaccharide), habitual food consumption (e.g., coffee, alcohol), and treatment-related compliance.

Quantitative data were analyzed by one-way analysis of variance (ANOVA); any differences in group mean values (i.e., before, during, and after MBP consumption) were then analyzed by Dunnett's multiple comparison test. For qualitative data, the number of occurrences outside the standard range was counted, and the differences in group mean values before, during, and after MBP consumption were analyzed using the chi-square test. Data from all 30 participants were included in the analysis; although over the 17 days of the study, some participants did not consume the MBP supplemented beverage every day. Four participants did not consume the beverage on one day (i.e., 300 mg MBP), and 1 participant did not consume the beverage on 2 days (600 mg MBP). In addition, for 6 participants, "some examinations were delayed for several days" (not otherwise specified).

No significant changes in group mean body weights, fat percentages, blood pressures, or hematology (Table 11), serum immunology (Table 12), or urinalysis (Table 13) parameters were observed. Upon review of the individual participant data, none were indicative of any clinical abnormalities.

Table 11. Hematology Results in 30 Healthy Adult Men Before, During, and After Daily Consumption of MBP

Parameter (Units)	Standard Range	Pre-treatment		During Treatment				7 Days Post-treatment	
				After 2 Days		After 16 Days			
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
WBC (/ μ L)	3500-9700	5707	1355	5716	1320	5898	1506	5932	1667
RBC ($\times 10^4$ / μ L)	438-577	498	28	492	29	493	29	488	32
Hb (g/dL)	13.6-18.3	15.3	0.8	15.0	0.8	15.2	0.8	15.1	0.8
Ht (%)	40.4-51.9	46.5	2.2	45.3	2.1	47.0	2.1	46.7	2.4
Platelet ($\times 10^4$ / μ L)	14.0-37.9	25.5	4.6	25.5	4.5	25.4	3.7	24.9	4.7

NOTES:

Data source: Snow Brand Milk Products 2000a.

ABBREVIATIONS:

WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin; Ht, hematocrit.

Table 12. Serum Immunology Results in 30 Healthy Adult Men Before, During, and After Daily Consumption of MBP

Parameter (Units)	Standard Range	Pre-treatment		During Treatment				7 Days Post-treatment	
				After 2 Days		After 16 Days			
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
IgG (mg/dL)	820-1740	1279	228	1253	232	1231	216	1217	216
IgE (IU/mL)	<250	291	309	296	312	269	296	280	322
RAST-Milk ^(a) (Ua/mL)	<0.34	30	NA	30	NA	30	NA	30	NA

NOTES:

Data source: Snow Brand Milk Products 2000a.

(a) RAST-Milk data are expressed as the number of subjects within the standard range (i.e., <0.34).

ABBREVIATION:

NA: Not applicable.

Table 13. Urinalysis Results in 30 Healthy Adult Men Before, During, and After Daily Consumption of MBP

Parameter (Units)	Standard Range	Pre-treatment		During Treatment				7 Days Post-treatment	
		Mean	SD	After 2 Days		After 16 Days		Mean	SD
				Mean	SD	Mean	SD		
Protein ^(a)	(-) or (±)	30	NA	30	NA	30	NA	30	NA
Sugar ^(a)	(-) or (±)	30	NA	30	NA	30	NA	30	NA
pH	4.8-7.5	6.0	0.8	5.9	0.7	6.2	0.6	6.0	0.6
Urobilinogen ^(a)	(±)	29	NA	30	NA	30	NA	29	NA
Bilirubin ^(a)	(-)	30	NA	30	NA	30	NA	30	NA
Ketone ^(a)	(-)	29	NA	30	NA	30	NA	30	NA
Occult blood ^(a)	(-)	28	NA	29	NA	30	NA	29	NA
Na (g/L)	NA	3.6	1.2	3.4	1.2	3.3	1.2	3.4	1.3
K (g/L)	NA	3.5	1.1	3.2	1.5	3.0	1.0	3.3	1.2
Cl (g/L)	NA	7.0	2.1	6.1	2.2	6.0	2.1	6.1	2.3
Ca (g/L)	NA	0.14	0.07	0.13	0.09	0.12	0.06	0.14	0.07
IP (g/L)	NA	0.84	0.35	0.88	0.53	0.71	0.39	1.03	0.59
Mg (g/L)	NA	0.08	0.05	0.07	0.05	0.07	0.05	0.09	0.06

NOTES:

Data source: Snow Brand Milk Products 2000a.

(a) These data are expressed as the number of participants within the specified standard range.

ABBREVIATIONS:

Na, sodium; K, potassium; Cl, chloride; Ca, calcium; IP, inorganic phosphorous; Mg, magnesium; NA, not available.

Of the serum biochemistry parameters evaluated, there were only five parameters that had significant differences relative to pre-treatment values (Table 14). Group mean creatinine levels were decreased at Exams 2 and 3 (i.e., 2 and 16 days after the initiation of MBP ingestion, respectively); however, the group mean creatinine values were within the standard range at all exams. Free cholesterol levels were increased at Exam 4 (i.e., 7 days post-termination of ingestion); however, the group mean pre-feeding value (i.e., Exam 1) was at the high end of the standard range, and the change at Exam 4 was not considered biologically significant. Potassium levels were increased after 2 days of ingestion (i.e., Exam 2), but group mean potassium values were within the standard range at all exams. Chloride levels were increased at Exams 2 and 4 (i.e., after 2 days of ingestion and 7 days post-termination of ingestion), but group mean chloride values were within the standard range at all exams. Magnesium levels were decreased after 2 and 16 days of ingestion (i.e., at Exams 2 and 3), but all values were within the standard range. For all parameters, review of individual data did not reveal any clinical abnormalities.

Table 14. Serum Biochemistry Results in 30 Healthy Adult Men Before, During, and After Daily Consumption of MBP

Parameter (units)	Standard Range	Pre-treatment		During Treatment				7 Days Post-treatment	
		Mean	SD	After 2 Days		After 16 Days		Mean	SD
				Mean	SD	Mean	SD		
Total protein (g/dL)	6.5-8.2	7.3	0.3	7.2	0.4	7.2	0.4	7.2	0.3
A/G	1.3-2.0	1.69	0.22	1.73	0.18	1.68	0.2	1.73	0.19
AST (U/L)	10-40	23	6	23	7	21	5	23	6
ALT (U/L)	5-45	22	8	20	7	20	8	20	8
LDH (U/L)	220-430	315	45	304	46	307	49	317	53
ALP (U/L)	104-338	187	53	189	53	189	52	184	57
γ -GTP (U/L)	16-73	37	24	35	22	35	22	35	24
LAP (U/L)	30-78	52	8	52	7	51	7	52	8
Total bilirubin (mg/dL)	0.2-1.0	0.8	0.2	0.6	0.2	0.7	0.2	0.8	0.3
Creatinine (mg/dL)	0.8-1.3	1.1	0.1	1.0**	0.1	1.0**	0.1	1.0	0.1
Urea nitrogen (mg/dL)	8-20	15.6	3.4	15.8	4.4	14.9	3.5	16.7	5.0
Uric acid (mg/dL)	<7	5.6	1.0	5.8	1.0	5.8	1.1	5.7	0.9
CPK (U/L)	50-230	155	76	160	102	169	85	169	107
Total cholesterol (mg/dL)	150-219	196	28	190	25	193	27	194	26
Free cholesterol (mg/dL)	25-60	56	12	60	10	55	9	66**	11
Cholesterol ester (mg/dL)	100-185	140	22	130	20	139	22	128	22
HDL (mg/dL)	41-80	62	16	61	17	63	17	62	16
LDL (mg/dL)	70-139	117	28	110	22	117	27	113	27
Triglycerides (mg/dL)	50-149	85	58	119	178	85	50	88	51
Apo AI (mg/dL)	119-155	157	28	161	28	163	31	154	26
Apo AII (mg/dL)	25.9-35.7	33.4	4.8	33.3	5.6	33.8	4.3	33.2	4.1
Apo B (mg/dL)	73-109	91	25	90	21	92	24	87	24
Apo E (mg/dL)	2.7-4.3	3.9	1	3.9	1.3	3.9	0.8	3.9	0.9
Na (mEq/L)	135-145	143	1	142	1	142	1	143	1
K (mEq/L)	3.5-5.0	3.8	0.3	4.1**	0.4	3.9	0.3	3.8	0.3
Cl (mEq/L)	98-108	101	2	102**	2	101	2	102*	2
Ca (mEq/L)	4.1-5.0	4.7	0.1	4.6	0.1	4.6	0.1	4.6	0.2
IP (mg/dL)	2.5-4.2	3.7	0.5	3.5	0.4	3.5	0.5	3.7	0.5
Mg (mg/dL)	1.7-2.6	2.24	0.13	2.14**	0.13	2.16*	0.12	2.22	0.11

NOTES:

Data source: Snow Brand Milk Products 2000a.

*Significantly different from pre-treatment, $p < 0.05$; **Significantly different from pre-treatment, $p < 0.01$.

ABBREVIATIONS:

A/G, albumin/globulin ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; γ -GTP, gamma glutamyl transpeptidase; LAP, leucine amino peptidase; CPK, creatine phosphokinase; HDL, high density lipoprotein; LDL, low density lipoprotein; Apo, apoprotein; Na, sodium; K, potassium, Cl, chloride; Ca, calcium; IP, inorganic phosphorous; Mg, magnesium.

Seventeen of the 30 study participants did not report adverse physical events during the study. Table 15 presents symptoms and adverse events reported by 13 of the study participants over the duration of the study. The symptoms were considered transient and not treatment-related.

Table 15. Symptoms Reported During 17 Days of MBP Consumption by 13 of 30 Healthy Adult Men

Symptom	Participant No.												
	1	2	4	6	8	9	10	15	19	26	27	28	30
Abdominal pain	1				2								4
Diarrhea	1			1				2	1	1			
Loose stools		1											
Vomiting		1											
Headache		1				1							
Physical deconditioning			2									2	
Fatigue				3			1				5		
Languor				2									
Anorexia								1					
Runny nose											7		
Sneezing											7		

NOTES:

Data source: Snow Brand Milk Products 2000a.

Data are the number of reports of each symptom per participant over duration of study. Seventeen of the 30 participants reported no symptoms.

Based on these results, no adverse health effects (based on subjective symptoms, hematology, clinical chemistry, serum immunology, and urinalysis) were associated with the consumption of 300 mg MBP per day by healthy adult men for 17 days.

In a trial² to determine the effects of MBP consumption on bone formation in human males (Toba *et al.* 2001), 30 healthy male participants ingested 300 mg MBP per day via consumption of a beverage for 16 days. Blood and urine were collected from each participant for analysis before the study and after 16 days of MBP consumption, in order to monitor biochemical markers of bone metabolism. In serum, the following parameters were examined: calcium, osteocalcin, and procollagen I carboxy-terminal propeptide (PICP); in urine, calcium and cross-linked N-teleopeptidases of type of collagen (NTx) were monitored. Each participant had a physical examination during each week of the study.

There was no statistically significant difference in group mean body weight or-group mean body mass index before and after 16 days of MBP consumption. In serum biochemistry, group mean osteocalcin, a biochemical marker of bone formation, was statistically significantly increased after 16 days of MBP consumption; however, calcium and PICP were not different. In urine biochemistry, group mean NTx, a biochemical measure of bone resorption, was statistically significantly decreased after 16 days of MBP consumption; however calcium was not different (Table 16). In addition, urinary NTx excretion was correlated with serum osteocalcin

² This appears to have the same participants as the Snow Brand Milk Products 2000a study.

concentration after 16 days of MBP consumption (correlation coefficient = 0.6457, $p < 0.0001$), but not before consumption (correlation coefficient = 0.0641, $p = 0.7366$) (Table 17).

These results suggest that 300 mg MBP per day for 16 days promoted bone formation and suppressed bone resorption in healthy adult men.

Table 16. Biochemistry in 30 Healthy Male Adults Before and After Daily Consumption of MBP

Parameter (Units)	Pre-treatment		After Treatment	
	Mean	SD	Mean	SD
Serum				
Calcium (mmol/L)	2.3	0.1	2.3	0.1
Osteocalcin (ng/mL)	3.7	1.8	5.4*	1.8
PICP (ng/mL)	122.3	37.0	130.0	44.1
Urine				
Calcium (mmol/mmol Cr)	0.21	0.11	0.23	0.10
NTx (nmol/mmol Cr)	31.5	10.2	26.8*	9.6

NOTES:
 Data source: Toba *et al.* 2001.
 *Statistically significantly difference from pre-treatment, $p < 0.0001$.
 ABBREVIATIONS:
 PICP, procollagen I carboxy-terminal propeptide; NTx, cross-linked N-telopeptidases of type I collagen; Cr, creatinine.

Table 17. Relationship Between Urinary NTx Excretion and the Serum Osteocalcin Concentration Before and After 16 Days of MBP Consumption

	Pre-treatment	p Value	After Treatment	p Value
Correlation coefficient	0.0641	0.7366	0.6457	<0.0001

NOTES:
 Data source: Toba *et al.* 2001.
 Differences are considered significant if $p < 0.05$.
 ABBREVIATIONS:
 NTx, cross-linked N-telopeptidases of type I collagen.

In a randomized, placebo-controlled, double blind study, 34 healthy adult women consumed daily a beverage containing either 40 mg MBP (n=18) or a placebo (n=16) for 6 months (Snow Brand Milk Products 2000b). The study was conducted under the direction of medical doctors and the code of ethics of the World Medical Association (i.e., the Helsinki declaration). Participants were examined before study commencement (prior to MBP ingestion; Exam 1), after 3 months of MBP ingestion (Exam 2), and after 6 months of MBP ingestion (Exam 3). During each of the 3 examination periods, the following parameters were evaluated: body weights, subjective symptoms reporting, urinalysis, hematology, serum biochemistry, and serum immunology (IgE). Urinalysis parameters examined were Na, K, Cl, Ca, IP, and Mg.

Hematology parameters examined were WBC, RBC, Hb, Ht, and platelet count. Serum biochemical parameters examined were total protein, albumin, AST, ALT, LDH, ALP, γ -GTP, total bilirubin, creatinine, urea nitrogen, uric acid, total cholesterol, ester-type cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, Na, K, Cl, Ca, IP, and Mg. Statistics included analysis of the differences between treatment groups for each laboratory parameter by the t-test, except IgE. Because IgE was not measured in the placebo group, changes between 0 and 6 months in the treated group were evaluated using a paired t-test.

None of the 34 participants dropped out during the 6-month study period, and none of the participants reported symptoms of nausea, diarrhea, or allergies. There were no treatment-related significant differences between treated and placebo groups in group mean body weights or hematology parameters examined (Table 18). Group mean Hb values were significantly different at all exams (i.e., 0, 3, and 6 months) in the MBP-treated group compared with the placebo group; however all group mean values were within the standard range. Additionally, upon examination of individual hematology parameter data, no clinically problematic cases were observed.

Group mean urinalysis values were not significantly different between placebo and MBP-treated individuals after 6 months in the study (Table 19). Group mean Cl was significantly decreased in the MBP-treated group at the Exam 2 compared with the placebo group; however, the biological significance of this difference is uncertain. Additionally, all group mean values were within the standard range, and upon examination of individual urinalysis data, no problematic cases were observed.

Group mean serum biochemistry values were not significantly different in MBP-treated individuals after 6 months of study compared with the placebo group (Table 20). In addition, there were no significant changes in IgE levels in the MBP-treated group after 6 months in the study (compared with pre-treatment). Group mean HDL cholesterol was significantly increased in the MBP-treated group at the 3-month exam compared with the placebo group; however the biological significance of this difference is uncertain, especially because there was no difference reported at the 6-month exam. Additionally, upon examination of individual serum biochemistry parameters, no clinically problematic cases were observed.

Based on these results, no adverse health effects (based on subjective symptoms, hematology, clinical chemistry, and urinalysis) were associated with the consumption of 40 mg per day of MBP by healthy adult women for 6 months.

Table 18. Hematology Results in Healthy Adult Women Before and After Daily Consumption of MBP for 6 Months

Parameter (Units)	Standard Range	Placebo ^(a)				MBP ^(b)			
		Before Treatment		After 6 Months		Before Treatment		After 6 Months	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
WBC (/μL)	3500-9700	5762	1482	5888	1972	5744	1251	5691	1664
RBC (x10 ⁴ /μL)	376-516	441	31	450	28	438	30	449	30
Hb (g/dL)	11.2-15.2	13.3	0.7	13.6	0.7	12.6*	0.9	13.0*	0.7
Ht (%)	34.3-45.2	41.5	2.4	43.3	2.1	40.1	2.5	42.4	1.8
Platelet (x10 ⁴ /μL)	14.0-37.9	27.7	5.7	30.5	6.5	26.8	4.8	29.2	5.6

NOTES:

Data source: Snow Brand Milk Products 2000b.

(a) n = 16

(b) n = 18

*Significantly different from placebo, p<0.05.

ABBREVIATIONS:

WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin; Ht, hematocrit.

Table 19. Urinalysis Results in Healthy Adult Women Before and After Daily Consumption of MBP for 6 Months

Parameter (Units)	Standard Range	Placebo ^(a)				MBP ^(b)			
		Before Treatment		After 6 Months		Before Treatment		After 6 Months	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Na (mEq/L)	137-256	139	58	147	56	120	54	125	57
K (mEq/L)	35-90	86	34	85	30	77	33	74	32
Cl (mEq/L)	140-260	173	57	184	64	131	70	148	65
Ca (mg/dL)	NA	9.9	7.2	9.9	5.4	11.3	9.8	9.4	5.8
IP (mg/dL)	NA	51	30	83	38	59	32	57	37
Mg (mg/dL)	NA	6.1	2.6	7.4	4.5	8.8	5.2	7.2	4.0

NOTES:

Data source: Snow Brand Milk Products 2000b.

(a) n = 16

(b) n = 18

ABBREVIATIONS:

Na, sodium; K, potassium; Cl, chloride; Ca, calcium; IP, inorganic phosphorous; Mg, magnesium; NA, not available.

Table 20. Serum Biochemistry Results in Healthy Adult Women Before and After Daily Consumption of MBP for 6 Months

Parameter (Units)	Standard Range	Placebo ^(a)				MBP ^(b)			
		Before Treatment		After 6 Months		Before Treatment		After 6 Months	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total protein (g/dL)	6.5-8.0	7.4	0.3	7.5	0.4	7.3	0.3	7.4	0.3
Albumin (g/dL)	4.3-5.5	4.7	0.2	4.7	0.2	4.6	0.2	4.7	0.2
AST (U/L)	10-37	21	5	22	5	19	9	20	5
ALT (U/L)	5-40	17	11	18	10	16	13	15	4
LDH (U/L)	107-220	152	19	167	22	158	31	174	30
ALP (U/L)	96-284	185	54	186	58	160	48	167	49
γ -GTP (U/L)	3-70	22	26	26	32	14	6	15	6
Total bilirubin (mg/dL)	0.3-1.2	0.7	0.3	0.6	0.2	0.7	0.3	0.6	0.2
Creatinine (mg/dL)	0.34-0.79	0.61	0.08	0.57	0.08	0.62	0.08	0.58	0.11
Urea nitrogen (mg/dL)	8-20	11	2	13	2	13	3	13	4
Uric acid (mg/dL)	2.5-6.0	4.2	0.8	4.3	0.8	4.0	0.5	4.1	0.6
Total cholesterol (mg/dL)	120-230	180	30	193	48	200	41	202	45
Cholesterol ester (IU/L)	3600- 7600	4563	706	4970	721	4447	713	4779	872
HDL (mg/dL)	50-75	71	10	72	15	77	13	81	15
LDL (mg/dL)	<130	96	25	100	31	110	34	106	35
Triglycerides (mg/dL)	40-130	63	40	72	79	63	21	60	22
Na (mEq/L)	138-147	139	1	141	2	139	1	141	3
K (mEq/L)	3.3-4.8	4.3	0.2	4.4	0.3	4.2	0.2	4.4	0.3
Cl (mEq/L)	98-110	102	1	104	2	102	1	103	2
Ca (mg/dL)	8.5-10.5	9.4	0.2	9.1	0.3	9.2	0.2	9.1	0.2
IP (mg/dL)	2.4-4.4	3.6	0.4	3.9	0.4	3.5	0.4	3.7	0.4
Mg (mg/dL)	1.8-2.55	2.0	0.1	2.1	0.1	2.0	0.1	2.1	0.1
IgE (IU/mL)	NA	NA	NA	NA	NA	127	145	156	220

NOTES:

Data source: Snow Brand Milk Products 2000b.

(a) n = 16

(b) n = 18

ABBREVIATIONS:

AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; γ -GTP, gamma glutamyl transpeptidase; HDL, high density lipoprotein; LDL, low density lipoprotein; Na, sodium; K, potassium; Cl, chloride; Ca, calcium; IP, inorganic phosphorous; Mg, magnesium; NA, not available.

In a peer-reviewed, randomized, placebo-controlled, double blind study, the effects of MBP on bone metabolism and BMD was evaluated in women (Aoe *et al.* 2001; Yamamura *et al.* 2002). Thirty-three healthy adult women consumed daily a beverage containing either 40 mg of MBP (n=17) or a placebo (n=16) for six months. The women were evaluated just prior to treatment (baseline; Exam 1) and after 3 and 6 months (Exams 2 and 3, respectively) of

treatment. At each evaluation, blood and urine samples were collected for biochemical analysis and urinalysis, and at 0 and 6 months, calcaneal BMD was evaluated. Also at the 3- and 6-month evaluations, each participant completed a 3-day food record.

Serum and urinary bone biomarkers were analyzed using repeated measures (ANOVA) adjusted with degrees of freedom (Huynh and Feldt 1976, cited in Aoe *et al.* 2001). In the treated and control groups, the relationship between calcaneal BMD at baseline and after 6-months was examined using regression analysis. Dietary records were analyzed using the Mann-Whitney U-test, and other results were compared using the t-test.

During the study, no participants dropped out or reported symptoms of bloating, diarrhea, or allergies. Calcaneal BMD values increased in both groups, but were only significantly increased in the MBP-treated group compared with the placebo group. The relationships between calcaneal BMDs at 0 and 6 months were linear for both groups. No differences between serum bone markers were observed between the 2 groups. Radial BMD gain over time was also significant in the MBP-treated group, but not in the placebo group compared to baseline values. There were decreases in urinary bone resorption marker values when comparing the MBP group with the placebo group: that is, the level of cross-linked N-telopeptidase of type I collagen excretion was decreased at 3 and 6 months and the mean excretion concentration of D-Pyr was decreased at 6 months.

There were no significant differences between group mean intakes of calcium, phosphorous, magnesium, vitamin D, vitamin K, or vitamin C. Additionally, there were no significant correlations between calcaneal BMD gain and intake of any vitamins or minerals in either placebo or MBP group. Accordingly, it appears that the increase in BMD in the MBP-treated group is independent of dietary intake of vitamins and minerals.

a) Conclusions

Under the conditions of use in clinical trials of MBP ingestion, the product was safe and well tolerated in men at doses up to 300 mg/day for 17 days and in women at doses of up to 40 mg/day for 6 months.

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