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[redacted]

SENMI EKISU Co., Ltd.
779-2 Noda, Hirano-Cho
Ohzu-City, Ehime
795-0021
Japan

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NOV 9 2010
Division of
Biotechnology and
GRAS Notice Review

October 29, 2010

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

Delivered via Courier

RE: GRAS NOTICE FOR HYDROLYZED SARDINE PROTEIN

Dear Dr. Martin:

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting in triplicate, as the notifier [SENMI EKISU Co. Ltd.; 779-2 Noda, Hirano-Cho; Ohzu-City, Ehime; 795-0021; Japan], a Notice of the determination, on the basis of scientific procedures, that hydrolyzed sardine protein, as defined in the enclosed documents, is GRAS under specific conditions of use as a food ingredient, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act.*

The Notice presents information setting forth the basis for the GRAS determination, which includes a comprehensive summary of the data available and reviewed by an independent panel of experts (the Expert Panel) in support of the safety of hydrolyzed sardine protein under the intended conditions of use. Each copy of the GRAS Notice includes the GRAS Notification dossier (***GRAS EXEMPTION CLAIM FOR HYDROLYZED SARDINE PROTEIN***), with the signed Expert Panel Consensus Statement comprising Appendix I of the dossier.

I trust that the enclosed Notice is acceptable. Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner. I look forward to receiving acknowledgement of receipt of this notice.

Sincerely,

(b) (6)

Katsuhiro Osajima, Ph.D.
President

Encl.

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Biotechnology and
GRAS Notice Review

GRAS EXEMPTION CLAIM FOR HYDROLYZED SARDINE PROTEIN

Summary of Data Concerning the Safety and GRAS Determination of Hydrolyzed Sardine Protein for Use as a Food Ingredient

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Submitted by:

SENMI EKISU Co., Ltd.
779-2 Noda, Hirano-Cho
Ohzu-City, Ehime
795-0021
Japan

October 29, 2010

GRAS EXEMPTION CLAIM FOR HYDROLYZED SARDINE PROTEIN

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GRAS EXEMPTION CLAIM FOR HYDROLYZED SARDINE PROTEIN

I. GRAS EXEMPTION CLAIM

I.A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

Hydrolyzed sardine protein has been determined by Senmi Ekisu Co., Ltd. (hereafter "Senmi") to be Generally Recognized as Safe (GRAS) for use in a variety of traditional food products, consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use in food, among experts qualified by scientific training and expertise. Therefore, the use of hydrolyzed sardine protein in food as described below is exempt from the requirement of premarket approval.

Signed,

(b) (6)

Oct. 29th, 2010

Katsuhiro Osajima, Ph.D.
President
SENMI EKISU Co., Ltd.

Date

I.B Name and Address of Notifier

Name: Katsuhiro Osajima
Position: President

SENMI EKISU Co. Ltd.
779-2 Noda, Hirano-Cho
Ohzu-City, Ehime
795-0021
Japan

Telephone: 0893-24-6878
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I.C Common Name of the Notified Substance

The common name for the notified substance is hydrolyzed sardine protein. Other common names include: purified sardine peptide, hydrolyzed fish protein isolate, fish protein hydrolysate. The trade name for the ingredient is VALTYRON®.

I.D Conditions of Intended Use in Food and Consumer Exposure

I.D.1 Current Regulatory Status

Currently, hydrolyzed sardine protein is not permitted for addition to food in the United States (U.S.). However, the U.S. Food and Drug Administration (FDA) was notified of and had no questions regarding the self-affirmed GRAS status of acid-extracted fish protein for use in food (GRN 000147; U.S. FDA, 2004) and has approved the use of hexane- and ethanol-extracted fish protein isolates for purposes of food supplementation (21 CFR §172.340; U.S. FDA, 2009). The FDA also has reviewed data on the safety of protein hydrolysates obtained from non-fish sources. Most recently, the FDA had no questions regarding the GRAS status of concentrated hydrolyzed milk protein (GRN 000199 – U.S. FDA, 2007). For food labeling purposes, the FDA requires that the common or usual name of the protein hydrolysate shall be specific to the ingredient and shall include the identity of the food source from which the protein was derived (21 CFR §102.22; U.S. FDA, 2009); therefore, these provisions will be adhered to in the labeling of hydrolyzed sardine protein as an ingredient for use in foods under the conditions of use specified herein.

Protein hydrolysates were in use in the U.S. prior to 1958 and hydrolyzed plant protein, vegetable protein, animal protein, and milk protein are presumed GRAS substances by the FDA or have prior sanction or approval for use in foods (as cited in FASEB, 1978). In an ongoing process of reviewing the safety of GRAS or prior sanctioned food substances as food ingredients, the Federation of American Societies for Experimental Biology (FASEB) was

contracted by the FDA to conduct an evaluation of the health aspects of protein hydrolysates for use as food ingredients and, specifically, for use as flavoring agents (FASEB, 1978). The Committee concluded that "while no evidence in the available information on acid hydrolyzed proteins, enzymatically hydrolyzed proteins, and yeast autolyzates demonstrate[d] a hazard to the public [at the then current intake levels associated with the use of such protein hydrolysates] as flavoring agents, uncertainties exist[ed] requiring that additional studies should be conducted". Specifically, in evaluating the safety of protein hydrolysates, the Committee expressed concern regarding possible developmental or teratogenic effects that have been associated with glutamate (glutamic acid), noting, however, that the dose levels at which adverse effects may be observed with protein hydrolysates may be greater than with glutamic acid alone. Accordingly, the Committee recommended that future Food Chemicals Codex (FCC) standards for protein hydrolysate products specify glutamic acid and aspartic acid content. The current FCC specification for acid hydrolysates of protein indicates a value of not more than 20% as glutamic acid and not more than 35% of the total amino acids (FCC, 2003). Consistent with the current FCC specifications for similar protein hydrolysates, glutamic acid is present in Senmi's hydrolyzed sardine protein at levels of less than 15% of total amino acids.

Most recently, the European Food Safety Authority (EFSA) issued a positive opinion regarding the safety of hydrolyzed sardine protein (EFSA, 2010). The EFSA Panel concluded that hydrolyzed sardine protein was safe for use as a food ingredient at use-levels of up to 0.6 g/serving in similar foods as those proposed in the U.S. (see Section I.D.4).

I.D.2 Daily Recommended Intake of Protein

Proteins have numerous cellular and organ functions in the body. Inadequate protein intakes have clinical implications and are associated with adverse effects on human health and normal development. In 2005, the Institute of Medicine (IOM) evaluated data on protein and individual amino acids to determine dietary reference values for these nutrients (IOM, 2005). A Recommended Dietary Allowance[1] (RDA) of 0.8 g/kg body weight was set for protein (good quality) for both adult males and females. Adequate intakes[2] (AI) of protein for infants (up to 6 months of age) and RDAs for individual age-groups as set by the IOM are summarized in Table I.D.2-1.

---

[1] The average daily dietary nutrient intake level sufficient to meet the nutrient requirement of nearly all (97 to 98%) healthy individuals in a particular life stage and gender group (IOM, 2005).

[2] The recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate – used when an RDA cannot be determined (IOM, 2005).

Table I.D.2-1 Recommended Dietary Allowance (RDA) Values for Protein (IOM, 2005)

Age	g/kg body weight		g/day	
Infants and young children				
0 to 6 months*	1.52		--	
7 to 12 months	1.2		11	
1 to 3 years	1.05		13	
4 to 8 years	0.95		19	
9 to 13 years	0.95		34	
Teenagers and adults	Males	Females	Males	Females
14 to 18 years	0.85	0.85	52	46
Adults (19 to >70 years)	0.80	0.80	56	46
Pregnancy	--	1.1	--	+25 g additional protein
Lactation	--	1.3	--	+25 g additional protein

* AI, Adequate Intake.

The IOM concluded that there were insufficient data to set a Tolerable Upper Intake Level (UL) for total protein or for any of the amino acids and advised that caution is warranted in using any single amino acid at levels significantly above that normally found in food.

The IOM also estimated background dietary protein intakes for the U.S. population using the Continuing Survey of Food Intakes by Individuals (CSFII) (1994-1996, 1998) (IOM, 2005). Mean and 90th percentile intakes of protein by the U.S. population are presented in Table I.D.2-2. The mean adult intake for protein ranged from approximately 56 g (adult females over the age of 70) to 104 g (19- to 30-year-old males) per day (IOM, 2005). At the 90th percentile, adult protein intakes ranged from 76 g/day for adult females over 70 years of age to 142 g/day for 19- to 30-year-old males. In infants and children, mean and 90th percentile intakes ranged from 15.9 to 62.5 and 21.9 to 81.9 g/day, respectively. The mean and 90th percentile total population intakes were estimated to be approximately 75 and 114 g protein/day, respectively.

Table I.D.2-2 Summary of the Usual Daily Intake of Protein (G), United States, CSFII (1994-1996, 1998) (IOM, 2005)

Population Group	Age Group (Years)	No. of Individuals Surveyed		Mean (g)		90th Percentile (g)	
Infants	0-6 months	596		15.9		21.9	
	7-12 months	530		28.3		42.5	
Children	1-3 years	3,949		50.9		68.4	
	4-8 years	3,935		62.5		81.9	
		Males	Females	Males	Females	Males	Females
Teenagers	9-13 years	595	606	79.1	65.3	101.0	83.6
	14-18 years	474	449	99.0	66.5	132.0	85.3
Adults	19-30 years	920	808	104.0	63.3	142.0	84.0
	31-50 years	1,806	1,690	99.4	64.9	135.0	86.0
	51-70 years	1,680	1,605	86.8	61.7	117.0	80.4
	>70 years	722	670	72.5	56.4	99.0	76.0
	Pregnancy	--	81	--	78.2	--	97.0
	Lactating	--	44	--	79.7	--	100.8
Total Population	All age groups combined	21,035		75.2		114.0	
	All age groups combined (+P/L)	21.159		75.3		114.0	

P/L, Pregnant and/or lactating.

Although the IOM could not set an UL for protein intake, it was noted that "the risk of adverse effects resulting from excess intakes of protein from foods appears to be very low at the highest estimated intake[s]" (IOM, 2005).

I.D.3 Background Exposure to Sardines and Sardine-Derived Protein in the Diet

In the U.S., sardines are included in the top 4 most popular canned fish products along with tuna, salmon, and shellfish based on *per capita* consumption values (Pritchard, 2004). Nonetheless, since the early 1900s, the *per capita* consumption of canned sardines in the U.S. has been declining steadily, with estimates of approximately 0.25 g/person/day (0.2 lb/person/ year) in 2008 compared to the highest recorded value of 1.7 g/person/day (1.4 lbs/person/year) in 1950 (Pritchard, 2004; USDA-ERS, 2010). Sardine peptides are natural components of sardines, resulting from normal protein digestion. Accordingly, sardine peptides as present in the hydrolyzed sardine protein ingredient, have a long-standing, well-established history of human consumption. While the exact peptide distribution generated as a result of sardine muscle hydrolysis using a bacterial enzyme (see Section II.B – Method of Manufacture) is likely to be different from that produced by human gastrointestinal proteases, the amino acid profile of the sardine peptides in Senmi's hydrolyzed sardine protein product from sardines is similar to that of the original muscle tissue.

Background consumption of protein from sardines specifically was calculated using the 2-day National Center for Health Statistics' (NCHS) 2003-2004 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2006; USDA, 2009). The exposure estimate was based on the approximation that sardines are typically composed of 20% protein. Based on the NHANES, the following food products were identified as containing sardines: cooked sardines, dried sardines, sardines canned in oil, sardines packed in water, sardines in tomato or mustard sauce, sardine sandwiches, and pizza with sardine topping. Based on the consumption of the listed sardine-containing foods, the all-person mean intake of sardine protein was estimated to be 15.14 mg/person/day (0.21 mg/kg body weight/day) for the total U.S. population, with the highest absolute intake observed in adult males (31.21 mg/person/day). Since sardines are only added to a very limited number of foods and are consumed by a relatively small portion of the whole population, these low *all-person* intakes are expected. Due to the small number of users identified within the total population and each population group, it was not possible to generate an estimate for all-person heavy (90th percentile) intakes for sardines and sardine protein. Conversely, the *all-user* mean intake of protein as a result of sardine consumption for the total U.S. population was considerably higher at 7.02 g/person/day (96.59 mg/kg body weight/day). At the 90th percentile, an all-user intake of 9.20 g/person/day or 151.57 mg/kg body weight/day was estimated. Absolute intakes and exposures on a body weight basis to protein derived from sardines by different age-groups of the U.S. population are summarized in Tables I.D.3-1 and I.D.3-2, respectively.

Table I.D.3-1 Summary of the Estimated Daily Background Intake of Sardine Protein In the U.S. by Population Group (NHANES 2003-2004 Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (mg)	90th Percentile (mg)	Mean (g)	90th Percentile (g)
Infant	0-2	0.1	1	8.16*	N/A	4.97	4.97
Child	3-11	0	0	N/A	N/A	N/A	N/A
Female Teenagers	12-19	0.3	3	26.61*	N/A	13.08	16.10
Male Teenagers	12-19	0	0	N/A	N/A	N/A	N/A
Female Adult	20 and Up	0.3	7	6.91*	N/A	4.71	7.45
Male Adult	20 and Up	0.3	6	31.21*	N/A	7.51	8.51
Total Population	All ages	0.2	17	15.14	N/A	7.02	9.20

N/A, Not applicable.
*Indicates an intake estimate that may not be statistically reliable, as the coefficient of variation (CV) of the mean is equal to or greater than 30%.

Table I.D.3-2 Summary of the Estimated Daily Per Kilogram Body Weight Background Intake of Sardine Protein In the U.S. by Population Group (NHANES 2003-2004 Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (mg/kg)	90th Percentile (mg/kg)	Mean (mg/kg)	90th Percentile (mg/kg)
Infant	0-2	0.1	1	0.57*	N/A	347.34	347.34
Child	3-11	0	0	N/A	N/A	N/A	N/A
Female Teenagers	12-19	0.3	3	0.50*	N/A	246.62	299.26
Male Teenagers	12-19	0	0	N/A	N/A	N/A	N/A
Female Adult	20 and Up	0.3	7	0.12*	N/A	80.78	122.25
Male Adult	20 and Up	0.3	6	0.32*	N/A	77.81	89.06
Total Population	All ages	0.2	17	0.21	N/A	96.59	151.57

N/A, Not applicable.
*Indicates an intake estimate that may not be statistically reliable, as the coefficient of variation (CV) of the mean is equal to or greater than 30%.

I.D.4 Intended Use of Hydrolyzed Sardine Protein and Levels of Use in Foods

Senmi intends to market hydrolyzed sardine protein as a food ingredient in the U.S. in selected food categories, including beverages and breakfast cereals, frozen dairy desserts and mixes, milk and milk products, fish products, pastas, hard and soft candy, soups and soup mixes, and processed fruits and vegetables and fruit and vegetable juices. Hydrolyzed sardine protein is proposed for use in foods and beverages at use-levels of up to 0.6 g/serving (up to 30%). The individual intended food-uses and use-levels are summarized in Table I.D.4-1. Hydrolyzed sardine protein is not intended for use in any meat, poultry, or egg products. In the case of the soup category, all the foodcodes included to derive the intake estimates were inspected to ensure that none were representative of meat- or poultry-containing soups that are under the jurisdiction of the U.S. Department of Agriculture (USDA).

Although as described in Section II.C.1 the powdered product is characterized by a yellowish white color, it does not impart any coloring properties to foods or beverages under the intended conditions of use.

Table I.D.4-1 Summary of the Individual Proposed Food Uses and Use-Levels for Hydrolyzed Sardine Protein in the United States

Food Category	Proposed Food-Use	Sardine Peptide Level (g/RACC)	RACC (g)	Use-Levels (%)
Breakfast Cereals	Instant and Hot Regular Cereals	0.6	240	0.25
	Ready-to-Eat Cereals	0.6	15	4.0
			30	2.0
			55	1.1
Fish Products	Frozen Multicourse Fish-Based Meals	0.6	240	0.25
Frozen Dairy Desserts and Mixes	Frozen Yogurt	0.6	120	0.5
Grain Products and Pastas	Frozen Multicourse Grain Product and Pasta-Based Meals	0.6	240	0.25
Hard Candy	Hard Candy (Functional)	0.6	2 to 15	4.0 to 30.0
Milk and Milk Products	Drinkable Yogurt	0.6	240	0.25
	Fermented Milk	0.6	240	0.25
	Milk-based Meal Replacements and Protein Beverages made from Dry Powders	0.6	240*	0.25
	Milk-Based Beverages made from Dry Powders	0.6	240*	0.25
	Yogurt	0.6	225	0.27
Processed Fruits and Fruit Juices	Flavored Water	0.6	240	0.25
	Fruit Drinks and Ades	0.6	240	0.25
Processed Vegetables and Vegetable Juices	Frozen Multicourse Vegetable-Based Meals	0.6	240	0.25
	Vegetable Juices	0.6	240	0.25
Soft Candy	Soft Candy and Chocolate Bars	0.6	40	1.5
Soups and Soup Mixes	Canned, Condensed, and Dehydrated Soups	0.6	245*	0.24

RACC, Reference Amounts Customarily Consumed Per Eating Occasion (21 CFR §101.12) (U.S. FDA, 2009).
* As consumed.

I.D.5 Estimated Dietary Consumption of Hydrolyzed Sardine Protein Based upon Intended Food Uses

Estimates for the intake of hydrolyzed sardine protein were based on the proposed food-uses and use-levels in conjunction with food consumption data included in the 2003-2004 NHANES (CDC, 2006; USDA, 2009). Approximately 88% of the total U.S. population was identified as potential consumers of hydrolyzed sardine protein from the proposed food-uses (7,250 actual users identified). Children aged 3 to 11 reported the greatest percentage of users of any population group at 98.4%. On an all-user basis, the mean intake of Senmi's hydrolyzed sardine protein by the total population from all proposed food-uses was estimated to be 1.3 g/person/day. The heavy consumer (90th percentile) all-user intake of Senmi's hydrolyzed sardine protein by the total population from all proposed food-uses was 2.8 g/person/day. On a

body weight basis, the mean and 90th percentile all-user intakes of Senmi's hydrolyzed sardine protein by the total population were 27 and 62 mg/kg body weight/day, respectively. On a per user basis, the highest mean and 90th percentile all-user intakes were observed to occur in children at 1.9 and 3.8 g/person/day, respectively, equivalent to 69 and 146 mg/kg body weight/day, respectively. On a body weight basis, the mean and 90th percentile all-user intakes of hydrolyzed sardine protein by infants were the greatest at 73 and 156 mg/kg body weight/day, respectively (see Tables I.D.5-1 and I.D.5-2).

It should be noted, however, that the specified food uses are not intended to be marketed for infants (*i.e.*, hydrolyzed sardine protein is not intended for addition to infant formula/food); thus, the actual infant consumption of hydrolyzed sardine protein is expected to be highly limited, and although an estimate of the consumption of hydrolyzed sardine protein on a body weight basis in infants from all proposed food uses has been included for completeness of this report, it is considered to be a gross over-estimate of the actual expected intake of hydrolyzed sardine protein by infants from its addition to food. This is supported by the fact that the lowest intakes on an absolute basis were determined to be in infants. Furthermore, this type of intake methodology is generally considered to be "worst case" as a result of several conservative assumptions made in the consumption estimates. For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use. In addition, it is well established that the length of a dietary survey affects the estimated consumption of individual users. Short-term surveys, such as the typical 2- or 3-day dietary surveys, overestimate the consumption of food products that are normally consumed relatively infrequently.

Table I.D.5-1 Summary of the Estimated Daily Intake of Hydrolyzed Sardine Protein from All Proposed Food Uses in the U.S. by Population Group (NHANES 2003-2004 Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (g)	90th Percentile (g)	Mean (g)	90th Percentile (g)
Infant	0-2	66.1	615	0.7	1.7	0.9	1.9
Child	3-11	98.4	1,267	1.8	3.7	1.9	3.8
Female Teenager	12-19	92.9	922	1.2	2.6	1.4	2.7
Male Teenager	12-19	91.4	913	1.7	3.5	1.8	3.6
Female Adult	20 and Up	89.7	1,909	1.0	2.2	1.2	2.3
Male Adult	20 and Up	84.2	1,624	1.1	2.4	1.3	2.7
Total Population	All Ages	87.7	7,250	1.2	2.6	1.3	2.8

Table I.D.5-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of Hydrolyzed Sardine Protein from All Proposed Food Uses in the U.S. by Population Group (NHANES 2003-2004 Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (mg/kg)	90th Percentile (mg/kg)	Mean (mg/kg)	90th Percentile (mg/kg)
Infant	0-2	66.1	615	55	131	73	156
Child	3-11	98.4	1,267	68	144	69	146
Female Teenager	12-19	92.9	922	22	48	24	49
Male Teenager	12-19	91.4	913	27	63	30	67
Female Adult	20 and Up	89.7	1,909	15	30	17	33
Male Adult	20 and Up	84.2	1,624	13	31	16	33
Total Population	All Ages	87.7	7,250	24	55	27	62

I.D.6 Comparison of Background Dietary Protein Intakes, Sardine-Derived Protein Intakes, and Sardine Peptide Intakes from Senmi's Hydrolyzed Sardine Protein Product

A comparison of the all-user background dietary protein intakes and intakes of sardine proteins to the estimated intakes of sardine peptides from Senmi's hydrolyzed sardine protein under the proposed conditions of use is provided in Table I.D.6-1.

Table I.D.6-1 Comparison of the All-User Total U.S. Population Intakes of Protein from All Dietary Sources, Protein from Sardines, and Sardine Peptides from Senmi's Hydrolyzed Sardine Protein

Source of Protein	Mean		90th Percentile	
	g/day	mg/kg body weight	g/day	mg/kg body weight
Background protein intake from all dietary sources	75.3[a]	1,074[b]	114.0[a]	1,628[b]
Background protein intake from sardines[c]	7.0	97	9.2	152
Estimated intake of sardine peptides from Senmi's ingredient[d]	1.1	23	2.4	53

[a] Values obtained from the Institute of Medicine (IOM, 2005). Based on the Continuing Survey of Food Intakes by Individuals (CSFII) (1994-1996, 1998) (IOM, 2005).
[b] Assuming body weight of 70 kg.
[c] Calculated using National Health and Nutrition Examination Surveys (NHANES) 2003-2004 (all-user consumption).
[d] Values in Tables I.D.5-1 and I.D.5-2 adjusted by 85% to account for peptide content only.

I.E Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30, hydrolyzed sardine protein has been determined by Senmi to be GRAS on the basis of scientific procedures (U.S. FDA, 2009). This determination is based on the views of experts who are qualified by scientific training and experience to evaluate the

safety of hydrolyzed sardine protein as a component of food [see Appendix I, entitled "**EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF VALTYRON®, A SARDINE PEPTIDE PRODUCT, FOR USE IN FOODS**"].

At the request of Senmi, an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened on September 18, 2007 to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether the intended uses of hydrolyzed sardine protein as a food ingredient are safe and suitable and would be GRAS based on scientific procedures.

The Panel consisted of the following qualified scientific experts: Jack Bend, Ph.D. (University of Western Ontario), Robert Bush, M.D. (University of Wisconsin), and Gary Williams, M.D. (New York Medical College).

The Expert Panel convened on behalf of Senmi, independently and collectively, critically evaluated the data and information summarized herein and concluded that the intended uses in traditional foods described herein for hydrolyzed sardine protein, meeting appropriate food-grade specifications and manufactured according to current Good Manufacturing Practice (cGMP), are safe and suitable and GRAS based on scientific procedures. It is also the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion.

Therefore, Senmi has concluded that hydrolyzed sardine protein is GRAS under the intended conditions of use on the basis of scientific procedures. Senmi's hydrolyzed sardine protein is GRAS based on scientific procedures for its intended uses in food; therefore, it is excluded from the definition of a food additive and thus may be marketed and sold for the uses designated above in the U.S. without the promulgation of a food additive under Title 21 of the CFR.

I.F Availability of Information

The detailed data and information that serve as a basis for this GRAS determination will be provided to the United States Food and Drug Administration (FDA) upon request, or are available for the FDA's review and copying during reasonable business hours at the offices of:

SENMI EKISU Co. Ltd.
779-2 Noda, Hirano-Cho
Ohzu-City, Ehime
795-0021
Japan

Should the FDA have any questions or additional information requests regarding this notification, Senmi will supply these data and information.

II. DETAILED INFORMATION ABOUT THE IDENTITY OF THE SUBSTANCE

II.A Identity

II.A.1 Chemical Description

Senmi's hydrolyzed sardine protein contains peptides (*i.e.,* ≥85%) obtained by enzymatic hydrolysis of raw muscle tissue from sardines. The peptides comprising Senmi's hydrolyzed sardine protein product are composed of 2, 3, 4, or 5 amino acids (see Table II.A.1-1 for amino acid profile of Senmi's hydrolyzed sardine protein product) and include among others valine-tyrosine (Val-Tyr), valine-tryptophan (Val-Trp), isoleucine-tyrosine (Ile-Tyr), and isoleucine-tryptophan (Ile-Trp).

Common or Usual Name:	Hydrolyzed sardine protein; purified sardine peptide; hydrolyzed fish protein isolate; fish protein hydrolysate
Chemical Name:	Not Applicable
Chemical Abstracts Service (CAS) Number:	Not Applicable
Empirical Formula:	Not Applicable
Molecular Weight:	Not Applicable
Structural Formula:	Not applicable
Appearance:	Yellowish-white powder

Table II.A.1-1 Amino Acid Profile for Senmi's Hydrolyzed Sardine Protein Product

Amino Acid	Hydrolyzed Sardine Protein	
	g/32 g	Percent of total amino acids
Aspartic acid (aspartate) and asparagine (Asx)	3.03	9.5
Threonine (Thr)	1.43	4.5
Serine (Ser)	0.90	2.8
Glutamic acid (glutamate) and glutamine (Glx)	3.69	11.5
Proline (Pro)	2.69	8.4
Glycine (Gly)	2.41	7.5
Alanine (Ala)	1.49	4.7
Cysteine (Cys)	<0.01[1]	0
Valine (Val)	2.02	6.3
Methionine (Met)	0.67	2.1
Isoleucine (Ile)	1.79	5.6
Leucine (Leu)	3.08	9.6
Tyrosine (Tyr)	1.39	4.3
Phenylalanine (Phe)	1.45	4.5
Histidine (His)	1.05	3.3
Lysine (Lys)	2.92	9.1
Arginine (Arg)	1.51	4.7
Tryptophan (Trp)	0.47	1.5
Total	**32**	**100**

[1] Below limit of detection.

II.B Method of Manufacture

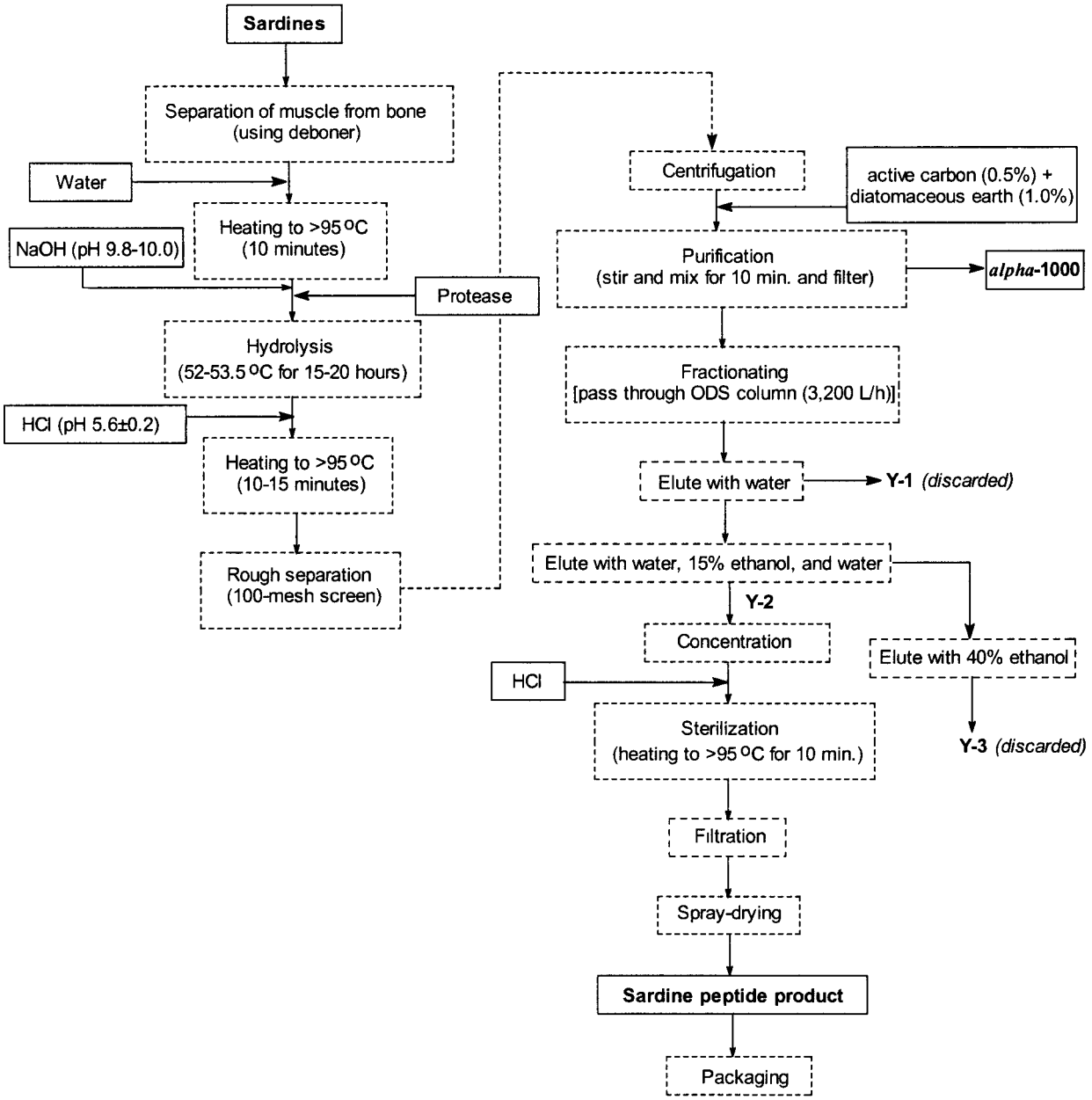
Senmi's hydrolyzed sardine protein product is manufactured from sardines caught off the coast of California, which are frozen for transport and storage. The flesh of the sardines is obtained by removing the skin and bones using a mechanical deboner and is subsequently frozen until further processing. Prior to use, the frozen flesh is pulverized and transferred to a 1,500-L tank to which water is added. The flesh is heated to near boiling temperatures (95 to 99°C) for 10 minutes to inactivate the autolytic enzymes present in the flesh and allowed to cool in a sealed tank to about 52 to 53°C. Hydrolysis of the flesh protein is initiated by adding sodium hydroxide to adjust the pH (9.8 to 10.0) and the hydrolytic protease enzyme (Alcalase® 2.4L) to the raw material to a final concentration of 0.7% (w/w). Hydrolysis proceeds for up to 20 hours following which hydrochloric acid is added and the temperature is increased to 95 to 99°C to inactivate the enzyme. Subsequently, the contents of the tank are filtered through a vibratory screen (100 mesh sieve) to remove any remaining bones and undecomposed residues. Next, the filtrate is continuously centrifuged (8,000 rpm) to remove any insoluble solid matter and is batch-type centrifuged (15,000 rpm) to further remove any fine residues. Activated charcoal and diatomaceous earth are added to the fluid for purification purposes. The fluid is subjected to precision filtration to remove the majority of enzyme residues (approximately 85%) remaining in the fluid and the resulting precursor peptide product (peptide α-1000) is inspected for quality.

The precursor product consists of organic acids, amino acids, and 200 to 300 di-, tri-, tetra-, and penta-peptides.

Once the fluid passes the interim inspection, it is filtered through a cartridge filter (0.45 µm pores) and diluted with deionized water. The diluted hydrolysate is fractioned by passing it through an octadecylsilica (ODS) column packed with 1,600 L of brominated styrene divinylbenzene (DVB) copolymers (Sepabeads® SP207), which absorb hydrophobic peptides. The column eluate and the eluate following rinsing of the column with deionized water (non-absorbed material) are discarded (Y-1 fraction). Deionized water is passed again through the column and this time the eluate is collected and retained. The adsorbed peptides are further eluted from the column using a 15% aqueous ethanol solution and added to the eluate retained following the second water wash. Again, deionized water is passed through the column and also added to the already collected eluate (Y-2 fraction). Finally, the column is rinsed with a 40% aqueous ethanol solution to remove strongly adsorbed materials and is discarded (Y-3 fraction). The fractionation removes approximately 85% of the enzyme residues remaining after the filtration steps (not more than 5% of the enzyme preparation remains).

The Y-2 fraction is concentrated using a vacuum condenser and the pH is adjusted using hydrochloric acid. The concentrate is sterilized by heating, and is then cooled and filtered through a 200-mesh screen. The filtered concentrate is then powderized using a spray drier to produce the commercial hydrolyzed sardine protein product. A schematic overview of the manufacturing process for Senmi's hydrolyzed sardine protein product is presented in Figure II.B-1.

Figure II.B-1 Schematic Overview of the Manufacturing Process for Senmi's Hydrolyzed Sardine Protein Product



All processing aids used in the manufacture of hydrolyzed sardine protein are used in compliance with appropriate federal regulations, as indicated in Table II.B-1.

Table II.B-1 Raw Materials and Processing Aids Used in the Manufacture of Senmi's Hydrolyzed Sardine Protein

Material	Use	Regulatory Status
Raw Materials		
Sardines [frozen sardines caught in California (U.S.), commonly known as California sardines (South American pilchard or *Sardinops sagax*)]	Source of sardine peptides	Food
Processing Aids		
Alcalase® 2.4 L FG derived from the submerged fermentation of *Bacillus licheniformis*	Catalyze the hydrolysis of the peptide bonds of protein in sardine muscle tissue	The specifications for the protease used in the production of hydrolyzed sardine protein are consistent with those set forth by JECFA and the FCC for enzyme preparations. In accordance with 21 CFR §184.1027, Alcalase® 2.4 L FG derived from non-pathogenic and non-toxicogenic *B. licheniformis* is affirmed as GRAS with no limitation except cGMP for use in the manufacture of alcoholic beverages, nutritive sweeteners, and protein hydrolysates (U.S. FDA, 2009).
Sodium Hydroxide	pH adjustment	In accordance with 21 CFR § 184.1763, sodium hydroxide is permitted for use in food with no limitations other than Cgmp (U.S. FDA, 2009).
Hydrochloric Acid	pH adjustment	Under 21 CFR §182.1057 hydrochloric acid is GRAS when used as a buffer and neutralizing agent in accordance with cGMP (U.S. FDA, 2009).
Activated carbon	Purification and decolorizing agent	No federal regulations specific to the intended use were identified. Similar uses of activated carbon are considered GRAS for purification and clarification of wine as per 27 CFR §24.246 (U.S. ATTTB, 2009).
Diatomaceous earth	Purification aid	Diatomaceous earth is permitted for use as a food additive in feed and drinking water of animals (21 CFR 573.340 - U.S. FDA, 2009).
Ethanol	Ion-exchange eluent	Ethanol is permitted for use as a solvent in the processing of fish protein isolate with residual levels of 3.5% (21 CFR §172.340 - U.S. FDA, 2009).
SEPABEADS® SP207 (brominated styrene DVB copolymers)	Ion exchange resin (purification aid)	Sulfonated copolymers of styrene and DVB are permitted for use by the U.S. Food and Drug Administration as ion exchange resins for the purification of food (21 CFR § 173.25 - U.S. FDA, 2009).

CFR, Code of Federal Regulation; cGMP, current Good Manufacturing Practice; DVB, Divinylbenzene; GRAS, Generally Recognized as Safe.

II.C Specifications for Food Grade Material

II.C.1 Product Specifications and Analysis

Senmi's hydrolyzed sardine protein is characterized by a peptide content of not less than 85% and a Val-Tyr content specifically in the range of 0.10 to 0.16%. Chemical, physical, and microbiological specifications for Senmi's hydrolyzed sardine protein product are presented in Table II.C.1-1. Since Senmi's hydrolyzed sardine protein is prepared from natural sources several microbiological specifications similar to those included for other food ingredients also have been specified to ensure safety of its use in food. Analysis of non-consecutive representative batches (Lot Nos. BZ1.07, DZ1.06, and GY3.06) demonstrated compliance with final product physical, chemical, and microbiological specifications. Please note that for this set of lot samples, lead analysis was conducted only as total heavy metals. Lead analysis specifically was conducted only on GY3.06, as well as on 4 additional sample lots (Lot Nos. DX03.07, DY09.07, DZ6.06, and LY0.05) as part of heavy metal analysis that also included analysis for copper, arsenic, cadmium, and mercury (see Section II.C.2).

Table II.C.1-1 Product Specifications for Hydrolyzed Sardine Protein

Specification Parameter	Specification Requirement	Method of Analysis
Appearance	Yellowish white powder	Visual examination
Peptide	Not less than 85%	Kjeldahl method[1]
Val-Tyr	0.10 to 0.16%	HPLC method[2]
Moisture	Not more than 8.0%	Atmospheric heat drying method[1]
Ash	Not more than 10.0%	Direct ashing method[1]
Sodium	Not more than 2.0%	Atomic absorption spectrometric method[1]
Lead (Pb)	Not more than 0.1 ppm	Lead limit test (Atomic-absorption spectrometric method)[1]
Standard plate count	Not more than 1,000 CFU/g	Microbial Limit Tests[1]
Coliform count	Negative	Microbial Limit Tests[1]
Yeasts and molds	Not more than 300 CFU/g	Microbial Limit Tests[1]

As_2O_3 , Arsenic oxide; CFU, Colony-forming units; HPLC, High-performance liquid chromatography; Val-Tyr, Valine-Tyrosine.
[1] Japanese Specifications and Standards for Food Additives 7th Edition (2000).
[2] Senmi's internal method.

II.C.2 Additional Analyses

The three representative sample lots of Senmi's hydrolyzed sardine protein (Lot Nos.BZ1.07, DZ1.06, and GY3.06) also were assayed for residual levels of ethanol. Analytical results demonstrated ethanol levels of less than 500 ppm in the final product.

Additionally, since hydrolyzed sardine protein is produced from sardines, GY3.06 and 4 additional lots (Lot Nos. DX3.07, DY9.07, DZ6.06, and LY0.05) also were tested for various environmental contaminants, including heavy metals (as mentioned above), pesticides, dioxins

and dioxin-like polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). Analysis for potential residues of perfluorooctane sulfonate (PFOS) and other fluorochemicals and brominated flame retardants (BFRs) was performed on a homogeneous blend of the 5 sample lots of Senmi's hydrolyzed sardine protein product in a ratio of 1:1:1:1:1 (w/w). Levels of these chemical contaminants were often below the limits of detection or quantification reported for the analytical method (see Table II.C.2-1) and, where FDA action levels or guidelines were identified, the levels were below the permitted limits. Levels of mercury in the hydrolyzed sardine protein product were shown to be in compliance with the action level of 1 ppm set for methyl mercury in the edible portion of fish by the U.S. FDA (2000). The FDA recommended limited consumption of fish with dioxin concentrations greater than 25 ppt, and no consumption of fish with dioxin concentrations greater than 50 ppt (ATSDR, 1998). Additionally, the FDA recommends not consuming fish with 2,3,7,8-tetrachloro-*p*-dibenzo-dioxin (TCDD), which is considered to be the most toxic of the known dioxins and furans, at levels of greater than 50 ppt. 2,3,7,8-TCDD was not detected (<0.01 ng/kg) in the sample analysis of the hydrolyzed sardine protein products. Collectively, the above analyses indicate absence of any external contaminants arising from the marine environment indigenous to the starting material (*i.e.*, California sardines), which is consistent with the fact that the hydrolyzed sardine protein, having undergone column chromatography, is a highly purified ingredient. Therefore, while these potential environmental contaminants were considered in light of the source material, specific limits for these were not included in the product specifications as presented in Table II.C.1-1. However, should the origin of the source material change in the future, Senmi would monitor levels of any potential contaminants specific to the new environment and, if needed, revise the specifications accordingly.

Table II.C.2-1 Summary of Additional Environmental Contaminant Analysis

Parameter	Manufacturing Lot No.				
	DX3.07	DY9.07	DZ6.06	GY3.06	LY0.05
Heavy Metals (ppm)					
Copper (Cu)	1.08	0.89	1.03	0.60	0.68
Arsenic (As)	1.47	1.80	2.07	1.84	1.95
Cadmium (Cd)	(0.008)[1]	0.017	(0.008)[1]	(0.008)[1]	(0.008)[1]
Mercury (Hg)	<0.005	<0.005	<0.005	<0.005	<0.005
Lead (Pb)[2]	(0.006)[1]	<0.005	<0.005	<0.005	<0.005
PFOS and related fluorochemicals	<LOD (1 µg/kg)				
BFRs[3]	<LOQ (0.01 to 15 µg/kg depending on analyte)				
Pesticides[4]	<LOD[5]	<LOD[5]	<LOD[5]	<LOD[5]	<LOD[5]
Dioxins and Dioxin-like PCBs[6]					
Sum of *ortho*-PCB	0.02	0.02	0.02	0.02	0.02
Sum of non-*ortho*-PCB	<0.01	0.01	0.01	<0.01	0.01
Sum of dioxins and furans	0.05	0.03	0.03	0.03	0.03
Sum of WHO-TEQs (upper)	*0.08*	*0.06*	*0.06*	*0.06*	*0.06*
Benzo(a)pyrene (µg/kg)	<0.04	<0.04	<0.04	<0.04	<0.06

BFRs, brominated flame retardants; LOD, limit of detection; LOQ, limit of quantification; PCBs, polychlorinated biphenyls; PFOS, perfluorooctane sulfonate; WHO-TEQ, World Health Organization Toxic Equivalent.
[1] Values in brackets are below the limit of quantification, but above the limit of detection.
[2] Complies with product specification for lead of not more than 0.5 ppm.
[3] Includes a series of polybrominated diphenyl ethers, polybrominated biphenyls, tetrabromobisphenol A, and hexabromocyclododecane.
[4] The 5 manufacturing batches of the hydrolyzed sardine protein product were screened for a broad spectrum of common pesticides.
[5] 0.01 to 0.05 mg/kg depending on analyte.
[6] Expressed as WHO-TEQ in ng/kg whole (Upperbound concentrations, calculated on the assumption that all non detected compounds are present at the limit of quantification.

The manufacturing process employed by Senmi has control measures in place to minimize the potential for microbial growth and contamination in the final product. Specifically, the manufacturing process for Senmi's hydrolyzed sardine protein product involves a sterilization stage prior to spray drying. In addition to the analysis conducted to demonstrate compliance with the set microbiological specifications, the 5 sample lots (Lot Nos. LY0.05, DZ6.06, GY3.06, DX3.07, and DY9.07) were additionally assayed for the potential presence of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Salmonella*. Results of the analyses revealed levels of less than 10 CFU/g or absence of these microorganisms in the final product.

A single lot of Senmi's hydrolyzed sardine protein product (Lot No. BZ1.07) was analyzed for mineral content. Analysis revealed detectable levels of sodium (1.83%) (consistent with product

specifications – see Table II.C.1-1), potassium (0.23%), calcium (0.21%), magnesium (0.13%), and phosphorus (0.27%).

II.C.3 Stability

Stability of Senmi's hydrolyzed sardine protein product was evaluated following a long-term storage period of 41 months. Samples from 3 lots of Senmi's hydrolyzed sardine protein product were stored in vacuum metalizing polyethylene bags at 25°C for up to 41 months. Senmi's hydrolyzed sardine protein product samples were analyzed at 0 (study initiation), 8, 18, 26, and 41 months for physical properties (appearance and color) and Val-Tyr content. No changes in the physical attributes of the hydrolyzed sardine protein samples were observed throughout the testing period. The Val-Tyr content of Senmi's hydrolyzed sardine protein product remained stable for the duration of the storage period (0.157 to 0.158% at baseline compared to 0.155 to 0.159% at 41 months).

III. SELF-LIMITING LEVELS OF USE

No self-limiting levels of use were identified for hydrolyzed sardine protein.

IV. BASIS FOR GRAS DETERMINATION

IV.A Introduction

The determination that Senmi's hydrolyzed sardine protein product is GRAS is based on scientific procedures. The exposure to sardine peptides from the proposed food uses of the hydrolyzed sardine protein is estimated to be 1.1 g/day at the mean, and 2.4 g/day at the 90th percentile. In comparison, background dietary protein intakes from all sources are reported to be 75 and 114 g/day at the mean and 90th percentile, respectively (IOM, 2005), and background protein intakes from sardines specifically were determined to be 7.0 and 9.2 g/day, respectively.

The safety of Senmi's hydrolyzed sardine protein product is based on the endogenous occurrence of Val-Tyr, one of the many peptides identified in Senmi's hydrolyzed sardine protein product, and other peptides formed as a normal part of protein digestion, on the general understanding of peptide absorption and metabolic fate following uptake from the gastrointestinal tract, as well as studies conducted specifically to assess the systemic fate of Val-Tyr in animals and humans following oral administration (Matsui *et al.*, 2000, 2002a,b, 2003, 2004). The results of a battery of published standard toxicological studies (*in vitro* and *in vivo* mutagenicity/genotoxicity assays and acute and 28-day animal toxicity studies) (Osajima *et al.*, 2009) further support the safety of the hydrolyzed sardine protein. While the test material used in the *in vitro* standard bacterial assays (*Salmonella typhimurium* and *Escherichia coli*), as well as in the *in vivo* micronucleus assay was the final product of commerce, the closely-related

precursor product, peptide α-1000, obtained during the manufacturing process (see Section II.B - Method of Manufacture) was the test material used in the acute (LD_{50}) and 28-day animal studies.

As described in Section II.B, the final product of commerce is eluted by passing the precursor, peptide α-1000, through ODS chromatography columns. With the exception of highly water and ethanol soluble peptides, which are removed with the first and last wash of the column, respectively (Y-1 and Y-3 fractions, respectively), the fraction that comprises the final hydrolyzed sardine protein product (Y-2) contains the same peptides as those identified in peptide α-1000. While only approximately 32% of the peptide α-1000 mixture makes up the commercial Y-2 fraction, the relative amino acid composition is largely maintained between peptide α-1000 and Y-2. A comparison of the amino acid compositions of the final hydrolyzed sardine protein product and the precursor, peptide α-1000, are presented in Table IV.A-1. Given the comparative nature of the amino acid profile of the final hydrolyzed sardine protein product and the precursor, peptide α-1000, studies conducted with peptide α-1000 were considered to be appropriate for the assessment of the safety of Senmi's hydrolyzed sardine protein product.

Table IV.A-1 Comparison of the Amino Acid Composition of the Final Hydrolyzed Sardine Protein Product and the Precursor, Peptide α-1000[a]

Amino acid	Hydrolyzed Sardine Protein[b] (% total amino acid)	Peptide α-1000[c] (% total amino acid)
Aspartic acid (aspartate) and asparagine (Asx)	9.47	9.72
Threonine (Thr)	4.47	4.96
Serine (Ser)	2.81	3.70
Glutamic acid (glutamate) and glutamine (Glx)	11.53	18.28
Proline (Pro)	8.41	5.07
Glycine (Gly)	7.53	6.84
Alanine (Ala)	4.66	6.73
Cysteine (Cys)	<0.01[d]	<0.01[d]
Valine (Val)	6.31	5.61
Methionine (Met)	2.09	1.99
Isoleucine (Ile)	5.59	4.25
Leucine (Leu)	9.62	7.88
Tyrosine (Tyr)	4.34	3.07
Phenylalanine (Phe)	4.53	3.00
Histidine (His)	3.28	4.16
Lysine (Lys)	9.12	10.37
Arginine (Arg)	4.72	3.73
Tryptophan (Trp)	1.47	0.65
Total	**100**	**100**

[a] Obtained from Senmi Ekisu Co., Ltd.
[b] Comprising an amino acid composition of not less than 85 g/100 g.
[c] Comprising an amino acid composition of not less than 80 g/100 g.
[d] Below limit of detection (0.01 g/100 g).

Additionally, as discussed in Section II.B, peptide α-1000 also is inspected routinely for quality and compliance with set specifications (see Table IV.A-2 for peptide α-1000 specifications and a comparison to the specifications for the final hydrolyzed sardine protein product). The hydrolyzed sardine protein product has a slightly higher peptide content than peptide α-1000 (85% *versus* 80%). Peptide α-1000 also contains fat and has a higher sodium content than the final product of commerce.

Table IV.A-2 Comparison of the Specifications for Precursor, Peptide α-1000, and the Commercial Hydrolyzed Sardine Protein Product (Y-2)

Specification Parameter	Specification	
	Final Hydrolyzed Sardine Protein Product (Y-2)	Peptide α-1000
Appearance	Yellowish-white powder	Yellowish-white powder
Moisture	≤8.0%	≤8.0%
Ash	≤10.0%	≤10.0%
Peptide content	≥85%	≥80%
Total fat	NA	≤10.0%
Sodium (Na)	≤2.0%	≤10.0% (as NaCl)
Microbiological		
Standard plate count	≤1,000 CFU/g	≤10,000 CFU/g
Coliform count	Negative	Negative
Yeast and molds	≤300 CFU/g	≤300 CFU/g

CFU, Colony-forming units; NA, Not applicable; NaCl, Sodium chloride.

The safety of Senmi's hydrolyzed sardine protein product was further substantiated in a series of published single dose and repeat-administration human studies up to 13 weeks in duration in which study participants consumed test preparations containing 500 mg to 4 g of the hydrolyzed sardine protein product (6 g in single-dose administration studies) (Kawasaki *et al.*, 2000, 2002; Matsui *et al.*, 2002a,b; Kajimoto *et al.*, 2003, 2005a,b).

The weight of the available scientific evidence supports the safety of the proposed uses of hydrolyzed sardine protein. A summary of these data is presented herein.

IV.B Endogenous Occurrence and Biological Function

The daily turnover (synthesis and degradation) for protein in humans is estimated to be at least 250 g of protein (Waterlow, 1984) and, on a body weight basis compared to young adults, is greater in infancy than during later stages of life. In addition to supplying energy and fulfilling other vital cellular and organ functions, proteins and specifically peptides, the natural break-down products of protein metabolism, also have been associated with additional biological and physiological properties (mineral binding, immunomodulatory, regulation of serum cholesterol levels, antioxidative, antimicrobial, blood pressure normalization *etc.*) (Gardner, 1983, 1984; Kitts and Weiler, 2003; Zaloga and Siddiqui, 2004; Rutherfurd-Markwick and Moughan, 2005; Hartmann and Meisel, 2007). Peptides with biological properties usually comprise 2 to 20 amino acids and are produced endogenously by gastrointestinal proteases during normal protein digestion or as a result of food processing (Roberts *et al.*, 1999; Li *et al.*, 2002; Vermeirssen *et al.*, 2004).

Val-Tyr, one of several peptides identified in Senmi's hydrolyzed sardine protein product, has been identified to occur endogenously in plasma as a primary metabolite of angiotensin I (ANG I) in rodents and in humans (Kawasaki *et al.*, 2000). Breakdown of ANG I, II, and III to Val-Tyr has been shown *in vitro* and in plasma samples from spontaneously hypertensive rats (Matsufuji *et al.*, 1995; Matsui *et al.*, 1997a,b). Depending on whether healthy subjects were lying down or standing up to evoke an increase in blood pressure, Val-Tyr was identified in plasma at levels in the range of 111 to 447 fmol/mL (Matsui *et al.*, 1997a,b). Val-Tyr levels were shown to be lower in mildly hypertensive subjects compared to normotensive individuals, suggesting that Val-Tyr plays a role in blood pressure regulation (Matsui *et al.*, 1999).

Several peptides from various dietary sources (*e.g.*, bonito, salmon, sardines, tuna, pork, chicken, oyster, fermented fish sauce) including Val-Tyr have been shown to inhibit the activity of the angiotensin I converting enzyme (ACE) and thus to inhibit ANG I to ANG II conversion (Matsui *et al.*, 1993; Matsufuji *et al.*, 1994; Seki *et al.*, 1995a; Ichimura *et al.*, 2003; Vercruysse *et al.*, 2005). Examined *in vivo* in hypertensive mice and rats, administration of Val-Tyr was associated with reductions in blood pressure and was accompanied by increased plasma and organ levels of Val-Tyr (Matsufuji *et al.*, 1995; Matsui *et al.*, 2003, 2004).

IV.C Metabolic Fate and Pharmacokinetics

Senmi's hydrolyzed sardine protein product consists primarily of short-chain peptides of 2, 3, 4, or 5 amino acids in length. Unlike earlier contentions that proteins and smaller peptides are entirely hydrolyzed to free amino acids prior to absorption, it is now more widely accepted that a small portion of peptides also may be taken up intact into the enterocytes from the luminal phase of the small intestine by processes independent of those responsible for amino acid absorption (Friedrich, 1982; Gardner, 1982; Webb, 1990; Ziv and Bendayan, 2000). Specifically, a proton (H^+)-dependent peptide transporter (PepT1) has been identified in the tissues of the small intestine of humans (Ganapathy *et al.*, 1995; Tsuji and Tamai, 1996). Once inside the intestinal epithelial cells, peptides may be hydrolyzed by intracellular peptidases or released intact across the basolateral membrane into the circulation (Friedrich, 1982; Yang *et al.*, 1999). Transport of peptides across the basolateral membrane is thought to occur *via* transporter-mediated transport, transcellular transport (passive diffusion and endocytosis), or paracellular transport (*via* tight junctions between cells) (Gardner, 1984, 1998; Shimizu *et al.*, 1997). Following systemic absorption, peptides are subject to hydrolysis in the blood or other organ tissues, including the liver and kidneys (Lochs *et al.*, 1988, 1990; Webb, 1990; Gardner, 1998). Peptides that are resistant to hydrolysis in the renal tissues are excreted in the urine (Gardner, 1998; Minami *et al.*, 1992).

In mice and rats, plasma levels of Val-Tyr increased significantly in comparison to pre-treatment values following oral administration of the dipeptide (Matsui *et al.*, 2003, 2004). Following absorption, the dipeptide was shown to be eliminated rapidly from plasma and distributed to a

wide range of major organs, including the liver, kidneys, heart, and lungs (Matsui *et al.*, 2003, 2004). Likewise, in human studies conducted with normotensive subjects and participants with mild hypertension, significant and dose-dependent increases were observed in plasma Val-Tyr levels in comparison to baseline values (Matsui *et al.*, 2000, 2002a,b). In normotensive subjects, maximum plasma Val-Tyr levels (C_{max}) of 933 ± 201, 1,192 ± 72, and 1,934 ± 145 fmol/mL were obtained following ingestion of 3, 6, and 12 mg of Val-Tyr (provided as a drink), respectively, compared to baseline levels of 159 ± 11 fmol/mL (Matsui *et al.*, 2000, 2002a). Mildly hypertensive subjects exhibited C_{max} Val-Tyr levels of 1,245 ± 26.9 and 2,041 ± 148 fmol/mL after ingestion of a drink containing 6 and 12 mg of Val-Tyr, respectively (Matsui *et al.*, 2002b). Under the intended conditions of use of the hydrolyzed sardine protein and Val-Tyr levels of up to 0.16% in the product, exposure to Val-Tyr from the consumption of hydrolyzed sardine protein would be up to approximately 6 mg/day (based on the highest 90th percentile consumption estimate of 3.8 g/day for hydrolyzed sardine protein in children). In comparison, control group plasma levels of Val-Tyr ranged from 163 to 243 fmol/mL. In both study groups, peak plasma levels were attained approximately 2 hours following ingestion of the drinks and the elimination half-life ($t_{1/2}$) was estimated to be approximately 3 hours. While the study results confirmed dose-dependent absorption of intact Val-Tyr, as a percentage of the highest administered dose (12 mg of Val-Tyr), only 0.014% of Val-Tyr was absorbed and detected in the plasma of human subjects, with the remainder likely hydrolyzed to individual amino acids prior to absorption (Matsui *et al.*, 2002a).

Based on the data obtained for Val-Tyr, one of the peptides identified in Senmi's hydrolyzed sardine protein product, and the general understanding of peptide metabolism, it is expected that a large portion of the peptides in Senmi's product will be hydrolyzed to the individual constituent amino acids prior to absorption and only a small fraction of intact peptides will be available for systemic uptake.

Since absorption of peptides may occur *via* passive processes, a potential exists for increased absorption of peptides in infants in whom the gastrointestinal epithelium is not yet fully developed and/or in individuals with reduced peptidase activity (*e.g.*, Celiac disease, chemotherapy) or increased intestinal permeability (*e.g.*, Crohn's disease) (Gardner, 1982, 1983). However, it should be noted that peptide formation is a naturally occurring process related to the normal digestion of protein, the background dietary intake of which is considerably higher than the estimated intake of sardine peptides from the proposed use of Senmi's product. Therefore, exposure to peptides formed during the first stages of protein digestion will be greater than exposure to sardine peptides from the consumption of Senmi's product. Furthermore, Senmi does not intend to market foods to which its hydrolyzed sardine protein product has been added directly for infants (*i.e.*, not intended for addition to infant formula/foods) and therefore there are no anticipated concerns for adverse effects in this population group. Thus, it is expected that consumption of Senmi's hydrolyzed sardine protein product under the proposed conditions of use would not pose a greater risk to infants or

individuals with pathologies of the gastrointestinal tract that affect the handling of peptides beyond what is associated with the intake of protein in general.

IV.D Toxicological Studies

IV.D.1 Acute Toxicity Studies

Male SD (Crj:CD) rats were randomly assigned to 2 groups (4 rats/group) and administered a single dose of 10,000 mg/kg body weight of peptide α-1000 (peptide content 82.44%) or water (solvent control) by oral gavage (Osajima *et al.,* 2009). Following treatment administration, the animals were observed for a period of 7 days and body weight measurements were collected on Days 1, 3, and 7 of the study. On Day 7, all surviving animals were necropsied and the major organs of the thoracic and abdominal cavities, as well as the brain were subjected to macroscopic evaluation. All animals were reported to survive the 7-day observation period. Mild salivation was observed in 2 of the peptide α-1000-treated animals immediately following dose administration, which resolved approximately 1-hour post-administration. Salivation was noted to occur only briefly following dosing and as such may have been attributable to administration of a bolus dose by gavage, rather than to the test material *per se*. Otherwise, no significant clinical observations related to peptide α-1000 exposure were observed. In comparison to the control group, no significant differences in body weight and no macroscopic changes were reported in the test animals. Since administration of a single bolus dose of peptide α-1000 at a level of 10,000 mg/kg body weight was not associated with any adverse effects in male rats under the conditions of this study, the median lethal dose (LD_{50}) of peptide α-1000 in male rats was estimated to be greater than 10,000 mg/kg body weight.

IV.D.2 Sub-Chronic Toxicity Studies

A repeat-dose oral toxicity study of peptide α-1000 (peptide content 82.44%) was conducted with male and female SD (Crj:CD) rats (Osajima *et al.,* 2009). A total of 48 rats (6/sex/group) were administered peptide α-1000 at dose levels of 0 (negative control), 1,250, 2,500, or 5,000 mg/kg body weight/day by oral gavage for a period of 28 days. The negative control group animals were treated with the solvent (*i.e.,* water for injection). Food and water were provided *ad libitum* for the entire duration of the study period. Animals were observed daily for clinical signs of toxicity and mortality, whereas body weights were recorded on a weekly basis on Days 1, 7, 14, 21, and 28. Daily food intake was calculated on the basis of weekly food consumption values. A 24-hour urine sample was taken approximately 1 week prior to the end of the administration period and analyzed for volume, pH, protein, glucose, ketone bodies, occult blood, and bilirubin. Prior to necropsy, blood was collected from the abdominal aorta and analyzed for red blood cell count (RBC), white blood cell count (WBC), hemoglobin content (Hb), hematocrit value (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), total protein, albumin, albumin/globulin ratio, glucose, total cholesterol (T-Ch), triglyceride (TG), total bilirubin,

blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). Absolute and relative organ weights were recorded for the lungs, heart, liver, spleen, and kidneys. Organs of the gastrointestinal tract, as well as the pancreas, lungs, heart, liver, spleen, and kidneys were preserved in buffered 10% formalin solution and histopathological examinations were performed on any organs with gross lesions observed during macroscopic examination.

All animals survived until the end of the study period and no abnormalities in general condition were observed. No significant differences were noted in the food consumption, final body weights, and weight gain of peptide α-1000-treated animals compared to controls. Reductions in water consumption were observed in male and female test animals, but the differences did not reach statistical significance in comparison to controls and in males were not dose-related. In females, the decrease in water consumption appeared to be mediated by considerably lower intakes in one female at each of the mid- and high-dose levels. Water intakes of all other female animals in these groups were reported to be within the normal range. Hematology, clinical chemistry, and urinalysis parameters of test animals also were comparable to controls. With the exception of statistically significant increases in the relative and absolute left kidney weights in males in the high-dose group, no other significant organ weight variations were observed relative to the control group. Macroscopic examination revealed hemorrhage in the glandular stomach of 1 female in the mid-dose group (*i.e.*, 2,500 mg/kg body weight/day). In light of the incidental occurrence of this effect in only a single female at the mid-dose level, this was not considered to be related to the administration of peptide α-1000, but rather, may have occurred as a result of gavage error.

Although no gross lesions were observed in male rats examined macroscopically, left kidneys from control and high-dose animals were examined microscopically as a result of the increase in both absolute and relative weights. Renal histopathology revealed an increased presence of eosinophilic microparticles and infiltration of lymphocytes and fibroblasts into the interstitium in both controls and high-dose animals (*i.e.,* 5,000 mg/kg body weight). Thickening of the basal membrane, along with the presence of calcium-like deposits in the left renal tubules and thickening of the Bowman's capsule, also were sporadically identified in the left kidneys of both negative controls and high-dose rats. Notably, necrosis of the tubular epithelium was only observed in the control animals. Since the renal changes occurred with similar incidence and severity (slight) in both the control and test animals, these changes were not considered to be attributable to the test material; furthermore, these renal changes appeared to be consistent with male-specific spontaneous nephropathy commonly observed in rats (Hard and Khan, 2004) and were not considered to be relevant in an assessment of the safety of sardine peptides as intended for consumption by humans.

Based on the results of this study demonstrating an absence of any adverse effects related to the oral administration of peptide α-1000 at dose levels of up to 5,000 mg/kg body weight, the highest dose level tested was determined to be the no-observed-adverse-effect level (NOAEL)

in rats under the conditions of this study. As discussed in Section IV.A., 100 g of peptide α-1000 yields approximately 32 g of the final commercial hydrolyzed sardine peptide product. Thus, 5,000 mg/kg body weight/day of peptide α-1000 provides 1,600 mg/kg body weight/day of the commercial hydrolyzed sardine protein ingredient and thus supports the safety of the ingredient under the proposed conditions of use (146 mg/kg body weight/day in children; highest estimated intake at the 90th percentile). Furthermore, given the comparative nature of the amino acid profile of peptide α-1000 and the final ingredient and the fact that the majority of the peptides will be hydrolyzed to the constituent amino acids, the results of this study also indicate that no safety concerns are likely at dose levels of up to 5,000 mg/kg body weight/day of the final commercial hydrolyzed sardine protein ingredient.

Senmi's sardine peptide preparation was administered to 10-week old male stroke-prone spontaneously hypertensive (SPSH) rats, a non-obese, hyperglycemic, and hypertensive strain, at a dose level of 0 (untreated control) or 1,000 mg/kg body weight/day (6 and 7 rats/group, respectively) in the drinking water for a period of 4 weeks (Otani *et al.*, 2009). An additional group of SPSH rats was provided captopril in the drinking water (8 mg/kg body weight/day; n=6). A group of untreated Wistar-Kyoto rats also was included in the study. Following the 4-week treatment period, no differences were observed in food intake, body weights, or levels of total cholesterol, triglyceride, or free fatty acids between the untreated and sardine peptide-treated SPSH rats. In comparison to untreated SPSH rats, SPSH rats provided sardine peptides in the drinking water exhibited significantly reduced relative heart weights, which were still significantly higher than those of Wistar-Kyoto rats, but less so than in the untreated SPSH rats. However, no variations in either absolute or relative heart weights were observed in the 28-day study conducted by Osajima *et al.* (2009) in which SD (Crj:CD) rats were administered sardine peptides at dose levels of up to 5,000 mg/kg body weight/day. Sardine peptide-treated SPSH rats also showed significant reductions in systolic blood pressure, similar to those observed in rats treated with captopril, compared to untreated SPSH rats. After the 4-week treatment period, no differences were observed in fasting plasma glucose levels among any of the SPSH groups of rats; however, all 3 groups exhibited significantly higher blood glucose levels than the Wistar-Kyoto rats. Likewise, no differences were observed in fasting plasma insulin levels in any of the SPSH groups of rats. Fasting plasma insulin levels of the SPSH rats were, however, lower than those of the Wistar-Kyoto rats. Thirty (30) minutes following administration of a glucose tolerance test, peak plasma glucose levels of the sardine peptide- and captopril-treated SPSH rats were significantly lower than those of the untreated SPSH rats. Conversely, glucose loading was associated with only minimal increases in insulin levels in SPSH rats, with no significant variations observed among the different treatment groups. In comparison, a significant increase in plasma insulin levels was observed in the Wistar-Kyoto rats following administration of the glucose tolerance test. Since the reductions in plasma glucose levels in sardine peptide-treated SPSH rats were not paralleled by increased levels of insulin, the study authors speculated that sardine peptides may instead have helped to improve insulin resistance in SPSH rats.

IV.D.3 Mutagenicity and Genotoxicity Studies

Hydrolyzed Sardine Protein

Results of the *in vitro* mutagenicity assays and the *in vivo* genotoxicity study of Senmi's hydrolyzed sardine protein product are summarized in Table IV.D.3-1 and discussed in greater detail below.

Table IV.D.3-1 Summary of *In vitro* and *In vivo* Mutagenicity/Genotoxicity Studies on Hydrolyzed Sardine Protein

Test System	Type	Metabolic Activation	Concentration(s)/ Dose levels	Result	Reference
In vitro					
Salmonella typhimurium TA98, TA100, TA1535, and TA1537	Rev. Mut	+/-S9	0 or 313 to 5,000 µg/plate[1]	Negative	Osajima *et al.* (2009)
Escherichia coli WP2*uvr*A	Rev. Mut	+/-S9	0 or 313 to 5,000 µg/plate[1]	Negative	Osajima *et al.* (2009)
In vivo					
Mouse [Crj:CD-1 (ICR); male] bone marrow	MN	NA	0, 250, 500, 1,000, or 2,000 mg/kg bw[2]	Negative[3]	Osajima *et al.* (2009)

MN, micronucleus; NA, not applicable; Rev. Mut, reverse mutation; +S9, with metabolic activation; -S9, without metabolic activation.
[1]Sardine peptide was dissolved in sterilized 0.1 mol/L Na-phosphate buffer to make a 50 mg/mL solution.
[2]Mice were treated twice at 24-hour intervals. Animals were killed 24 hours following administration of the last dose.
[3] A statistically significant reduction in the ratio of polychromatic erythrocytes (PCE):total number of erythrocytes was observed at the 1,000 and 2,000 mg/kg body weight dose levels in comparison to the negative control.

The potential mutagenicity of Senmi's hydrolyzed sardine protein product (peptide content 89.1%) was assessed in a standard battery of *S. typhimurium* strains and in one strain of *E. coli* (Osajima *et al.,* 2009). Tests in both prokaryotic systems were performed with Senmi's hydrolyzed sardine protein product at concentrations ranging from 313 to 5,000 µg/plate, with or without metabolic activation (±S9). 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2), sodium azide (NaN_3), 9-aminoacridine (9-AA), and 2-aminoanthracene (2-AA), were tested as positive controls, whereas the solvent, sodium-phosphate buffer (Na-phosphate), served as the negative control. Evaluated using the Ames assay, with and without metabolic activation (±S9) at concentrations of up to 5,000 µg/plate, Senmi's hydrolyzed sardine protein product consistently tested negative for mutagenic activity in all strains of *S. typhimurium* tested (Osajima *et al.,* 2009). Likewise, when tested with *E. coli*, Senmi's hydrolyzed sardine protein product failed to significantly increase the number of revertant colonies compared to the solvent control at concentrations of up to 5,000 µg/plate, with and without metabolic activation.

The potential clastogenicity of Senmi's hydrolyzed sardine protein product was evaluated *in vivo* under the conditions of a micronucleus assay in mouse erythroblasts (Osajima *et al.,* 2009).

Hydrolyzed sardine protein (peptide content 85.63%) was administered orally to groups of male Crj:CD-1 (ICR) mice (5 animals/group) at dose levels of 0, 250, 500, 1,000, or 2,000 mg/kg body weight by gavage. Both a negative and positive control group were included in this study. Mice in the negative control group were orally administered the carboxymethyl cellulose sodium (CMC-Na) solvent solution (*i.e.,* 0.5% CMC-Na), whereas positive controls were treated with 2 mg/kg body weight of mitomycin C (MMC) *via* intraperitoneal injection. Mice were treated once daily for 2 consecutive days, at 24-hour intervals, and were killed 24 hours after the final dose was administered. The femoral bone was removed and bone marrow cells were examined to determine the prevalence of micronucleated polychromatic erythrocytes (MNPCE), and the ratio of polychromatic erythrocytes (PCE) to the total number of erythrocytes, PCE + normochromatic erythrocytes (NCE). The prevalence of MNPCE obtained from animals in the sardine peptide-treated groups was comparable to that in the negative control group. A dose-dependent decrease was observed in the PCE ratio, such that at the two highest dose levels (*i.e.*, 1,000 and 2,000 mg/kg body weight groups) the decrease was statistically significant in comparison to the negative control group (*i.e.,* 40.52 and 40.08%, respectively, compared to 49.96%). A further reduction in the PCE ratio was observed in the positive control group. Furthermore, it was noted by the study authors that both the prevalence of MNPCE and ratio of PCE to total erythrocytes observed in the negative and positive control groups in the study were within the range of historical data.

Valine-Tyrosine (Val-Tyr)

The potential mutagenicity of Val-Tyr (99% purity), a dipeptide found in Senmi's hydrolyzed sardine protein product, also was evaluated under the conditions of the Ames assay in a standard battery of *S. typhimurium* strains [Yamaguchi, 1999 (unpublished)]. Additionally, potential mutagenic activity also was examined in *E. coli.* Val-Tyr was tested at concentrations of up to 5,000 µg/plate with and without metabolic activation. In comparison to the solvent control, no differences were observed in the number of revertant colonies in any of the bacterial strains incubated in the presence of Val-Tyr, irrespective of metabolic activation.

The potential genotoxicity of the purified dipeptide, Val-Tyr (99% purity), was assessed *in vivo* in mice (Kimura, 2004 [unpublished]). Groups of 5, 8-week-old male Crj:CD-1 (ICR) mice received oral gavage administration of Val-Tyr at dose levels of 0 (negative and positive controls), 250, 500, 1,000, or 2,000 mg/kg body weight for 2 consecutive days at 24-hour intervals. Animals were killed 24 hours following administration of the last dose and the bone marrow was removed for examination of erythrocytes for micronucleus formation. The prevalence of MNPCE in Val-Tyr-treated mice was comparable to that observed in negative controls. As in the study performed with Senmi's hydrolyzed sardine protein product, the ratio of PCE to the total number of erythrocytes in animals administered Val-Tyr decreased in a dose-dependent fashion relative to the negative control value, with values in groups of animals receiving the 2 highest dose levels attaining statistical significance. Negative and positive control group values

of MNPCE prevalence and the ratio of PCE to total erythrocytes were within the range of historical values.

Summary

Neither the hydrolyzed sardine protein product, nor Val-Tyr exhibited any mutagenic potential in a standard battery of *Salmonella* (TA98, TA100, TA1535, and TA1537) or *E. coli* (WP2*uvr*A) in the presence or absence of metabolic activation at concentrations of up to 5,000 mg/plate. Likewise, examined *in vivo* under the conditions of the mouse micronucleus assay, neither the hydrolyzed sardine protein product nor isolated Val-Tyr produced any chromosomal aberrations indicative of clastogenic properties.

In both mouse micronucleus assays conducted with Senmi's hydrolyzed sardine protein product and with the dipeptide Val-Tyr, dose-related reductions were observed in the ratio of PCE to the total number of erythrocytes in test animals relative to the negative control group, with values in groups of animals treated with the 2 highest dose levels (1,000 and 2,000 mg/kg body weight) attaining statistical significance and, therefore, indicating a potential effect on erythropoiesis. However, no statistically significant differences were observed in any hematological parameters (including red blood cell count, hemoglobin content, and hematocrit value) following gavage administration of peptide α-1000 at dose levels of up to 5,000 mg/kg body weight/day compared to the negative control group in the repeat-dose 28-day rat study (Osajima *et al.*, 2009). Therefore, consumption of Senmi's hydrolyzed sardine protein product under the proposed conditions of use is not expected to be associated with any adverse effects on normal blood cell development.

IV.D.4 Developmental and Reproductive Toxicity Studies

No studies related specifically to the potential developmental and reproductive toxicity of sardine peptides were identified in the publically available literature.

In utero exposure to pharmaceutical ACE inhibitors has been reported to be associated with fetopathy, which is speculated to be partly due to the direct action of the re-circulated ACE inhibitors on the renin-angiotensin system in the fetus and partly to fetal ischemia ensuing as a result of maternal hypotension and reduced fetal-placental blood flow and oxygen/nutrient delivery to the baby (Solhaug *et al.*, 2004; Cooper *et al.*, 2006; Quan, 2006). More recently, an increased risk for congenital malformations as a result of first trimester exposure to ACE inhibitors also has been reported (Cooper *et al.*, 2006; Quan, 2006). Consequently, the use of ACE inhibitor drugs is contraindicated during pregnancy. Since some dietary peptides are postulated to mediate blood pressure changes *via* a similar mechanism as ACE inhibitor drugs, the potential for Senmi's hydrolyzed sardine protein product to mediate similar developmental effects was considered for the purposes of this safety assessment.

Although the underlying mechanism of action of dietary peptides related to the blood pressure mediating effects is hypothesized to be similar to that of pharmaceutical ACE inhibitors, the bioactivity of the dietary peptides is significantly lower than that of the drugs. Moreover, in order to attain therapeutically efficacious plasma levels of pharmaceutical ACE inhibitors following oral ingestion, most are given as pro-drugs that are converted to the active form by de-esterification in the liver as a result of the first-pass effect (Barr, 1994). Conversely, short-chain peptides such as those in Senmi's product are subjected to extensive hydrolysis beginning in the gastrointestinal tract to individual amino acids, with only a small fraction escaping hydrolysis prior to uptake.

The adverse fetal effects of ACE inhibitor pharmaceutical agents have also been partly related to the ensuing hypotension in the mother. However, unlike the more bioactive pharmaceutical ACE inhibitory agents, human studies have shown consistently that Senmi's hydrolyzed sardine protein product only assists in the maintenance of healthy blood pressure in individuals with above-normal blood pressure and does not mediate reductions beyond the normal physiological range. Moreover, unlike pharmaceutical ACE inhibitors, which are intended to treat diagnosed medical hypertension with many different etiologies, Senmi's product is not intended for use as a therapeutic agent. It has been suggested that the adverse effects observed in infants born of mothers who had taken ACE inhibitor drugs during pregnancy also could be related to preexisting maternal disease conditions rather than the pharmaceutical agents *per se*, especially considering that some of the characteristic effects of ACE inhibitory fetopathy also are common complications of hypertensive pregnancies (Barr, 1994).

Further support for the absence of any adverse developmental effects related to the consumption of hydrolyzed sardine protein can be derived from the results of a developmental/reproductive toxicity study conducted with a similar protein hydrolysate product that has also been associated with ACE inhibitory properties. Specifically, Kurosaki *et al.* (2005) conducted a study to assess the potential effects of *Lactobacillus helveticus*-fermented milk powder on fertility and reproductive performance in rats. The results of this study were discussed as part of the GRAS notice submitted to the FDA for concentrated hydrolyzed milk protein obtained by milk fermentation or enzymatic hydrolysis of casein (GRN 000199 – U.S. FDA, 2007). Two tripeptides, L-valyl-L-prolyl-L-proline (VPP) and L-isoleucyl-L-prolyl-L-proline (IPP), have been identified in hydrolyzed milk protein that, like the peptides in hydrolyzed sardine protein, may mediate blood pressure lowering effects in individuals with above-normal blood pressure by inhibiting ACE activity (Nakamura *et al.*, 1995). The tripeptides, VPP and IPP, are contained in the milk powder fermented with *L. helveticus* one of the subjects of the GRAS notice and the test material administered in the reproductive toxicology study.

Although Senmi's hydrolyzed protein product is obtained from a non-milk source (*i.e.*, sardines), it mediates similar blood pressure reductions in humans as those observed with *L. helveticus*-fermented sour milk products containing VPP and IPP. Specifically, in human studies where

120 to 160 g of sour milk cultured with *L. helveticus* was provided for oral administration, systolic blood pressure (SBP) blood pressure changes in the range of 5 to 15 mmHg were observed in mildly hypertensive subjects (>140 mmHg) and 3 to 8 mmHg in diastolic blood pressure (DBP) (>90 mmHg) (Kajimoto *et al.*, 2001; Hirata *et al.*, 2002; Mizushima *et al.* 2004). The sour milk beverages contained approximately 2 to 3 grams of protein in general and 2 to 3 mg of VPP and 1.2 to 1.6 mg IPP specifically. Since any potential concerns regarding the developmental safety of Senmi's hydrolyzed sardine protein are primarily related to its postulated physiological function of blood pressure regulation, the results of a study conducted with another proteinaceous ingredient with similar physiological attributes were considered to be supportive of the developmental safety of Senmi's product, even though the protein hydrolysates are obtained from different sources. Specifically, no adverse effects on reproductive performance or fetal development related to the oral administration of *L. helveticus*-fermented milk powder at dose levels of up to 2,000 mg/kg body weight/day (equivalent to approximately 3.3 mg/kg body weight/day of VPP and IPP) were observed in Sprague-Dawley rats (Kurosaki *et al.*, 2005). The milk powder was provided to males beginning 4 weeks prior to mating and continuing until the end of the mating period. Females were administered the milk powder starting 2 weeks prior to mating through Day 20 of lactation. Based on the results of the study conducted with fermented milk powder, which mediates blood pressure changes similar to those observed with hydrolyzed sardine protein, it can be inferred that hydrolyzed sardine protein also is unlikely to adversely affect fetal development.

Additionally, protein, which is broken down to peptides and amino acids as part of the normal digestive process, is considered essential for fetal development. It is well recognized that the demand for protein increases during pregnancy and lactation (IOM, 2005). Accordingly, the IOM has recommended that pregnant and lactating women consume 25 g of additional protein per day during these periods. In comparison to current daily protein intakes from all dietary sources of women during pregnancy and lactation (*i.e.*, 78.2 to 79.7 and 97.0 to 100.8 g/day, respectively) (IOM, 2005) and the all-user background intakes of sardine protein by adult females (7.45 g/day), the daily mean and 90th percentile peptide intakes estimated from the all-user consumption of Senmi's hydrolyzed sardine protein product by adult women (*i.e.*, 1.0 and 2.0 g/day, respectively) are considerably lower. Likewise, the mean and 90th percentile all-user intakes of peptides estimated from the proposed uses of Senmi's hydrolyzed sardine protein product in foods in infants (0 to 2 years of age) (*i.e.*, 0.8 and 1.6 g/day, respectively; values presented in Table I.D.5-1 are adjusted by 0.85 to account for peptide content only) were approximately 10- to 60-fold lower than the daily intake of all dietary protein reported for infants up to 12 months of age by the IOM (*i.e.*, 15.9 to 28.3 and 21.9 to 42.5 g/day, respectively) (IOM, 2005). However, as previously mentioned, Senmi does not intend to market foods with added hydrolyzed sardine protein for infants. Protein hydrolysates of plant, vegetable, animal, or milk origin have a long history of use in the U.S. Furthermore, peptides are not only a product of normal digestion of protein, but Val-Tyr specifically also is produced endogenously by the body

as a result of ANG I breakdown and likely functions as part of the renin-angiotensin system in the proper maintenance of blood pressure.

Given the low bioactivity of Senmi's hydrolyzed sardine protein product and its limited effect to only assist in the maintenance of above-normal blood pressure to levels within the normal physiological range, coupled with the fact that peptides are natural break-down products of protein digestion and Val-Tyr is a naturally-occurring metabolite of ANG I degradation, it is not anticipated that consumption of food products containing Senmi's hydrolyzed sardine protein ingredient during pregnancy would be associated with any adverse developmental effects in neonates. The results of a study conducted in rats showing no adverse effects on development or reproduction following oral administration of a related protein hydrolysate product with similar effects on blood pressure further corroborate this conclusion.

IV.E Human Studies

Several studies, including single- and repeat-dose administration studies, were conducted in humans to evaluate the potential physiological effects and the safety of Senmi's hydrolyzed sardine protein product and hydrolyzed sardine protein products manufactured by Senmi using slightly modified methods (Kawasaki *et al.*, 2000, 2002; Matsui *et al.*, 2002a,b; Kajimoto *et al.*, 2003, 2005a,b). The results of these studies are summarized in Table IV.E-1. The studies were primarily designed to investigate the potential physiological effects of sardine peptides, and Val-Tyr specifically, on maintaining normal blood pressure levels; however, each study also included extensive safety-related endpoints (biochemical, hematological, and urinalysis parameters). Furthermore, study subjects were monitored for the occurrence of any adverse reactions during the treatment periods, including the development of a dry cough, which is a reported side effect of pharmaceutical ACE inhibitors resulting from increased levels of bradykinin. Study participants comprised not only individuals with above-normal blood pressure (otherwise healthy), but also normotensive subjects. The results of these studies were reviewed solely in the context of the ingredient's safety. For purposes of the determination of the GRAS status of the hydrolyzed sardine protein product, blood pressure and heart rate were considered only as end-points of safety and not efficacy.

Two single-dose trials were conducted with 5 normotensive and 12 mild-hypertensive male subjects (Matsui *et al.*, 2002a,b). Following ingestion of 6 g of a sardine peptide product manufactured by Senmi using a method similar to that described herein, no significant variations were observed in urinalysis and none of the study participants experienced any adverse side effects. SBP and DBP remained unchanged in both groups of study subjects (Matsui *et al.*, 2002a,b).

In the 6 studies involving repeated consumption of sardine protein hydrolysates over a course of 2 to 13 weeks, a total of 194 male and female subjects (25 normotensive and 169 with above normal blood pressure) participated (Kawasaki *et al.*, 2000, 2002; Kajimoto *et al.*, 2003,

2005a,b). In the studies involving repeated consumption of the hydrolyzed sardine protein products, no clinically significant disturbances were noted in standard hematological, clinical chemistry, or urinalysis parameters following daily ingestion of the hydrolyzed sardine protein products at dose levels of 500 mg for 12 to 13 weeks (Kawasaki *et al.*, 2002; Kajimoto *et al.*, 2003, 2005a) or 1.5 to 4 g for 2 to 4 weeks (Kawasaki *et al.*, 2000, 2002; Kajimoto *et al.*, 2005b). Since ACE inhibitors have been reported to stimulate dry coughing as a result of bradykinin activation (Israili and Hall, 1992), the development of a cough and any other allergic reactions was monitored specifically in all of the human studies. None of the study participants in any of the studies reported developing a cough or any other adverse effects while ingesting hydrolyzed sardine protein. Treatment with hydrolyzed sardine protein mediated reductions in blood pressure (both SBP and DBP) only in individuals with high-normal or above normal blood pressure at baseline following repeat-dosing and in no case was blood pressure decreased to levels below the normal range (Kawasaki *et al.*, 2000, 2002; Kajimoto *et al.*, 2003, 2005a,b). Decreases in SBP ranged from approximately 4 to 6% (approximately 6 to 8 mmHg) in individuals with high-normal blood pressure (individuals with blood pressure in the range of 130 to 140 mmHg) and from approximately 4 to 11% (approximately 6 to 18 mmHg) in hypertensive subjects (individuals with blood pressure >140 mmHg). Reductions in DBP were less consistently observed and were rarely significant in comparison to the control group, ranging from approximately 2 to 8% (approximately 1 to 8 mmHg) in subjects with above normal blood pressure. No significant variations in either SBP or DBP were reported in subjects enrolled in these studies with normal blood pressure.

Of the total 8 studies conducted to assess the safety of sardine peptide consumption in humans, 3 repeat-dose studies and 1 acute study included normotensive individuals (Kawasaki *et al.*, 2002; Matsui *et al.*, 2002a; Kajimoto *et al.*, 2005b). In the acute study, sardine peptides were administered at a dose level of 6 g as a bolus dose (Matsui *et al.*, 2002a), while in the repeat-dose studies sardine peptides were administered either at dose levels of 500 mg for 13 weeks (Kawasaki *et al.*, 2002) or 1.5 g for up to 4 weeks (Kawasaki *et al.*, 2002; Kajimoto *et al.*, 2005b). While the repeat-dose studies were conducted at dose levels below the estimated daily intake (3.8 g/day for children at the 90th percentile), the acute study was conducted at levels that were significantly higher than the estimated exposure even at the more conservative 90th percentile level. The acute study in normotensive subjects supports the safety of the ingredient and the lack of a hypotensive effect in individuals with normal blood pressure. Given that the pharmacokinetic data (see Section IV.C) demonstrate a rapid clearance of the peptides ($t_{1/2}$ approximately 3 hours) from the plasma, indicating a lack of potential to bioaccumulate, the acute studies at the higher dose levels provide reassurance that no adverse effects on blood pressure will occur at the levels greater than the estimated daily intake. No changes in heart rate were observed in any of the study participants consuming the hydrolyzed sardine protein products.

Table IV.E-1 Summary of Studies in Humans Conducted Using Hydrolyzed Sardine Protein Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Placebo-controlled trial	5 N-BP males (22 to 26 years; mean age 23.6 ± 0.6; BP 114±7/62±4 mmHg)	Single dose	0 (control), 3, 6, or 12 mg VY/day (as 0, 1.5, 3, and 6 g hydrolyzed sardine protein/day, respectively[1]), provided as a drink	i) SBP, DBP, and HR; ii) clinical chemistry (plasma levels of ANG I, ANG II, aldosterone, and VY); and, iii) urinalysis (Na^+, K^+, and Crea).	i) No significant Δ in SBP, DBP, or HR following ingestion of the test drink compared to baseline levels. ii) Dose-dependent ↑ in plasma VY; no Δ in plasma levels of ANG I, ANG II, and aldosterone following test drink ingestion. iii) No effect on urinary Na^+, K^+, or Crea levels. No adverse effects reported.	Matsui *et al.* (2002a)
Placebo-controlled trial	12 (4/group) mild-H males (39 to 59 years of age; mean 48.6 ± 2.8; BP 145.9±3.5/93.6±2.1 mmHg)	Single dose	0 (control), 6, or 12 mg VY/day (as 0, 3, and 6 g hydrolyzed sardine protein/day, respectively[1]), provided as a drink	i) SBP, DBP, and HR; ii) clinical chemistry (plasma VY); and, iii) urinalysis (Na^+, K^+, and Crea).	i) No significant Δ in SBP, DBP, or HR following ingestion of the test drink compared to baseline levels. ii) Dose-dependent ↑ in plasma VY. iii) No effect on urinary Na^+, K^+, or Crea levels. No adverse effects reported.	Matsui *et al.* (2002b)
Un-blinded ingestion trial	25 (23M, 2F; mean age 44.9±13.9); 5 MH subjects (BP 160-180/100-110 mmHg), 6 mild-H subjects (BP 140-160/90-100 mmHg), 6 HN-BP subjects (BP 130-140/80-90 mmHg), and 8 N-BP subjects (BP <130/<80 mmHg).	2 weeks	500 mg Senmi's hydrolyzed sardine protein (providing 0.4 mg VY) 3x per day (total 1,500 mg Senmi's hydrolyzed sardine protein and 1.2 mg VY per day), provided as 195 g vegetable juice	i) SBP and DBP; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (total protein, albumin, total bilirubin, AST, ALT, ALP, γ-GTP, LAP, LDH, BUN, Crea, CPK, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Fe^{2+}, Ch, HDL-Ch, LDL-Ch, Tg, glucose, HbA_{1c}); iv) urinalysis (parameters not specified); and, v) physical examinations; body weights.	i) No Δ in SBP, DBP, and HR in N-BP. ↓ SBP and DBP (compared to baseline) in mild-H subjects, ↓ SBP (compared to baseline), but not in DBP or HR in MH subjects and HN-BP subjects. ii, iii, and iv) Hematology, urinalysis, and clinical chemistry unremarkable (all statistically significant variations within physiological range). v) No subjective/objective symptoms or adverse effects reported, including coughing. No Δ in body weight.	Kawasaki *et al.* (2002)

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Table IV.E-1 Summary of Studies in Humans Conducted Using Hydrolyzed Sardine Protein Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Randomized, double-blind, placebo controlled trial	29 (27M, 2F) subjects with HN-BP and mild-H (mean BP 146/91-92 mmHg) Treatment group: 17 (16M, 1F) (Mean age 45.5 ± 3.2; 4 HN-BP and 13 mild-H) Control group: 12 (11M, 1F) (Mean age 48.8 ± 3.0)	4 weeks	Treatment group: 3 mg VY (as 2 g of protein from a sardine protein hyrolysate) 2x per day (total 4 g of a sardine peptide hydrolysate per day), provided as a 100 mL drink Control group: 100 mL drink provided 2x per day	i) SBP, DBP, and HR; ii) clinical chemistry (ANG I, ANG II, VY, and aldosterone); iii) urinalysis (Na^+, K^+, Na^+/K^+ ratio, and Crea); and, iv) physical examination.	i) Significant ↓ in SBP and DBP of HN-BP and mild-H test subjects compared to baseline levels; no Δ in HR. ii) Significant ↑ in plasma VY and ANG I levels compared to baseline values, but not compared to placebo control. Significant ↓ in plasma ANG II and aldosterone compared to baseline values, but not compared to placebo control. Unspecified change in serum chloride levels in test subjects. iii) No effect on urinary Na^+, K^+, Na^+/K^+ ratio, or Crea levels. iv) No adverse effects, including no cough.	Kawasaki *et al.* (2000)
Randomized, double-blind, placebo controlled trial	44 subjects with N-BP (118/73 mmHg), HN-BP (134/82 mmHg), or mild-H (148/87 mmHg) Treatment group: 22 (9M, 13F; mean age 55.4±8.0; 6 N-BP, 9 HN-BP, and 7 mild-H) Control group: 22 (10M, 12F; mean age 57.0±6.4; 5 N-BP, 8 HN-BP, and 9 mild-H)	4 weeks	Treatment group: 500 mg Senmi's hydrolyzed sardine protein/day (providing 0.4 mg VY) 3x per day (total 1,500 mg Senmi's hydrolyzed sardine protein and 1.2 mg VY per day), provided as a 350 mL tea beverage Control group: 350 mL tea beverage without Senmi's hydrolyzed sardine protein 3x per day	i) SBP, DBP, and HR; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (plasma renin, BUN, Crea, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Ch, HDL-Ch, Tg, total protein, albumin, A/G, blood glucose, AST, ALT, ALP, γ-GTP, LDH, total bilirubin, HbA_{1c}); iv) urinalysis (*i.e.*, Na^+, K^+, Cl^-, Crea, pH, specific gravity, protein, glucose, urobilinogen, and occult blood); and v) physical examinations; body weight and BMI.	i) Significant ↓ in SBP of test subjects compared to baseline and control values. Stratified analysis: ↓ in SBP (compared to controls), but not in DBP or HR in HN-BP; no Δ in SBP, DBP, or HR in subjects with N-BP or mild-H compared to controls. ii and iii) No Δ between control and test group[2]; all statistically significant Δ between pre- and post-treatment values within physiological range. iv) Protein and occult blood in one single female; likely result of sample contamination due to menstruation. ↓ in K^+ in test subjects compared to controls 2 weeks after study end. v) Mild GI symptoms and skin itching reported by both test and control subjects; no cough and no significant Δ in body weights or BMI.	Kajimoto *et al.* (2005b)

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Table IV.E-1 Summary of Studies in Humans Conducted Using Hydrolyzed Sardine Protein Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Double-blind, placebo controlled trial	114 subjects with HN-BP (BP 131-132/80-81 mmHg) or mild-H (BP 146-147/88 mmHg) Treatment group: 55 (24M, 31F; mean age 50.3±12.0) Control group: 59 (27M, 32F; mean age 50.1±10.3)	12 weeks	Treatment group: 500 mg Senmi's hydrolyzed sardine protein/day (providing 0.4 mg VY), provided as a 350 mL tea beverage Control group: 350 mL tea beverage/day without Senmi's hydrolyzed sardine protein	i) SBP, DBP, and HR; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (plasma rennin, BUN, Crea, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Ch, HDL-Ch, Tg, total protein, albumin, A/G, blood glucose, AST, ALT, ALP, γ-GTP, LDH, total bilirubin, HbA_{1c}); iv) urinalysis (*i.e.,* Na^+, K^+, Cl^-, Crea, pH, specific gravity, protein, glucose, urobilinogen, and occult blood); and, v) physical examinations; body weight and BMI.	i) ↓ in SBP and DPB in test subjects compared to baseline values and placebo-control values (SBP only); no Δ in HR. Stratified analysis: mild-H - ↓ in SBP and DBP compared to controls and baseline (DBP only) at Weeks 10 and 12 and Week 8, respectively; HN-BP - ↓ in SBP compared to control at Weeks 4-10 and to baseline at Week 10; no Δ in DBP. ii and iii) No Δ between control and test group; all statistically significant Δ between pre- and post-treatment values within physiological range. iv) Significant ↓ Na^+, K^+, and Cl^- in test subjects compared to controls; changes in qualitative parameters mild and transient and within physiological range of variations; occult blood and protein in samples from females likely result of sample contamination due to menstruation. v) Subjects reported mild GI effects in both control and test groups; no cough; ↓ in body weight and BMI of test subjects compared to baseline values.	Kajimoto *et al.* (2005a)

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Table IV.E-1 Summary of Studies in Humans Conducted Using Hydrolyzed Sardine Protein Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Randomized double-blind placebo controlled trial	88 subjects with HN-BP, mild-H, or MH Treatment group: 44 (26M, 18F; mean age 49±11; BP 148.3/92.1 mmHg) Control group: 44 (29M, 15F; mean age 50±10; BP 147.6/92.1 mmHg)	12 weeks	Treatment group: 500 mg Senmi's hydrolyzed sardine protein/day (0.4 mg VY), provided as 4 tablets Control group: 4 tablets/day without Senmi's hydrolyzed sardine protein	i) SBP, DBP, and HR; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (ANG II; ACE; aldosterone; plasma rennin activity and conc't, BUN, Crea, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Ch, HDL-Ch, Tg, total protein, albumin, A/G, blood glucose, AST, ALT, ALP, γ-GTP, LDH, total bilirubin, HbA_{1c}); iv) urinalysis (*i.e.,* Na^+, K^+, pH, specific gravity, protein, glucose, urobilinogen, and occult blood); and, v) physical examinations; body weight and BMI.	i) ↓ in SBP of test subjects compared to baseline and placebo and in DBP of test subjects compared to placebo (at 12 weeks). No change in HR. Stratified analysis: mild-H - ↓ in SBP compared to baseline at study end and several other interim weeks; ↓ DBP compared to baseline and control values at Week 8. HN-BP - ↓ in SBP compared to control values and baseline at study end and several other interim weeks and ↓ in DBP compared to control values. ii) ↓ in MCV of test group compared to placebo; all other statistically significant Δ only between pre- and post-treatment values and within physiological range. iii) No Δ between control and test group; Ch ↑ above upper end of normal range in both test and placebo subjects (at Week 8); all other statistically significant Δ between pre- and post-treatment values within physiological range (ANG II ↑ 1.7x from baseline in test group[3]). iv) ↓ in K^+ compared to baseline. No other Δ between test subjects and controls, and pre- and post-treatment values. v) ↓ in BMI of test subject 4 weeks after study end compared to baseline. No adverse effects related to test diet identified (no cough).	Kajimoto *et al.* (2003)

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Table IV.E-1 Summary of Studies in Humans Conducted Using Hydrolyzed Sardine Protein Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Randomized, double-blind, placebo controlled	63 subjects with mild-H/HN-BP (140-142/88 mmHg) and N-BP (111-113/71-72 mmHg) Treatment group: 31 subjects: 20 (16M, 4F) mild-H or HN-BP (mean age 51.8±9.4) and 11 (9M, 2F) N-BP (mean age 47.1±11.9) Control group: 32 subjects: 20 (17M, 3F) mild-H or HN-BP (mean age 52.0±9.9) and 12 (9M, 3F) N-BP (mean age 43.8±11.2)	13 weeks	Treatment group: 500 mg hydrolyzed sardine protein/day (providing 0.4 mg VY) in 195 g vegetable juice Control group: 195 g vegetable juice without hydrolyzed sardine protein	i) SBP and DBP; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (total protein, albumin, total bilirubin, AST, ALT, ALP, γ-GTP, LAP, LDH, BUN, Crea, CPK, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Fe^{2+}, Ch, HDL-Ch, LDL-Ch, Tg, glucose, insulin, HbA_{1c}, 1,5-AG); iv) urinalysis (parameters not specified); and, v) physical examinations; body weight.	i) ↓ SBP and DBP in mild-H and HN-BP subjects compared to baseline levels and placebo (SBP only). No significant change in SBP or DBP in N-BP subjects compared to baseline levels or placebo. ii, iii, and iv) Hematology, urinalysis, and clinical chemistry unremarkable (all statistically significant variations within physiological range). v) No subjective/objective symptoms or adverse effects reported (no dry cough). No changes in body weight.	Kawasaki *et al.* (2002)

ACE, Angiotensin I converting enzyme; 1,5-AG, 1,5-Anhydroglucitol; A/G, Albumin to globulin ratio; ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; ANG I, Angiotensin I; ANG II, Angiotensin II; AST, Aspartate aminotransferase; BMI, Body mass index; BP, Blood pressure; BUN, Blood urea nitrogen; Ca^{2+}, Calcium; Ch, Cholesterol (total); Cl^-, Chloride; CPK, Creatine phosphokinase; Crea, Creatinine; DBP, Diastolic blood pressure; F, Female; Fe^+, Iron; GI, Gastrointestinal; γ-GTP, gamma-Glutamyl transpeptidase; Hb, Hemoglobin; HbA_{1c}, Hemoglobin A_{1c}; HDL-Ch, High-density lipoprotein-cholesterol; HN-BP, High-normal blood pressure; HR, Heart rate; Ht, Hematocrit; LAP, leukocyte alkaline phosphatase; K^+, potassium; LDH, lactate dehydrogenase; LDL-Ch, Low-density lipoprotein-cholesterol; M, Male; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; MH, Moderate hypertension; mild-H, Mild hypertension; Mg^{2+}, Magnesium; Na^+, Sodium; N-BP, Normal blood pressure; RBC, Red blood cells; SBP, Systolic blood pressure; Tg, Triglycerides; UA, Uric acid; VY, Valine-tyrosine; WBC, White blood cells; Δ, variation.

[1] Assuming hydrolyzed sardine protein products contain 0.2% Val-Tyr.

[2] Hemoglobin A_{1c} levels in test subjects significantly lower compared to controls at study beginning and study end.

[3] The authors noted that markers of the renin-angiotensin-aldosterone system are highly variable and susceptible to many exogenous factors, including seasonal changes, which may have influenced the ANG II levels between study beginning and end.

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IV.F Other Considerations of Safety

IV.F.1 Potential for Drug Interaction

One pre-clinical study was identified in which the potential for an interaction of Val-Tyr with the ACE inhibitor, captopril, was examined in 16-week-old male SH rats (Matsui *et al.*, 2006). Administered individually once by gavage, captopril (2.5 mg/kg body weight) or Val-Tyr (25 mg/kg body weight) mediated a similar reduction in blood pressure (*i.e.*, 23 and 20 mmHg, respectively) compared to baseline values 3 hours post-dosing, which was maintained for 9 hours following administration of the test compounds. However, when captopril and Val-Tyr were administered jointly, no significant reduction was observed in blood pressure compared to baseline values. Co-consumption of Val-Tyr and a pharmaceutical ACE inhibitor therefore did not exert a synergistic effect in regard to blood pressure reductions; however, the combined consumption of the peptide and captopril reduced plasma levels of captopril below the effective range. Results of additional *in vivo* studies evaluating plasma captopril levels following co-administration with Val-Tyr and *in vitro* permeability assays suggested that Val-Tyr acted as a competitive inhibitor of captopril uptake, but that the transporter responsible for the uptake possessed a greater affinity for captopril than Val-Tyr. Based on the results of this study and considering that Val-Tyr is only one of several di- and tri-peptides normally present in peptide products that may exhibit similar properties, the authors suggested that the intake of foods that are rich in peptides should be limited in hypertensive individuals treated with ACE inhibitors such as captopril.

Under the intended conditions of use of the hydrolyzed sardine protein ingredient, resulting in dietary exposures of 146 mg/kg body weight/day (90th percentile in children), the corresponding intake of Val-Tyr, which is specified in the ingredient at levels of up to 0.16%, is 0.23 mg/kg body weight/day. Therefore, the potential for a similar interaction in humans is minimized by the fact that exposure to Val-Tyr from the consumption of hydrolyzed sardine protein would be at least 100 times below the effective inhibitory dose in rats. Considering further that sardine peptides from the use of hydrolyzed sardine protein under the conditions of use proposed herein were estimated to only minimally contribute to the overall dietary protein intake and the intake of protein from sardines specifically, and that the amino acids comprising the peptides in Senmi's hydrolyzed sardine protein product have a history of background dietary consumption due to their natural presence in sardine tissue, the potential for drug interactions to occur under the intended conditions of use of the hydrolyzed sardine protein product in the defined food products is expected to be negligible and would not pose a risk to human health. The potential for drug-interaction is further reduced by the fact that the short-chain peptides derived from Senmi's product will be consumed as an ingredient of food and beverages. Incorporation of Senmi's hydrolyzed sardine protein into a food or beverage matrix is expected to be associated with a different digestion and absorption profile following consumption than that for a pharmaceutical ACE inhibitor such as captopril, which has demonstrated a lower absorption

when taken together with food and should be taken on an empty stomach (PDR, 2006). Therefore, there is no anticipated potential for interaction of Senmi's sardine peptide product with the blood pressure lowering drug, captopril, or any other pharmaceuticals of similar use.

IV.F.2 Allergenicity

Fish proteins, including proteins derived from sardines, are well-recognized food allergens and have been reported to be responsible for anaphylactic reactions in both children and adults (Helbling *et al.*, 1996; Koyama *et al.*, 2006). Specifically, a number of major allergens have been identified in seafood that are associated with the induction of allergic reactions, including tropomyosin (muscle protein) in shrimp and Gad c 1 (parvalbumin) in codfish (Lehrer *et al.*, 2003). However, when the potential allergenicity of short-chain sardine peptides (average length: 2.67 amino acids) was evaluated in Harley guinea pigs using the active systemic anaphylaxis (ASA) test and the passive cutaneous anaphylaxis (PCA) test, negative responses were obtained (Seki *et al.,* 1995b).

The size and digestibility of a protein are typically recognized as critical determinants of its allergenic potential (Lehrer *et al.*, 2002). Most food allergens are between 10 and 70 kDa (equivalent to approximately 90 to 600 amino acids in length), with 3 to 5 kDa determined to be the minimum molecular mass required to elicit immunogenicity and allergenicity (Van Beresteijn *et al.*, 1994; Huby *et al.*, 2000). In shrimp tropomyosin, 5 major IgE-binding regions have been identified, containing a total of 8 distinct epitopes (Lehrer *et al.*, 2003). The length of the peptide responsible for the IgE-binding varied from 8 to 15 amino acids in length. Given that Senmi's hydrolyzed sardine protein product is a protein hydrolysate that contains peptides of not more than 5 amino acids in length, the results of the ASA and PCA tests performed with guinea pigs are not unexpected despite the fact that some fish proteins are recognized food allergens. The lack of sardine peptide-related allergenicity is confirmed by the absence of any reported effects in human studies. Specifically, none of the study participants consuming hydrolyzed sardine protein products for up to 13 weeks (Section IV.E) developed compound-related signs or symptoms of allergic reactions. Furthermore, since the approval of sardine peptides in Japan as Foods for Specified Health Uses (FOSHU), no incidences of allergic reactions have been reported. Overall, it is therefore not anticipated that Senmi's hydrolyzed sardine protein product would produce allergenic reactions under the intended conditions of use in food. However, despite the lack of allergenic potential of Senmi's hydrolyzed sardine protein product, it would be prudent to assume that the ingredient could elicit an allergic reaction in a sensitive individual due to the source being sardine. Proper food labeling should alert the fish-allergenic consumer to the presence of a fish-derived ingredient.

IV.G Summary and Basis for GRAS Conclusion

Senmi's hydrolyzed sardine protein product is proposed for use in foods and beverages at use-levels of up to 0.6 g/serving. Under the conditions of intended use, an assessment of the

estimated intake of the hydrolyzed sardine protein product on an all-user basis provided a mean intake by the total U.S. population from all-proposed food-uses of 1.3 g/person/day or 27 mg/kg body weight/day. For the heavy consumer (90th percentile), an intake of 2.8 g/person/day (62 mg/kg body weight/day) was estimated. Assuming that protein contributes approximately 20% to the overall composition of sardines, mean and 90th percentile all-user intakes of 7.02 and 9.20 g/person/day were estimated for protein consumption from sardines for the total U.S. population. In comparison, mean and 90th percentile intake of protein from all food sources for the total U.S. population has been estimated to be several-fold higher (*i.e.*, 75.3 and 114.0 g/person/day, respectively) than the estimated intakes of peptides from the consumption of foods containing added sardine-derived peptides (*i.e.*, 1.1 and 2.4 g/person/day, respectively). Thus, at the proposed use levels, peptides from sardines would not contribute significantly to the overall protein intake of humans.

Protein is a macronutrient that occurs as part of the daily human diet. Amino acids are normal constituents of protein, 9 of which have been classified as essential, meaning that they can only be obtained from the diet. The IOM has established RDA values of 56 and 46 g protein/day (0.8 g protein/kg body weight/day) for adult males and females, respectively. The estimated exposure to sardine peptides from hydrolyzed sardine protein under the intended conditions of use is well below current daily protein intakes and the RDAs established for protein by the IOM.

Amino acids, di-peptides, and other small peptides are normal breakdown products of protein digestion. Many biologically-active peptides of dietary origin have been identified and are produced in the gastrointestinal tract following consumption of protein-containing foods. Additionally, peptides also are synthesized endogenously to carry out various physiological functions. Val-Tyr, one of the di-peptides identified specifically in Senmi's hydrolyzed sardine protein product, is an endogenously-occurring product of ANG metabolism (Matsufuji *et al.*, 1995; Matsui *et al.*, 1997a,b, 1999). *In vitro*, Val-Tyr has been demonstrated to competitively inhibit ACE, which is responsible for the conversion of ANG I to ANG II.

Dose-dependent increases in plasma levels are observed following oral ingestion of Val-Tyr (Matsui *et al.*, 2000, 2002a,b). Following absorption, Val-Tyr is quickly cleared from plasma and distributes rapidly to major organs (Matsui *et al.*, 2003, 2004). However, in relation to the total administered dose, absorption of unhydrolyzed peptides is minimal. Thus, it is expected that the majority of the peptides in Senmi's hydrolyzed sardine protein product will be hydrolyzed to individual amino acids and utilized by the body in well-recognized pathways. Only a very small fraction of the peptides are expected to be absorbed intact. Peptides that are absorbed systemically can be hydrolyzed in various body tissues or are excreted in the urine.

The safety of the hydrolyzed sardine protein product is confirmed by a series of pre-clinical toxicity studies, as well as several human studies assessing the safety of dose levels relevant to the conditions of intended use of the hydrolyzed sardine protein product in foods. In rats, an LD_{50} of greater than 10,000 mg/kg body weight/day was estimated for peptide α-1000, indicating

very low oral toxicity. A NOAEL of 5,000 mg/kg body weight, the highest dose level tested, was determined for peptide α-1000 in male and female rats under the conditions of a 28-day oral toxicity study. Since 32% of peptide α-1000 comprises the final product, the results of this study also indicate a NOAEL of at least 1,600 mg/kg body weight/day for the final hydrolyzed sardine protein ingredient; however, since the amino acid profiles of the precursor and final product are largely comparable and the majority of the peptides are expected to be hydrolyzed to the amino acids prior to absorption or shortly thereafter in plasma, the final hydrolyzed sardine protein ingredient is also likely not to be associated with any toxicologically significant adverse effects at 5,000 mg/kg body weight/day. A study also was conducted in which significant reductions in blood glucose levels were observed following glucose loading in a hypertensive and hyperglycemic strain of rat which was administered sardine peptides at a dose level of 1,000 mg/kg body weight/day for 4 weeks. In human studies in which sardine peptides were consumed at dose levels of 1.5 g/day for up to 4 weeks or 500 mg/day for up to 13 weeks, no changes in blood glucose levels were observed. Neither Senmi's hydrolyzed sardine protein product nor Val-Tyr exhibited any mutagenic or genotoxic activities *in vitro* in standard bacterial test strains or *in vivo* in bone marrow cells of orally treated mice, respectively.

The significant decreases in the ratio of PCEs to total erythrocytes observed in the mouse micronucleus assays at dose levels of 1,000 and 2,000 mg/kg body weight of Senmi's hydrolyzed sardine protein product and Val-Tyr following only 2 consecutive treatments were not confirmed in the 28-day rat toxicity study or any of the studies conducted in humans, as evidenced by the lack of any variations in hematological parameters, including hematocrit levels, between groups treated with peptide α-1000 or Senmi's hydrolyzed sardine protein product and controls. In the human studies in which indices of hematological relevance were examined, significant variations were observed only between pre- and post-treatment values and often occurred in both placebo and test group subjects. Apart from a significant reduction in MCV in study participants consuming 500 mg of Senmi's hydrolyzed sardine protein product daily for 12 consecutive weeks compared to the placebo-control values, which increased relative to baseline levels (Kajimoto *et al.*, 2003), no differences in any other hematological parameters were observed between test and placebo subjects in the human studies (Kajimoto *et al.*, 2003, 2005a,b). Results of the repeat-dose rat toxicity study and human studies therefore demonstrate an absence of any potential adverse effects on erythropoiesis related to the oral consumption of Senmi's hydrolyzed sardine protein product.

Dietary peptides are thought to mediate blood pressure changes *via* a similar mechanism as pharmaceutical ACE inhibitors, which are contraindicated for use during pregnancy. No studies were identified that specifically assessed the potential effect of sardine peptides on reproduction or development. However, given the limited ability of Senmi's hydrolyzed sardine protein product to lower only above-normal blood pressure to levels within the normal physiological range, coupled with the fact that peptides are natural break-down products of protein digestion and Val-Tyr is a naturally-occurring metabolite of ANG I degradation, it is not anticipated that

consumption of food products containing Senmi's hydrolyzed sardine protein ingredient during pregnancy would be associated with any adverse developmental effects in neonates. Furthermore, the safety of protein hydrolysates is supported by a long history of use. The present IOM recommendations for increased protein intake by women during pregnancy and lactation (25 g in addition to the female adult RDA for protein) and current infant protein intakes from all dietary sources are considerably higher (up to 60-fold) than estimated intakes of sardine peptides from hydrolyzed sardine protein at the proposed use levels. Thus, exposure to sardine peptides at the specified use levels is not expected to result in any adverse effects related to reproduction or development. This conclusion is further supported by the results of a reproductive/developmental rat toxicity study which showed absence of any adverse effects related to the treatment of animals with a milk-derived protein hydrolysate product that also has been postulated to mediate blood pressure changes in mildly hypertensive individuals by ACE inhibition.

A series of human studies was conducted, involving the administration of single doses of up to 6 g of a hydrolyzed sardine protein product (Matsui *et al.*, 2002a,b), as well as repeat-administration studies with daily exposure to Senmi's hydrolyzed sardine protein product and products prepared by Senmi using a slightly modified procedure at dose levels ranging from 500 mg to 4 g over the course of 2 to 13 weeks (Kawasaki *et al.*, 2000, 2002; Kajimoto *et al.*, 2003, 2005a,b). These studies consistently demonstrated an absence of any adverse effects associated with the oral consumption of hydrolyzed sardine protein, including a lack of any responses indicative of allergic reactions and the absence of any changes in standard clinical chemistry and urinalysis parameters. In addition, studies conducted specifically to assess the potential for short-chain peptides derived from sardines to induce active systemic or passive cutaneous anaphylactic reposes in guinea pigs were negative (Seki *et al.,* 1995b). As such, there is no reason to expect that consumption of Senmi's hydrolyzed sardine protein product would elicit an allergic reaction. Nevertheless, product labeling indicating that the ingredient is derived from sardines as a potential warning to individuals with fish allergies should alert the fish-allergic consumer.

In the human studies, significant reductions in blood pressure were observed only in subjects with higher than normal blood pressure at baseline, and not in normotensive individuals. Although in the longer term human trials normotensive subjects consumed the sardine peptides at dose levels that were below the highest estimated dietary intakes under the intended use conditions (0.5 to 1.5 g/day *versus* 3.8 g in children at the 90th percentile), since the peptides are cleared rapidly from the circulation and there is no indication of accumulation, the single dose study conducted with normotensive subjects who exhibited no blood pressure changes following consumption of 6 g of sardine peptides provides support that the ingredient would not lead to blood pressure reduction in individuals with normal blood pressure even following repeated consumption at the estimated dietary intakes. Furthermore, the observed reductions in blood pressure in individuals with above normal blood pressure did not result in levels below the

normal range. Although peptides are considered likely to be absorbed *via* the same transport mechanism as hypertensive drugs, consumption of Senmi's hydrolyzed sardine protein product under the intended conditions of use resulting in negligible intakes of Val-Tyr (approximately 0.2 mg/kg body weight/day) that are several orders of magnitude below an effective inhibitory dose level of Val-Tyr observed in rats, is unlikely to interfere with the uptake of pharmaceutical agents used for the treatment of high blood pressure.

The data and information summarized in this report demonstrate that Senmi's hydrolyzed sardine protein product, meeting appropriate food-grade specifications and manufactured in accordance with cGMP, is GRAS for the intended uses in traditional food products as described herein based on scientific procedures.

The Expert Panel convened on behalf of Senmi, independently and collectively, critically evaluated the data and information summarized above, and concluded that the intended uses of Senmi's hydrolyzed sardine protein product (VALTYRON®), produced consistently in accordance with cGMP and meeting appropriate food grade specifications described herein, are safe and suitable. Furthermore, the Expert Panel unanimously concluded that the intended uses of hydrolyzed sardine protein are GRAS based on scientific procedures. It is also Senmi's opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, Senmi has concluded that hydrolyzed sardine protein is GRAS under the intended conditions of use on the basis of scientific procedures. Senmi's hydrolyzed sardine protein product is GRAS based on scientific procedures for its intended uses in food; therefore, it is excluded from the definition of a food additive and thus may be marketed and sold for the uses designated above in the U.S. without the promulgation of a food additive under Title 21 of the CFR.

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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Section Title
101—Food labeling	101.12	Reference amounts customarily consumed per eating occasion
102—Common or usual name for nonstandardized foods	102.22	Protein hydrolysates
109— Unavoidable contaminants in food for human consumption and food-packaging material	109.30	Tolerances for polychlorinated biphenyls (PCB's)
170— Food additives	170.30	Eligibility for classification as generally recognized as safe (GRAS)
172— Food additives permitted for direct addition to food for human consumption	172.340	Fish protein isolate
173—Secondary direct food additives permitted in food for human consumption	173.25	Ion-exchange resins
182—Substances generally recognized as safe	182.1057	Hydrochloric acid

Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
184—Direct food substances affirmed as generally recognized as safe	184.1027	Mixed carbohydrase and protease enzyme product
	184.1763	Sodium hydroxide
573—Food additives permitted in feed and drinking water of animals	573.340	Diatomaceous earth

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APPENDIX I

EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF HYDROLYZED SARDINE PROTEIN (VALTYRON®) FOR USE IN FOODS

EXPERT PANEL CONSENSUS STATEMENT: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF HYDROLYZED SARDINE PROTEIN (VALTYRON®) FOR USE IN FOODS

September 14, 2010

INTRODUCTION

At the request of Senmi Ekisu Co., Ltd. (hereafter Senmi) an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information on hydrolyzed sardine protein (VALTYRON®) and determine whether, under the conditions of intended use as a food ingredient, hydrolyzed sardine protein manufactured by Senmi would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Panel consisted of the below-signed qualified scientific experts: Jack Bend, Ph.D. (University of Western Ontario), Robert Bush, M.D. (University of Wisconsin), and Gary Williams, M.D. (New York Medical College).

The Panel, independently and collectively, critically examined a comprehensive package of scientific information and data compiled from the literature and other published sources through September 2007 (updated through May 2010). In addition, the Panel evaluated other information deemed appropriate or necessary, including published and unpublished technical and safety data provided by Senmi. The information evaluated by the Panel included data pertaining to the method of manufacture and product specifications of Senmi's hydrolyzed sardine protein, supporting analytical data, intended use levels of the ingredient in specified food products, consumption estimates for all intended uses, background intakes of protein, and a comprehensive assessment of the available scientific literature on the safety of sardine peptides and peptides in general.

The Panel was originally convened on September 18, 2007 to critically evaluate the data and information and at the time, unanimously concluded that under the then proposed conditions of intended use in traditional foods Senmi's hydrolyzed sardine protein (VALTYRON®) meeting appropriate food-grade specifications, was safe. The Panel further concluded that the uses were GRAS based on scientific procedures. Subsequently, Senmi modified the intended food uses of hydrolyzed sardine protein, with changes including the removal of certain food categories. These changes in food uses have, depending on the population group, resulted in a reduction or no change to the intake estimates of hydrolyzed sardine protein and therefore do not impact on the safety of the ingredient. As a result, the Panel was requested to re-examine the data and information supporting the GRAS status of hydrolyzed sardine protein and consider any new data that had become available since the original GRAS determination. In

September of 2010, following review of new and existing data, the Panel re-affirmed that under the new conditions of intended use in traditional foods, Senmi's hydrolyzed sardine protein meeting appropriate food-grade specifications is safe. As previously, the Panel further concluded that the uses are GRAS based on scientific procedures. A summary of the basis for the Panel's conclusion, excluding confidential data and information, is provided below. The data presented herein includes all the information previously considered by the Panel, as well as any new information that had become available since the Panel was originally convened in 2007. This consensus statement supersedes the consensus statement dated June 09, 2008.

MANUFACTURING AND COMPOSITION

Senmi's hydrolyzed sardine protein is manufactured by adding an exogenous hydrolytic enzyme (protease from *Bacillus licheniformis*) to pulverized sardine muscle tissue to break down the muscle protein into smaller peptides. The enzyme catalyzes the hydrolysis of the peptide bonds that join together the individual amino acids of the muscle tissue protein. In the United States (U.S.), protease enzyme preparations obtained from non-pathogenic strains of *B. licheniformis* are affirmed as GRAS for use in the manufacture of protein hydrolysates (21 CFR §184.1027; U.S. FDA, 2009). Once hydrolysis is complete, the enzyme is inactivated by heating and the crude peptide mixture (*i.e.*, peptide α-1000) is purified by fractionation *via* passage through a chromatography column and sequential elution with water or ethanol. As a result of the purification and fractionation procedures, not more than 5% of the enzyme preparation remains in the final product. The final fraction (termed Y-2) is concentrated, sterilized, spray-dried, and packaged.

Approximately 100 g of peptide α-1000 yields 32 g of the final hydrolyzed sardine protein. While the commercial product (Y-2) is concentrated in certain peptides, analysis of the amino acid composition of peptide α-1000 and the commercial product revealed that the relative ratio of individual amino acids (as a % of total amino acids) is retained following the extraction procedure. The final product is characterized by a peptide content of not less than 85% and conforms to the established physical, chemical, and microbiological product specifications listed below in Table 1. Analyses of representative lots demonstrated compliance with final product specifications. All equipment and processing aids (*i.e.,* pH-adjusting agents and extractant) used in the manufacture of the ingredient are appropriate for food use.

Table 1 Product Specifications for Senmi's Hydrolyzed Sardine Protein		
Specification Parameter	**Specification Requirement**	**Method of Analysis**
Appearance	Yellowish white powder	Visual examination
Peptide	Not less than 85%	Kjeldahl method[1]
Val-Tyr	0.10 to 0.16%	HPLC method[2]
Moisture	Not more than 8.0%	Atmospheric heat drying method[1]
Ash	Not more than 10.0%	Direct ashing method[1]
Sodium	Not more than 2.0%	Atomic absorption spectrometric method[1]
Lead (Pb)	Not more than 0.1 ppm	Lead limit test (AAS method)[1]
Arsenic (as As_2O_3)	Not more than 10 ppm	Gutzeit method[1]
Standard plate count	Not more than 1,000 CFU/g	Microbial Limit Tests[1]
Coliform count	Negative	Microbial Limit Tests[1]
Yeasts and molds	Not more than 300 CFU/g	Microbial Limit Tests[1]

As_2O_3 = Arsenic oxide; AAS = Atomic absorption spectrometry; CFU = Colony-forming units; HPLC = High-performance liquid chromatography; Val-Tyr = Valine-Tyrosine
[1] Japanese Specifications and Standards for Food Additives 7th Edition (2000).
[2] Senmi's internal method.

Representative sample batches of Senmi's hydrolyzed sardine protein also were assayed for residual levels of ethanol. Analytical results demonstrated ethanol levels of less than 500 ppm in the final product. Additionally, since the peptide product is produced from sardines, sample manufacturing lots also were tested for various environmental contaminants, including copper, cadmium, and mercury, in addition to lead and arsenic, perfluorooctane sulfonate (PFOS) and related fluorochemicals, brominated flame retardants (BFRs), pesticides, dioxins and dioxin-like polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). Levels of these chemical contaminants were often below the limits of detection or quantification reported for the analytical method and, where U.S. Food and Drug Administration (FDA) action levels or guidelines were identified, the levels were below the permitted limits. The results of stability studies conducted with Senmi's hydrolyzed sardine protein indicated that the ingredient as packaged for commercial use is stable for up to 41 months.

CURRENT REGULATORY STATUS OF SARDINE PEPTIDES

Currently, sardine peptides are not permitted for addition to food in the U.S. However, the FDA was notified of and had no questions regarding the self-affirmed GRAS status of acid-extracted fish protein for use in food (GRN 000147; U.S. FDA, 2004) and has approved the use of hexane- and ethanol-extracted fish protein isolates for purposes of food supplementation (21 CFR §172.340; U.S. FDA, 2009). For food labeling purposes, the FDA requires that the common or usual name of the protein hydrolysate shall be specific to the ingredient and shall include the identity of the food source from which the protein was derived (21 CFR §102.22; U.S. FDA, 2009); therefore, these provisions will be adhered to in the labeling of sardine peptides as an ingredient for use in foods under the conditions of use specified herein.

Protein hydrolysates were in use in the U.S. prior to 1958 and hydrolyzed plant protein, vegetable protein, animal protein, and milk protein are presumed GRAS substances by the U.S. FDA or have prior sanction or approval for use in foods (as cited in FASEB, 1978). In an ongoing process of reviewing the safety of GRAS or prior sanctioned food substances as food ingredients, the Federation of American Societies for Experimental Biology (FASEB) was contracted by the FDA to conduct an evaluation of the health aspects of protein hydrolysates for use as food ingredients and, specifically, for use as flavoring agents (FASEB, 1978). The Committee concluded that "while no evidence in the available information on acid hydrolyzed proteins, enzymatically hydrolyzed proteins, and yeast autolyzates demonstrate[d] a hazard to the public [at the then current intake levels associated with the use of such protein hydrolysates] as flavoring agents, uncertainties exist[ed] requiring that additional studies should be conducted". Specifically, in evaluating the safety of protein hydrolysates, the Committee expressed concern regarding possible developmental or teratogenic effects that have been associated with glutamate (glutamic acid), noting, however, that the dose levels at which adverse effects may be observed with protein hydrolysates may be greater than with glutamic acid alone. Accordingly, the Committee recommended that future Food Chemicals Codex (FCC) standards for protein hydrolysate products specify glutamic acid and aspartic acid content. The current FCC specification for acid hydrolysates of protein indicates a value of not more than 20% as glutamic acid and not more than 35% of the total amino acids (FCC, 2003). Consistent with the current FCC specifications for similar protein hydrolysates, glutamic acid is present in Senmi's hydrolyzed sardine protein at levels of less than 15% of total amino acids.

INTENDED USE AND ESTIMATED EXPOSURE

Senmi intends to market hydrolyzed sardine protein as a food ingredient in a variety of foods including beverages and breakfast cereals, frozen dairy desserts and mixes, milk and milk products, fish, products, pastas, hard and soft candy, soups and soup mixes, and processed fruits and vegetables and fruit and vegetable juices. Hydrolyzed sardine protein is proposed for use in foods and beverages at use-levels of up to 0.6 g/serving (up to 30%). The individual proposed food-uses and use-levels for Senmi's hydrolyzed sardine protein are summarized in Table 2. Food codes representative of each proposed food use were chosen from the National Center for Health Statistics' (NCHS) 2003-2004 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2006; USDA, 2009) and were grouped in food use categories according to Title 21, Section §170.3 of the *Code of Federal Regulations* (U.S. FDA, 2009). Serving sizes were assigned according to Title 21, Section §101.12, Reference Amounts Customarily Consumed Per Eating Occasion (RACC) (U.S. FDA, 2009).

Table 2	Summary of the Individual Proposed Food Uses and Use-Levels for Senmi's Hydrolyzed Sardine Protein in the United States			
Food Category	**Proposed Food-Use**	**Sardine Peptide Level (g/RACC)**	**RACC (g)**	**Use-Levels (%)**
Breakfast Cereals	Instant and Hot Regular Cereals	0.6	240	0.25
	Ready-to-Eat Cereals	0.6	15	4.0
			30	2.0
			55	1.1
Fish Products	Frozen Multicourse Fish-Based Meals	0.6	240	0.25
Frozen Dairy Desserts and Mixes	Frozen Yogurt	0.6	120	0.5
Grain Products and Pastas	Frozen Multicourse Grain Product and Pasta-Based Meals	0.6	240	0.25
Hard Candy	Hard Candy (Functional)	0.6	2 to 15	4.0 to 30.0
Milk and Milk Products	Drinkable Yogurt	0.6	240	0.25
	Fermented Milk	0.6	240	0.25
	Milk-based Meal Replacements and Protein Beverages made from Dry Powders	0.6	240*	0.25
	Milk-Based Beverages made from Dry Powders	0.6	240*	0.25
	Yogurt	0.6	225	0.27
Processed Fruits and Fruit Juices	Flavored Water	0.6	240	0.25
	Fruit Drinks and Ades	0.6	240	0.25
Processed Vegetables and Vegetable Juices	Frozen Multicourse Vegetable-Based Meals	0.6	240	0.25
	Vegetable Juices	0.6	240	0.25
Soft Candy	Soft Candy and Chocolate Bars	0.6	40	1.5
Soups and Soup Mixes	Canned, Condensed, and Dehydrated Soups	0.6	245*	0.24

RACC = Reference Amounts Customarily Consumed Per Eating Occasion (21 CFR §101.12) (U.S. FDA, 2009).
* As consumed.

Approximately 88% of the total U.S. population was identified as potential consumers of hydrolyzed sardine protein from the proposed food-uses (7,250 actual users identified). The consumption of all foods containing hydrolyzed sardine protein by the total U.S. population yielded mean estimates of daily intake (all-user basis) of 1.3 g/person/day. Based on total population body weight approximations, this would correspond to an exposure of 27 mg/kg body weight/day. Assessment of heavy consumers (90th percentile, all-user basis) indicated exposures of 2.8 g/person/day or 62 mg/kg body weight/day on a body weight basis (see Tables 3 and 4).

The highest 90th percentile all-user intake of hydrolyzed sardine protein on an absolute basis was observed to occur in male teenagers at 3.8 g/person/day, equivalent to 67 mg/kg body

weight/day. On a body weight basis the highest 90th percentile all-user intake was observed in infants at 156 mg/kg body weight/day.

Table 3 Summary of the Estimated Daily Intake of Senmi's Hydrolyzed Sardine Protein from All Proposed Food Uses in the U.S. by Population Group (NHANES 2003-2004 Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (g)	90th Percentile (g)	Mean (g)	90th Percentile (g)
Infant	0-2	66.1	615	0.7	1.7	0.9	1.9
Child	3-11	98.4	1,267	1.8	3.7	1.9	3.8
Female Teenager	12-19	92.9	922	1.2	2.6	1.4	2.7
Male Teenager	12-19	91.4	913	1.7	3.5	1.8	3.6
Female Adult	20 and Up	89.7	1,909	1.0	2.2	1.2	2.3
Male Adult	20 and Up	84.2	1,624	1.1	2.4	1.3	2.7
Total Population	All Ages	87.7	7,250	1.2	2.6	1.3	2.8

Table 4 Summary of the Estimated Daily Per Kilogram Body Weight Intake of Senmi's Hydrolyzed Sardine Protein from All Proposed Food Uses in the U.S. by Population Group (NHANES 2003-2004 Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (mg/kg)	90th Percentile (mg/kg)	Mean (mg/kg)	90th Percentile (mg/kg)
Infant	0-2	66.1	615	55	131	73	156
Child	3-11	98.4	1,267	68	144	69	146
Female Teenager	12-19	92.9	922	22	48	24	49
Male Teenager	12-19	91.4	913	27	63	30	67
Female Adult	20 and Up	89.7	1,909	15	30	17	33
Male Adult	20 and Up	84.2	1,624	13	31	16	33
Total Population	All Ages	87.7	7,250	24	55	27	62

This type of intake methodology is generally considered to be 'worst case' as a result of several conservative assumptions made in the consumption estimates. For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use. In addition, it is well established that the length of a dietary survey affects the estimated consumption of individual users. Short-term surveys, such as the typical 2- or 3-day dietary surveys, overestimate the consumption of food products that are normally consumed relatively infrequently.

While children and infants were identified to have the greatest all-user 90th percentile intakes of sardine peptides on a body weight basis, with values of 146 and 156 mg/kg body weight/day, respectively (Table 4), it should be noted that the specified food uses are not intended to be marketed for infants; thus, the actual infant consumption of hydrolyzed sardine protein is expected to be highly limited, and although an estimate of the consumption of hydrolyzed sardine protein on a body weight basis in infants from all proposed food uses has been included for completeness of this report, it is considered to be a gross over-estimate of the actual expected intake of sardine peptides by infants from its addition to food. This is supported by the fact that the lowest intakes on an absolute basis were determined to be in infants.

INFORMATION TO SUPPORT THE GRAS STATUS OF SENMI'S HYDROLYZED SARDINE PROTEIN

The safety of Senmi's hydrolyzed sardine protein is based on its composition and the general understanding of peptide absorption and metabolic fate following uptake from the gastrointestinal tract, as well as studies conducted specifically to assess the systemic fate of Val-Tyr, one of the many peptides identified in Senmi's hydrolyzed sardine protein, in animals and humans following oral administration (Matsui *et al.*, 2000, 2002a,b, 2003, 2004). Additionally, the potential toxicity of hydrolyzed sardine protein and a closely related manufacturing precursor product (peptide α-1000) were evaluated in a battery of toxicological studies, including *in vitro* and *in vivo* mutagenicity/genotoxicity assays and acute and sub-chronic (28-day) animal toxicity studies (Osajima *et al.,* 2009). A series of single-dose and repeat-administration human studies up to 13 weeks in duration in which study participants consumed test preparations containing 500 mg to 4 g of Senmi's hydrolyzed sardine protein serves to further substantiate the safety of the food ingredient (6 g in single-dose administration studies) (Kawasaki *et al.*, 2000, 2002; Matsui *et al.*, 2002a,b; Kajimoto *et al.*, 2003, 2005a,b). The safety of hydrolyzed sardine protein under the conditions of use described herein additionally is supported by considerably higher background intake levels of dietary protein in general and protein from sardines specifically, as well as by the endogenous production of peptides by humans. Using all of these data to support the safety of the ingredient, sardine peptides are GRAS based on scientific procedures. A summary of this information is provided below.

BACKGROUND DIETARY INTAKES OF PROTEIN FROM ALL DIETARY SOURCES AND FROM SARDINES SPECIFICALLY

The Institute of Medicine (IOM) evaluated data on protein and individual amino acids and set a Recommended Dietary Allowance[1] (RDA) of 0.8 g/kg body weight for protein (good quality) for both adult males and females (56 and 46 g/day, respectively) (IOM, 2005). For infants, an adequate intake (AI)[2] of 1.52 g/kg body weight was established. Young children (7 months to 13 years) were recommended to consume 0.95 to 1.52 g protein/kg body weight/day (or 11 to 34 g/day). During periods of pregnancy or lactation, the IOM recommended that women increase their daily protein intake by 25 g.

The IOM also estimated background dietary protein intakes for the U.S. population using the Continuing Survey of Food Intakes by Individuals (CSFII) (1994-1996, 1998) (IOM, 2005). Mean adult intakes for protein ranged from approximately 56 g (adult females over the age of 70) to 104 g (19- to 30-year-old males) per day (IOM, 2005). At the 90th percentile, adult protein intakes ranged from 76 g/day for females over 70 years of age to 142 g/day for 19- to 30-year-old males. The mean and 90th percentile total population intakes were estimated to be approximately 75 and 114 g protein/day, respectively. In infants and children, mean and 90th percentile intakes ranged from 15.9 to 62.5 and 21.9 to 81.9 g/day, respectively. Although the IOM could not set a Tolerable Upper Intake Level (UL) for protein intake, it was noted that "the risk of adverse effects resulting from excess intakes of protein from foods appears to be very low at the highest estimated intake[s]" (IOM, 2005).

Background consumption of protein from sardines specifically was calculated using NHANES 2003-2004 (CDC, 2006; USDA, 2009). The exposure estimate was based on the approximation that sardines are typically composed of 20% protein. Since sardines are only added to a very limited number of foods and are consumed by a relatively small portion of the whole population, the all-person mean consumption of sardine protein for the total U.S. population was low (15.14 mg/person/day); however, the all-user mean intake for the total U.S. population was considerably higher at 7.02 g/person/day (96.6 mg/kg body weight/day). At the 90th percentile, an all-user intake of 9.2 g/person/day or 151.57 mg/kg body weight/day was estimated for sardine protein.

A comparison of the background dietary protein intakes and intakes of sardine proteins to the estimated intakes of Senmi's sardine peptides under the proposed conditions of use is provided in Table 5.

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[1] The average daily dietary nutrient intake level sufficient to meet the nutrient requirement of nearly all (97 to 98%) healthy individuals in a particular life stage and gender group (IOM, 2005).
[2] The recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate – used when an RDA cannot be determined (IOM, 2005).

Table 5	Comparison of the All-User Total U.S. Population Intakes of Protein from All Dietary Sources, Protein from Sardines, and Senmi's Sardine Peptides			
Source of Protein	Mean		90th Percentile	
	g/day	mg/kg body weight	g/day	mg/kg body weight
Background protein intake from all dietary sources	75.3[a]	1,074[b]	114.0[a]	1,628[b]
Background protein intake from sardines[c]	7.0	97	9.2	152
Estimated intake of Senmi's sardine peptides[d]	1.1	23	2.4	53

[a] Values obtained from IOM (2005). Based on (CSFII 1994-1996, 1998).
[b] Assuming body weight of 70 kg.
[c] Calculated using NHANES 2003-2004 (all-user consumption).
[d] Values in Tables 3 and 4 adjusted by 85% to account for peptide content only.

ENDOGENOUS OCCURRENCE AND BIOLOGICAL FUNCTION

The daily turnover (synthesis and degradation) for protein in humans is estimated to be at least 250 g of protein (Waterlow, 1984) and, on a body weight basis compared to young adults, is greater in infancy than during later stages of life. In addition to supplying energy and fulfilling other vital cellular and organ functions, proteins and specifically peptides, the natural break-down products of protein metabolism, also have been associated with additional biological and physiological properties (mineral binding, immunomodulatory, regulation of serum cholesterol levels, antioxidative, antimicrobial, blood pressure normalization *etc.*) (Gardner, 1983, 1984; Kitts and Weiler, 2003; Zaloga and Siddiqui, 2004; Rutherfurd-Markwick and Moughan, 2005; Hartmann and Meisel, 2007). Peptides with biological properties usually comprise 2 to 20 amino acids and are produced endogenously by gastrointestinal proteases during normal protein digestion or as a result of food processing (Roberts *et al.*, 1999; Li *et al.*, 2002; Vermeirssen *et al.*, 2004).

Val-Tyr, one of several peptides identified in Senmi's hydrolyzed sardine protein, occurs endogenously in plasma as a primary metabolite of angiotensin I (ANG I) in rodents and in humans (Kawasaki *et al.*, 2000). Breakdown of ANG I, II, and III to Val-Tyr has been shown *in vitro* and in plasma samples from spontaneously hypertensive rats (Matsufuji *et al.*, 1995; Matsui *et al.*, 1997a,b). Depending on whether healthy subjects were lying down or standing up to evoke an increase in blood pressure, Val-Tyr was identified in plasma at levels in the range of 111 to 447 fmol/mL (Matsui *et al.*, 1997a,b). Val-Tyr levels were shown to be lower in mildly hypertensive subjects compared to normotensive individuals, suggesting that Val-Tyr plays a role in blood pressure regulation (Matsui *et al.*, 1999).

Several peptides from various dietary sources (*e.g.*, bonito, salmon, sardines, tuna, pork, chicken, oyster, fermented fish sauce) including Val-Tyr have been shown to inhibit the activity of the angiotensin I converting enzyme (ACE) and thus to inhibit ANG I to ANG II conversion (Matsui *et al.*, 1993; Matsufuji *et al.*, 1994; Seki *et al.*, 1995a; Ichimura *et al.*, 2003; Vercruysse *et al.*, 2005). Examined *in vivo* in hypertensive mice and rats, administration of Val-Tyr was

associated with reductions in blood pressure and was accompanied by increased plasma and organ levels of Val-Tyr (Matsufuji *et al.*, 1995; Matsui *et al.*, 2003, 2004).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION (ADME)

Senmi's hydrolyzed sardine protein consists primarily of short-chain peptides of 2, 3, 4, or 5 amino acids in length. The digestion, absorption, and metabolism of peptides from sardine muscle tissue are expected to be similar to that of other dietary proteins and peptides. Unlike earlier contentions that proteins and smaller peptides are entirely hydrolyzed to free amino acids prior to absorption, it is now more widely accepted that a small portion of peptides also may be taken up into the enterocytes from the luminal phase of the small intestine and hydrolyzed in the intestinal cells or released intact across the basolateral membrane into the circulation (Gardner, 1982; Ziv and Bendayan, 2000). The uptake of peptides and amino acids appears to occur by separate processes (Friedrich, 1982; Webb, 1990). Peptide absorption has been suggested to occur *via* one of 3 possible mechanisms: transporter-mediated transport, transcellular transport (passive diffusion and endocytosis), or paracellular transport (*via* tight junctions between cells) (Gardner, 1984, 1998; Shimizu *et al.*, 1997). Two proton-dependent peptide transporter proteins (PepT1 and PepT2) that may mediate uptake of peptides into the intestinal cells and release into the circulation, respectively, have been identified (Gardner, 1984, 1998; Tsuji and Tamai, 1996; Shimizu *et al.*, 1997). Peptides that escape intracellular hydrolysis and are absorbed systemically are subjected to hydrolysis in the blood or other organ tissues, including the liver and kidneys (Lochs *et al.*, 1988, 1990; Webb, 1990; Gardner, 1998). Peptides that are resistant to hydrolysis in the renal tissues are excreted in the urine (Minami *et al.*, 1992; Gardner, 1998).

In mice and rats, plasma levels of Val-Tyr increased significantly in comparison to pre-treatment values following oral administration of the dipeptide (Matsui *et al.*, 2003, 2004). Following absorption, the dipeptide was shown to be eliminated rapidly from plasma ($t_{½}$ approximately 3 hours) and distributed to a wide range of major organs, including the liver, kidneys, heart, and lungs (Matsui *et al.*, 2003, 2004). Likewise, in human studies, significant and dose-dependent increases were observed in plasma Val-Tyr levels in comparison to baseline values (Matsui *et al.*, 2000, 2002a,b). Maximum plasma concentrations of up to approximately 2,000 fmol/mL were attained approximately 2 hours following ingestion of up to 12 mg of the dipeptide (*versus* approximately 160 fmol/mL at baseline). The elimination $t_{1/2}$ was estimated to be approximately 3 hours. While the study results confirmed dose-dependent absorption of intact Val-Tyr, as a percentage of the highest administered dose (12 mg of Val-Tyr), only 0.014% of Val-Tyr was absorbed and detected in the plasma of human subjects, with the remainder likely hydrolyzed to individual amino acids prior to absorption.

Based on the data obtained for Val-Tyr, one of the peptides identified in Senmi's sardine hydrolyzed sardine protein, and the general understanding of peptide metabolism, it is therefore expected that a large portion of the peptides in Senmi's hydrolyzed sardine protein would be

hydrolyzed to the individual constituent amino acids prior to absorption and only a small fraction of intact peptides would be available for systemic uptake. Following absorption, amino acids are utilized by the body in well-recognized metabolic pathways. Peptides that are absorbed intact would be again subjected to hydrolysis in the plasma or in organ tissues following systemic distribution and any peptides escaping hydrolysis would be excreted in the urine.

Since absorption of peptides may occur *via* passive processes, it has been noted that peptide absorption may be greater in infants in whom the epithelium of the gastrointestinal tract is not yet fully developed or in individuals with conditions leading to reduced peptidase activity or increased intestinal permeability; however, given that peptides are formed naturally during the process of normal protein digestion and that the intake of sardine peptides from Senmi's product is estimated to be considerably lower than the background dietary intake of proteins, which are broken down to peptides during the first stages of digestion, it is expected that consumption of Senmi's hydrolyzed sardine protein under the proposed conditions of use would not pose a greater risk to such individuals beyond what is associated with the intake of protein in general. Furthermore, Senmi does not intend to market foods to which sardine peptides have been added directly for infants (*i.e.*, not intended for addition to infant formula/foods) and therefore there are no anticipated concerns for adverse effects in this population group.

TOXICOLOGICAL STUDIES

A series of studies was conducted with the commercial hydrolyzed sardine protein product or a closely-related precursor product obtained during the manufacturing process of the commercial preparation (*i.e.*, peptide α-1000[3]) to evaluate potential acute toxicity and toxicity following repeated administration, as well as *in vitro* mutagenicity and *in vivo* genotoxicity of Senmi's product (Osajima *et al.,* 2009). These studies are described below. Table 6 presents a comparison of the physical, chemical, and microbiological specifications for peptide α-1000 and the commercial hydrolyzed sardine protein product and illustrates the relevance of the data generated using peptide α-1000 for its use in establishing the safety of Senmi's product. The final commercial product is only slightly more concentrated in overall peptide content than peptide α-1000 (80 *vs.* 85%). In total, approximately 32% of the peptide α-1000 mixture makes up the commercial Y-2 fraction following removal of the highly water and ethanol soluble peptides with the first and last wash of the column, respectively (Y-1 and Y-3 fractions, respectively). The relative ratio of amino acids, however, is largely retained between the initial peptide mixture (peptide α-1000) and the final product obtained following fractionation (Y-2) and as such also is reflective of the amino acid composition of the raw material (sardines). Accordingly, studies conducted with peptide α-1000 were considered to be appropriate for the evaluation of the safety of the final commercial hydrolyzed sardine protein product.

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[3] The final sardine peptide product is eluted by passing peptide α-1000 through ODS chromatography columns and extracting fractions of different solubilities with water and ethanol.

Table 6	Comparison of the Specifications for the Precursor (Peptide α-1000) and the Commercial Hydrolyzed Sardine Protein Product (Y-2)	
Specification Parameter	**Specification**	
	Sardine Peptide (Y-2)	**Peptide α-1000**
Appearance	Yellowish, white powder	Yellowish, white powder
Moisture	Not more than 8.0%	Not more than 8.0%
Ash	Not more than 10.0%	Not more than 10.0%
Peptide content	Not less than 85%	Not less than 80%
Total fat	N/A	Not more than 10.0%
Sodium (Na)	Not more than 2.0%	Not more than 10.0% (as NaCl)
Microbiological Parameters		
Standard plate count	Not more than 1,000 CFU/g	Not more than 10,000 CFU/g
Coliform count	Negative	Negative
Yeast and molds	Not more than 300 CFU/g	Not more the 300 CFU/g

CFU = Colony-forming units; NA = Not applicable.

Acute Studies

No early deaths were observed following single bolus dose administration of peptide α-1000 at a dose level of 10,000 mg/kg body weight to a group of 4 male SD (Crj:CD) rats and, thus, the LD_{50} in rats was estimated to be greater than the treatment dose (Osajima *et al.,* 2009). All animals were observed to gain weight during the 7-day observation period that followed treatment and pathological examination of animals at the end of the study period did not reveal any abnormalities. The salivation that was observed in 2 of the 4 treated animals immediately following dosing was considered to be likely the result of administering a large bolus dose of the test material by gavage.

Subchronic Studies

To assess the potential toxicity following repeat dosing, a 28-day study was conducted in which male and female SD (Crj:CD) rats (6/sex/group) were treated orally with peptide α-1000 at dose levels of 0, 1,250, 2,500, or 5,000 mg/kg body weight/day by gavage (Osajima *et al.,* 2009). All animals survived until study termination and no abnormalities in the general condition and behavior, food consumption, body weights and weight gains, hematology and clinical chemistry, or urinalysis were observed. Slight reductions observed in the water consumption of test males were neither dose-dependent, nor statistically significant. In females, the decreases particularly at the mid- and high-dose levels, appeared to be driven by isolated incidences of considerably reduced water intakes in single animals. Furthermore, differences in water intakes were not accompanied by any significant changes in any of the measured urinalysis parameters. Gross examination of rats revealed hemorrhage in the glandular stomach of a single mid-dose female (2,500 mg/kg body weight/day), which in light of the sporadic nature of this finding was considered to be likely due to gavage error. The statistically significant increases in absolute

and relative kidney weights that were observed in high-dose males (5,000 mg/kg body weight/day) occurred in the presence of other renal variations (inflammatory changes, hyaline droplet degeneration, thickening of the Bowman's capsule and basal membrane, calcium-like deposits) and appeared to be consistent with male-specific spontaneous nephropathy commonly observed in rats (Hard and Khan, 2004). Similar changes, as well as vacuolar hyaline cast formation, and degeneration and necrosis of the tubular epithelium were observed in control animals and all of the renal lesions were judged to be only of slight severity. Accordingly, the changes observed in the male kidneys were determined to be unrelated to treatment with peptide α-1000 and were not considered to be relevant in an assessment of the safety of sardine peptide as intended for consumption by humans. Based on the results of this study, which demonstrated an absence of any adverse effects related to the oral administration of peptide α-1000 at dose levels of up to 5,000 mg/kg body weight, the highest dose level tested was determined to be the no-observed-adverse-effect level (NOAEL) in rats under the conditions of this study.

Senmi's sardine peptide preparation was administered to male stroke-prone spontaneously hypertensive (SPSH) rats, a non-obese, hyperglycemic, and hypertensive strain, at a dose level of 0 (untreated control) or 1,000 mg/kg body weight/day in the drinking water for a period of 4 weeks (Otani *et al.*, 2009). An additional group of SPSH rats was provided captopril in the drinking water (8 mg/kg body weight/day). A group of untreated Wistar-Kyoto rats also was included in the study. Following the 4-week treatment period, no differences were observed in food intake, body weights, or levels of total cholesterol, triglyceride, or free fatty acids between the untreated and sardine peptide-treated SPSH rats. In comparison to untreated SPSH rats, SPSH rats provided with sardine peptides in the drinking water exhibited significantly reduced relative heart weights, which were still significantly higher than those of Wistar-Kyoto rats, but less so than in the untreated SPSH rats. However, no variations in either absolute or relative heart weights were observed in the 28-day study conducted by Osajima *et al.* (2009) in which SD (Crj:CD) rats were administered sardine peptides at dose levels of up to 5,000 mg/kg body weight/day. Sardine peptide-treated SPSH rats also showed significant reductions in systolic blood pressure, similar to those observed in rats treated with captopril, compared to untreated SPSH rats. After the 4-week treatment period, fasting plasma glucose and insulin levels were comparable among the SPSH groups of rats; however, all 3 groups exhibited significantly higher glucose levels and lower insulin levels than the Wistar-Kyoto rats. Thirty (30) minutes following administration of a glucose tolerance test, peak plasma glucose levels of the sardine peptide- and captopril-treated SPSH rats were significantly lower than those of the untreated SPSH rats. Conversely, insulin levels increased only slightly in SPSH rats, with no significant variations observed among the different treatment groups. In comparison, a significant increase in plasma insulin levels was observed in the Wistar-Kyoto rats following administration of the glucose tolerance test. Since the reductions in plasma glucose levels in sardine peptide-treated SPSH rats were not paralleled by increased levels of insulin, the study authors speculated that sardine peptides may instead have helped to improve insulin resistance in SPSH rats.

Mutagenicity/Genotoxicity Studies

Product-specific studies were conducted to assess the potential mutagenicity and genotoxicity of Senmi's hydrolyzed sardine protein. hydrolyzed sardine protein did not exhibit any mutagenic potential in a standard battery of *Salmonella* (TA98, TA100, TA1535, and TA1537) or *Escherichia coli* (WP2*uvr*A) in the presence or absence of metabolic activation at concentrations of up to 5,000 mg/plate (Osajima *et al.,* 2009). Examined *in vivo*, oral gavage administration of hydrolyzed sardine protein on 2 consecutive days at dose levels of 250, 500, 1,000, or 2,000 mg/kg body weight did not produce any clastogenic effects in bone marrow cells of male Crj:CD-1 (ICR) mice (5 animals/group) in comparison to controls (Osajima *et al.,* 2009). Specifically, the prevalence of micronucleated polychromatic erythrocytes (MNPCEs) in treated animals was comparable to that of controls and was within the historical range. The absence of *in vitro* mutagenicity in standard bacterial tester strains and *in vivo* genotoxicity (mouse micronucleus assay) in assays performed with the purified dipeptide, Val-Tyr (Yamaguchi, 1999 [unpublished]; Kimura, 2004 [unpublished]), further confirm the lack of any mutagenic or genotoxic properties for hydrolyzed sardine protein.

In both mouse micronucleus assays conducted with Senmi's hydrolyzed sardine protein and with the dipeptide Val-Tyr, dose-related reductions were observed in the ratio of PCE to the total number of erythrocytes in test animals relative to the negative control group, with values in groups of animals treated with the two highest dose levels (1,000 and 2,000 mg/kg body weight) attaining statistical significance and, therefore, indicating a potential effect on erythropoiesis. However, no statistically significant differences were observed in any hematological parameters (including red blood cell count, hemoglobin content, and hematocrit value) following gavage administration of peptide α-1000 at dose levels of up to 5,000 mg/kg body weight/day compared to the negative control group in the repeat-dose 28-day rat study (Osajima *et al.,* 2009). Therefore, consumption of Senmi's hydrolyzed sardine protein under the proposed conditions of use is not expected to be associated with any adverse effects on normal blood cell development.

Reproductive and Developmental Studies

In utero exposure to pharmaceutical ACE inhibitors has been reported to be associated with fetopathy, which is speculated to be partly due to the direct action of the re-circulated ACE inhibitors on the renin-angiotensin system in the fetus and partly to fetal ischemia ensuing as a result of maternal hypotension and reduced fetal-placental blood flow and oxygen/nutrient delivery to the baby (Solhaug *et al.*, 2004; Cooper *et al.*, 2006; Quan *et al.*, 2006). More recently, an increased risk for congenital malformations as a result of first trimester exposure to ACE-inhibitors also has been reported (Cooper *et al.*, 2006; Quan *et al.*, 2006). Consequently, the use of ACE inhibitor drugs is contraindicated during pregnancy. Since some dietary peptides are postulated to regulate blood pressure levels *via* a similar mechanism as ACE

inhibitor drugs, the potential for Senmi's hydrolyzed sardine protein to mediate similar developmental effects was considered for the purposes of this safety assessment.

Although the underlying mechanism of action of dietary peptides related to the blood pressure mediating effects is hypothesized to be similar to that of pharmaceutical ACE-inhibitors, the bioactivity of the dietary peptides is significantly lower than that of the drugs. Moreover, in order to attain therapeutically efficacious plasma levels of pharmaceutical ACE inhibitors following oral ingestion, most are given as pro-drugs that are converted to the active form by de-esterification in the liver as a result of the first-pass effect (Barr, 1994). Conversely, short-chain peptides such as those in Senmi's product are subjected to extensive hydrolysis beginning in the gastrointestinal tract to individual amino acids, with only a small fraction escaping hydrolysis prior to uptake.

The adverse fetal effects of ACE-inhibitor pharmaceutical agents have also been partly related to the ensuing hypotension in the mother. However, unlike the more bioactive pharmaceutical ACE inhibitory agents, human studies have shown consistently that Senmi's hydrolyzed sardine protein only assists in the maintenance of healthy blood pressure in individuals with above-normal blood pressure and does not mediate reductions beyond the normal physiological range. Moreover, unlike pharmaceutical ACE inhibitors, which are intended to treat diagnosed medical hypertension with many different etiologies, Senmi's product is not intended for use as a therapeutic agent. It has been suggested that the adverse effects observed in infants born of mothers who had taken ACE inhibitor drugs during pregnancy also could be related to preexisting maternal disease conditions rather than the pharmaceutical agents *per se*, especially considering that some of the characteristic effects of ACE inhibitory fetopathy also are common complications of hypertensive pregnancies (Barr, 1994).

Additionally, protein, which is broken down to peptides and amino acids as part of the normal digestive process, is an essential nutrient required for proper development. It is well recognized that the demand for protein increases during pregnancy and lactation (IOM, 2005). Accordingly, the IOM has recommended that pregnant and lactating women consume 25 g of additional protein per day during these periods. In comparison to current daily protein intakes from all dietary sources of women during pregnancy and lactation (*i.e.*, 78.2 to 79.7 and 97.0 to 100.8 g/day, respectively) (IOM, 2005) and the all-user background intakes of sardine protein by adult females (7.45 g/day), the daily mean and 90th percentile peptide intakes estimated from the all-user consumption of Senmi's sardine peptides by adult women (*i.e.*, 1.0 and 2.0 g/day, respectively) are considerably lower. Likewise, the mean and 90th percentile all-user intakes of peptides estimated from the proposed uses of Senmi's hydrolyzed sardine protein product in foods in infants (0 to 2 years of age) (*i.e.*, 0.8 and 1.6 g/day, respectively; values presented in Table 4 are adjusted by 0.85 to account for peptide content only) were approximately 10- to 60-fold lower than the daily intake of all dietary protein reported for infants up to 12 months of age by the IOM (*i.e.*, 15.9 to 28.3 and 21.9 to 42.5 g/day, respectively) (IOM, 2005). However,

as previously mentioned, Senmi does not intend to market foods with the sardine peptide products for infants. Protein hydrolysates of plant, vegetable, animal, or milk origin have a long history of use in the U.S. Furthermore, peptides are not only a product of normal digestion of protein, but Val-Tyr specifically also is produced endogenously by the body as a result of ANG I breakdown and likely functions as part of the renin-angiotensin system in the proper maintenance of blood pressure.

Given the low bioactivity of Senmi's hydrolyzed sardine protein and its limited effect to only assist in the maintenance of above-normal blood pressure to levels within the normal physiological range, coupled with the fact that peptides are natural break-down products of protein digestion and Val-Tyr is a naturally-occurring metabolite of ANG I degradation, it is not anticipated that consumption of food products containing Senmi's sardine peptide ingredient during pregnancy would be associated with any adverse developmental effects in neonates.

HUMAN STUDIES

The safety of hydrolyzed sardine protein was evaluated in a number of human studies, involving single- and repeat-dose administration (Kawasaki *et al.*, 2000, 2002; Matsui *et al.*, 2002a,b; Kajimoto *et al.*, 2003, 2005a,b). The designs and results of these studies are summarized in detail in Attachment 1. The studies were primarily designed to investigate the potential physiological effects of sardine peptides, and Val-Tyr specifically, on maintaining normal blood pressure levels; however, each study also included extensive safety-related endpoints (biochemical, hematological, and urinalysis parameters). Furthermore, study subjects were monitored for any adverse reactions during the treatment periods, including the development of a dry cough, which is a reported effect of pharmaceutical ACE inhibitors resulting from increased levels of bradykinin. Study participants comprised not only individuals with above-normal blood pressure (otherwise healthy), but also normotensive subjects. The results of these studies were reviewed solely in the context of the ingredient's safety. For purposes of the determination of the GRAS status of hydrolyzed sardine protein, blood pressure and heart rate were considered as end-points of safety only.

Two single dose trials were conducted with 5 normotensive and 12 mild-hypertensive male subjects (Matsui *et al.*, 2002a,b). Following ingestion of 6 g of hydrolyzed sardine protein manufactured by Senmi using a method similar to that described herein, no significant variations were observed in urinalysis and none of the study participants experienced any adverse side effects. Systolic and diastolic blood pressure (SBP and DBP) remained unchanged in both groups of study subjects (Matsui *et al.*, 2002a,b). A total of 194 male and female subjects (25 normotensive and 169 with above normal blood pressure) participated in the 5 studies involving repeated consumption of sardine protein hydrolysates over a course of 2 to 13 weeks (Kawasaki *et al.*, 2000, 2002; Kajimoto *et al.*, 2003, 2005a,b). No clinically significant disturbances were noted in standard hematological, clinical chemistry, or urinalysis parameters following daily ingestion of Senmi's hydrolyzed sardine protein or peptide products

manufactured by Senmi using slightly modified methods at dose levels of 500 mg for 12 to 13 weeks (Kawasaki *et al.*, 2002; Kajimoto *et al.*, 2003, 2005a) or 1.5 to 4 g for 2 to 4 weeks (Kawasaki *et al.*, 2000, 2002; Kajimoto *et al.*, 2005b). Any differences that were observed between pre- and post-treatment values remained within the normal physiological range of variation and often occurred in both control and placebo group subjects. Since ACE inhibitors have been reported to stimulate dry coughing as a result of bradykinin activation (Israili and Hall, 1992), the development of a cough was monitored specifically in the human studies. None of the study participants in any of the studies reported developing a cough or any other adverse effects that were related to the consumption of the test material. Repeat-dosing with hydrolyzed sardine protein mediated reductions in blood pressure (both SBP and DBP) to within the normal range and only in individuals with high-normal or above normal blood pressure at baseline (Kawasaki *et al.*, 2000, 2002; Kajimoto *et al.*, 2003, 2005a,b). Decreases in SBP ranged from approximately 4 to 6% (approximately 6 to 8 mmHg) in individuals with high-normal blood pressure (individuals with blood pressure in the range of 130 to 140 mmHg) and from approximately 4 to 11% (approximately 6 to 18 mmHg) in hypertensive subjects (individuals with blood pressure >140 mmHg) following consumption of hydrolyzed sardine protein. Reductions in DBP were less consistently observed and were rarely significant in comparison to the control group, ranging from 2 to 8% (approximately 1 to 8 mmHg) in subjects with above normal blood pressure. No significant variations in either SBP or DBP were reported in subjects enrolled in these studies with normal blood pressure. Likewise, no changes in heart rate were observed in any of the study participants consuming hydrolyzed sardine protein.

ADDITIONAL DOCUMENTATION TO SUPPORT SAFETY

Fish proteins, including proteins derived from sardines, are well-recognized food allergens and have been reported to be responsible for anaphylactic reactions in both children and adults (Helbling *et al.*, 1996; Koyama *et al.*, 2006). Specifically, a number of major allergens have been identified in seafood that are associated with the induction of allergic reactions, including tropomyosin (muscle protein) in shrimp and Gad c 1 (parvalbumin) in codfish (Lehrer *et al.*, 2003). However, as previously indicated, none of the study participants consuming hydrolyzed sardine protein for up to 13 weeks in any of the human studies described above developed compound-related signs or symptoms of allergic reactions. Furthermore, negative responses were obtained in Hartley guinea pigs subjected to the active systemic anaphylaxis (ASA) test and the passive cutaneous anaphylaxis (PCA) test to assess the potential allergenicity of short-chain sardine peptides (average length 2.67 amino acids) (Seki *et al.,* 1995b).

The size and digestibility of protein are typically recognized as critical determinants of its allergenic potential (Lehrer *et al.*, 2002). Most food allergens are between 10 and 70 kDa (equivalent to approximately 90 to 600 amino acids in length), with 3 to 5 kDa determined to be the minimum molecular mass required to elicit immunogenicity and allergenicity (van Beresteijn *et al.*, 1994; Huby *et al.*, 2000). In shrimp tropomyosin, 5 major IgE-binding regions have been

identified, containing a total of 8 distinct epitopes (Lehrer *et al.*, 2003). The length of the peptide responsible for the IgE-binding varied from 8 to 15 amino acids in length. Given that Senmi's product is a protein hydrolysate that contains peptides of not more than 5 amino acids in length, combined with the results of the ASA and PCA tests performed with guinea pigs and the absence of any allergic reaction in the human studies, Senmi's hydrolyzed sardine protein is not expected to elicit allergenic reactions. Moreover, proper labeling of foods containing Senmi's hydrolyzed sardine protein should alert the fish-allergic consumer to the presence of a fish-derived ingredient.

Based on the results of a pre-clinical study conducted with rats which demonstrated that combined administration of Val-Tyr and captopril (an ACE inhibitor) attenuated captopril's blood pressure-lowering effect and suggested that Val-Tyr is a competitive inhibitor of captopril uptake, the study authors concluded that the intake of foods that are rich in peptides should be limited in hypertensive individuals treated with ACE inhibitors (Matsui *et al.*, 2006). Considering, however, that under the conditions of use proposed herein sardine peptides from Senmi's product were estimated to only minimally contribute to the overall dietary protein intake and the intake of protein from sardines specifically, and that the amino acids comprising the peptides in Senmi's product have a history of background dietary consumption due to their natural presence in sardine tissue, the potential for drug interactions to occur under the intended conditions of use of hydrolyzed sardine protein in the defined food products is expected to be negligible and would not pose a risk to human health. The potential for drug-interaction is further reduced by the fact that the short-chain peptides derived from Senmi's product will be consumed as an ingredient of food and beverages. Incorporation of Senmi's sardine peptides into a food or beverage matrix is expected to be associated with a different digestion and absorption profile following consumption than that for a pharmaceutical ACE-inhibitor such as captopril, which has demonstrated a lower absorption when taken together with food and should be taken on an empty stomach (PDR, 2006). Therefore, there is no anticipated potential for interaction of Senmi's hydrolyzed sardine protein with the blood pressure lowering drug, captopril, or any other pharmaceuticals of similar use.

SUMMARY

Senmi intends to market short-chain peptides obtained from sardines as an ingredient in a variety of food products including breakfast cereals, milk and milk products, soups and soup mixes, sweets, and vegetable drinks. Senmi's hydrolyzed sardine protein, VALTYRON®, is manufactured by enzymatic hydrolysis of sardine muscle and subsequent water and ethanol extraction of peptides. Based on the specifications for the final product, peptides (di-, tri-, tetra-, and penta-peptides) comprise at least 85% of the overall composition, with Val-Tyr identified at levels in the range of 0.10 to 0.16%. While the extraction procedure removes highly hydrophilic and hydrophobic peptides, the amino acid composition of the final product is reflective of the amino acid profile of the sardine starting material. The enzyme used to carry out the hydrolytic

degradation of the protein is a protease obtained from *B. licheniformis*, which is recognized as GRAS by the FDA for use in the manufacture of protein hydrolysate products. Several down-stream purification steps are employed during the manufacturing of hydrolyzed sardine protein to remove residues of the biocatalyst and other processing aids used during the production process from the final product. The final product has been analyzed for the presence of several contaminants that are known to persist in the marine environment (*i.e.*, pesticides, heavy metals, PCBs, *etc.*). The analytical results revealed compliance with current FDA action and tolerance levels that have been set for some of these environmental contaminants.

Protein is a macronutrient that occurs as part of the daily human diet. Amino acids are normal constituents of protein, 9 of which have been classified as essential, meaning that they can only be obtained from the diet. The IOM has established RDA values of 56 and 46 g protein/day (0.8 g protein/kg body weight/day) for adult males and females, respectively. Amino acids, di-peptides, and other small peptides are normal breakdown products of protein digestion. Many biologically-active peptides of dietary origin have been identified and are produced in the gastrointestinal tract following consumption of protein-containing foods. Additionally, peptides also are synthesized endogenously to carry out various physiological functions. Val-Tyr, one of the di-peptides identified specifically in Senmi's sardine muscle hydrolysate product, is an endogenously-occurring product of ANG metabolism (Matsufuji *et al.*, 1995; Matsui *et al.*, 1997a,b, 1999). *In vitro*, Val-Tyr has been demonstrated to competitively inhibit ACE, which is responsible for the conversion of ANG I to ANG II.

Senmi's hydrolyzed sardine protein is proposed for use in foods and beverages at use-levels of up to 0.6 g/serving. Under the conditions of intended use, an assessment of the estimated intake of hydrolyzed sardine protein on an all-user basis provided a mean intake by the total U.S. population from all-proposed food-uses of 1.3 g/person/day or 27 mg/kg body weight/day. For the heavy consumer (90th percentile), an intake of 2.8 g/person/day (62 mg/kg body weight/day) was estimated. Sardines are part of the normal human diet and have a long history of safe consumption, although *per capita* intakes of canned sardines have declined over the last few decades from 1.7 to 0.25 g/person/day. Assuming that protein contributes 20% to the overall composition of sardines, mean and 90th percentile all-user intakes of 7.02 and 9.20 g/person/day were estimated for protein consumption from sardines for the total U.S. population. In comparison, mean and 90th percentile intake of protein from all food sources for the total U.S. population has been estimated to be several-fold higher (*i.e.*, 75.3 and 114.0 g/person/day, respectively) than the estimated intakes of peptides from the consumption of foods containing added sardine-derived peptides (*i.e.*, 1.1 and 2.4 g/person/day, respectively). Thus, at the proposed use-levels, peptides from sardines would not contribute significantly to the overall protein intake of humans.

In comparison to baseline plasma levels of Val-Tyr in the range of approximately 100 to 250 fmol/mL (up to 450 fmol/mL in the upright position) in human subjects (Matsui *et al.*,

1997a,b, 2000, 2002a,b), dose-dependent increases in plasma levels were observed following oral ingestion of Val-Tyr (up to 1,934 to 2,041 fmol/mL following 12 mg of Val-Tyr) (Matsui *et al.*, 2000, 2002a,b). Following systemic absorption, Val-Tyr is quickly cleared from plasma and observed to distribute rapidly to major organs (Matsui *et al.*, 2003, 2004). However, in relation to the total administered dose, absorption of unhydrolyzed peptides is minimal. Thus, it is expected that comparable to the process of protein digestion following consumption of unprocessed sardines, the majority of the peptides in Senmi's product will be hydrolyzed to individual amino acids and utilized by the body in well-recognized pathways. Only a very small fraction of the peptides would be expected to be absorbed intact. Peptides that are absorbed systemically can be hydrolyzed in various body tissues or are excreted in the urine.

In rats, an LD_{50} of greater than 10,000 mg/kg body weight/day was estimated for peptide α-1000, a closely related manufacturing precursor of Senmi's commercial product, indicating a very low order of oral toxicity. A NOAEL of 5,000 mg/kg body weight, the highest dose level tested, was determined for peptide α-1000 in male and female rats under the conditions of a 28-day oral toxicity study. In a 4-week study in which a hypertensive and hyperglycemic strain of rat was administered sardine peptides at a dose level of 1,000 mg/kg body weight/day, a significant reduction in blood glucose levels was observed following glucose loading. However, in human studies in which sardine peptides were consumed at dose levels of 1.5 g/day for up to 4 weeks or 500 mg/day for up to 13 weeks, no changes in blood glucose levels were observed. Neither Senmi's hydrolyzed sardine protein nor Val-Tyr specifically exhibited any mutagenic or genotoxic activities *in vitro* in standard bacterial test strains and *in vivo* in bone marrow cells of orally treated mice, respectively.

Dietary peptides are thought to mediate blood pressure changes *via* a similar mechanism as pharmaceutical ACE inhibitors, which are contraindicated for use during pregnancy. While no studies were identified that specifically assessed the potential effect of sardine peptides on reproduction or development, given the low bioactivity of Senmi's hydrolyzed sardine protein and its limited effect to lower only above-normal blood pressure to levels within the normal physiological range, coupled with the fact that peptides are natural break-down products of protein digestion and Val-Tyr is a naturally-occurring metabolite of ANG I degradation, it is not anticipated that consumption of food products containing Senmi's sardine peptide ingredient during pregnancy would be associated with any adverse developmental effects in neonates. Furthermore, the safety of protein hydrolysates is supported by a long history of use. The present IOM recommendations for increased protein intake by women during pregnancy and lactation (25 g in addition to the female adult RDA for protein) and current infant protein intakes from all dietary sources are considerably higher (up to 60-fold) than estimated intakes of sardine peptides at the proposed use-levels. Thus, exposure to sardine peptides at the specified use-levels is not expected to result in any adverse effects related to reproduction or development.

The results of a series of human studies involving administration of single doses of up to 6 g of a sardine peptide hydrolysate product (Matsui *et al.*, 2002a,b), as well as repeat-administration studies with daily exposure to Senmi's hydrolyzed sardine protein and products prepared by Senmi using a slightly modified procedure at dose levels ranging from 500 mg to 4 g over the course of 2 to 13 weeks (Kawasaki *et al.*, 2000, 2002; Kajimoto *et al.*, 2003, 2005a,b) consistently demonstrated an absence of any adverse effects associated with the oral consumption of sardine peptides, including a lack of any responses indicative of allergic reactions and the absence of any changes in standard clinical chemistry and urinalysis parameters. Significant reductions in blood pressure were observed only in subjects that exhibited higher than normal blood pressure at study beginning and not in normotensive individuals. Furthermore, the observed reductions in blood pressure did not result in levels below the normal range. In addition, studies conducted specifically to assess the potential for short-chain peptides derived from sardines to induce active systemic or passive cutaneous anaphylactic reposes in guinea pigs were negative (Seki *et al.,* 1995b). As such, there is no reason to expect that consumption of Senmi's hydrolyzed sardine protein would elicit an allergic reaction. Nevertheless, product labeling indicating that the ingredient is derived from sardines as a potential warning to individuals with fish allergies should alert the fish-allergic consumer. Although peptides are considered likely to be absorbed *via* the same transport mechanism as hypertensive drugs, consumption of Senmi's hydrolyzed sardine protein under the intended conditions of use is not expected to interfere with the uptake of pharmaceutical agents used for the treatment of high blood pressure.

The significant decreases in the ratio of PCEs to total erythrocytes observed in the mouse micronucleus assays at dose levels of 1,000 and 2,000 mg/kg body weight of Senmi's hydrolyzed sardine protein and the dipeptide, Val-Tyr, following only 2 consecutive treatments were not confirmed in either the 28-day rat toxicity study or any of the studies conducted in humans as evidenced by the lack of any variations in hematological parameters, including hematocrit levels, between groups treated with peptide α-1000 or Senmi's hydrolyzed sardine protein and controls. In the human studies in which indices of hematological relevance were examined, significant variations were observed only between pre- and post-treatment values and often occurred in both placebo and test group subjects. Apart from a significant reduction in MCV in study participants consuming 500 mg of Senmi's hydrolyzed sardine protein daily for 12 consecutive weeks compared to the placebo-control values, which increased relative to baseline levels (Kajimoto *et al.*, 2003), no differences in any other hematological parameters were observed between test and placebo subjects in the human studies (Kajimoto *et al.*, 2003, 2005a,b). Results of the repeat-dose rat toxicity study and human studies, therefore, demonstrate an absence of any potential adverse effects on erythropoiesis related to the oral administration of Senmi's hydrolyzed sardine protein by humans.

When viewed in its entirety, the scientific evidence presented herein indicates that under the conditions of intended use in foods, Senmi's hydrolyzed sardine protein would not produce any

adverse health effects. Sardine peptides are produced by Senmi in accordance with current Good Manufacturing Practices and meet appropriate food-grade specifications. Sardines have a long history of consumption as part of the normal human diet. Peptides are produced naturally during protein digestion and also are synthesized endogenously. The estimated exposure to sardine peptides under the intended conditions of use is well below current daily protein intakes and the RDAs established for protein by the IOM. Following oral consumption, peptides are primarily hydrolyzed to individual amino acids that comprise the sardine raw material, with only a small fraction taken up intact. The safety of hydrolyzed sardine protein is confirmed by a series of animal toxicity studies, as well as several human studies assessing the safety of dose levels relevant to the conditions of intended use of hydrolyzed sardine protein in foods.

CONCLUSION

We, the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that the proposed uses of hydrolyzed sardine protein (VALTYRON®), meeting appropriate food-grade specifications, are safe and suitable.

We further conclude that hydrolyzed sardine protein is Generally Recognized as Safe (GRAS) based on scientific procedures under the conditions of intended use in foods specified herein.

It is our opinion that other qualified experts would concur with this conclusion.

(b) (6)

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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Section Title
101—Food labeling	101.12	Reference amounts customarily consumed per eating occasion
102—Common or usual name for nonstandardized foods	102.22	Protein hydrolysates
170— Food additives	170.30	Eligibility for classification as generally recognized as safe (GRAS)
172— Food additives permitted for direct addition to food for human consumption	172.340	Fish protein isolate
184—Direct food substances affirmed as generally recognized as safe	184.1027	Mixed carbohydrase and protease enzyme product

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ATTACHMENT 1

SUMMARY OF HUMAN STUDIES CONDUCTED USING SARDINE PEPTIDES

Table A1-1 Summary of Single-Dose Studies in Humans Conducted Using Sardine Peptide Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Placebo-controlled trial	5 N-BP males (22 to 26 years; mean age 23.6 ± 0.6; BP 114±7/62±4 mmHg)	Single dose	0 (control), 3, 6, or 12 mg VY/day (as 0, 1.5, 3, and 6 g hydrolyzed sardine protein/day, respectively[1]), provided as a drink	i) SBP, DBP, and HR; ii) clinical chemistry (plasma levels of ANG I, ANG II, aldosterone, and VY); and, iii) urinalysis (Na^+, K^+, and Crea).	i) No significant Δ in SBP, DBP, or HR following ingestion of the test drink compared to baseline levels. ii) Dose-dependent ↑ in plasma VY; no Δ in plasma levels of ANG I, ANG II, and aldosterone following test drink ingestion. iii) No effect on urinary Na^+, K^+, or Crea levels. No adverse effects reported.	Matsui *et al.* (2002a)
Placebo-controlled trial	12 (4/group) mild-H males (39 to 59 years of age; mean 48.6 ± 2.8; BP 145.9±3.5/93.6±2.1 mmHg)	Single dose	0 (control), 6, or 12 mg VY/day (as 0, 3, and 6 g hydrolyzed sardine protein/day, respectively[1]), provided as a drink	i) SBP, DBP, and HR; ii) clinical chemistry (plasma VY); and, iii) urinalysis (Na^+, K^+, and Crea).	i) No significant Δ in SBP, DBP, or HR following ingestion of the test drink compared to baseline levels. ii) Dose-dependent ↑ in plasma VY. iii) No effect on urinary Na^+, K^+, or Crea levels. No adverse effects reported.	Matsui *et al.* (2002b)

ANG I, Angiotensin I; ANG II, Angiotensin II; BP, Blood pressure; Crea, Creatinine; DBP, Diastolic blood pressure; HR, Heart rate; K^+, potassium; mild-H, Mild hypertension; Na^+, Sodium; N-BP, Normal blood pressure; SBP, Systolic blood pressure; VY, Valine-tyrosine; Δ, variation.
[1] Assuming hydrolyzed sardine protein products contain 0.2% Val-Tyr.

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Table A1-2 Summary of Repeat-Dose Studies in Humans Conducted Using Sardine Peptide Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Un-blinded ingestion trial	25 (23M, 2F; mean age 44.9±13.9); 5 MH subjects (BP 160-180/100-110 mmHg), 6 mild-H subjects (BP 140-160/90-100 mmHg), 6 HN-BP subjects (BP 130-140/80-90 mmHg), and 8 N-BP subjects (BP <130/<80 mmHg).	2 weeks	500 mg Senmi's hydrolyzed sardine protein (providing 0.4 mg VY) 3x per day (total 1,500 mg Senmi's hydrolyzed sardine protein and 1.2 mg VY per day), provided as 195 g vegetable juice	i) SBP and DBP; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (total protein, albumin, total bilirubin, AST, ALT, ALP, γ-GTP, LAP, LDH, BUN, Crea, CPK, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Fe^{2+}, Ch, HDL-Ch, LDL-Ch, Tg, glucose, HbA_{1c}); iv) urinalysis (parameters not specified); and, v) physical examinations; body weights.	i) No Δ in SBP, DBP, and HR in N-BP. ↓ SBP and DBP (compared to baseline) in mild-H subjects, ↓ SBP (compared to baseline), but not in DBP or HR in MH subjects and HN-BP subjects. ii, iii, and iv) Hematology, urinalysis, and clinical chemistry unremarkable (all statistically significant variations within physiological range). v) No subjective/objective symptoms or adverse effects reported, including coughing. No Δ in body weight.	Kawasaki *et al.* (2002)
Randomized, double-blind, placebo controlled trial	29 (27M, 2F) subjects with HN-BP and mild-H (mean BP 146/91-92 mmHg) Treatment group: 17 (16M, 1F) (Mean age 45.5 ± 3.2; 4 HN-BP and 13 mild-H) Control group: 12 (11M, 1F) (Mean age 48.8 ± 3.0)	4 weeks	Treatment group: 3 mg VY (as 2 g of protein from a sardine protein hyrolysate) 2x per day (total 4 g of a sardine peptide hydrolysate per day), provided as a 100 mL drink Control group: 100 mL drink provided 2x per day	i) SBP, DBP, and HR; ii) clinical chemistry (ANG I, ANG II, VY, and aldosterone); iii) urinalysis (Na^+, K^+, Na^+/K^+ ratio, and Crea); and, iv) physical examination.	i) Significant ↓ in SBP and DBP of HN-BP and mild-H test subjects compared to baseline levels; no Δ in HR. ii) Significant ↑ in plasma VY and ANG I levels compared to baseline values, but not compared to placebo control. Significant ↓ in plasma ANG II and aldosterone compared to baseline values, but not compared to placebo control. Unspecified change in serum chloride levels in test subjects. iii) No effect on urinary Na^+, K^+, Na^+/K^+ ratio, or Crea levels. iv) No adverse effects, including no cough.	Kawasaki *et al.* (2000)

Table A1-2 Summary of Repeat-Dose Studies in Humans Conducted Using Sardine Peptide Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Randomized, double-blind, placebo controlled trial	44 subjects with N-BP (118/73 mmHg), HN-BP (134/82 mmHg), or mild-H (148/87 mmHg) Treatment group: 22 (9M, 13F; mean age 55.4±8.0; 6 N-BP, 9 HN-BP, and 7 mild-H) Control group: 22 (10M, 12F; mean age 57.0±6.4; 5 N-BP, 8 HN-BP, and 9 mild-H)	4 weeks	Treatment group: 500 mg Senmi's hydrolyzed sardine protein/day (providing 0.4 mg VY) 3x per day (total 1,500 mg Senmi's hydrolyzed sardine protein and 1.2 mg VY per day), provided as a 350 mL tea beverage Control group: 350 mL tea beverage without Senmi's hydrolyzed sardine protein 3x per day	i) SBP, DBP, and HR; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (plasma renin, BUN, Crea, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Ch, HDL-Ch, Tg, total protein, albumin, A/G, blood glucose, AST, ALT, ALP, γ-GTP, LDH, total bilirubin, HbA_{1c}); iv) urinalysis (*i.e.,* Na^+, K^+, Cl^-, Crea, pH, specific gravity, protein, glucose, urobilinogen, and occult blood); and v) physical examinations; body weight and BMI.	i) Significant ↓ in SBP of test subjects compared to baseline and control values. Stratified analysis: ↓ in SBP (compared to controls), but not in DBP or HR in HN-BP; no Δ in SBP, DBP, or HR in subjects with N-BP or mild-H compared to controls. ii and iii) No Δ between control and test group[1]; all statistically significant Δ between pre- and post-treatment values within physiological range. iv) Protein and occult blood in one single female; likely result of sample contamination due to menstruation. ↓ in K^+ in test subjects compared to controls 2 weeks after study end. v) Mild GI symptoms and skin itching reported by both test and control subjects; no cough and no significant Δ in body weights or BMI.	Kajimoto *et al.* (2005b)

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Table A1-2 Summary of Repeat-Dose Studies in Humans Conducted Using Sardine Peptide Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Double-blind, placebo controlled trial	114 subjects with HN-BP (BP 131-132/80-81 mmHg) or mild-H (BP 146-147/88 mmHg) Treatment group: 55 (24M, 31F; mean age 50.3±12.0) Control group: 59 (27M, 32F; mean age 50.1±10.3)	12 weeks	Treatment group: 500 mg Senmi's hydrolyzed sardine protein/day (providing 0.4 mg VY), provided as a 350 mL tea beverage Control group: 350 mL tea beverage/day without Senmi's hydrolyzed sardine protein	i) SBP, DBP, and HR; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (plasma rennin, BUN, Crea, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Ch, HDL-Ch, Tg, total protein, albumin, A/G, blood glucose, AST, ALT, ALP, γ-GTP, LDH, total bilirubin, HbA_{1c}); iv) urinalysis (*i.e.,* Na^+, K^+, Cl^-, Crea, pH, specific gravity, protein, glucose, urobilinogen, and occult blood); and, v) physical examinations; body weight and BMI.	i) ↓ in SBP and DPB in test subjects compared to baseline values and placebo-control values (SBP only); no Δ in HR. Stratified analysis: mild-H - ↓ in SBP and DBP compared to controls and baseline (DBP only) at Weeks 10 and 12 and Week 8, respectively; HN-BP - ↓ in SBP compared to control at Weeks 4-10 and to baseline at Week 10; no Δ in DBP. ii and iii) No Δ between control and test group; all statistically significant Δ between pre- and post-treatment values within physiological range. iv) Significant ↓ Na^+, K^+, and Cl^- in test subjects compared to controls; changes in qualitative parameters mild and transient and within physiological range of variations; occult blood and protein in samples from females likely result of sample contamination due to menstruation. v) Subjects reported mild GI effects in both control and test groups; no cough; ↓ in body weight and BMI of test subjects compared to baseline values.	Kajimoto *et al.* (2005a)

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Table A1-2 Summary of Repeat-Dose Studies in Humans Conducted Using Sardine Peptide Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Randomized double-blind placebo controlled trial	88 subjects with HN-BP, mild-H, or MH Treatment group: 44 (26M, 18F; mean age 49±11; BP 148.3/92.1 mmHg) Control group: 44 (29M, 15F; mean age 50±10; BP 147.6/92.1 mmHg)	12 weeks	Treatment group: 500 mg Senmi's hydrolyzed sardine protein/day (0.4 mg VY), provided as 4 tablets Control group: 4 tablets/day without Senmi's hydrolyzed sardine protein	i) SBP, DBP, and HR; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (ANG II; ACE; aldosterone; plasma rennin activity and conc't, BUN, Crea, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Ch, HDL-Ch, Tg, total protein, albumin, A/G, blood glucose, AST, ALT, ALP, γ-GTP, LDH, total bilirubin, HbA_{1c}); iv) urinalysis (*i.e.,* Na^+, K^+, pH, specific gravity, protein, glucose, urobilinogen, and occult blood); and, v) physical examinations; body weight and BMI.	i) ↓ in SBP of test subjects compared to baseline and placebo and in DBP of test subjects compared to placebo (at 12 weeks). No change in HR. Stratified analysis: mild-H - ↓ in SBP compared to baseline at study end and several other interim weeks; ↓ DBP compared to baseline and control values at Week 8. HN-BP - ↓ in SBP compared to control values and baseline at study end and several other interim weeks and ↓ in DBP compared to control values. ii) ↓ in MCV of test group compared to placebo; all other statistically significant Δ only between pre- and post-treatment values and within physiological range. iii) No Δ between control and test group; Ch ↑ above upper end of normal range in both test and placebo subjects (at Week 8); all other statistically significant Δ between pre- and post-treatment values within physiological range (ANG II ↑ 1.7x from baseline in test group[2]). iv) ↓ in K^+ compared to baseline. No other Δ between test subjects and controls, and pre- and post-treatment values. v) ↓ in BMI of test subject 4 weeks after study end compared to baseline. No adverse effects related to test diet identified (no cough).	Kajimoto *et al.* (2003)

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Table A1-2 Summary of Repeat-Dose Studies in Humans Conducted Using Sardine Peptide Products with Endpoints Related to Efficacy and Safety

Study Design	Study Population	Test Duration	Daily Dose Level, Route	Endpoints Evaluated	Results Related to Sardine Peptide Treatment	Reference
Randomized, double-blind, placebo controlled	63 subjects with mild-H/HN-BP (140-142/88 mmHg) and N-BP (111-113/71-72 mmHg) Treatment group: 31 subjects: 20 (16M, 4F) mild-H or HN-BP (mean age 51.8±9.4) and 11 (9M, 2F) N-BP (mean age 47.1±11.9) Control group: 32 subjects: 20 (17M, 3F) mild-H or HN-BP (mean age 52.0±9.9) and 12 (9M, 3F) N-BP (mean age 43.8±11.2)	13 weeks	Treatment group: 500 mg hydrolyzed sardine protein/day (providing 0.4 mg VY) in 195 g vegetable juice Control group: 195 g vegetable juice without hydrolyzed sardine protein	i) SBP and DBP; ii) hematology (WBC, RBC, Hb, Ht, MCV, MCH, MCHC, and platelet count); iii) clinical chemistry (total protein, albumin, total bilirubin, AST, ALT, ALP, γ-GTP, LAP, LDH, BUN, Crea, CPK, UA, Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Fe^{2+}, Ch, HDL-Ch, LDL-Ch, Tg, glucose, insulin, HbA_{1c}, 1,5-AG); iv) urinalysis (parameters not specified); and, v) physical examinations; body weight.	i) ↓ SBP and DBP in mild-H and HN-BP subjects compared to baseline levels and placebo (SBP only). No significant change in SBP or DBP in N-BP subjects compared to baseline levels or placebo. ii, iii, and iv) Hematology, urinalysis, and clinical chemistry unremarkable (all statistically significant variations within physiological range). v) No subjective/objective symptoms or adverse effects reported (no dry cough). No changes in body weight.	Kawasaki *et al.* (2002)

ACE, Angiotensin I converting enzyme; 1,5-AG, 1,5-Anhydroglucitol; A/G, Albumin to globulin ratio; ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; ANG I, Angiotensin I; ANG II, Angiotensin II; AST, Aspartate aminotransferase; BMI, Body mass index; BP, Blood pressure; BUN, Blood urea nitrogen; Ca^{2+}, Calcium; Ch, Cholesterol (total); Cl^-, Chloride; CPK, Creatine phosphokinase; Crea, Creatinine; DBP, Diastolic blood pressure; F, Female; Fe^+, Iron; GI, Gastrointestinal; γ-GTP, *gamma*-Glutamyl transpeptidase; Hb, Hemoglobin; HbA_{1c}, Hemoglobin A_{1c}; HDL-Ch, High-density lipoprotein-cholesterol; HN-BP, High-normal blood pressure; HR, Heart rate; Ht, Hematocrit; LAP, leukocyte alkaline phosphatase; K^+, potassium; LDH, lactate dehydrogenase; LDL-Ch, Low-density lipoprotein-cholesterol; M, Male; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; MH, Moderate hypertension; mild-H, Mild hypertension; Mg^{2+}, Magnesium; Na^+, Sodium; N-BP, Normal blood pressure; RBC, Red blood cells; SBP, Systolic blood pressure; Tg, Triglycerides; UA, Uric acid; VY, Valine-tyrosine; WBC, White blood cells; Δ, variation.

[1] Hemoglobin A_{1c} levels in test subjects significantly lower compared to controls at study beginning and study end.

[2] The authors noted that markers of the renin-angiotensin-aldosterone system are highly variable and susceptible to many exogenous factors, including seasonal changes, which may have influenced the ANG II levels between study beginning and end.

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SUBMISSION END

From: Ashley Roberts Intertek
To: Carlson, Susan;
Subject: RE: GRN 360, hydrolyzed sardine protein
Date: Wednesday, January 19, 2011 6:10:50 PM

Dear Susan,

Sorry for the delay; just do not know where the time goes???

I have looked in to the issue below and the numbers in Table I.D.6-1 that compares the intake of protein from the diet, protein from sardine consumption and protein from the sardine peptide hydrolysate I believe are correct. I think the confusion lies because the numbers in Table I.D.5-1 and I.D.5-2 are actually corrected for the fact that they represent the protein hydrolysate (amino acids) while the numbers in Table I.D.6-1 represent the amount in terms of peptide where the numbers from Tables I.D.5-1 and I.D.5-2 are corrected by a factor of 85% that represents the water loss during the formation of peptide bonds. This information is found in a footnote to the Table.

The confusion arises because the numbers in Tables I.D.5-1 and I.D.5-2 for male adults are the same as found in Table I.D.6-1 following correction of the all user general population numbers for the 85% factor.

Please let me know if this is now any clearer?

Many Thanks

Ashley

From: ••••• •••••
To: Carlson, Susan;
cc: Dr.Ashely Roberts;
Subject: Re: Information request from U.S. FDA, re: GRAS Notice
Date: Monday, December 20, 2010 11:11:35 PM

Dear Dr.Susan Carlson

Thank you very much for your e-mail informing us that our company's GRAS Notice has been filed.
Regarding your question on the product category " frozen multicourse fish-based meals" in our GRAS
Notice, I would like to confirm with you that catfish are not included in the product category.

At the same time, I would like to introduce Dr. Ashely Roberts, a vice president of CANTOX HEALTH
SCIENCE INTERNATIONAL, as a representative from our company for GRAS notification for our
product hydrolyzed sardine protein. It is ,therefore,very much appreciated if you could understand
that CANTOX will be dealing with further questions regarding the GRAS notification for our product.

Best regards,

Katsuhiro Osajima,Ph.D.
President
SENMI EKISU CO., LTD.