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SRA International, Inc.

May 5, 2006

Document Receiving
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

REC'D MAY 5 2006

Attention:
Dr. Robert Martin, Deputy Div. Director
Center for Food Safety and Nutrition
Office of Operations
Office of Food Additive Safety
DBGNR, HFS-255, Rm 2045

Re: Milk-Derived Tripeptides (MDT) – GRAS Notification

Dear Dr. Martin:

This document serves as the covering letter for a submission to the U.S. Food and Drug Administration (USFDA) Generally Recognized as Safe (GRAS) Notification process.

Attached please find three (3) copies of the submission for Milk-Derived Tripeptides (MDT), substances that have been declared GRAS by scientific procedure.

If you have any questions, please do not hesitate to contact me at the SRA offices.

Most Sincerely,

Bruce K. Bernard, Ph.D.
President SRA International Inc.
Authorized Representative for
Calpis Co., Ltd.

BKB/km
Attachments 3

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GRAS Exemption Claim

Submitted: May 5, 2006

Notifer:

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PURPOSE STATEMENT

The purpose of this submission is to notify the Food and Drug Administration that the use of the milk-derived tripeptides (MDT) L-valyl-L-prolyl-L-proline (VPP, Val-Pro-Pro), and L-isoleucyl-L-prolyl-L-proline (IPP, Ile-Pro-Pro), as an ingredient in four food categories at specified maximum usage levels has been determined to be GRAS by scientific procedures by the notifier and that this particular use of this substance is exempt from the premarket approval requirements of the Federal Food Drug and Cosmetic Act. The food categories and maximum usage levels are:

Food Category	Maximum Usage Level
Orange juice	21ppm
Yogurt including yogurt drinks (smoothies)	100ppm
Nutrition bar	102ppm
Margarine spread	364ppm

For purposes of food product ingredient labeling, these milk-derived tripeptides will be labeled as: casein hydrolysate (milk), hydrolyzed casein (milk), hydrolyzed protein (milk), milk-derived peptides, hydrolyzed milk protein, milk protein hydrolysate, hydrolyzed milk casein.

Signature of Notifier's Agent

5 May 2006

Date

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Executive Summary

Calpis Co., LTD. authorized the convening a GRAS panel, hereafter referred to as SRA GRAS panel, to evaluate the use of two milk-derived tripeptides (MDT) L-valyl-L-prolyl-L-proline (VPP, Val-Pro-Pro) and L-isoleucyl-L-prolyl-L-proline (IPP, Ile-Pro-Pro) in four food categories at specified maximum use levels. The members of this GRAS Panel are qualified by scientific training and experience to evaluate the safety of the substances added to food.

The four food categories and maximum intended use levels are:

Category	PPM Level
Orange juice	21
Yogurt (including yogurt drinks [smoothies])	100
Nutritional bars	102
Margarine spread	364

The SRA GRAS panel reviewed information related to the physical and chemical properties of the compounds, manufacturing processes (via enzymatic and milk fermentation), product specifications, information on the safety of VPP and IPP including information published and/or publicly available studies, estimates of current consumption of VPP and IPP and the potential increased dietary exposure as a result this intended use. Based on this review, the SRA GRAS panel concluded that the use of VPP and IPP in the above identified food categories and maximum usage level is GRAS under the intended conditions of use.

The SRA GRAS panel summarized their critical evaluation of the available information as follows:

- MDT are naturally occurring in a number of commonly consumed foods (e.g., milk, milk products [particularly cheeses]).

- A broad range of animal and human toxicity studies demonstrate the safety of these milk-derived tripeptides under the proposed conditions of human use.

- The oral administration of MDT, up to 400 mg/kg body weight (bw)/day (the highest level tested) to rats for 28 days, did not elicit any adverse effects.

- MDT at doses up to 3.3 mg/kg bw/day (the highest dose tested) did not elicit any adverse reproductive or developmental effects in rats.

- Normal human volunteers ingested 28 grams of the tablets containing 29.1 mg of milk-derived tripeptides daily for 7 consecutive days and there were no reported adverse effects including clinical chemistry and hematology.

- The intended conditions of use could result in a maximum consumption of 12.0 mg/person/day or 0.20 mg/kg bw/day of the MDT for a 60 kg person.

1 Administrative Information

1.1 Name and Address of the Notifier

Calpis Co., Ltd.
4-1, 2-chome,
Ebisu-minami,
Shibuya-ku, Tokyo 150-0022
Japan

1.2 Name and address of the Agent

Dr. Bruce K. Bernard, President
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After May 15th please address correspondence to:

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1.3 Common or Usual Name of Substance of the Notified Substances

L-valyl-L-prolyl-L-proline (VPP, Val-Pro-Pro);

L-isoleucyl-L-prolyl-L-proline (IPP, Ile-Pro-Pro),

1.4 Conditions of Use

1.4.1 Use

The food categories and associated usage levels are presented below.

Food Category	Usage Level (ppm)
Orange Juice	21
Yogurt including drinks (smoothies)	100
Nutritional Bars	102
Margarine Spread	364

1.4.2 Exposure Calculations

1.4.2.1 Data Sources and Methods

The Applicant retained an independent consulting firm, Exponent (Washington D.C.), to estimate the dietary intake of milk-derived tripeptides from the foods proposed for enrichment (see above) for the total US population as well as for US population subgroups (i.e. children 7-12 years, women 13-49 years, and adults 55+ years).

Dietary consumption was estimated using the data from the 1994-96, 1998 Continuing Survey of Food Intakes by Individuals (CSFII) (USFDA, 2000) and Exponent's Foods Analysis and Residue Evaluation Program. Both per capita and per user (Eaters Only, EO) intakes were estimated.

1.4.2.2 Calculated Intake Levels

Employing the consumption survey data identified above, it was determined that if milk-derived tripeptides were employed in the four (4) food groups and at the usage levels previously identified, the mean daily per user (EO) intake in the total US population would be 5.3 mg/person/day. The E.O. 90th percentile for the same group would be 12.0 mg/person/day. In the subgroups for which additional information was sought, the mean daily E.O. intake ranged from 4.2 mg/person/day among children 7-12 years to 5.3 mg/person/day among women 13-49 years. Subgroup intakes among higher E.O. consumers (90th percentile) ranged from 9.1 mg/person/day among children 7-12 years to 12.0 mg/person/day among women 13-49 years.

1.4.3 Consumption of Tripeptides from Other Sources

Tripeptides are consumed in a number of natural foods, particularly dairy products. The best data available on levels of tripeptides in foods and consumption of those foods relates to cheese. Table 1.4.3 from the U.S. Dairy Export Council shows that the average individual living in the U.S. consumes 12,757 g of cheese per year.

Table 1.4.3 Consumption of dairy products in 1996[1]

	consumption/person/year	
	lbs	g
Total cheese	28.1	12,757
Cheddar	9.21	4,181
Parmesan	0.29	132
Romano	0.19	86
Swiss	1.14	518
Blue	0.18	82
Flavored milk & drinks	10.4	4,722

[1] From U.S. Dairy Export Council.
Note: Majority of cheese consumed is processed cheese.

This total quantity of cheese consumed (12,757 g/yr) is the sum of a wide variety of different types of cheese (e.g., Cheddar, Parmesan, Romano, Swiss, Blue). Not only does the concentration of tripeptides vary across cheese type, but it also varies significantly across brands within the same cheese variety. Thus, it is very difficult to estimate the consumption of tripeptides from cheese consumption.

To obtain at least a *guesstimate* of tripeptides consumption from cheese, we can use the following *hypothetical scenario*. The consumption of Cheddar cheese is the greatest contributor to total cheese consumption (approximately 25% of total). Thus, assuming 100% of the cheese consumed was Cheddar and the Cheddar consumed had the highest concentration of tripeptides in that grouping (there are higher concentrations of tripeptides in some other cheeses) the total consumption of tripeptides would be 2,413 mg/person/yr. This is equal to 6.6 mg/person/day. Clearly, the consumption of tripeptides from cheese is less than 6.6 mg/person/day because all consumed cheese is not Cheddar and certainly most is not of the highest tripeptides concentration.

1.4.4 Quantitative Analysis Method of Tripeptides in Cheese

1.4.4.1 Analytical method for VPP and IPP in cheese

Extraction Procedure

A 5.0 g cheese sample, which had been allowed to warm to room temperature, was weighted into 50 ml beaker. A 30 ml of a solution of dichlromethane:methanol (75:8) is added, and the solids chopped to small particles with a spatula. The resulting mixture is transferred to a 250 ml round bottom flask (rbf). The original beaker is washed with the remaining dichlromethane:methanol solution and added to the rbf. The rbf is alternatively sonicated and stirred with a magnetic stir bar until solids are dispersed uniformly. 40 ml water is added, sonicated for 1 minute, then stirred for 15 minutes. Aliquots of 2 x 1ml are transferred to Eppendorf tubes and centrifuged at 10,000 rpm (relative centrifugal force: 10,286) for 10 minutes on an Eppendorf model 5417R centrifuge. Clear liquid is pipetted from the two Eppendorf tubes and transferred to an autosampler vial.

LC-MS Analysis

All chromatography was controlled via a Hewlett/Packard G1946A system. A Waters symmetry C18 column (150mm x 3.9mm id) was employed for the quantitative analysis of VPP and IPP. The solvent system employed in the analysis consisted of a) 0.1% TFA in water and b) 0.1% TFA in acetonitrile. Tripeptides (VPP and IPP) were analyzed using an elution gradient, 100% of A (t=0 min), 100% of A (t=1min), 100% of B (t=10 min). A post-run time of 2 minutes allows the column to re-equilibrate to 100% of A before beginning the next analysis. VPP and IPP were quantified using the synthesized peptides (VPP and IPP) as the standard. Five microliters were applied onto the column and analyzed at 70°C using a flow rate of 1.0 ml/min. A mass spectrometer with an electrospray sources was optimized for analysis in positive ion mode using a selective ion monitoring at m/z 312.1 and 326.1 for VPP and IPP, respectively.

The estimation consumption of milk-derived tripeptides (VPP and IPP) from various cheeses is presented in Table 1.4.4.1 below.

Table 1.4.4.1 Estimation of Tripeptides (VPP and IPP) Consumption from Cheese

Type	Brand	Tripeptides*1 µg/g	Cheese consumption/person/year*2 g	Tripeptides (VPP and IPP) consumption/person/year*3 mg
Cheddar	Mona Lisa	189.15	4,181	791
	Black Diamond	94.56	4,181	395
	Tilamok	41.43	4,181	173
	Kaukauna	9.66	4,181	40
Parmesan	Empiria	88.52	132	12
	Villaparma	31.90	132	4
	Digiorno	11.00	132	1
	Kraft	10.30	132	1
Romano	Pinna	32.94	68	2
	Dominick's	18.41	68	1
	Villaparma	21.60	68	1
	Digirno	15.99	68	1
	Hautly	14.80	68	1
Swiss	Kaukaura	32.52	518	17
	Schnucks	31.77	518	16
	Kraft	11.73	518	6
	Kraft	7.82	518	4
Blue	Stella	556.88	82	46
	Treasure Cave	222.46	82	18

*1: Applicant's internal report (unpublished)
*2: From U.S. Dairy Export Council
*3: Estimated amount of tripeptides (VPP and IPP) consumed in only high tripeptides content cheese.

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1.4.5 Factors Acting to Limit Consumption

There are a number of factors that will limit consumption. These include:

- **Taste**
  Products containing more MDT that those recognized as GRAS may develop an undesirable taste.

- **Cost**
  Products containing MDT will cost significantly more than non-enhanced products, thereby discouraging consumption beyond levels considered to be GRAS.

1.5 Basis of GRAS determination and availability of materials reviewed

The basis of the GRAS determination is through scientific procedures. The SRA GRAS panel was a five-member panel comprised of internationally known experts in the following areas: clinical medicine, clinical and comparative pathology, medicinal chemistry, toxicology, and nutrition. The members are past-presidents of their respective professional associations and editors-in-chief of professional journals.

The materials and information reviewed will be sent to FDA upon request and/or are available for the FDA's review and copying at reasonable times at the office of our Agent:

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Washington, D.C. 20036

After May 15th please address correspondence to:

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2 Identity

2.1 Definitions

MDT----- Milk-derived tripeptides: A conceptual term describing the combination of L-valyl-L-prolyl-L-proline (VPP) and L-isoleucyl-L-prolyl-L-proline (IPP) produced from casein protein.

2.2 Physical and Chemical Information

Table 2.2-1 Chemical Identity of Substances

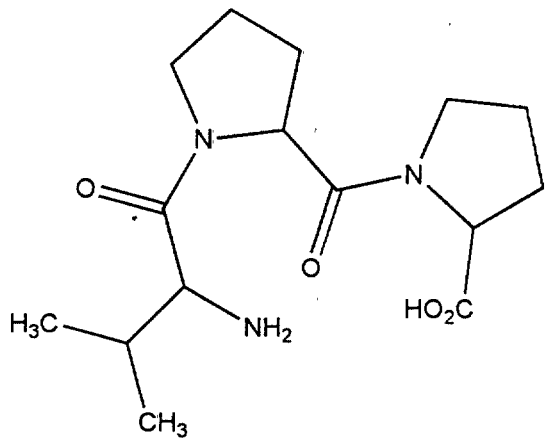
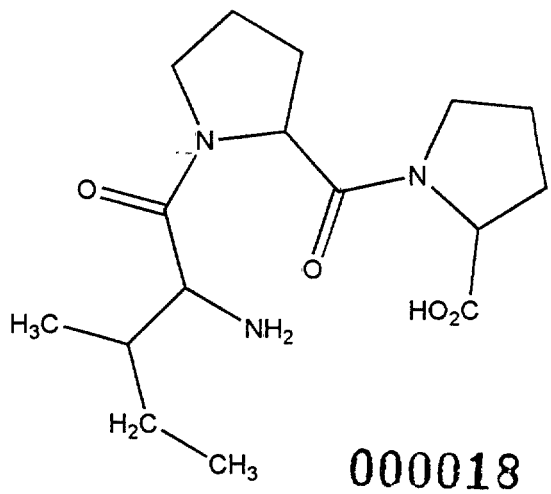
Common Name	Val-Pro-Pro (VPP)	Ile-Pro-Pro (IPP)
Chemical Name	L-valyl-L-prolyl-L-proline	L-isoleucyl-L-prolyl-L-proline
CAS Number	58872-39-2	26001-32-1
Synonyms	VPP	IPP
Molecular Formula	$C_{15}H_{25}N_3O_4$	$C_{16}H_{27}N_3O_4$
Chemical Structure		

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Table 2.2-2 Physical and Chemical Properties

Property	VPP	IPP
Color	White	White
Form	Powder	Powder
Molecular Weight	311.4	325.3
Melting Point	198.5 °C	125.9 °C
pKa	pKa1=3.61, pKa2=8.27	pKa1=3.63, pKa2=8.27
Optical Rotation	$[a]^{20}_{D}$.-103.5° (C=0.5, methanol, 100mm)	$[a]^{20}_{D}$.-100.0° (C=0.5, methanol, 100mm)

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2.3 Manufacturing Information

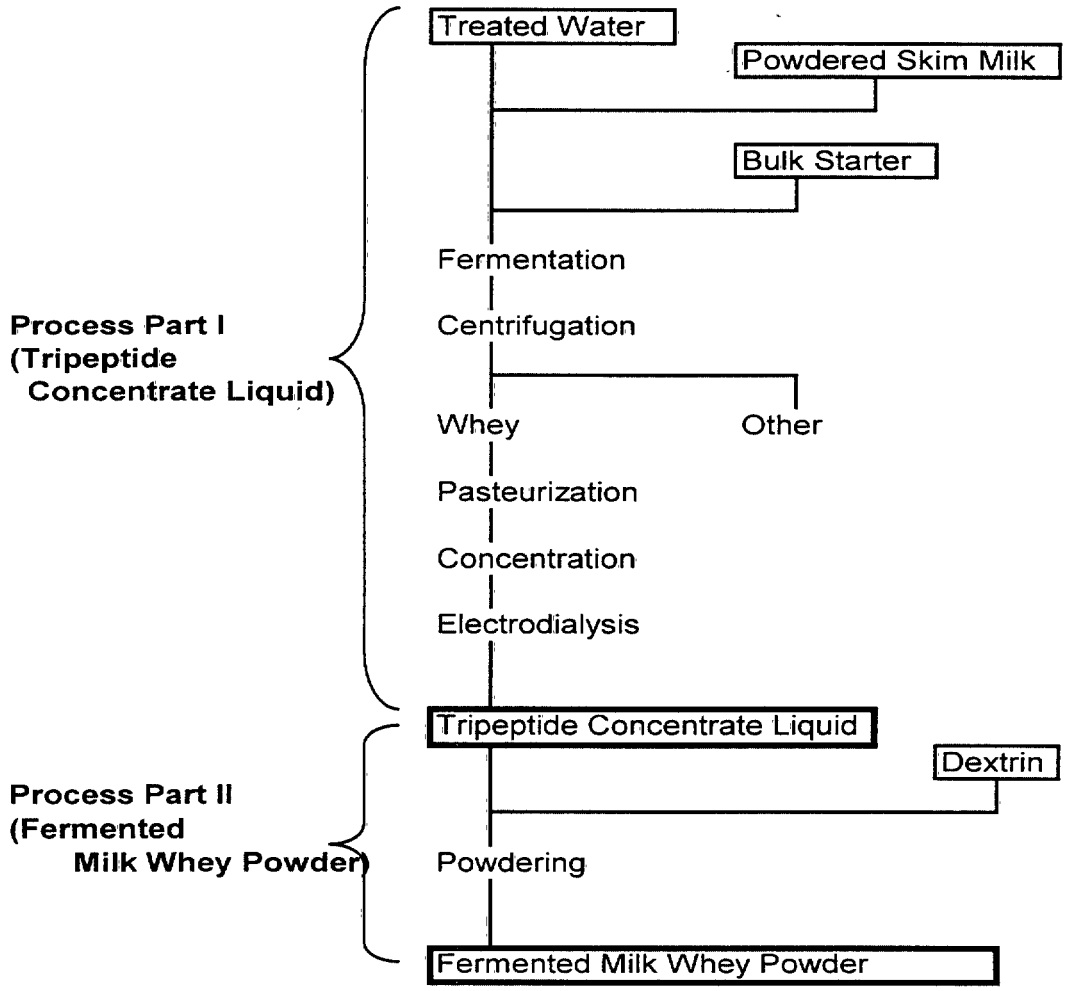
The powder containing VPP and IPP are manufactured via milk fermentation or an enzymatic process. In both methods a tripeptide concentrated liquid is made and then filtered and dried. In order to differentiate between the two processes and the resultant tripeptide containing powders, powder created via milk fermentation is referred to as *Lactobacillus helveticus* fermented milk powder. The powder manufactured via the enzymatic process is referred to as casein hydrolysate powder.

2.3.1 Milk Fermentation

2.3.1.1 Process

The first step of the manufacturing process is to produce a concentrated whey liquid by reconstituting powdered skim milk with distilled water (reconstitution rate is 8.9%) and adding *L.helveticus* as the starter bacteria. The liquid then undergoes fermentation, centrifugation, pasteurization and concentration. Maltodextrin is added to the liquid, which is then sterilized, filtered and powdered. All ingredients are food grade materials or better. Figure 2.3.1.1 illustrates the manufacturing process.

FIGURE 2.3.1.1 **Tripeptide Fermentation Powder Manufacturing process by fermentation method**



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2.3.1.2 Tripeptide Powder Product Specifications

Table 2.3.1.2-1 Specifications for *L.helveticus* fermented milk powder

Quality Standard	Specification
Volume (kg)	10.00±0.05
Taste and Flavor	Pleasant aroma, taste and flavor with no obvious irregularities
Appearance	White to pale yellow coloration with no foreign matter or other irregularities in the product or container
Acidity W/W% (as Lactic Acid)	2.89 – 4.47
Tripeptides (mg/100g)	115 - 215
Moisture W/W%	Less than 5
Powder Density	0.46±0.06
Mold (cells/g)	Less than 100
Yeast (cells/g)	Less than 100
Coliforms	Negative
Total Bacteria Count (cells/g)	Less than 3000

Table 2.3.1.2-2 *L.helveticus* Fermented Milk Powder Product Analysis Data (Average of data from 3 Lots)

Items	Results (Detection Limits)	Test Methods
Tripeptide (mg/100g)	175	HPLC[1]
Yeasts (cells/g)	1	Japanese Food Hygiene Law standards method
Molds (cells/g)	0	Japanese Food Hygiene Law standards method
Coliforms (cells/g)	Negative (0 cells/g)	Japanese Food Hygiene Law standards method
Aerobic plate count (cells/g)	60	Japanese Food Hygiene Law standards method
Total nitrogen (TN) (%)	1.27	Kjeldahl method
α -Amino nitrogen (AN) (m%)	314	Van Slyke method
AN/TN	247	Calculation
Ammonia Nitrogen (%)	Not detected (at 0.01 %)	Ion Chromatography
Glutamic Acid (%)	0.35	Amino Acid Auto Analyzer
Heavy Metal (as Pb) (ppm)	Not detected (at 1 ppm)	Sodium Sulfide Colorimetry
Lead (ppm)	Not detected (at 0.05 ppm)	Atomic Absorption Spectrophotometry
Protein (%)	8.1	Kjeldahl method
Moisture (%)	2.3	Vacuum oven method
Fat (%)	1.1	Acid hydrolysis method
Ash (%)	1.7	Ashing method
Carbohydrate (%)	86.7	100-[Moisture+Protein+Fat+Ash]
Dietary Fiber (%)	0.8	AOAC method
Sodium (mg/100g)	3	Atomic absorption spectrophotometry
Cadmium (ppm)	Not detected (at 0.01 ppm)	Atomic absorption spectrophotometry

1) Aihara et al., J. Am. Coll. Nutr., 24(4), 257-265 (2005)

2.3.2 Enzymatic Method

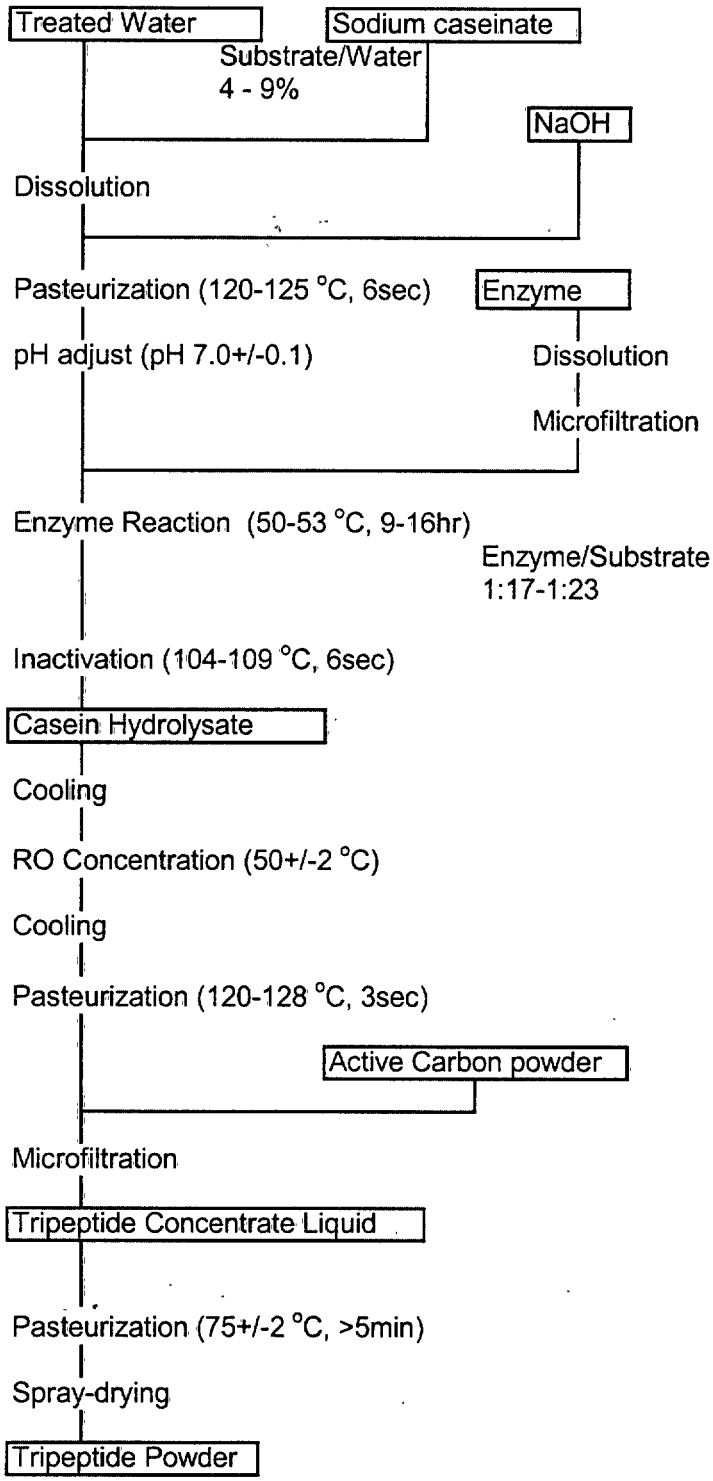
2.3.2.1 Process

The starting material used is casein solution or sodium caseinate solution depending on the solubility of the ingredient by equipment in the manufacturing plant (i.e. sodium caseinate has better solubility, while casein is dissolved only in hot and alkalified water). Furthermore, concentration of casein or sodium caseinate is variable flexibly depending on production efficiency of tripeptide within the range of the product specification. The solution is pasteurized to eliminate microorganisms originally contained in the starting material. Sodium hydroxide is added to adjust pH to 7. Enzyme is then added for hydrolysis in accordance with good manufacturing practice.

The hydrolysis is terminated by pasteurization. The solution undergoes reverse-osmosis (RO) concentration, pasteurization, carbon filtration, and microfiltration (MF) followed by spray drying. Figure 2.3.2.1 below illustrates the manufacturing process.

Figure 2.3.2.1

Tripeptide Powder
Manufacturing process by enzymatic method



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2.3.2.2 Enzymatic Tripeptide Powder Product Specifications

Table 2.3.2.2-1 Specifications for MDT containing Casein Hydrolysate Powder

Quality Standard	Specifications
Volume (kg)	15 ± 0.5
Taste and Flavor	Pleasant aroma, taste and flavor with no obvious irregularities
Appearance	White to pale yellow coloration with no foreign matter or other irregularities in the liquid
Tripeptides (mg/100g)	430-650
Moisture (W/W%)	Less than 7
Powder Density (g/ml)	0.70±0.10
Heavy Metal (as Pb) (mg/Kg)	<10
Mold (counts/g)	Not more than 100
Yeast (counts/g)	Not more than 100
Total Bacteria Count (cells/g)	Not more than 3000
Coliforms	Negative

Table 2.3.2.2-2 MDT Containing Casein Hydrolysate Powder Product Analysis Data (Average of data from 5 Lots)

Items	Results (Detection Limit)	Test Methods
Tripeptide (mg/100g)	575	LC/MS[1]
Yeasts (cells/g)	0	Japanese Food Hygiene Law standards method
Molds (cells/g)	0	Japanese Food Hygiene Law standards method
Coliforms (cells/g)	Negative (0 cells/g)	Japanese Food Hygiene Law standards method
Aerobic plate count (cells/g)	314	Japanese Food Hygiene Law standards method
Total nitrogen (TN) (%)	13	Kjeldahl method
α-Amino nitrogen (AN) (m%)	6200	Van Slyke method
AN/TN	490	Calculation
Ammonia Nitrogen (%)	0.3	Ion Chromatography
Glutamic Acid (%)	2.28	Amino Acid Auto Analyzer
Heavy Metal (as Pb) (ppm)	Not detected	Sodium Sulfide Colorimetry
Lead (ppm)	Not detected (at 0.05 ppm)	Atomic Absorption Spectrophotometry
Protein (%)	81	Kjeldahl method
Moisture (%)	7	Vacuum oven method
Fat (%)	0.2	Acid hydrolysis method
Ash (%)	4.3	Ashing method
Carbohydrate (%)	7.8	100-[Moisture+Protein+Fat+Ash]
Dietary Fiber (%)	0.5	AOAC method
Sodium (mg/100g)	1270	Atomic absorption spectrophotometry
Cadmium (ppm)	Not Detected (at 0.01 ppm)	Atomic absorption spectrophotometry

1)Matsuura et al., Milchwissenschaft, 60, 24-27 (2005)

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3 Toxicological Data

3.1 Introduction

The results of investigations conducted over the past 3 decades have afforded some scientific evidence that sour milk, long consumed as a nutritional supplement by Asian cultures, may contain components possessing normotensive activity. Normotensive activity appears to reside in two tripeptides, L-valyl-L-prolyl-L-proline (VPP) and L-isoleucyl-L-prolyl-L-proline (IPP). These tripeptides are produced by the action of *Lactobacillus helveticus* on reconstituted powdered skim milk.

These two tripeptides have been available for human consumption in Japan since 1997. The product or its active components have been assessed for toxicological potential.

The toxicity data reviewed is based on studies employing one or more of 3 specific types of tripeptide containing powders: 1) synthetic L-valyl-L-prolyl-L-proline (VPP), 2) a powdered casein hydrolysate with *Aspergillus oryzae* protease containing two tripeptides, L-valyl-L-prolyl-L-proline (VPP) and L-isoleucyl-L-prolyl-L-proline (IPP) and 3) Powdered *L. helveticus* fermented milk prepared from reconstituted skim milk which is pasteurized and fermented with *Lactobacillus helveticus*.

Table 3.1 TOXICOLOGY STUDY OVERVIEW

Type of Study	Species and Strain	Method of Administration	Duration of Dosing	Material Dose (mg/kg/d[a])	VPP + IPP/dose (mg/kg/d)	Results	Reference
Single-Dose Toxicity							
Casein hydrolysate tripeptide-containing powder	Sprague Dawley Rats (5/sex/group)	Oral gavage	Single	2000	12	No evidence of treatment associated toxicity.	Maeno et al. 2005b
L. helveticus fermented milk powder	Sprague Dawley Rats (5/sex/group)	Oral gavage	Single	0, 2000, 4000	0, 2.9, 5.8	No evidence of treatment associated toxicity	Maeno et al. 2005b
Synthesized VPP	Sprague Dawley Rats (5/sex/group)	Oral gavage	Single	0, 40, 200, 400	0, 40, 200, 400	No evidence of treatment associated toxicity	Maeno et al. 2005b
Repeat-Dose Toxicity							
L. helveticus fermented milk	Sprague Dawley Rats (10/sex/group)	Oral gavage	4 weeks	0. 500, 1000, 2000	0, 0.7, 1.5 2.9	The MTD is > 2000mg/kg/d and there are no specific target organs.	Maeno et al. 2005b
Synthesized VPP	CD Rats (20/sex/group)	Oral	8 weeks	0, 2, 8, 16	0, 2, 8, 16	The NOEL is 16 mg/kg/d and the MTD is ≥ 16 mg/kg/d.	Nakamura et al. 2005
Synthesized VPP	Beagle Dogs (20/sex/group)	Oral	8 weeks	0, 2, 8, 16	0, 2, 8, 16	The NOEL is 16 mg/kg/d and the MTD is ≥ 16 mg/kg/d.	Nakamura, et al. 2005

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Type of Study	Species and Strain	Method of Administration	Duration of Dosing	Material Dose (mg/kg/d[a])	VPP + IPP/dose (mg/kg/d)	Results	Reference
Casein hydroylsate tripeptide-containing powder	Crj:CD(SD)IG SPF Rats (12/sex/group)	Oral gavage	13 weeks	0, 40, 200, 1000	0, 0.2, 1.2, 6.0	The NOAEL and the MTD is > 1000 mg/kg/d and there are no specific target organs.	Mizuno et al. 2005b
Genotoxicity							
Micronucleus (in vivo)							
Casein hydroylsate tripeptides-containing powder	Sprague Dawley Rats (5M/group)	Oral gavage	2 days	0, 500, 1000, 2000 Neg Ctrl: water Pos Ctrl: cyclophosphamide, ip, (10 mg/kg)	0, 3.0, 6.0, 12.0	Does not possess the potential to induce micronuclei formation	Matsuura et al. 2005
L. helveticus fermented milk	ICR Mice (5M/group)	Oral gavage	Single	0, 500, 1000, 2000 Neg Ctrl: medicinal grade water Pos Ctrl: Mitomycin C, ip (2 mg/kg)	0, 0.8, 1.6, 3.3	Does not possess the potential to induce micronuclei formation	Matsuura et al. 2005

000030

Type of Study	Species and Strain	Method of Administration	Duration of Dosing	Material Dose (mg/kg/d[a])	VPP + IPP/dose (mg/kg/d)	Results	Reference
Cytotoxicity/ Clastogenicity (in vitro)							
Casein hydrolysate tripeptides-containing powder	Chinese Hamster Lung Cells	In vitro	6 hrs (w and w/o S9) 24 hrs (w/o S9)	1250, 2500, 5000 ug/mL Pos Ctrls: Mitomycin C w/o metabolic activation & benzo[a]pyrene w/ metabolic activation	7.446, 14.89, 29.79 µg/mL	Does not demonstrate the potential for the induction of cytotoxicity or clastogenic activity	Maeno et al. 2005a
L. helveticus fermented milk	Chinese Hamster Lung Cells	In vitro	6 hrs (w and w/o S9) 24 hrs and 48 hrs (w/o S9)	1250, 2500, 5000 µg/mL Pos Ctrls: Mitomycin C w/o metabolic activation & benzo[a]pyrene w/ metabolic activation	2.096, 4.193, 8.385 µg/mL	Does not demonstrate the potential for the induction of cytotoxicity or clastogenic activity.	Maeno et al. 2005a

000031

Type of Study	Species and Strain	Method of Administration	Duration of Dosing	Material Dose (mg/kg/d[a])	VPP + IPP/dose (mg/kg/d)	Results	Reference
Bacterial gene mutation							
Synthesized VPP	*Salmonella typhimurium* (TA1535, TA1537, TA98, TA100), *E. coli*, WP2 (*uvr*A) With (5% and 10% S-9) and without activation	In vitro	-	156.2, 312.5, 625, 1250, 2500, 5000 µg/plate Pos Ctrls: sodium azide, 2-aminoanthracene, 9-aminoacridine HCl, 2-nitrofluorene, N-ethyl-N'-nitro-N-nitrosoguanidine	0.1562, 0.3125, 0.625, 1.25, 2.5, 5.0 mg/plate	With and without metabolic activation, there is no mutagenic potential	Bernard et al. 2005
Reproductive and Developmental Toxicity							
Fertility/Embryogenesis							
L. helveticus fermented milk powder	Sprague Dawley Rats (21/sex/group)	Oral	M: 28 days pre-mating F: 14 days pre-mating – GD7	0, 500, 1000, 2000	0, 0.8, 1.6, 3.3	The NOAEL is 2000 mg/kg/d and MTD > 2000 mg/kg/d	Kurosaki et al. 2005

000032

Type of Study	Species and Strain	Method of Administration	Duration of Dosing	Material Dose (mg/kg/d[a])	VPP + IPP/dose (mg/kg/d)	Results	Reference
Embryo/Fetal Development							
L. helveticus fermented milk powder	Sprague Dawley Rats (21F/group)	Oral	GD7 – GD17	0, 500, 1000, 2000	0, 0.8, 1.6, 3.3	At 1000 and 2000 mg/kg/d there is a significantly decreased implantatation index but comparable to historical implantation indices	Kurosaki et al. 2005
Maternal Function & Pre/Post Natal Development							
L. helveticus fermented milk powder	Sprague Dawley Rats (22F/group)	Oral	GD7 – LD20	0, 500, 1000, 2000	0, 0.8, 1.6, 3.3	No evidence of toxicity in either the Fo and F1 generation. The NOAEL for both maternal and fetal development is 2000 mg/kg/d.	Kurosaki et al. 2005

a – unless otherwise specified. For single dose toxicity and repeated dose toxicity, the highest NOEL (No Observed Effect Level) is underlined.

000033

3.2 Acute Toxicity

Table 3.2 Acute Toxicity Study Results

Type of Study	Species and Strain	Route	Material Dose (mg/kg/ bw)	VPP + IPP/dose (mg/kg/ bw)	VPP+ IPP LD_{50} (mg/kg bw)	Reference
Casein hydrolysate tripeptide-containing powder	Sprague Dawley Rats (5/sex/group)	Oral gavage	2000	12	>12	Maeno et al. 2005b
L. helveticus fermented milk powder	Sprague Dawley Rats (5/sex/group)	Oral gavage	0, 2000, 4000	0, 2.9, 5.8	>5.8	Maeno et al. 2005b
Synthesized VPP	Sprague Dawley Rats (5/sex/group)	Oral gavage	0, 40, 200, 400	0, 40, 200, 400	>400	Maeno et al. 2005b

The table above presents the results of acute toxicity studies. The panel noted the lack of toxicity in these studies employing each of the 3 discrete tripeptides-containing test articles. There was no evidence of either systemic or local (GI tract) toxicity with any of the test articles. Gross observation of rats administered as much as 12 mg/kg/bw of VPP+IPP in powdered casein hydrolysate, 5.8 mg/kg/bw of VPP+IPP in powdered *L.helveticus* fermented milk, or 400 mg/kg/bw of synthesized VPP revealed no evidence of treatment-associated toxicity. The authors concluded that it is probable that rats could have tolerated higher oral doses of all test substances. The studies employing casein hydrolysate tripeptide-containing powder and *L.helveticus* fermented milk powder were performed under Good Laboratory Practice standards. The study employing synthesized VPP was not performed under Good Laboratory Practice standards and there was no Quality Assurance inspection.

3.3 Short-term Toxicity Studies

3.3.1 28-Day Oral Gavage Toxicity Study in Rats

The panel reviewed a 28-day study of toxicity of VPP+IPP in powdered *L.helveticus* fermented milk. Test material was administered via oral gavage to groups of Sprague Dawley rats (10/sex) at doses of 0, 0.7, 1.5, or 2.9 mg/kg bw/day VPP+IPP for 28 consecutive days (Maeno et al. 2005b). This study was performed according to Good Laboratory Practice standards.

There was no treatment-related effect on body weight, body weight gain, food consumption, mean absolute and relative (to body weight) organ weights, clinical chemistry values, urinary volume and excretion of electrolytes in any dose group, nor any evidence of treatment-related ocular changes. Soft stools and perianal fecal smudging were occasionally observed in dosed but not control animals.

Some male rats exhibited proteinuria and ketonuria (5, 5, 4, 3 in the control, low-, mid- and high-dose groups, respectively). These changes were observed in both control and dosed animals The urinary sediment in both treated and control rats showed the presence of white blood cells and epithelial cells: males: 3, 2, 3, 2 (control, low-, mid- and high-dose respectively); females: 4, 1 ,2 (control, low- and high-dose groups respectively) for white blood cells.

There were statistically significant alterations were increases in hematocrit and hemoglobin concentrations (3.8–5.2%) in the low- and mid-dose male groups. There was no indication of similar changes in the high-dose rats of either sex. The authors concluded the lack of a dose-response relationship indicated that these changes are not test article related. No significant changes in female hematological parameters were observed.

High-dose females had slightly (1.7%) and significantly lower mean serum chloride concentration than those of the control group. The authors noted the decrease was within normal variation and of doubtful toxicological significance. A single high-dose male had elevated AST and ALT values; however, this did not measurably affect the mean values for the group. During necropsy the animal with elevated AST and ALT values was noted to have a swollen liver with adhesions to the diaphragm. No other animal had macroscopic changes of the liver but a second High-dose male did have grossly observable white spots on the surface of a kidney. Macroscopic changes were not observed in either control or dosed female rats.

The mean absolute and relative organ weights of dosed animals were not distinguishable from those of controls.

Two high-dose males exhibited epithelial changes in the proximal renal tubules but these changes are known to occur spontaneously in male rats. One control male also exhibited the tubular lesion. The panel agreed with the authors' conclusion that the low incidence of renal changes in the high-dose group to be unrelated to the administration of powdered *L.helveticus* fermented milk.

The High-dose male rat that had exhibited liver swelling with adhesions at necropsy had microscopic hemorrhage and focal thickening of the liver capsule in the areas of adhesions. Livers from the nine remaining high-dose males and all (10) high-dose females were normal in microscopic appearance. The authors did not consider the hepatic changes in the single high-dose male to be treatment-related.

The authors concluded that a) the maximally tolerated oral dose (MTD) of VPP+IPP in powdered *L.helveticus* fermented milk administered for 28 consecutive days is greater than 2.9 mg/kg bw/day and b) the results of this study do not support identification of specific target organs for VPP+IPP toxicity.

3.3.2 Eight-week study via Oral Administration in Rats

Synthesized tripeptide, VPP, was administered in the diet to CD rats (Charles River, UK), 20/sex/group, in concentrations estimated to deliver daily doses of 0, 2, 8, or 16 mg/kg of the tripeptide for 8 weeks (Nakamura et al. 2005). This study was not conducted under Good Laboratory Practice standards, nor was there a Quality Assurance inspection.

There was no treatment-related effect on body weight, body weight gain, or food consumption. During week 6, mid- and high-dose females exhibited a statistically significant decrease (32%, $p<0.05$) in efficiency of food utilization (EFU), the mean EFU values for the overall study were unaffected by administration of VPP.

There were no ocular effects attributable to the administration of synthesized VPP. Urinalysis did not reveal any statistically significant or meaningful differences between dosed and control animals.

While there were hematological changes observed in synthesized VPP-dosed rats, there is no evidence of a dose-response relationship for these changes. High-dose males and mid- and high-dose females had significantly reduced mean numbers of large unstained cells. The reductions ranged from 26% in males to as high as 39% in mid-dose females but only 1% in high-dose females. Mid- and high-dose females also exhibited significantly reduced mean monocyte counts (54 and 8%, respectively) but the mean monocyte counts in groups of dosed males were not different from that in the control group. Mean cell hemoglobin concentration was slightly (approximately 1%) but significantly ($p<0.05$) reduced in all groups of dosed females but there was no similar decrease in males.

There were sporadic changes in clinical chemistry parameters in rats receiving synthesized VPP-dosed rats. High-dose males had an increase in serum γ-GT and a 29% decrease in ornithine carbamyl transferase but these parameters were unaffected

in females. Total bilirubin was reduced by 33% in all dosed female groups but this parameter was unaffected in males. Small (1%) increases in serum sodium concentrations in all dosed females were statistically significant ($p<0.05$) but there was no similar change in dosed males. Mean serum calcium concentration was increased (3%) in high-dose females but significantly decreased (2-3%) in mid- and high-dose males. The high-dose was associated with a significant ($p<0.05$) 6-8% increase in serum glucose concentration in both males and females but there was no evidence of a dose-response relationship. The changes were considered of doubtful toxicological significance.

Statistically significant decreases were observed in mean absolute and relative (to brain) male pituitary weights in mid- and high-dose animals while mean relative (to brain) kidney weights were also decreased in mid- and high-dose males. The authors concluded that the decrease in pituitary weights were due to random variability, given (1) the magnitude of decreases were the same in both groups, (2) there were no similar changes noted in females and (3) the lack of corroborating microscopic evidence.

A small decrease in kidney weights relative to brain was observed in males. This was an isolated finding in that a) no differences were observed in females, b) the findings were not corroborated by evidence of microscopic renal changes in males, and c) there were no changes in the absolute weights of either kidneys or brains in either sex. Therefore this finding was considered unlikely to be related to the administration of VPP.

Increased mean uterus weights relative to body weight in mid- and high-dose groups were statistically significant. However, mean absolute uterine weights were not statistically affected. A 2- to 3-fold incidence of dilation of uterus was observed in these groups. The authors concluded that while this may offer an explanation for the difference in relative weight difference, it was unlikely due to administration of synthesized VPP.

Macroscopic examination of animals during necropsy revealed a small number of spontaneous changes commonly observed in rats which were not attributable to administration of synthesized VPP. Microscopic examination of approximately 50 organs and tissues from dosed and control rats revealed only changes considered to be spontaneous in nature.

The oral administration of up to 16 mg/kg bw/day of VPP to male and female CD rats for 56 consecutive days produced no physical, biochemical or pathological evidence of treatment-associated changes. Statistically significant variations in hematologic and biochemical parameters in both species were small, lacked dose-response relationships and were generally observed in one sex but not the other.

The authors concluded that the no-observable-toxic-effect-level of synthesized VPP in rats is ≥16 mg/kg bw/day and the maximally tolerated dosed is >16 mg/kg bw/day.

3.3.3 13-week Oral Gavage Study in Rats

The Panel reviewed a 91-day study performed in male and female Crj:CD(SD)IG SPF rats, Charles River, Japan, Inc. (Mizuno et al. 2005b). Twelve animals/sex/group were dosed once daily via gavage administration of vehicle control (water for injection), or powdered casein hydrolysate (casein hydrolysate with *Aspergillus oryzae* protease) containing 0, 0.2, 1.2, 6.0 mg/kg bw VPP plus IPP suspended in water for injection. This study was conducted according to Good Laboratory Practice standards.

The organs of all animals in the vehicle Control and high-dose groups were sectioned and stained with Hematoxylin-and-eosin. Grossly abnormal organs (lungs of animal 1 mid-dose male; kidney of 1 low-dose female; and spleen of 1 mid-dose female) were also sectioned and stained. The organs/tissues from other low- and mid-dose groups were not examined because treatment-associated changes were not observed in the high-dose group.

There were no statistically significant differences in weekly mean body weights amongst the experiment groups. Male rats in the mid- and high-dose groups gained significantly more bodyweight (approximately 11% in both groups) during the first week of dosing ($p<0.01$). This was the only significant difference in mean bodyweight gain in either sex during the entire study.

Low-dose males were observed to consume significantly less food than controls during the final 7 weeks of the study (6-10% each week, $p<0.05$). The mid- or high-dose level rats were not similarly affected and no female groups exhibited statistically significant changes in food consumption. Therefore, the changes observed in the low-dose group were not considered to be related to the test article. The authors concluded that the administration of VPP+IPP in powdered casein hydrolysate had no treatment effect on body weights or food consumption.

The low-dose male group demonstrated significantly lower (66%) eosinophil count compared to controls. However, this difference was related to an increase in mean eosinophil count in the control group. The mean low-dose male eosinophil count was similar to counts in other groups of males and in all groups of females. All other mean values for assessed hematologic and blood coagulation parameters for each treatment group were within normal limits. There were no statistically significant differences between groups. The authors concluded there were no observed hematological changes attributable to administration of VPP+IPP in powdered casein hydrolysate.

There was a slight but statistically significant ($p<0.01$) increases in mean serum chloride concentrations in mid- and high-dose males as compared to the control group. These differences were small (<2%) and the absolute values were actually similar to that in the control group. Similar changes in chloride ion concentrations were not observed in the casein hydrolysate-dosed females. Therefore, the study authors considered the small changes observed in dosed males to be unrelated the test article.

The presence of crystals, white blood and epithelial cells, and urobilinogen was observed in urine of control and treated groups and both sexes. There was no evidence of a dose-response relationship. The authors concluded that there were no urinary changes attributable to the administration of VPP+IPP in powered casein hydrolysate.

Ophthalmic changes occurred in both dosed and control animals during the pretreatment as well as the final week of the study. The most frequently encountered change, particulate opacity of the lens, was present in all treatment groups of both sexes (males, 4/6, 5/6, 6/6, and 6/6; females, 3/6, 2/6, 2/6, and 3/6 in vehicle control, low-, mid-, and high-dose groups, respectively). These ophthalmic changes were viewed by the authors as naturally occurring and unrelated to administration of the test article.

There was a significant decreased (8%, $p<0.05$) in mean kidney to body weight ratios in low-dose males. Mean absolute kidney weights were unaffected in either sex. Low-dose females exhibited a significant *increase* in both mean absolute and mean relative uterus weights (46% increases, $p<0.01$). These changes occurred only in low-dose groups with no dose-response relationship and were not attributed to the administration of VPP+IPP in powdered casein hydrolysate. All other absolute and relative organ weights were similar in dosed and control animals of both sexes.

Necropsy revealed the occurrence of two high-dose males with hepatodiaphragmatic nodules and a mid-dose male with a brown patch on its lung. One high-dose male had a small testis and epididymis (unilateral). One control female had a white patch on its liver, a mid-dose female had an accessory spleen and a low-dose female had a kidney cyst. None of these findings were considered attributable to VPP+IPP in powdered casein hydrolysate administration.

A few microscopic changes were observed are recognized as naturally occurring changes in rats and none could be attributed to the administration of VPP+IPP in

powdered casein hydrolysate. Their incidences were low and similar in both control and treated groups.

The authors concluded that the daily oral administration of VPP plus IPP in powdered casein hydrolysate manufactured from casein hydrolysate with *A. oryzae* protease was well tolerated by male and female rats. VPP+IPP doses of 0, 0.2, 1.2, and 6.0 mg/kg bw/day produced no evidence suggestive of either systemic or local toxicity. Based on the results of this toxicity study, it is concluded that the no-observed-adverse-effect level of VPP plus IPP in powdered casein hydrolysate in male and female rats is >6 mg/kg bw/day. The maximum tolerated oral dose in rats is anticipated to be >6 mg/kg bw/day in both rat sexes and there was no evidence of specific target organs of toxicity for VPP+IPP in powdered casein hydrolysate in the rat.

3.3.4 Eight-week Study via Oral Administration in Dogs

In this study, beagle dogs (5/sex/group) were administered synthesized VPP in hard gelatin capsules, 7 days/week for 8 consecutive weeks (Nakamura et al. 2005). Capsules were individualized for each dog and the amount in each capsule was calculated to deliver daily doses of 0, 2, 8, or 16 mg/kg bw of VPP based on the most recently recorded individual animal body weight. This study was not conducted under Good Laboratory Practice standards, nor was there a Quality Assurance inspection.

There were no clinical signs attributable to administration of synthesized VPP. Clinical signs observed equally in control and treated animals included loose stool, lacrimation, dermal rashes, abrasions and scabs, and occasional emesis. There was no treatment-related effect on bodyweight gain or food consumption. Electrocardiograms administered prior to treatment and 2- and 24-hours following administration of VPP (week 7) demonstrated no treatment-related changes in wave intervals or amplitudes.

Urinalysis revealed a statistically significant increase in mean specific gravity in all groups of dosed males during week 8 (control: 1042 g/L, low dose: 1046 g/L, mid-dose:

1045 g/L, high dose: 1049 g/L). Mean specific gravities of dosed females were quantitatively higher but statistically the same as controls (control: 1043 g/L, low dose: 1049 g/L, mid-dose: 1044 g/L, high dose: 1045 g/L).

There were no significant differences in hematological parameters observed in male dogs. During Treatment Week 7, females treated with synthesized VPP had statistically significant ($p \leq 0.05$) reductions (31-40%) in mean neutrophil counts. The authors took note that control value during treatment week 7 was 36% higher than that observed during the pretreatment period (Week-1) while the values for dosed dogs were the same at both observations. The authors concluded that the statistically significant reduction in mean neutrophil counts in dosed females is most likely related to the increase in the mean count of controls rather than a decrease in dosed animals.

Organ weight data were analyzed by sex and then with sexes combined. There was a statistically significant increase (35-45%, $p<0.05$) in all High-dose group female ovary weight parameters (absolute and relative weights). Ovary weights were higher in the low- and mid-dose groups (28 and 13% respectively), but these differences were not statistically significant. The absence of a dose-response supports the authors' conclusion that the increase is not toxicologically significant.

When the sexes were pooled, statistically significant decreases (20-30%, $p \leq 0.05$) were observed in absolute and relative thyroid weights in high-dose dogs. The 28% ($p<0.05$) reduction in thyroid gland weight (sexes combined) in the high-dose dog group was most likely related to the nearly 38% reduction in mean absolute thyroid weight in high-dose males. That numerical decrease did not achieve statistical significance when male data were separately analyzed. The increase in the combined sexes group, although statistically significant, is without toxicological meaning.

The authors noted that macroscopic examination of each dog yielded a small number of deviations in organ coloration, small hemorrhagic areas, cysts, and evidence of organ

congestion which were distributed throughout all groups. They considered these changes unrelated to administration of synthesized VPP.

An increased incidence of heart valve hemocysts were observed in treated males (3/5 and 2/5 in the low- and high-dose groups respectively). However, this change was also seen in a control male (1/5). Given the lack of a dose-response relationship, the authors interpreted this finding to be without toxicological significance. A high incidence (80-100%) of renal papillary mineralization was observed in both sexes of control and dosed dogs and cytoplasmic vacuolation of the proximal convoluted tubules was also evenly distributed among dosed and control dogs. The severities of these renal changes were judged to be minimal in all cases.

All females were considered to have immature ovaries, most likely due the relatively young age of the animals at study initiation (approximately 25 weeks). Mean ovarian weight parameters in the high-dose group were significantly elevated (approximately 35%, $p<0.05$) and those parameters were numerically but not significantly elevated in the low- and mid-dose groups (28 and 13%, respectively). The authors considered the absence of a dose-response relationship supported the authors' conclusion that the increase in the high-dose group is without toxicological significance.

At least one dog in all but the mid-dose female group exhibited minimal to slight thymic involution/atrophy. While the numerical incidence of the change appeared to be somewhat higher in mid- and high-dose males (3/5, 60% in each group), the severity of the change was generally less than that observed in the 1/5 (20%) male control dog.

There was no evidence of local (stomach and GI tract) or systemic toxicity.

The oral administration of up to 16 mg/kg bw/day of VPP to male and female dogs for 56 consecutive days produced no physical, biochemical or pathological evidence of treatment-associated changes. Statistically significant variations in hematologic and biochemical parameters in both species were small, lacked relationships to doses and

were generally observed in one sex but not the other. The authors considered the no-observable-toxic-effect level of synthesized VPP in dogs is ≥16 mg/kg bw/day and that the maximally tolerated dosed is >16 mg/kg bw/day.

3.4 Mutagenicity Studies

In the mutagenicity tests summarized below, there were no indications of any mutagenic potential of any of the three test articles at the doses utilized.

Table 3.4 Mutagenicity Study Results

Test System	Test Substance	Test Object	Concentration (VPP + IPP)	Results	Reference	GLP	QA Inspect.
Micronucleus formation	Casein hydrolysate tripeptides-containing powder	Sprague Dawley Rats	0, 3.0, 6.0, 12.0 mg/kg bw gavage, 2 d	Negative	Matsuura et al. 2005a	Yes	Yes
Micronucleus formation	*L. helveticus* fermented milk	ICR Mice (5M/group)	0, 0.8, 1.6, 3.3 mg/kg bw, gavage, single dose	Negative	Matsuura et al. 2005a	No	No
Cytotoxicity/ Clastogenicity	Casein hydrolysate tripeptides-containing powder	Chinese Hamster Lung Cells	7.446, 14.89, 29.79 µg/mL	Negative	Maeno et al. 2005a	Yes	Yes
Cytotoxicity/ Clastogenicity	*L. helveticus* fermented milk	Chinese Hamster Lung Cells	2.096, 4.193, 8.385 µg/mL	Negative	Maeno et al. 2005a	No	No

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Test System	Test Substance	Test Object	Concentration (VPP + IPP)	Results	Reference	GLP	QA Inspect.
Reverse mutation[1]	Synthesized VPP	*S. typhimurium* TA98, TA100, TA1535, TA1537,	0.1562, 0.3125, 0.625, 1.25, 2.5, 5.0 mg/plate	Negative	Bernard et al. 2005	Yes	Yes
Reverse mutation[1]	Synthesized VPP	*E. coli*, WP2 (*uvr*A)	0.1562, 0.3125, 0.625, 1.25, 2.5, 5.0 mg/plate	Negative	Bernard et al. 2005	Yes	Yes

[1] In the presence and absence of metabolic activation

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3.5 Fertility and Reproductive Toxicity

The objective of these experiments was to study the effects of VPP+IPP in powdered fermented milk with *Lactobacillus helveticus* on reproduction. Specific experiments were conducted in male and female Sprague Dawley rats to assess effects of the powder on: 1) mating behavior and fertility in males and females, 2) fetal development in pregnant rats and 3) pre- and post-natal development in offspring of dams dosed from gestation day 7 through lactation day 20. These studies were not conducted under Good Laboratory Standards; a Quality Assurance inspection was performed on all three segments of the studies. (Kurosaki et al. 2005)

3.5.1 Fertility and Embryogenesis

One hundred sixty eight male and female Sprague-Dawley rats (Crj:CD(SD)IGS, Charles River Japan) were randomly assigned to one of four study groups (21 rats/sex/group). Males were administered daily gavage doses of powdered *L. helveticus* fermented milk containing 0, 0.8, 1.6, or 3.3 mg/kg bw of VPP+IPP for 28 consecutive days prior to mating and throughout the mating period. Administration of the same daily gavage doses to females began 14 days before the mating trials and continued until gestation day 7. The first day of dosing was defined as treatment day 1.

One male and one female from the same dose group were housed together after test article administration (treatment day 29 for the male and treatment day 15 for the female). They cohabited for a maximum of 2 weeks during which time daily vaginal smears were employed to determine the presence of sperm.

After successful mating, defined as a positive smear result, males were euthanized using carbon dioxide and necropsied. On gestation day 13, females were similarly euthanized and subjected to complete necropsy and examination of ovarian and uterine contents.

There were no clinical signs of systemic toxicity during the pre-mating period or in pregnant females. One male in the control group exhibited loose stool on dosing day 2 but at no other time. All animals survived until the scheduled termination of the study.

There was no effect on individual body weights (BW) or body weight gain (BWG) during the study. Food consumption in successfully mated females was not affected. There were small, non-significant variations (absent dose-response relationships) in absolute organ weights. Relative organ weights were not affected by administration of the test article.

Necropsy results (males after successful mating and females after sacrifice on gestation day 13) revealed no grossly observable signs of toxicity. Findings in males included dilated renal pelvis in a vehicle control group animal, white spots in the epididymis of a single animal in the Low-Dose group and a thyroid gland nodule in a Mid-Dose group animal. The only observed abnormality in females was a rudimental uterine horn in one control animal. Macroscopic findings in bilateral organs were consistently unilateral.

Males in the mid-dose group exhibited an increased fibrous tissue in the thyroid gland. Mid- and high-dose males had a spermatic granuloma in the epididymal tails and dilated renal pelvis was found in one control group male. One female in the control group had a rudimental uterine horn, although no microscopic changes were observed in the uterus.

All pairs, except for a single low-dose pair, successfully copulated during the initial mating trial. Both of the unsuccessful partners successfully mated with new partners during a second mating trial.

One female in the control, low- and high-dose groups exhibited infertility. No reproductive abnormalities were observed during macro- and microscopic examination.

The mean numbers of estrous cycles required for pregnancy, and the copulation and fertility indices were comparable between the vehicle control and treated groups.

The implantation index range from 94 to 97.4% in the low- and high-dose groups respectively. The percent of fetuses alive on gestation day 13 was greater than 90% in all groups and the mean incidence of dead fetuses per dam ranged from 0.6 in the high-dose group to 1.2 in the low-dose group.

The assessment of mating behavior and fertility produced no signs of systemic toxicity. Body weights, food consumption and absolute and relative organ weights were similar in groups of control and dosed animals. Similarly, there was no effect on either mating behavior or fertility. Although a single pair of rats failed to copulate during the two-week co-habitation period, both of the animals were successful when paired with new partners.

Examination of ovarian and uterine contents of female rats sacrificed on gestation day 13 yielded no evidence of embryotoxicity. The mean numbers of implant sites per impregnated female were similar in all experimental groups as were the mean numbers of living and dead fetuses in each group. Macroscopic examination at the necropsy of all experimental animals revealed few changes, none of which were considered attributable to administration of VPP plus IPP in powdered *L.helveticus* fermented milk.

The fertility of a single male dosed with the 500 mg/kg bw/day dose of powdered *L. helveticus* fermented milk (containing 0.8 mg/kg bw/day of VPP+IPP) was not proven during the mating phase of the experiment. At necropsy this animal exhibited atrophied seminiferous tubules and his epididymal ducts contained degenerated spermatozoa. These microscopic findings explain the animal's infertility but the relationship of this finding in one animal to the administration of test article is unclear.

The authors concluded that the maximally tolerated dose of VPP plus IPP in powdered *L.helveticus* fermented milk in breeding rats is greater than 3.3 mg/kg bw/day. The No

Observable Adverse Effect dose of VPP plus IPP in powdered *L.helveticus* fermented milk on female rats was >3.3 g/kg bw/day (Kurosaki et al. 2005).

3.5.2 Embryo and Fetal Development

Female and male Sprague-Dawley rats (Crj:CD(SD)IGS, Charles River Japan) were co-habited for 17 hours following acclimation to laboratory conditions. The females were examined for the presence of vaginal sperm, which was an indicator of successful copulating. A positive vaginal smear was defined as day 0 of gestation. The mating and sperm checks continued until a total of 84 sperm positive females were obtained. The females were weighed and randomly assigned to one of four experimental groups, each comprised of 21 sperm positive females and designated to receive daily oral doses of VPP plus IPP in powdered *L.helveticus* fermented milk in doses of 0, 0.8, 1.6, 3.3 mg/kg bw. The actual numbers of pregnant animals were 20, 19, 20 and 20 in control, low-, mid- and high-dose groups, respectively. Administration of the test article to pregnant females began on gestation day 7 and continued through gestation day 17 (11 consecutive days).

Pregnancies were terminated on gestation day 20 by cesarean section. Abnormal or altered organs and the corresponding organs of vehicle control animals were removed and stored in 10% neutral buffered formalin solution.

Each uterus was removed and examined for evidence of embryonic or fetal death. The number of live fetuses was determined. Dead embryos or fetuses were classified as having been an implantation site, placental remnant, resorption (early or late), macerated fetus or dead fetus. For the dams with no implantations, the uterus was incised and examined with ammonium sulfide staining. If the stain confirmed the absence of implantation sites, the animal was considered infertile. Ovaries of all pregnant rats were examined and the number of *corpora lutea* counted. Implantation and post-implantation loss indices were computed.

Live fetuses were separated according to sex, weighed and examined for external anomalies. Fetuses with grossly observable external malformations were fixed in 10% neutral buffered formalin solution without being subjected to either skeletal or visceral examination. With the exception of any externally malformed fetuses, about the half of each litter was allocated to skeletal examination, and the remaining animals to visceral examination.

Visceral and skeletal observations were performed on the fetuses of the vehicle control and high-dose groups. A significant increase in ossification numbers of fore- and hind limb phalanges was observed in the high-dose group. Therefore, similar examinations were also performed on the fetuses from the low- and mid-dose groups.

There were no clinical signs that suggested either systemic or local toxicity. All pregnant animals survived until their scheduled day of cesarean section (gestation day 20). There were no spontaneous abortions or premature births. All animals gained weight during the study and mean body weights of the treatment groups were comparable to those of vehicle controls throughout the pregnancy period. Food consumption was comparable across both control and treated groups.

Gross examination revealed few observable changes. One mid-dose dam exhibited a recessed area in her right kidney. In the high-dose group, one female presented with an enlarged spleen, while another in that group exhibited an inguinal, subcutaneous mass. No additional gross lesions were observed. Since each of the changes occurred in single animals of diverse groups none were considered to be attributable to administration of the test article.

All fetuses were alive and the mean number (12.4-14.8) and percent of live fetuses (89.1-96.3) were similar in all groups. The numbers of *corpora lutea* were also similar in all animals.

The implantation indices for mid- and high-dose groups were significantly reduced (12 and 6%, $p < 0.01$ and 0.05, respectively) although there was no evidence of dose dependency in the magnitude of the difference. There was no treatment related effect on the sex ratio of pups or mean fetal body weights across the groups. There was a one fetus in the high-dose observed to have been edematous.

The most frequent change was the presence of thymic remnants in the necks of 10 fetuses from Control dams and 9 fetuses from high-dose dams. One fetus from a control dam exhibited dilation of the cerebral ventricles but this change was not observed in dosed animals. One fetus in the control group and 2 in the high-dose group exhibited persistent left umbilical arteries. None of these changes are considered attributable to administration of the test article.

There were no skeletal abnormalities observed in fetuses from either the control or the high-dose group. The presence of a fourteenth rib was the most frequently observed skeletal variation. It was observed in 12 fetuses from 8 different Control dams and in 13 fetuses from 9 different dams in the high-dose group. Four fetuses from different dams in the high-dose group demonstrated a shortened thirteenth rib. This variation (mean incidence 2.4% per affected dam) was not observed in fetuses from the control dams. The small difference in incidences between control and the high-dose group was not statistically significant. Additionally, while this is somewhat elevated relative to historical control background data of 1.4%, in 5 studies in this strain of rats, the difference was not considered to be treatment related because it was not statistically different from controls.

During assessment of ossification of fore- and hind limb phalanges small but statistically significant (5-12%, Student's t test, $p<0.05$) increases in the numbers of ossification sites were observed in fetuses from dosed females relative to vehicle controls; this triggered a skeletal evaluation of the low- and mid-dose fetuses. The complete data set was re-analyzed by analysis of variance and no statistically significant differences were observed among experimental groups, including the 3.3 mg/kg bw/day group.

Powdered *L.helveticus* fermented milk containing VPP and IPP at doses of 1.8 and 3.3 mg/kg bw/day was associated with a significantly decreased implantation index. The authors looked at historical control data from contemporary experiments conducted in this strain of rats. Implantation indices in the mid- and high-dose treated animals (86.9% and 92.1% respectively), were comparable to the historical control data from 6 earlier studies (90.9±5.7%) in 80 vehicle control dams from the previous 4 years. Based on the historical control implantation indices the small differences observed in this study were most likely attributable to an abnormally high index in concurrent control animals (98.4%) rather than to administration of the test substance.

The authors concluded that given the historical control implantation indices, the small differences observed in this study were most likely attributable to an abnormally high index in concurrent control animals rather than to administration of the test substance (Kurosaki et al. 2005).

3.5.3 Maternal function and pre- and post-natal development

Female and male Sprague-Dawley rats (Crj:CD(SD)IGS, Charles River Japan) were co-habited for 17 hours following acclimation to laboratory conditions. The females were examined for the presence of vaginal sperm, which was an indicator of successful copulation. This was defined as day 0 of gestation. The mating and sperm checks continued until a total of 88 sperm positive females were obtained. The females were weighed and randomly assigned to one of four experimental groups, each comprised of 22 sperm positive females. Administration of the test article to pregnant females began on gestation day 7 and continued daily through lactation day 20. Females delivered their offspring spontaneously. The doses of VPP and IPP in powdered *L.helveticus* fermented milk were 0, 0.8, 1.8, 3.3 mg/kg bw/day.

Five additional mated but untreated females were maintained to obtain their female offspring for possible use during the assessment of reproductive performance of the F_1 generation. The 5 untreated mated females were allowed to deliver their offspring (F_1 animals), which were then culled to 5 pups per litter on postnatal day 4. At weaning the maternal and unneeded F_1 animals were sacrificed without necropsy. Seven of the untreated, F_1 animals were used for re-mating purposes to test mating behavior and fertility of treated F_1 males that were unsuccessful in their initial cohabitation trials.

Each litter was randomly culled to 8 pups (4/sex) on postnatal day 4. These offspring were weaned on postnatal day 21, and 1 animal per sex from each litter was randomly selected for observations of behavior (emotionality and learning tests), organ weights and macropathological examination on postnatal day 48. An additional 1 animal of each sex from each litter was selected for assessing body weights (after weaning), sexual maturity, reproductive capability, and macropathological examination after mating. The remaining animals were sacrificed and subjected to macropathological examination at weaning.

After weaning, F_1 animals (lactation day 21) the dams were killed using carbon dioxide asphyxiation inhalation and examined macroscopically. Uteri were removed and the number of implantations determined.

At parturition, offspring were examined for mortality and external abnormalities, sexed, and individually weighed. During the lactation period, the offspring were observed daily and weighed on postnatal days 0, 4, 7, 14 and 21. Stillbirths and pups that died during the lactation period were subjected to necropsy, including renal cross sectioning. Pups culled on postnatal day 4 were sacrificed using carbon dioxide and macroscopically examined for vertical cross-section images of the kidney. The live birth index (percent of implant sites resulting in live births), viability index on day 4 (percent of live births that survived until day 4) and the lactation index (percent of pups at day 4 surviving until weaning) were computed.

Offspring were observed for achievement of the following landmarks of physical development: pinna detachment (both pinna), Incisor eruption (upper incisor), and eye opening (both eyelids).

One male and one female from each litter was designated for assessment of behavioral and sensory development and were tested for achievement of landmarks of sensory functional development and cumulative percentage achievements were calculated for each day of observation.
Two animals of each sex were selected for subsequent testing. The remaining pups (2 of each sex per litter) were killed using carbon dioxide and subjected to macropathological examination. Any kidneys that presented with macroscopic abnormalities were removed and fixed/stored in 10% neutral buffer formalin solution.

On postnatal days 26-29, one male and one female pup per litter were employed in an open-field test for assessment of emotional reactivity. The water T-maze was employed to assess learning.

F_1 animals used for behavioral observations were sacrificed and examined macroscopically (including examination of kidney) on postnatal day 48.

Observations for achievement of physical development were made in one male and one female per litter maintained for use in mating trials. The landmarks were: vaginal opening and cleavage of the balanopreputial gland observed on postnatal days 34 and 41.

On postnatal day 70 (10 weeks old), male and female off-spring in the same experimental groups were individually co-habited, to avoid sibling mating, for up to 2 weeks. The females were examined daily for sperm in the vagina and mated females were separated from males. Males and females unsuccessful in mating were cohabited with new partners. Females unsuccessful in mating were housed with successful males, and unsuccessful males were housed with untreated females. Mating trials were terminated if animals were unsuccessful after 4 trials.

All males and unsuccessful females were sacrificed at the end of the mating period. Successfully mated females were sacrificed on gestation day 13 and examined.

Uteri from the mated females were removed to examine the pregnancy status (number of implantations and live fetuses). If no implantation sites were observed, the uterine horn was incised and tested with 10% ammonium sulfate stain. The presence of implantation was regarded as a sign of pregnancy. *Corpora lutea* were counted and copulation, fertility and implantation indices and percent of post-implantation loss were computed.

There was no effect on survival of the maternal animals. There were no abortions or premature deliveries. Clinical signs exhibited by pregnant females were nonspecific and did not demonstrate a dose-response relationship. One female in the high-dose presented on gestation day 6 with an upper jaw injury. This resulted in a transient effect on food consumption and body weight gain, however, that animal delivered and reared

her pups normally. One female in the mid-dose group exhibited fur loss on her chest between gestation day 14 and lactation day 19.

There was no effect on mean body weights of F_0 rats during pregnancy and lactation. There was no significant affect on food consumption. Mean food consumption on gestation days 9 and 10 was reduced in the high-dose group due to the aforementioned upper jaw injury. This reduction was not statically significant. This injury also reflected in a transient but non-significant reduction in the high-dose group mean body weight on gestation day 7.

All animals delivered live offspring. The mean gestation period ranged from 22.1 to 22.5 days. The numbers of stillborn pups were not significantly different (3, 3, 2, 7 in the control, low, mid- and high-dose groups respectively). Sex ratios were similar for each experimental group as were mean body weights of fetuses. No offspring were observed to have grossly observable abnormalities.

There was no effect on survival (percent of offspring surviving between postnatal days 1 and 4 were 97.9, 97.8, 98.6, and 97.6 in control, low-, mid- and high-dose groups respectively). Each group contained 176 offspring after culling on postnatal day 4. All but a single control pup survived until weaning on lactation day 21. F_0 animals were sacrificed at weaning of the F_1 generation and macroscopic examination revealed no treatment-associated changes. Observations of weaned F_1 animals revealed no differences between groups with respect to spontaneous behavior.

There was no effect on F_1 animals after weaning in either rate or magnitude of body weight gain.

There were no significant differences in the number of animals achieving the measured developmental end-points nor the time at which the end-points were achieved. Similarly, physical development relating to sexual maturity was also unaffected.

Achievement of cleavage of the balanopreputial gland of males and vaginal opening of females did not differ between the control and treatment groups.

There was a significant decrease in the percent of animals achieving the mid-air righting reflex in both sexes of offspring of the high-dose group on day 13 after birth. Males from mid-dose group exhibited a decrement in achievement of the mid-air righting reflex on day 14. Times to achievement of righting reflex on a lateral surface, negative geotaxis and auditory startle reflex did not differ between the control and treatment groups.

There was no treatment related effects observed in the open-field emotionality test; ambulation and the incidence of defecation were unaffected by animal's earlier exposures to the test article.

During the assessments of learning ability in the water maze, it was observed that failure of rats to reach the goal within the arbitrary 3-minute limit was rare. In three trials, only 2 rats fail: mid-dose males, 3rd trial, day 2; low-dose females, 4th trial, day 2 and high-dose females, 1st trial, day 2. Males in the low-dose group exhibited an increased number of errors during the fourth trial of the third day of exposure to the maze. However, there was no evidence of a dose-response relationship on that day and error rates were similar for control and treated animals of either sex for the rest of the experiment. High-dose males exhibited a prolonged mean time to reach the goal during the fourth trial of the third day of exposure to the maze. There was no trend toward prolongation in the other treatment groups and there was no evidence of a decrement during trials on day 3.

With regard to reproductive performance of the F_1 generation animals, there were no significant differences in copulation and fertility indices between the control and treated groups. Initially, 7 pairs were not successful in copulating (1 each in the control, low- and mid-dose groups and 4 in the high dose-group); all animals were successful after re-mating with a different partner. There were 4 infertile females that had mated during

the initial mating (1 each in the low- and mid-dose groups and 2 in the high-dose group) and during the re-mating trial one of the replaced, untreated females that was a partner of one of the high-dose males was infertile.

There were no abnormal clinical signs observed in any of the females and body weights during pregnancy did not differ between the vehicle control and treated groups.

There were no significant differences in the numbers of *corpora lutea*, and implantations and the mean implantation index ranged from 14.9 in low-dose group to 15.5 in the mid-dose group. All pregnant rats carried live fetuses and the mean number of dead fetuses was less than 1 per litter, ranging from 0.5 in low-dose group to 0.9 in the control group.

There were no macroscopic changes observed in the offspring that died at delivery, during lactation, or those culled on postnatal day 4.

At weaning, dilation of the renal pelvis was observed in 2 male and 1 female control group offspring and in 1 low-dose and 2 high-dose males. Changes were unilateral in most animals but the single affected high-dose female exhibited a bilateral change.

In the offspring necropsied on post natal day 48 (used for behavioral observations), unilateral dilated renal pelvis was observed in 4, 4, 3 and 2 male offspring in control, low-, mid- and high-dose groups, respectively; unilateral discolored renal plaques in 1 male in each of the treatment groups; unilateral cystic renal cortex in 1 male each in the low- and mid-dose groups; and small seminal vesicles (bilateral) in 1 high-dose male. Female offspring exhibited, unilateral dilated renal pelvis (1 animal from low-dose group); and bilateral dilated renal pelvis in 1 animal from mid-dose group.

One male offspring in the high-dose group that swam in the test maze on the "training" day was found dead in the next morning. Necropsy revealed dark-red lungs and the death of this animal was attributed to inhalation of water during swimming.

For the offspring tested for reproductive capabilities, necropsy revealed dilated renal pelvis (unilateral) in 4, 5, 1 and 3 males from the control, low-, mid- and high-dose groups respectively; unilateral discolored renal plaques in 1, 2 and 1 males from control, low- and high-dose groups; unilateral small seminal vesicle in 1 male each in the control and low-dose groups; and bilateral small seminal vesicle in one high-dose male. In females, unilateral dilated renal pelvis was observed in 2 low-dose and 1 mid-dose animals; unilateral discolored plaques in a control animal; bilateral dilated renal pelvis in 1 female in the control and high-dose groups, respectively; and bilateral cystic renal cortex in one control group animal. These findings were all found in animals that had been pregnant.

Significantly decreased absolute weights of male and female brain, male prostate and female uterus were observed in F_1 animals in the high-dose group. The decrease in absolute brain weight appeared dose related down to 1000 mg/kg powdered *L.helveticus* fermented milk (FM) for males and to 500 mg/kg for females; and the decrease in absolute prostate weight down to 1000 g/kg FM for males. A significantly decreased relative adrenal weight was observed for males in high-dose, but there was no trend toward reduction in the other treatment groups.

The authors concluded that administration of VPP + IPP in powdered *L.helveticus* fermented milk to pregnant and lactating dams produced no evidence of toxicity in either the F_0 or F_1 generation. There were no external abnormalities in the F_1 animals. There were no treatment related effects on F_1 generation live births sex ratio, body weights at birth, pup viability, or clinical signs during lactation and after weaning. Attainment of landmarks of physical, sexual and sensory maturity were unaffected by administration of test article.

There was a decreased percent achievement of air righting in male and female F_1 animals in the high-dose group on postnatal day 13 and in males from the mid-dose group on post natal day 14 during the sensory function test. All pups in the treated

groups achieved 100% of the criteria no later that the control group (post natal day 16). Therefore, these findings were not considered to be of toxicological significance.

There were a significantly increased number of errors in the water T-maze learning test in pups delivered by dams in the low-dose group, and a significantly prolonged mean time to reach the goal in male pups from the high-dose group dams on the 4th trial of the final day of the experiment. Due to the absence of a dose-relationship, this increased error rate was not considered to be treatment. The authors note that the prolongation of mean time to reach the goal observed in the high-group was due to a single rat that took 161 seconds during the 4th trial which disproportionately increased the group mean. When this score is eliminated, the group mean was similar to mean times of all of the other groups (21.6 seconds). The authors concluded that the learning ability was not affected by administration of the test article.

Dilation of the renal pelvis was observed in all experimental groups and is considered to be naturally occurring change in this strain of rats. The authors concluded that the decrease in the absolute weights of male and female brain, male prostate and female uterus and the increase relative male adrenal weight in the high-dose group were not considered to be treatment related as the change was either in the absolute or relative weight, and these organs were macroscopically unaffected.

The authors concluded that administration of VPP and IPP in powdered *L.helveticus* fermented milk during pregnancy and lactation did not adversely affect pre- or postnatal development of offspring. The No Observable Adverse Effect dose of VPP and IPP in powdered *L.helveticus* fermented milk for both the maternal organism and embryo-fetus has reported to be 3.3 mg/kg bw/day.

3.6 Observations in Humans

3.6.1 Seven-Day Study in Healthy Volunteers

A double-blind, placebo-controlled study with 20 healthy subjects was conducted to examine the potential biological effects of milk-derived tripeptides, L-valyl-L-prolyl-L-proline (VPP) and L-isoleucyl-L-prolyl-L-proline (IPP), derived from reconstituted skim milk fermented with *L.helveticus*. The study design included a two-day pre-intake observation period, a seven-day consumption period and a four-day post-intake observation period (Yasuda et al. 2001).

The subjects, which ranged in age from 20-65 years old, were divided into two groups, treated and placebo (10 subjects/group, 5 male, 5 female). All subjects consumed 14 tablets/day in the morning for seven days. Each tablet consumed by the treated group contained 1.26 mg VPP and 0.82 mg IPP (total 2.08 mg/tablet, 29.1 mg/day); the tablets consumed by the placebo group contained no VPP or IPP.

Height, body weight (BW), body mass index (BMI) measurements and blood (hematology [WBC, RBC, Hb, Ht, MCV, MCH, MCHC, platelet count], and clinical pathology [total protein, A/G ratio, uric acid, uric nitrate, creatinine, Na, Cl, K, Ca, GOT, GPT, ALP, γ-GTP, LDH, total bilirubin, total cholesterol, HDL-cholesterol, triglyceride {TG}, blood sugar] and urine analyses [urobilinogen, protein and glucose] were performed once before consumption, and days 1 and 4 of the post-intake observation period.

Medical examinations were performed on day 2 of the pre-consumption period, day 4 of the consumption period and days 1 and 4 of the post-consumption period.

Blood pressure (BP, systolic [SBP] and diastolic [DBP]) and pulse rate (PR) were measured on all days except for day 3 of the post-intake observation period (12 times in total). The subject rested for 10 minutes prior to the measurements.

The subjects recorded, in a log provided for this purpose, symptoms they experienced during treatment. The symptoms were rated as mild, (awareness of symptoms, but easily tolerated and no medicine was required), moderate (uncomfortable, but does not prevent daily activity, need to take medicine), or severe (unable to perform usual activity, need to be hospitalized).

During the pre-consumption period, there were no meaningful differences between the treated and placebo groups with regard to age, gender, height, BW, SBP, DBP, PR or BMI.

With regard to SBP, the placebo group demonstrated significant reductions on consumption Days 1, 5 and 7 and post-consumption day 4 (compared to its own baseline). No significant change in treated group SBP (compared to its baseline) was noted at any interval. On consumption day 5, there was a significant difference in SBP between the treated and placebo groups (112.8 ± 6.5 vs 103.9 ± 7.8, $p<0.05$).

DBP was decreased in both groups on consumption day 7 (compared to their respective baselines); however, there were not differences between the groups.

No significant changes in PR or BMI were observed at any observation interval.

Significant within group hematology differences were observed in Ht, MCV, and MCHC values in both placebo and treated groups when baseline and post-consumption day 4 values were compared. In additional, the treated group exhibited a significant decrease in hemoglobin ($p<0.05$) on post-consumption day 4. The only difference between treated and placebo values occurred in WBC values on pre-consumption day 1; the treated group value was higher (4.9 ± 1.0 versus 6.1 ± 1.6, $p<0.05$). All of the values reported herein were within normal physiological ranges.

Significant within group clinical pathology differences were observed in creatinine, chloride and calcium values in both placebo and treated groups when baseline and post-consumption day 4 values were compared. In additional, the treated group exhibited a significant increase in ALP ($p<0.05$) on post-consumption day 4 and the placebo group demonstrated a significant decrease in BUN and increase in calcium on post-consumption day 1 and decreases in A/G ratio, TG on post-consumption day 4. The only difference between treated and placebo values occurred on post-consumption day 4 of the post-observation period where ALP in the treated groups was significantly higher (162.0 ± 42.9 versus 196.7 ± 43.5, $p<0.05$). All of the values reported in these comparisons were within normal physiological ranges.

Each tablet contained the same amount of lactose (0.75 g) and sorbitol (0.49 g). The relatively large intake of indigestible saccharides often produces abdominal symptoms of flatulence, abdominal pain and diarrhea. The amount of food consumed with the tablets and individual variability influences the degree of symptoms experienced. There were 24 reports of flatulence and 3 of acceleration of gastrointestinal movement in the treated group as compared to 7 and 20 reports, respectively, in the placebo group. There were 2 reports of watery feces in the placebo group. Upon further investigation, it was determined the subject that reported watery feces had consumed a very light breakfast (juice and half of a peach).

SRA EVALUATION

This study was conducted to examine the potential biological effects of milk-derived tripeptides, L-valyl-L-prolyl-L-proline (VPP) and L-isoleucyl-L-prolyl-L-proline (IPP), derived from reconstituted skim milk fermented with *L.helveticus.*

The strengths of the study included its double-blind design, inclusion of a concurrent placebo control, randomization of an adequate number of subjects, pre-consumption and post-consumption measurement periods, seven-day repeated dosing, and adequate and repeated physiological measurements.

The study could have included a larger number of subjects and the daily consumption in this study was higher than the estimated consumption level being considered by the Expert GRAS Panel.

3.6.2 Two-Week Study in Healthy Volunteers

A two-week, double-blind, placebo-controlled study with 43 healthy subjects was conducted to examine the potential biological effect of tripeptides (L-valyl-L-prolyl-L-proline [VPP] and L-isoleucyl-L-prolyl-L-proline [IPP]) derived from reconstituted skim milk fermented with *L.helveticus* (Kajimoto et al. 2001b).

The subjects, which ranged in age from 20-46 years old, were divided into two groups, placebo (22 subjects [10 male, 12 female]) and treatment (21 subjects [10 male, 11 female]). All subjects received six tablets to be taken in the morning for two weeks. Each tablet contained 1.31 mg VPP and 0.75 mg IPP (2.06 mg/tablet, 12.36 mg/day) or placebo. The two-week test period (consumption) was preceded by a five-day baseline period (no-consumption) and followed by a seven-day observation period (no consumption).

Various physiological evaluations of each subject occurred throughout the study. A medical examination was performed at four time points during the study: on the day prior to initiation of consumption, twice during the consumption period and on the final observation day.

Blood pressure (BP) including both systolic and diastolic blood pressure (SBP, DBP) and pulse rate (PR) were evaluated at nine intervals: three times before consumption, four times during the consumption period and twice following cessation of consumption. Subjects rested for 20 minutes prior to the evaluations; BP was measured three times at each interval. The median SBP and DBP were recorded.

Body weight (BW) was measured weekly. Height was measured once; on the day prior to initiation of consumption

Blood and urinary analyses at three intervals: on the day prior to initiation of consumption (baseline), on the last day of consumption and on the last day of the observation period. The hematology parameters analyzed included: WBC, RBC, Hb, Ht, and platelet count. The clinical pathology parameters evaluated included: total protein, A/G ratio, urea nitrogen, creatinine, Na, Cl, K, Ca, GOT, GPT, ALP, γ-GTP, LDH, total bilirubin, urate, total cholesterol, triglyceride, HDL-cholesterol, fasting blood sugar, and HbA_{1c},

Prior to test article consumption, no significant differences were observed between the placebo and treatment groups with regard to age, gender, height, BW or body mass index (BMI), SBP, DBP, and PR.

No statistically significant difference in SBP, DBP, PR or BMI was observed either between groups or compared to baseline in any phase of the study.

Normal variation in hematology and clinical pathology measures were observed in both groups. Significant increases in HbA_{1c}, creatinine, and calcium and decreases of total protein, HDL-cholesterol, sodium and potassium were observed in the treated group. The placebo group demonstrated significant increases in blood sugar, creatinine, and chloride and significant decreases in sodium, albumin/globulin ratio and HDL-cholesterol. All changes were within normal physiological ranges.

Two subjects in the placebo group demonstrated an increase in uric protein. The authors considered this change not to be serious as the subjects were thought to have orthostatic proteinuria or proteinuria. Two incidences of occult blood occurred in each group. The symptoms, which occurred in female subjects following menstruation, were not present a few days later and were considered to not be of clinical significance.

There were no reports of dry cough, exanthema, skin itching, digestive discomfort, vertigo, dyspepsia and dizziness or headaches. Two females in the placebo group exhibited transient diarrhea and stomachache on Days 3 and 7 of treatment. The symptoms resolved naturally and were not considered treatment related.

SRA GRAS Panel Evaluation:

A total of 43 healthy subjects consumed 12.36 mg of VPP+IPP daily for two weeks.

The results suggest the absence of any observable (or reported) test article related biological effects on clinical symptoms, BW, BMI, SBP, DBP, PR, hematology and clinical pathology measurements. All values were within their normal ranges and differences between groups or within groups (compared to baseline) were due to normal biological variation.

These results suggest that daily consumption of 12.36 mg of VPP+IPP for two weeks produced no untoward biological effects. The study was robust with regard to the study design (e.g., double-blind, concurrent placebo control, multiple-doses), parameters measured and intervals at which they were measured. The study was limited by the number of subjects employed, and the absence of multiple dosage levels. The dosage employed in this study (12.36 mg/day) is approximately the anticipated 90% percentile Eaters Only average daily consumption of VPP+IPP, from the four food groups considered by the GRAS Expert Panel.

3.6.3 Four Week Study in Normotensive and Mildly Hypertensive Subjects

A double-blind placebo-controlled study was conducted to investigate the potential biological effects of consuming milk-derived tripeptides (from casein hydrolysate) (Sano et al. 2004). Forty-three subjects (21 male, 22 female, mean age 46.8±7.5 years old) were administered a 200 ml bottle of vegetable fruit drink containing either VPP and

IPP (1.47 mg/200 ml and 1.6 mg/200 ml, respectively) or placebo, three times per day for four weeks.

The subjects ranged from normotensive to mildly hypertensive (mean = 133.7±14.6 and 80.9±9.1 mmHg, systolic blood pressure [SBP] and diastolic blood pressure [DBP], respectively). Subjects were randomized across treatment (11 males, 11 females) and control (10 males, 11 females) groups. At baseline, there were no significant differences in age, SBP, DBP, pulse rate (PR), height, weight (BW) or BMI. The subjects received a medical examination weekly, beginning 2 weeks pre-dosing (baseline), during the 4-week dosing-period and for 2 weeks post-dosing. Blood pressure, pulse rate, weight, height, blood analysis and urinalysis were measured 2 weeks prior to treatment. Blood pressure, pulse rate, weight were measured weekly. Blood analysis and urinalysis were measured at the beginning, at the end of the dosing-period, and 2 weeks after the dosing-period thereafter.

Blood was analyzed for the following parameters: WBC, RBC, hemoglobin, hematocrit, GOT, GPT, (-GTP, LDH, ALP, total protein, albumin, A/G, neutral fat, total bilirubin, total cholesterol, HDL-cholesterol, glucose, HbA_{1c}, urea nitrogen, creatinine, uric acid, electrolytes (Na, Ca, K, Cl, Mg) and renin activity (PRA). Urine was analyzed for specific gravity, pH, glucose, protein, occult blood, urobilinogen, Na, K, and creatinine. Urinary excretion of sodium and potassium were calculated.

Following one week of treatment, there were no significant difference in systolic blood pressure (SBP) compared to baseline, (132.3±17.0 vs. 132.0±15.4 mmHg).

Following four weeks of treatment, the treated group exhibited a mild and statistically significant decrease in SBP compared to baseline (127.4±13.5 versus 132.3±17.0 mmHg, $p<0.05$). A similar decrease was observed in diastolic blood pressure at four weeks (76.8±7.1 versus 80.6±9.2 mmHg, $p<0.05$). These decreases in SBP and DBP were not associated with any marked changes in pulse rate, blood analysis, urinary analysis or adverse clinical events; all values, even if statistically

significant, were all within the normal range. Four subjects in the placebo group experienced nausea and diarrhea. The symptoms were transient and rapidly reversed. There were no sudden or excessive decreases in blood pressure.

SRA GRAS Panel Evaluation:

A total of 22 subjects with either high-normal, mildly hypertensive or normal blood pressure consumed 3.07 mg of VPP+IPP three times/day for 4 weeks.

The results demonstrate treatment related decreases in SBP and DBP in the mildly hypertensive but not high normal or normotensive individuals at 4 weeks. No differences in clinical symptoms occurred between any of the groups. Differences between treatment groups and their baselines or the placebo group in hematology and clinical pathology measurements were either not statistically significant, or the values were within the normal range.

These results suggest that daily consumption of 3.07 mg of VPP+IPP, three times a day for four weeks produced no untoward biological effects. The study was robust with regard to parameters measured and intervals at which they were measured and its design (e.g., double-blind). The study was limited in that it employed relatively few subjects the consumption level was below the level considered by the GRAS Panel.

3.6.4 Six-Week Study in Subjects with High-Normal or Mildly-Hypertensive Blood Pressure

A six-week, single-blind, placebo-controlled study with 131 subjects with high-normal blood pressure (systolic blood pressure [SBP] 120-139 mmHg) or mildly hypertensive (SBP 140-159 mmHg) was conducted to examine the effect of casein hydrolysate containing major angiotensin-I-converting enzyme inhibitory peptides L-valyl-L-prolyl-L-proline (VPP) and L-isoleucyl-L-prolyl-L-proline (IPP) on blood pressure (Mizuno et al. 2005a).

Each subject received two tablets containing one of four different doses of VPP and IPP (0, 1.8, 2.5, 3.6 mg) to be taken daily at breakfast. Blood pressure, pulse rate, body weight, and height were measured two weeks prior to dose administration (baseline), and at 0, 1, 3, and 6 weeks after the start of treatment. The subjects were randomly assigned to one of the four groups. There were no significant differences between the groups at baseline with regard to gender, age, heart parameters (SBP, DBP, pulse rate [PR]), body mass index (BMI), body weight (BW), and height (Hgt). Each high normal BP group contained twelve subjects and each mild hypertension group contained 21 subjects. There were 20 subjects with mild hypertension in the placebo group.

Medical examinations, including measurement of SBP, DBP, PR, BW and Hgt were administered at 2 weeks prior to treatment (baseline) and at 0, 1, 3, and 6 weeks after the start of treatment. BP was the average of three values taken after a 10 minute resting period.

Peripheral blood was analyzed at 0 and 6 weeks of the treatment period. The analysis included the both clinical chemistry (i.e. urea, nitrogen, creatinine, uric acid, electrolytes [Na^+, K^+, Cl^-, Ca^{2+}, and Mg^{2+}], GOT, GPT, ALK PHOS, (-GTP, LDH, total bilirubin, total, protein, albumin, albumin:globulin ratio, blood glucose, hemoglobin A_{1c}, total cholesterol, HDL-cholesterol, and triacylglycerol), and hematology measurements (i.e. peripheral blood [leukocyte count, erythrocyte count, mean corpuscular volume, mean corpuscular hemoglobin, platelet count]).

A few adverse clinical events were reported in all groups during the study. In the placebo group, six subjects reported cold-like symptoms, two experienced diarrhea, and one reported pruritis. In the groups that received the tripeptides, three subjects reported cold-like symptoms, two experienced diarrhea, one developed constipation, one reported pruritis. The symptoms were reported in both the placebo and treatment groups, all subjected recovered during treatment, and the condition were not considered by the authors to be related to treatment. There were no reports or observations of dry coughs, or skin or gastrointestinal symptoms.

BW and BMI significantly decreased in the placebo and 2.5 mg group compared to baseline at the one week determination. These parameters were not significantly altered in the other study groups.

Hematological parameters (i.e: albumin, albumin:globulin ratio, hemoglobin A_{1c}, and Na^+) were significantly altered in all groups compared to baseline at the six week period. In addition, the 2.5 mg group was observed to have a significantly higher K^+ concentration compared to baseline. All values were within the normal range and not considered by the authors to be abnormal variations.

SBP was significantly decreased in the 1.8 mg (VPP+IPP) group (5.8 mmHg; $p<0.01$) at six weeks compared to baseline. SBP was significant decreased in the groups receiving 2.5 and 3.6 mg VPP+IPP at the third week (3.4 and 4.1 mmHg; $p<0.05$ and $p<0.05$, respectively) and sixth week (6.2 and 9.3 mmHg, $p<0.001$ and $p<0.0001$, respectively) determinations.

There was a significant difference in SBP between the placebo group and the group receiving 3.6 mg VPP+IPP at six weeks. There were no significant differences in the groups receiving either 1.8 or 2.5 mg VPP+IPP as compared to the placebo group.

DBP and PR in the treated groups did not differ significantly compared to either their respective baselines or to the placebo group.

<u>SRA GRAS Panel Evaluation</u>:

A total of 99 subjects with either high-normal or mildly hypertensive blood pressure consumed various amounts of VPP+IPP (1.8, 2.5, 3.6 mg) daily for 6 weeks.

The results demonstrate a dose related decrease in SBP in the mildly hypertensive but not high normal individuals. No differences in clinical symptoms occurred between any

of the groups. Differences between treatment groups and their baselines or the placebo group in BMI, BW, DBP, PR, hematology and clinical pathology measurements were either not statistically significant, not dose-related, or occurred only at a single dose level and that value at that level was within the normal range.

These results suggest that daily consumption of 3.6 mg of VPP+IPP for six weeks produced no untoward biological effects. The study was robust with regard to numbers of subjects and study groups, parameters measured and intervals at which they were measured. The study was limited in that it employed a single-blind design and the highest consumption level was below the level considered by the GRAS Panel.

3.6.5 Eight-Week Study in Subjects with Mild or Moderate Hypertension

An eight-week, double-blind, placebo-controlled study with 81 subjects with mild or moderate hypertension (defined by WHO/ISH 1999, systolic blood pressure [SBP] 140-179 mmHg, diastolic blood pressure [DSP] 90-109 mmHg) was conducted to examine the potential effect of consuming milk-derived tripeptides (L-valyl-L-prolyl-L-proline [VPP] and L-isoleucyl-L-prolyl-L-proline [IPP]) on blood pressure (Kajimoto et al. 2001b). The tripeptides were derived from reconstituted skim milk fermented with *L.helveticus*. The subjects were not on any blood pressure regulating medication.

Each subject received two tablets to be taken at breakfast for eight weeks. Each tablet contained 1.26 mg VPP and 0.82 mg IPP or placebo. There was a two-week pre-observation (non-intake) period and a four-week post-observation (non-intake) period.

A medical examination was performed every two weeks. Blood pressure (SBP and DBP) and pulse rate (PR) were measured for 12 weeks (initiation – end of withdrawal) period, following a 30-minute rest period. Three blood pressure readings were taken at each interval and the median value was used.

Blood, urinary and BW measurements were taken prior to the start of treatment (baseline) and at the end of treatment (eight weeks). Clinical pathology measurements included total protein, A/G ratio, urea nitrogen, creatinine, Na, Cl, K, Ca, GOT, GPT, ALP, γ-GTP, LDH, total bilirubin, total cholesterol, triglyceride and fasting blood sugar. Hematology analyses included RBC, WBC, hemoglobin, hematocrit, and platelet measurements.

The subjects were randomized across one of the two groups, treated and placebo. This resulted in 39 subjects (30 male, 9 female) being placed in the placebo group and 42 in the test group (29 male, 13 female). At baseline (immediately prior to consuming the first dose), there were no significant differences between the groups with regard to gender, age, SBP, DBP, PR, BW or height, BMI, clinical pathology or hematology. Using this method, 39 subjects (30 male, 9 female) were placed in the placebo group and 42 in the test group (29 male, 13 female).

With regard to SBP, a statistically significant decrease was observed beginning at 2 weeks of treatment (-7.4±9.6 mmHg; $p<0.01$)

The magnitude of the difference in SBP increased at treatment week 4 (-8.9±11.3 mmHg, $p<0.01$), 6 (-12.7±11.1 mmHg; $p<0.001$) and at 8 (-12.4±10.9 mmHg, $p<0.001$) as compared to the placebo group or the baseline measurements. At 10 weeks, two weeks after cessation of treatment, the treated group continued to have a lower SBP, but the difference was no longer significant due to an increase in the treated group SBP. At 12 weeks, four weeks after treatment cessation, there was no difference between the SBP of the treated versus the placebo groups. There was no significant change in SBP in the placebo group throughout the study.

The changes in DBP were qualitatively the same but quantitatively less than that observed in SBP and described above. A significant decrease was observed in the treated group beginning at 2 weeks of treatment (-6.7±13.2 mmHg, $p<0.05$) with the magnitude of the decrease increasing at 6 weeks (-9.2±11.8 mmHg, $p<0.05$) and

8 weeks (-8.1±12.07 mmHg, N.S.). There was no significant change in DBP in the placebo group throughout the study.

There were no differences reported or observed in the frequency or type of clinical symptoms. There were a no reports of dry cough, exanthema, skin itching, digestive discomfort, vertigo, dyspepsia and dizziness or headaches, known side-effects of ACE-inhibitors.

No significant differences between treated and placebo groups were observed with regard to clinical pathology or hematology measurements.

SRA GRAS Panel Evaluation:

The study involved the evaluation of the potential biological effects of consuming milk-derived tripeptides (2.08 mg of VPP+IPP) daily for 8 weeks. Eighty-one individuals with mild or moderate hypertension randomized across two groups, treatment and placebo.

Consumption of the test material was related to a decrease in SBP in the treated individuals which was evidenced at all time periods during treatment (i.e. 2, 4, 6 and 8 weeks) and 2 weeks after treatment cessation. Qualitatively similar but quantitatively smaller effects were observed in DBP. The test material did not alter the frequency or magnitude of clinical symptoms and no symptoms were noted. The test article was not associated with changes in BW, BMI, PR, hematology or clinical pathology measurements.

These results suggest that daily consumption of 2.08 mg of VPP+IPP for eight weeks produced no untoward biological effects. The study was robust with regard to study design (i.e. double-blind, use of a concurrent placebo control, baseline and withdrawal

measurements), numbers of subjects, parameters measured and intervals at which they were measured. The study was limited in that it did not evaluate a possible dose-response relationship and the consumption level was below the level considered by the GRAS Panel.

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