ORIGINAL SUBMISSION

Morgan Lewis

GRN 000624

Morgan, Lewis & Bockius LLP
1111 Pennsylvania Avenue, NW
Washington, DC 20004
Tel. +1.202.739.3000
Fax: +1.202.739.3001
www.morganlewis.com

Gary L. Yingling
Senior Counsel
+1.202.739.5610
gyingling@morganlewis.com

January 19, 2016

VIA FEDERAL EXPRESS

Dr. Antonia Mattia
Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for D-allulose 3-epimerase from *Arthrobacter globiformis* M30 expressed in *Escherichia coli* K-12 W3110

Dear Dr. Mattia:

On behalf of Matsutani Chemical Industry Co. Ltd ("MCI"), we are submitting under cover of this letter three paper copies and one eCopy of MCI's generally recognized as safe ("GRAS") notification for its D-allulose 3-epimerase from *Arthrobacter globiformis* M30 expressed in *Escherichia coli* K-12 W3110. The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission. MCI has determined through scientific procedures that its D-allulose 3-epimerase enzyme preparation is GRAS for use as a processing aid in the production of D-allulose.

The *Escherichia coli* K-12 W3110 D-allulose 3-epimerase preparation is intended to be used in the production of D-allulose and other keto sugars. The enzyme is added during the epimerization of fructose to allulose. D-allulose is a low calorie sweetener, and acts as postprandial blood glucose regulator. The produced D-allulose is intended to be used as an ingredient in a broad range of food and beverage applications.

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Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36, this use of D-allulose 3-epimerase from *Arthrobacter globiformis* M30 expressed in *Escherichia coli* K-12 W3110 is exempt from premarket approval requirements of the Federal Food, Drug and Cosmetic Act, because the notifier has determined that such use is GRAS.

If you have any questions regarding this notification, or require any additional information to aid in the review of MCI's conclusion, please do not hesitate to contact me via email at gyingling@morganlewis.com or by telephone, (202)739-5610.

Sincerely,

(b) (6)

Gary L. Yingling

cc: Matsutani Chemical Industry Co. Ltd

GRAS NOTIFICATION FOR D-ALLULOSE 3-EPIMERASE FROM *ARTHROBACTER GLOBIFORMIS* M30 EXPRESSED IN *ESCHERICHIA COLI* K-12 W3110

Submitted by:

Matsutani Chemical Industry Co. Ltd.
5-3 Kita-Itami, Itami City,
Hyogo, 664-8508, Japan

TABLE OF CONTENTS

1. GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS

With this document, Matsutani Chemical Industry Co. Ltd. ("MCI") is submitting a GRAS notification for its D-allulose 3-epimerase enzyme preparation produced by *Escherichia coli* K-12 W3110 strain. D-allulose 3-epimerase is also known as D-psicose 3-epimerase. MCI has developed a brand name for its D-allulose 3-epimerase, known as "Matsurase FE. " In this submission, the term "Matsurase FE" is used instead of D-allulose 3-epimerase (D-AE) or D-psicose 3-epimerase (D-PE). MCI`s Matsurase FE epimerizes D-fructose to D-allulose. MCI produces the Matsurase FE preparations in liquid and powder form. The enzyme is widely present in nature and can be found in microorganisms.

Matsurase FE is a processing aid for the production of D-allulose or ketose sugars. The enzyme epimerizes D-fructose on C3 position to D-allulose. D-allulose is a low calorie sweetener, and acts as postprandial blood glucose regulator. The produced D-allulose is intended to be used as an ingredient in a broad range of food and beverage applications, such as cereals, chewing gum, confections & frostings, dressings for salads, jams & jellies, sugar, sugar substitutes (carrier), and various low- calorie or dietetic foods including low-calorie, reduced-calorie, sugar-free beverages(non-alcoholic), cereals, frozen dairy desserts (ice cream, soft serve, sorbet), yogurt and frozen yogurt, gelatins, pudding & fillings, hard candies, soft candies, and sweet sauces & syrups.

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36 (see 62 Fed. Reg. 18,938, April 17, 1997), MCI has determined that its Matsurase FE enzyme originated from *Arthrobacter globiformis* M30, expressed in *Escherichia coli* K-12 W3110 is a GRAS substance for the intended food applications and is therefore exempt from the requirement for premarket approval. Information on the enzyme and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme, its applicable conditions for use, MCI`s basis for its GRAS determination and the availability of supporting information and reference materials for FDA's review can be found here in Section 1. The production organism, *Escherichia coli* K-12 W3110 derived from K-12, has a long history of safe use. The safety of the enzyme and strain are discussed in Section 7. Section 2 describes the origin and development of the production strain. In Section 3, the enzyme activity is described in more detail. The safety of the materials used in manufacturing, and the manufacturing process itself is described in Section 4. Section 5 reviews the hygienic measurements, composition and specifications. Section 6 provides information on the mode of action, applications, use levels of enzyme, and residues in final food products. The safety studies outlined in Section 7 indicate that Matsurase FE preparations from *Escherichia coli* K-12 W3110 show no evidence of pathogenic or toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

Name and Address of Notifier

Notifier:
Haruyo Matsutani
President

Matsutani Chemical Industry Co. Ltd.
5-3 Kita-Itami, Itami City,
Hyogo, Japan 664-8508

Telephone +81-72-771-2001
Facsimile +81-72-770-4680
Email: haruyo-matsutani@matsutani.co.jp

Manufacturer:
Matsutani Chemical Industry Co. Ltd.
5-3 Kitaitami
Itami-city, Hyogo, Japan 664-8508
Telephone +81-72-771-2001
Facsimile +81-72-770-4680

Person Responsible for the Dossier:
Kazuhiro Okuma, Ph. D.
Senior Director

Matsutani Chemical Industry Co. Ltd.
5-3 Kitaitami
Itami-city, Hyogo, Japan 664-8508

Telephone +81-72-771-2032
Facsimile +81-72-771-2023
Email: k-okuma@matsutani.co.jp

1.1. Common or Usual Name of Substance

MCI`s Matsurase FE enzyme prepared from *Escherichia coli* K-12 W3110 is produced by submerged fermentation of a selected, pure culture of *Escherichia coli* K-12 W3110. The common or usual name of the substance is D-allulose 3-epimerase and D-psicose 3-epimerase. The common names allulose 3-epimerase and psicose 3-epimerase have been interchangeably used over the years. The enzyme is the same, despite this naming difference. It is produced in liquid form stabilized with sugar or sugar alcohol and powder form.

1.2. Applicable Conditions of Use

The *Escherichia coli* K-12 W3110 Matsurase FE preparation is intended to be used in the production of D-allulose and other keto sugars. The enzyme is added during the epimerization of

fructose to allulose. The concentration, pH, temperature conditions and incubation time are optimized to achieve the desired product concentration. The enzyme dosage level can vary from 10,000 to 30,000 FEU/kg allulose expressed in dry matter.

One Matsurase FE unit (FEU) is defined as the amount of enzyme that could epimerized 1 μmol of substrate per minute.

After the enzyme reaction, the reaction mixture containing enzyme is inactivated and product is separated and purified.

1.2.1. Food Products Used in

The Matsurase FE described in this dossier is applied specifically for the production of allulose. Allulose is intended to be used in a wide range of food applications include cereals, chewing gum, confections & frostings, dressings for salads, jams & jellies, sugar, sugar substitutes (carrier), and various low- calorie or dietetic foods including low-calorie, reduced-calorie, sugar-free beverages(non-alcoholic), cereals, frozen dairy desserts (ice cream, soft serve, sorbet), yogurt and frozen yogurt, gelatins, pudding & fillings, hard candies, soft candies, and sweet sauces & syrups.

1.2.2. Levels of Use

Matsurase FE can be used for epimerization of fructose to produce allulose. The average dosage of the enzyme depends on the process conditions and on the desired properties of the final product.

A typical use level would be 10,000 – 30,000 FEU per kg allulose dry matter, or 20-60 g of Matsurase FE preparation in liquid form described in this dossier per kg allulose dry matter.

1.2.3. Purposes

Matsurase FE produces allulose from fructose by epimerization, and also active on production of keto sugars. Allulose enhances taste, flavour and also improves food qualities (Sun, Y. *et al*., 2006).

1.2.4. Consumer Population

Matsurase FE is widely present in nature. Its presence was demonstrated in a number of microbial sources (Kim, H.J. et al 2006; Zhang, L. et al 2009; Mu,W. et al 2011; Zhang, W. et al 2013; Jai, M. et al 2014; Zhang, W. et al 2015).

Matsurase FE, the enzyme responsible for the conversion of the fructose to allulose, was extracted from *Escherichia coli* (W3110 derived from K-12) harboring DPE that originated from *Arthrobacter globiformis,* which is present in soil, and is considered non-toxic and non-pathogenic. The *E. coli* strain K-12, from which W3110 is derived, has been safely used in the production of chymosin enzyme, otherwise known as rennin, used in cheese production, for many years without known side effects. The production of rennin by *E. coli* K-12 is listed under

GRAS affirmation regulation 21 C.F.R. 184.1685. Furthermore, this bacterial strain was specifically discussed in a paper published (Beresford, T.P *et al* 2001), in which it is stated "*E. coli* K-12 has been used as a laboratory organism for over many years without reported incidents of infection and that it does not produce toxins that cause illness by ingestion, such as Shiga-like toxin produced by certain toxigenic strains *of E.coli. E.coli* K-12 and its derivatives have a history of safe use in the production of specialty chemicals and human drugs, and were exempted from EPA review under TSCA."

As is shown in Section 6.4 of this dossier, in the case of all enzyme used to produce allulose the amount of enzyme TOS[1] in the final food is expected to be approximately 18 – 898 mg/kg in a wide range of products, such as cereals, chewing gum, confections & frostings, dressings for salads, jams & jellies, sugar, sugar substitutes (carrier), and various low- calorie or dietetic foods including low-calorie, reduced-calorie, sugar-free beverages(non-alcoholic), cereals, frozen dairy desserts (ice cream, soft serve, sorbet), yogurt and frozen yogurt, gelatins, pudding & fillings, hard candies, soft candies, and sweet sauces & syrups. The estimation was made under the all enzyme to be carried into allulose even though they are used as processing aids. With worst case scenario as above, total TOS intake would be substantially below the No Observable Adverse Effect Level ("NOAEL"). Also, the enzyme is used as processing aids to convert fructose into allulose with highly purified processes like ion-exchange system.

It can thus be concluded, the substrate not found in general foods. Hence, there is no basis to believe that the use of Matsurase FE will have a significant effect on processed foods or on the human body. It is unlikely that the consumer population will be affected by the presence of Matsurase FE in food stuffs when used as processing aid.

1.3. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, MCI has determined, through scientific procedures, that its Matsurase FE enzyme preparation from *E. coli* K-12 W3110 is GRAS for use in the production of allulose. Allulose can be used as an ingredient in a wide range of foods and beverages such as cereals, chewing gum, confections & frostings, dressings for salads, jams & jellies, sugar, sugar substitutes (carrier), and various low- calorie or dietetic foods including low-calorie, reduced-calorie, sugar-free beverages(non-alcoholic), cereals, frozen dairy desserts (ice cream, soft serve, sorbet), yogurt and frozen yogurt, gelatins, pudding & fillings, hard candies, soft candies, and sweet sauces & syrups.

1.4. Availability of Information for FDA Review

The data and information that are the basis for MCI GRAS determination are available for the FDA's review, and copies will be sent to FDA upon request. Requests for copies and arrangements for review of materials cited herein may be directed to:

Yuma Tani
Matsutani Chemical Industry Co. Ltd.

[1] TOS: Total Organic Solids

5-3 Kitaitami
Itami-city, Hyogo, Japan 664-8508
Telephone +81-72-771-2032
Facsimile +81-72-771-2023
Email: yuma-tani@matsutani.co.jp

Copy to:
Gary L. Yingling
Morgan, Lewis & Bockius LLP
1111 Pennsylvania Avenue, NW
Washington, DC 20004-2541
Telephone: 202-739-5610
Email: gyingling@morganlewis.com

2. PRODUCTION MICROORGANISM

2.1. Name and Designation

The strain used for the production of Matsurase FE belongs to the species *Escherichia coli* K-12 W3110.

2.2. Source of the Organism

The wild-type strain was isolated from soil, and after classical stain improvement, the Matsurase FE production strain was deposited in National Institute of Technology and Evaluation "NITE" in Japan under accession number P-1111. Several members of the *Arthrobacter* family, including *Arthrobacter globiformis*, are present in the microflora of common produce items such as broccoli (Pagada, M. *et al* 2000). Enzymes derived from this bacterial family were the subject of GRAS Notification (GRN) No. 45, in which another sweetener, trehalose, was produced through the use of enzymes from *Arthrobacter* species. FDA issued a response of "FDA has no questions" to this GRN, supporting its position as safe for use in food production. Further, this species has used during citrus fermentations to remove limonin and reduce bitterness since 1997 (described in IFD/EFFCA Inventory of Microorganisms).

The Matsurase FE from wild-type strain was cloned and expressed in *Escherichia coli* K-12 W3110 derived from K-12. The K-12 strain of *E. coli* has been safely used in the production of chymosin enzyme, otherwise known as rennin, used in cheese production, for many years without known side effects. The production of rennin by K-12 was affirmed as GRAS by the FDA in 1990. Furthermore, this enzyme strain was specifically discussed in a paper published by the FDA in 2006 (Olempska-Beer, Z .S. *et al* 2006), in which it is stated "*E .coli* K-12 has been used as a laboratory organism for over 30 years without reported incidents of infection and that it does not produce toxins that cause illness by ingestion, such as Shiga-like toxin produced by certain toxigenic strains *of E. coli. E. coli* K-12 has a history of safe use in the production of specialty chemicals and human drugs, and was exempted from EPA review under TSCA."

The W3110 substrain has long been used as a research organism (Bachmann, 1972). This strain played a critical role in the understanding of the K-12 wild-type strain (Jensen, 1993). Because of the extensive use of this strain in research, it has been well characterized, and a highly accurate genomic sequence has been obtained for W3110 (Hayashi, et al., 2006).

2.2.1. Information on Reproductive Cycles (sexual/asexual) of the Classical Production Organism

Arthrobacteria are *Coryneform* bacteria. They are characterized by pleomorphism (variable shape) and Gram variability (staining positive or negative) although genetically they branch from the Gram-positive phylum *Actinobacteria*. They have a complex life cycle marked by two distinct stages. When the cultures are young, cells are slender rods that may stain Gram-negative. Jointed rods can be observed after about 1-2 days. By about 30 hours the cells have become very short, gram-positive rods and coccoids. *Arthrobacteria* are nonsporulating and are members of the actinomycete branch of the gram-positive bacteria.

Arthrobacteria form small colonies on blood agar, ranging in color from yellow to white and measuring 2 mm in diameter on average. They are widely distributed in soil. Due to their ubiquitous presence in soil and their ability to metabolize a variety of substances, *Arthrobacteria* have been discovered to degrade a variety of chemicals.

Matsurase FE production organism *Escherichia coli*, when undergoing cellular division, is using a means of asexual reproduction because there is no transfer of genetic material; the bacterium is merely making an exact copy of itself. This is the most prevalent form of reproduction for *E. coli*. The individual bacterium begins this process by elongation of the cell, followed by almost exact replication of the genome so there are two identical copies. A septum is formed, and the cell equally divides the cellular components and one copy of the parental genome gets placed in each side of the cell. The cell divides, leaving two copies of the original bacterium called daughter cells.

2.3. Strain Improvement

The wild-type strain was isolated from soil, and was screened for its ability to produce allulose. A strain improvement program, using classical colony isolation and selection techniques, yielded strain M30, which was identified as *Arthrobacter globiformis* M30. Further, the gene responsible for production of rare ketoses such as allulose was cloned and expressed in *Escherichia coli* K-12 W3110 derived from K-12.

2.4. Taxonomy

The formal classification of *Escherichia coli* is:

Kingdom	: Bacteria
Division	: Proteobacteria
Class	: Gamaproteobacteria
Order	: Enterobacteriales
Family	: Enterobacteriaceae
Genus	: Escherichia
Species	: *Escherichia coli*

2.5. Stability of Classical Production Organism in Terms of Relevant Genetic Traits

As stated in 2.4, the production organism was prepared by selection of bacterial using antibiotic resistance selectable marker genes on the expression vector. The isolated single colony is streaked and used as working cell banks. These working cell banks are in use through the present day, and have shown no indication of deterioration in growth rate and/or Matsurase FE activity.

2.6. Nature of Pathogenicity and Virulence, Infectivity, Toxicity and Vectors of Disease Transmission

E. coli K-12 has been used as a laboratory organism for over many years without reported incidents of infection and that it does not produce toxins that cause illness by ingestion, such as Shiga-like toxin produced by certain toxigenic strains of *E. coli*. *E. coli* K-12 is one of the most extensively studied bacteria. For further details on the safety of the production stain is referred to section 7.1.

2.7. Natural Habitat, Geographic Distributions and Climatic Characteristics of the Original Habitats

E. coli is commonly found in the lower intestine of warm-blooded organisms (endotherms). The descendant K-12 is used routinely in molecular biology as both a tool and a model organism.

2.8. Absence of the Production Organism in the Product

Good Manufacturing Practice is not the only reason to assure absence of the production organism in the final products. For many reasons, it is very important for each enzyme producer that the final commercial product does not contain viable production organisms. Therefore all traces of the production organism are removed during the manufacturing process (see Annex 1), ensuring that the enzyme preparation is free from the production organism *E. coli* K-12 W3110.

2.9. Absence of Toxins

For several decades, *E. coli* K12 has been used in the commercial production of chymotrypsin (Flamm, E. L. 1991). Chymosin is a milk-clotting enzyme also known as rennin. During this long history of uses, there has been no evidence that this enzyme would contain toxins derived from the species. The MCI *E .coli* K-12 derivative strain K-12 W3110 does not produce any known toxins under the production conditions. *E. coli* K-12 and its derivatives have a history of safe use in the production of specialty chemicals and human drugs and were exempted from EPA review under TSCA (EPA, 1997).

3. ENZYME IDENTITY

3.1. Enzyme Identity

- Systematic name : D-psicose 3-epimerase
- Common name : D-allulose 3-epimerase
- Other names : Matsurase FE, DAEase, DPEase
- Enzyme Commission No. : 5.1.3.30

Within the enzyme nomenclature, Matsurase FE belongs to the family of isomerases, part of the isomerases acting on carbohydrates and its derivatives.

3.2. Amino Acid Sequence

The amino acid sequence revealed that Matsurase FE has 289 amino acids. The molecular weight of purified D-AE was estimated to be 128,000 Da by gel filtration chromatography. The enzyme showed a single protein band with a molecular weight of about 3,2000 Da on SDS-PAGE. This indicates that the enzyme is a tetramer with four identical subunits (See Annex 2).

3.3. Enzymatic Activity

3.3.1. Main Enzymatic Activity

Matsurase FE epimerizes fructose to produce allulose. It also epimerizes both D, L-keto-hexoses as well as keto-pentoses and keto-tetroses.

MCI developed a simple, accurate and reproducible method to measure the Matsurase FE enzyme activity using fructose as substrate (see Annex 3). This method is also used for standardizing the enzyme preparation. The enzyme activity described in this method is expressed in so called FE Units (FEU). Under the described conditions, one FEU is defined as the amount of enzyme that could isomerize 1 μmol of substrate per minute.

The biochemical properties of Matsurase FE from *E. coli* K-12 W3110 have been investigated. Matsurase FE exhibits activity from pH 6-11. The temperature optimum, measured with D-allulose as substrate is 70 ^{o}C. No enzyme activity can be found at 80 ^{o}C or higher after 60 minutes.

3.3.2. Subsidiary Enzymatic Activities

Like any other living organism, the Matsurase FE production organism produces many other enzymes needed for the breakdown of nutrients and buildup of cell material. The Matsurase FE enzyme preparation will therefore contain minor, non-standardized amounts of these other enzymes. These amounts do not have an effect in the application.

4. MANUFACTURING PROCESS

4.1. Overview

Matsurase FE from *E. coli* W3110 is produced by a controlled submerged fermentation of a selected, pure culture of *E. coli* W3110. The production process includes the fermentation process, recovery (downstream processing) and formulation of the product. A flow sheet of the different steps involved is given in Annex 1.

4.2. Raw Materials

All raw materials meet predefined quality standards that are controlled by the Quality Assurance Department of MCI. The raw materials used for the fermentation and recovery of the product are suited for the intended use; this leads to the required safety status of the product. The safety is confirmed by the toxicological studies performed (see Section 7.4 of this dossier). The raw materials used for the formulation are of food grade quality and meet FCC specifications. The antifoam and flocculent are listed in the FDA September 11, 2003 letter to ETA as acceptable for use in enzyme manufacturing.

4.3. Fermentation Process

Biosynthesis of Matsurase FE occurs during the main fermentation. To produce the enzyme of interest, a submerged, aerobic fed batch fermentation process is employed, using a stirred tank fermentor. The fermentor is equipped with devices to measure for pH, temperature, oxygen and antifoam control, a top-mounted mechanical agitator and a bottom air sparger.

All fermentation equipment is carefully designed, constructed, operated, cleaned, and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and to confirm strain identity.

The fermentation process consists of three steps: inoculum preparation, seed fermentation and main fermentation. The whole process is performed in accordance with Good Food Manufacturing Practice (see Section 5.2).

Growth of the production organism and increase of enzyme production are checked at the end of the main fermentation by analysis of aseptically collected samples. After the fermentation has been stopped downstream processing will start.

4.4. Recovery Process

The enzyme is extracted from cell material by proper extraction method, and cell material is separated from the enzymes by means of a filtration process. A flocculent and filter aid is used to facilitate the process. Subsequently, the remaining particles are removed with filtrations.

4.5. Formulation and Standardization Process

The purified concentrate is stabilized with sugar or sugar alcohol and standardized at an enzyme activity more than 500 FEU/g and subsequently stored at 4 – 10° C.

4.6. Quality Control of Finished Product

The final Matsurase FE from *E. coli* K-12 W3110 meets the following specifications:

Parameter	**Specification limit**
Lead	Not more than 5 ppm
Total Coliforms	Not more than 10,000/g
Salmonella	Absent in 25 g
Escherichia coli	Absent in 25 g
Parameter	**Specification**
Appearance	Liquid or Powder
Matsurase FE activity	More than 500 FEU/g as liquid

5. COMPOSITION AND SPECIFICATIONS

5.1. Composition

The common starting material of Matsurase FE preparations before formulation is the ultra-filtration concentrate. Apart from the enzyme complex, the Matsurase FE preparations will also contain some substances derived from the microorganism and the fermentation medium. These harmless impurities consist of polypeptides, proteins, carbohydrates and salts. Since the enzyme is purified by filtrations, the amount of impurities derived from the micro-organism and the fermentation medium is very low.

The Total Organic Solids (TOS) of the Matsurase FE preparations and the Matsurase FE activities were determined for three different batches of the ultra-filtration concentrate:

Calculation of the TOS / 100 g

Batch number	Activity (U/g)	Ash (%)	Water (%)	TOS (%)	Activity/mg TOS (FEU/mg)
3T270116	637	0.10	97.94	1.96	32.5
3T270118	641	0.10	98.00	1.90	33.7
3T270120	664	0.09	97.95	1.96	33.9
MEAN	647.3	0.097	97.96	1.94	33.4

5.2. General Production Controls and Specifications

Quality standards require a strictly controlled fermentation process. Enzyme fermentation experience with the MCI in Japan has resulted in a solidly established Good Food Manufacturing Practice within the framework of a certified ISO 9000 system.

5.2.1. Technical Measures

The batches of primary seed material are prepared, preserved and stored in such a way that contamination and degeneration is avoided and genetic stability is secured. The vials are clearly labeled and strict aseptic techniques are applied during the recovery of the culture.

The fermentor is a contained system. Prior to inoculation, the fermentor is cleaned, rinsed and sterilized. Membrane valves, air filters and seals are regularly checked, cleaned and replaced if necessary. Only sterilized air is used in the fermentation. The sterilized nutrient medium and the complete biomass broth are transferred aseptically to the main fermentor. The methods used effectively prevent microbial contamination during fermentation. The preparation of sterile media and the cleaning of the equipment are described in Quality Assurance documents and are strictly followed.

Microbial contamination is prevented during downstream processing by several germ reduction filtrations. The filters are thoroughly cleaned before each production run.

5.2.2. Control Measures

After preparation of a new batch of primary seed material, samples are checked for identity, viability and microbial purity. If these parameters are correct, the strain is tested for production capacity. Only if the productivity and the product quality meet the required standards will the new batch of primary seed material be accepted for further production runs. Each time a vial from such a certified batch of primary seed material is used for production, the viability, purity and identity of the strain is checked.

The raw materials used for the fermentation and recovery of the product are suited for the intended use leading to the required safety status of the product. The raw materials meet redefined quality standards that are controlled by the Quality Assurance Department of MCI. The raw materials used for the formulation are of food grade quality, and meet FCC specifications.

At regular intervals during the seed fermentation, samples are taken aseptically for analysis of pH, and microbiological quality in the laboratory.

During the main fermentation, the dissolved oxygen content, pH, temperature, viscosity and microbial quality are monitored. If microbial controls show that significant contamination has occurred, the fermentation will be discontinued.

Also during downstream processing, samples are taken and checked for the level of microbial contamination. If these checks show that significant contamination has occurred, the downstream processing is discontinued.

The final product is subjected to extensive controls and complies with MCI specifications. See Section 4.6: Quality Control of Finished Product.

6. APPLICATION

6.1. Mode of Action

Matsurase FE epimerizes D-fructose on C3 position to D-allulose (Kim, 2006; Mu, 2011; Zhang, 2013; Jia, 2014; Zhang, 2015; Benesford, 2001). It also epimerizes ketohexoses, ketopentoses, and ketotetroses to respective keto sugars. Matsurase FE exhibited highest activity with D-allulose which is about 2-3 times higher than that with D-fructose.

6.2. Application

The Matsurase FE described in this dossier is applied mainly for the production of D-allulose. D-allulose is a low calorie sweetener, and acts as postprandial blood glucose regulator. D-allulose produced with Matsurase FE is intended to be used in a wide range of food and beverage applications, such as cereals, chewing gum, confections & frostings, dressings for salads, jams & jellies, sugar, sugar substitutes (carrier), and various low- calorie or dietetic foods including low-calorie, reduced-calorie, sugar-free beverages (non-alcoholic), cereals, frozen dairy desserts (ice cream, soft serve, sorbet), yogurt and frozen yogurt, gelatins, pudding & fillings, hard candies, soft candies, and sweet sauces & syrups.

6.3. Use Levels

Enzyme preparations are used in the amount which is needed. Matsurase FE can be used for isomerization of fructose to produce allulose. The average dosage of the enzyme depends on the process conditions and on the desired properties of the final product.

A typical use level would be 10,000 – 30,000 FEU per kg allulose dry matter, or 20- 60 g of Matsurase FE preparation described in this dossier per kg allulose dry matter.

6.4. Enzyme Residues in the Final Food

Allulose production enzyme is naturally present in microorganisms such as an *Athrobacter globiformis, Agrobacterium tumefaciens, Clostridium cellulolyticum, and Clostridium scidens*. It would appear that the enzyme Matsurase FE is a common in nature.

The substrate for Matsurase FE is D-fructose which is abundant in nature. D-fructose is epimerized to the product D-allulose. D-allulose can be found in natural products such as fruits and daily food in very minute amounts (Oshima, 2006).

It can be concluded that the substrate is a natural ingredient found in all plant foods, and the enzymatic conversion by Matsurase FE creates a reaction product which is also a constituent of the human diet in small amounts. Hence, there is no basis to believe that the use of Matsurase FE will have a significant effect on processed foods or on the human body.

In all applications, after the conversion of D-allulose or other keto-sugars, the reaction mixture is used as processing aids and ultra-filtered. Therefore, no enzyme activity is present in the finished product.

Even if all enzyme used in the reaction carried to the final product as worst case scenario, based on the information given in Sections 1.2.2, 5.1, GRN 498, and its Response letter, the following calculations can be made:

Final food	**Enzyme use levels in food ingredient**	**Maximum amount of allulose in final food**	**Maximum residual amount of enzyme in final food**	**Maximum amount of TOS in final food**
	FEU/kg allulose dm	**%**	**FEU/kg**	**mg TOS/kg**
Beverages (non-alcoholic), low calorie, reduced calorie, sugar-free	10,000 – 30,000	3.5	1,050	31.43
Cereals, regular Cereals, low calorie, reduced calorie, sugar-free	10,000 – 30,000 10,000 – 30,000	5 2	1,500 600	44.91 17.96
Chewing gum	10,000 – 30,000	50	15,000	449.10
Confections & Frostings	10,000 – 30,000	5	1,500	44.910
Frozen dairy desserts (ice cream, soft serve, sorbet), low calorie, reduced calorie, sugar-free	10,000 – 30,000	5	1,500	44.910
Yogurt and frozen yogurt, low calorie, reduced calorie, sugar-free	10,000 – 30,000	5	1,500	44.910
Dressings for salads	10,000 – 30,000	5	1,500	44.910
Gelatins, pudding & fillings, low calorie, reduced calorie, sugar-free	10,000 – 30,000	10	3,000	89.82
Hard Candies, low calorie, reduced calorie, sugar-free	10,000 – 30,000	50	15,000	449.10
Soft Candies, low calorie, reduced calorie, sugar-free	10,000 – 30,000	25	7,500	224.55

Jams & Jellies	10,000 – 30,000	10	3,000	89.82
Sugar	10,000 – 30,000	10	3,000	89.82
Sugar substitutes	10,000 – 30,000	100	30,000	898.20
Sweet sauces & syrups, low calorie, reduced calorie, sugar-free	10,000 – 30,000	10	3,000	89.82

6.4.1. Possible Effects on Nutrients

The enzyme is intended for use a processing aid to manufacture allulose. The allulose, and not the enzyme, are intended for use in final food products. A minimal residual amount of enzyme, if any, is anticipated in the final food, and no relevant nutritional effects are foreseen. Further, digestibility studies have suggested that the enzyme will be degraded by human digestion enzymes. MCI analyzed Matsurase FE by ExPASy Peptide Cutter[2] to show its digestibility. A search conducted with Matsurase FE's amino acid sequence to check digestibility by chymotrypsin and trypsin was performed. The results of the analysis, available in Annex 4, found there were many places that chymotrypsin and trypsin can cut to digest Matsurase FE. Therefore, any residual enzyme that is ingested is not expected to have any enzymatic activity.

[2] http://web.expasy.org/peptide_cutter/

7. SAFETY EVALUATION

7.1. Safety of the Production Strain

The safety of the production organism is paramount to assessing the probable degree of safety for enzyme preparations to be used in food production. According to the IFBC, food or food ingredients are safe to consume if they have been produced according to current Good Manufacturing Practices, from a nontoxigenic and nonpathogenic organism (Coulston and Kolbye, 1990). A nontoxigenic organism is defined as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances" (Pariza and Foster, 1983)

In 1990, FDA affirmed as GRAS the chymosin enzyme preparation derived from *E. coli* K-12 (21 CFR 184.1685; Flamm, 1991). The safety of chymosin preparation was primarily based on published evidence that *E. coli* K-12 has been used as a laboratory organism for over 30 years without reported incidents of infection and that it does not produce toxins that cause illness by ingestion, such as Shiga-like toxin produced by certain toxigenic strains of *E. coli*.

E. coli K-12, from which the production strain is derived, is one of the most extensively studied bacteria, its genome was sequenced in 1997 (Blattner et al., 1997). *E. coli* K-12 has a history of safe use in the production of specialty chemicals and human drugs and was exempted from EPA review under TSCA (EPA, 1997).

Moreover, review of the scientific literature reveals no evidence of the *E. coli* K-12 being a pathogen for animals or humans, and in fact has frequently been a model of non-pathogenicity in studies aimed at understanding the mechanism of pathogenic strains of *E. coli* (Metzgar, 2001; Blanc-Potard, 2002; Nelson, 2000). Further, research has shown that E. coli K12 W3110 does not survive in water or soil, rapidly decreasing in population counts due to bacterial death, suggesting there is no risk for persistence of the organism outside of the culture media (Bogosian, 1996). As a consequence, it can be concluded that *E. coli* K-12 can be regarded as non-pathogenic. Based on the above, it can be concluded that the Matsurase FE producing *Escherichia coli* K-12 W3110 is non-toxigenic, and presents no risk to the public through its use in producing the enzyme..

7.2. Safety of the Matsurase FE enzyme

Consumer safety of enzyme preparations is determined usually by three variables: the producing organism, the raw materials used in the production process and the production process itself. In certain cases the enzyme might be of concern as well.

The safety of the production process is embedded in current Good Manufacturing Practice (cGMP) and Hazard Analysis of Critical Points (HACCP). The raw materials used for the production of Matsurase FE meet predefined quality standards set by the FCC that are controlled by the Quality Assurance Department of MCI (see also 5.2).

The enzyme of Matsurase FE produced by E. coli K-12 W3110 has the IUBMB number 5.1.3.30. As described in 7.1, Matsurase FE produced by E. coli K-12 W3110 has already been used for food production for several decades. Since it is generally accepted that the commercial enzyme preparation Matsurase FE of E. coli K-12 W3110 is not toxic, and since it is a natural constituent of many organisms (see Section 6.4), it is not expected that Matsurase FE would have any toxic properties.

7.2.1. Allergenicity

Although virtually all allergens are proteins, only a small percentage of all dietary proteins are food allergens. Enzymes have a long history of safe use in food. Because of the direct, catalytic function of enzymes as processing aids, exposure of the enzyme associated with ingestion is typically very low and residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system (Grimble, G.K., 1994). MCI is unaware of the existence of any reports on sensitization to enzyme products in the final commercial food after ingestion.

The absence of food enzyme allergenicity has been confirmed by an extensive literature search and survey of producers' files of scientific information by Association of Manufacturers and Formulators of Enzyme Products (AMFEP). In the report generated by the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food (Annex 5), the expert panel concluded that the use of enzymes in food products do not present any unacceptable risk to consumers, and there is no scientific evidence to suggest that the small amounts of enzyme used in food will sensitize consumers or trigger allergic reactions. Even among people who ingest high daily doses of enzymes as digestive aids for many consecutive years, there are no reports of gastrointestinal allergy to enzymes. Recently, it was concluded that ingestion of food enzymes in general is not considered to be a concern with regards to food allergy (Bindslev-Jensen, C. *et al.*, 2006).

To further understand the potential risk of an allergic response from ingestion of Matsurase FE from *E. coli* K-12 W3110, the sequence of the enzyme was analyzed with an allergen sequence database, Allergen Database for Food Safety (ADFS).[3]

The search was conducted to determine if Matsurase FE's amino acid sequence matched known allergen sequence through the ADFS website. Searches were held with 8 consecutive amino acid sequence match, and 80 amino acid sliding window homology searches of more than 35%. As a result, there were no 8 consecutive amino acid sequence match and 80 amino acid sliding window search match through the site.

With regard to allergenicity of the fermentation media, Matsutani has concluded that the public data and information allow it to conclude that there is no published or unpublished data that suggest there is an allergen causing protein from the fermentation media in the finished enzyme product. Matsutani relies on the public statement released by the Enzyme Technical Association in 2005, following a survey of its members and scientific literature search of data regarding fermentation medium and allergenicity. The statement concludes that no allergens protein from the fermentation medium has been found in the finished enzyme, and states that regulatory

[3] Available at: http://allergen.nihs.go.jp/ADFS/index.jsp

bodies in both the EU and Japan have concluded that enzyme preparations do not pose an allergen risk that would require allergen labeling on the final product. Further, ETA points out that the typical manufacturing process of enzyme preparations includes a step to separate the biomass and fermentation media from the enzyme. This step ensures the enzyme product purity and stability, and would likely remove most proteins present in the fermentation media. A copy of the public statement from the ETA website is attached in Annex 6.

In addition, the Food Allergy Research and Resource Program (FARRP) issued a paper in August of 2013 which concluded similarly that the fermentation medium presented no risk to the public health. The paper points to the fact that the fermentation media is consumed during the enzymatic process. It is clear that any *de minimis* amount of fermentation media protein that survived the fermentation process will not cause a significant public health risk to the consumer. Consistent with the findings of ETA, FARRP also points to the fact that the proteins would likely be removed during the filtration of the enzyme product. Importantly, FARRP notes that at this time, there is no reliable assay that could be used to detect the presence of most allergen proteins in the final enzyme products, as the proteins would likely be degraded fragments that would not reach levels of quantitation available with current commercial ELISA assays. The full August 2013 statement is provided in Annex 7.

7.3. Safety of the Manufacturing Process

Matsurase FE preparation is produced in accordance with current Good Manufacturing Practices, using ingredients that are food grade, under conditions that ensure a controlled fermentation and are subject to testing to assure the enzyme product meets the stated specification. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

7.4. Safety Studies

This section describes the studies performed to evaluate the safety of Matsurase FE preparations produced with *E.coli* K-12 W3110. All safety studies were performed according to internationally accepted guidelines (OECD) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

7.4.1. Summary of Safety Studies

The following studies were performed with the Matsurase FE enzyme preparation:

- Sub-chronic oral toxicity study
- Stability of test substance
- Haemotology
- Pathology
- Bacterial reverse mutation test (Ames test)
- *In vitro* micronucleus test in cultured human lymphocytes

The safety studies for the *E.coli* K-12 W3110 Matsurase FE were performed with the same representative batch of unformulated UF concentrate, batch number 3T-2, containing 25,269 FEU/g and 89.4% TOS.

7.4.2. Results of the Safety Studies

A sub-chronic (13 week) oral toxicity study, stability of test substance, bacterial reverse mutation test, and *in vitro* micronucleus test in cultured human lymphocytes with the Matsurase FE was conducted at TNO, The Netherlands, in order to assess the toxicity potential of the test substance Matsurase FE.

A sub-chronic (13 week) oral toxicity study

The study was conducted in accordance with the following guidelines:

- OECD Guideline for the Testing of Chemicals 408. Repeated dose 90-day oral toxicity study in rodents, adopted 21st September 1998.
- B.26. Subchronic oral toxicity test. Repeated dose 90-day oral toxicity study in rodents. Annex 5D to Commission Directive 2001/59/EC, Official Journal of the European Communities, L225, 21.8.2001.

The study comprised four groups of 10 male and 10 female Wistar rats. One control group was kept on cereal based (VRF1 (FG)) diet. Three test groups received the test substance at dietary levels of 0.5%, 1.0% and 2.0% mixed with the VRF1 (FG) diet for 13 weeks. These dietary levels provided an overall mean intake of the test substance in the low, mid- and high-dose group of 0.3, 0.6 and 1.1 (males) or 1.3 (females) g/kg body weight/day, respectively.

The following parameters were evaluated in all animals:

- General clinical observations
- Neurobehavioral testing (detailed clinical observations, FOB and motor activity)
- Ophthalmoscopic examination
- Body weight
- Food consumption
- Hematology
- Chemical chemistry
- Urinalysis
- Organ weights of principal organs
- Macroscopic examination
- Histopathology of organs (control and high-dose group)
- Histopathology of all lesions

Results

There were no treatment-related clinical signs. Neurobehavioral observations and motor activity assessment did not indicate any neurotoxic potential of the test substance. Ophthalmoscopic examination did not reveal any treatment-related ocular changes. There were no treatment-related changes in body weight, feed intake or water intake. Hematology, conducted on all rats at necropsy, did not reveal any relevant changes in red blood cell variables, clotting potential or in total and differential white blood cell counts. Clinical chemistry was conducted on all rats at necropsy. No treatment-related effects were observed. Urinalysis was conducted on all rats in the

week prior to necropsy. The renal concentrating ability was not affected; the urinary density and volume were comparable between all groups. Semi-quantitative (dipstick) urinary measurements and microscopic examination of the urinary sediment did not reveal any treatment-related effects. There were no treatment-related differences in absolute organ weights or in organ-to- body weight ratios. Macroscopic examination at necropsy and microscopic examination of organs and tissues did not reveal any treatment-related findings.

Because Matsurase FE did not induce any adverse changes in any test group, the no-observed-adverse-effect level (NOAEL) was placed at the highest level tested, namely 2% diet (≥1.1 g/kg body weight/day).

Bacterial reverse mutation test (Ames test)

The objective of this study was to provide data on the potential mutagenicity of Matsurase FE, in four selected strains of Salmonella typhimurium, TA 1535, TA 1537, TA 98 and TA 100, and in the Escherichia coli mutant WP2 uvrA, in both the absence and presence of a metabolic activation system (S9-mix).

This study was conducted in accordance with the following guidelines:
· OECD guideline no. 471, Genetic Toxicology: Bacterial Reverse Mutation Test, adopted 21 July 1997

Results

One independent test was performed, which included two experiments. The experiment was repeated with strain TA 1535, because in the first experiment the negative control was outside acceptable range. For both experiments, a suspension of 50 mg/ml of the test substance in DMSO was prepared; this resulted in a homogeneous, turbid, brown suspension. Five concentrations (serial dilutions in DMSO) of the test substance ranging from 62 to 5000 µg/plate were tested.

Negative controls (vehicle) and positive controls were run simultaneously with the test substance. The test substance was not toxic to any strain, in both the absence and presence of S9-mix, as neither a dose related decrease in the mean number of revertants nor a clearing of the background lawn of bacterial growth compared to the negative controls was observed.

For both experiments, a precipitation of the test substance on the agar plates was observed at and above 1667 µg/plate. Precipitation of the test substance was observed at 5000 µg/plate with the unaided eye.

In all strains tested, in both the absence and presence of S9-mix, the test substance did not induce a more than 2-fold and/or dose related increase in the mean number of revertant colonies compared to the background spontaneous reversion rate observed with the negative control.

It is concluded that the results obtained in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, and in the Escherichia coli strain WP2 uvrA, in the absence and presence of

the S9-mix, indicate that the test substance Matsurase FE is not mutagenic under the conditions used in this study.

***In vitro* micronucleus test in cultured human lymphocytes**
The purpose of this study was to determine the potential of the test substance Matsurase FE to induce micronuclei in vitro in binucleated human lymphocytes.

The *in vitro* micronucleus test was used for the detection of chemicals that induce the formation of small membrane-bound DNA fragments such as micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. The assay thus has the potential to detect the activity of both clastogenic and aneugenic chemicals. The actin polymerization inhibitor cytochalasin B, added during the target mitosis, allows the identification of nuclei that have undergone one division as binucleates. At predetermined intervals after treatment, the cells are harvested, fixed and dropped onto microscopic slides. After staining, the slides are analyzed microscopically for the presence of micronuclei in binucleated cells.

The study plan was conducted in accordance with the following guideline:
· OECD guideline 487 for the testing of chemicals: *In Vitro* Mammalian Cell Micronucleus Test (MNvit); adopted 26 September 2014

From the results obtained in the in vitro micronucleus test it is concluded that, under the conditions used in this study, the test substance Matsurase FE was not clastogenic and/or aneugenic to cultured human lymphocytes.

7.4.3. Conclusion

- Summarizing the results obtained from the several toxicity studies the following conclusions can be drawn: Based on the results of sub-chronic oral toxicity study the No Observed Adverse Effect Level (NOAEL) of the Matsurase FE is 1.1 g/kg body weight/day (1.02 g TOS/kg body weight/day) for males which corresponds to 27,786 FEU/kg body weight/day, whereas 1.3 g/kg body weight/day for females, corresponds to 32,838 FEU/kg body weight/day.

- Taking together the results of these safety studies, Matsurase FE produced with *E. coli* K-12 W3110 is considered safe when manufactured with Good Manufacturing Practices.

7.5. Estimates of Human Consumption and Safety Margin

The Estimated Daily Intake (EDI) was calculated with Sections 6.4 and GRN 498 (Exponent exposure assessment)

7.5.1. Estimated Daily Intake with 2+ years age group

*Calculated with a person weighing 60 kg

Final food	Maximum residual amount of enzyme in final food (FEU/kg)	Maximum amount of TOS in final food (mg TOS/kg)	90th percentile intake level (g food/person/ day)	Maximum Estimated Daily Intake of enzyme (FEU/kg bw/day)*	Maximum Estimated Daily Intake of TOS (mg TOS/kg bw/day)*
Beverages (non-alcoholic), low calorie, reduced calorie, sugar-free	1,050	31.43	39.4	0.6895	0.0206
Cereals, regular Cereals, low calorie, reduced calorie, sugar-free	1,500 600	44.91 17.96	NA 3.2	NA 0.0320	NA 0.00164
Chewing gum	15,000	449.10	3.5	0.8750	0.04498
Confections & Frostings	1,500	44.910	1.3	0.0325	0.00167
Frozen dairy desserts (ice cream, soft serve, sorbet), low calorie, reduced calorie, sugar-free	1,500	44.910	5.5	0.1375	0.00707
Yogurt and frozen yogurt, low calorie, reduced calorie, sugar-free	1,500	44.910	8.9	0.2225	0.01144
Dressings for salads	1,500	44.910	2.0	0.0500	0.00257
Gelatins, pudding &	3,000	89.82	12.1	0.6050	0.03110

fillings, low calorie, reduced calorie, sugar-free					
Hard Candies, low calorie, reduced calorie, sugar-free	15,000	449.10	NA	NA	NA
Soft Candies, low calorie, reduced calorie, sugar-free	7,500	224.55	4.5	0.5625	0.02891
Jams & Jellies	3,000	89.82	2.2	0.1100	0.00565
Sugar	3,000	89.82	3.7	0.1850	0.00951
Sugar substitutes	30,000	898.20	4.5	2.2500	0.11565
Sweet sauces & syrups, low calorie, reduced calorie, sugar-free	3,000	89.82	4.0	0.2000	0.01028
Total	-	-	-	-	0.29111

7.5.2. Margin of Safety

The Margin of Safety for human consumption can be calculated by dividing the NOAEL by the EDI. As was shown in Section7.4, the 90-day oral toxicity study showed a NOAEL of 1.02 g TOS /kg Body Weight, corresponding with 0.29111 mg TOS/kg Body Weight/day. Consequently, the Margin of Safety is 3,504.

8. GRAS Conclusion

As documented in this notification, the strain *E. coli* K-12 W3110 has been evaluated on its safety (see also 7.1), and it was concluded that the strain is non-pathogenic and non-toxicogenic. The safety studies described in Section 7.4 of this dossier support the fact that the produced enzyme does not exhibit any toxic effects.

Therefore, consistent with the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36 (see 62 Fed. Reg. 18,938, April 17, 1997), MCI has determined that its Matsurase FE enzyme originated from *Arthrobacter globiformis* M30, expressed in *Escherichia coli* K-12 W3110 is a GRAS substance for the intended food applications and is exempt from the requirement for premarket approval.

9. LIST OF ANNEXES

Annex 1: Flow Chart of Manufacturing Process.

Annex 2: Amino acid sequence of the Matsurase FE from *Escherichia coli* K-12 W3110.

Annex 3: Determination of Matsurase FE activity.

Annex 4: Results of ExPASy Peptide Cutter Analysis

Annex 5: AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food

Annex 6: ETA Statement on the Allergenicity of Fermentation Media

Annex 7: Food Allergy Research and Resource Program Statement on the Allergenicity of Fermentation Media

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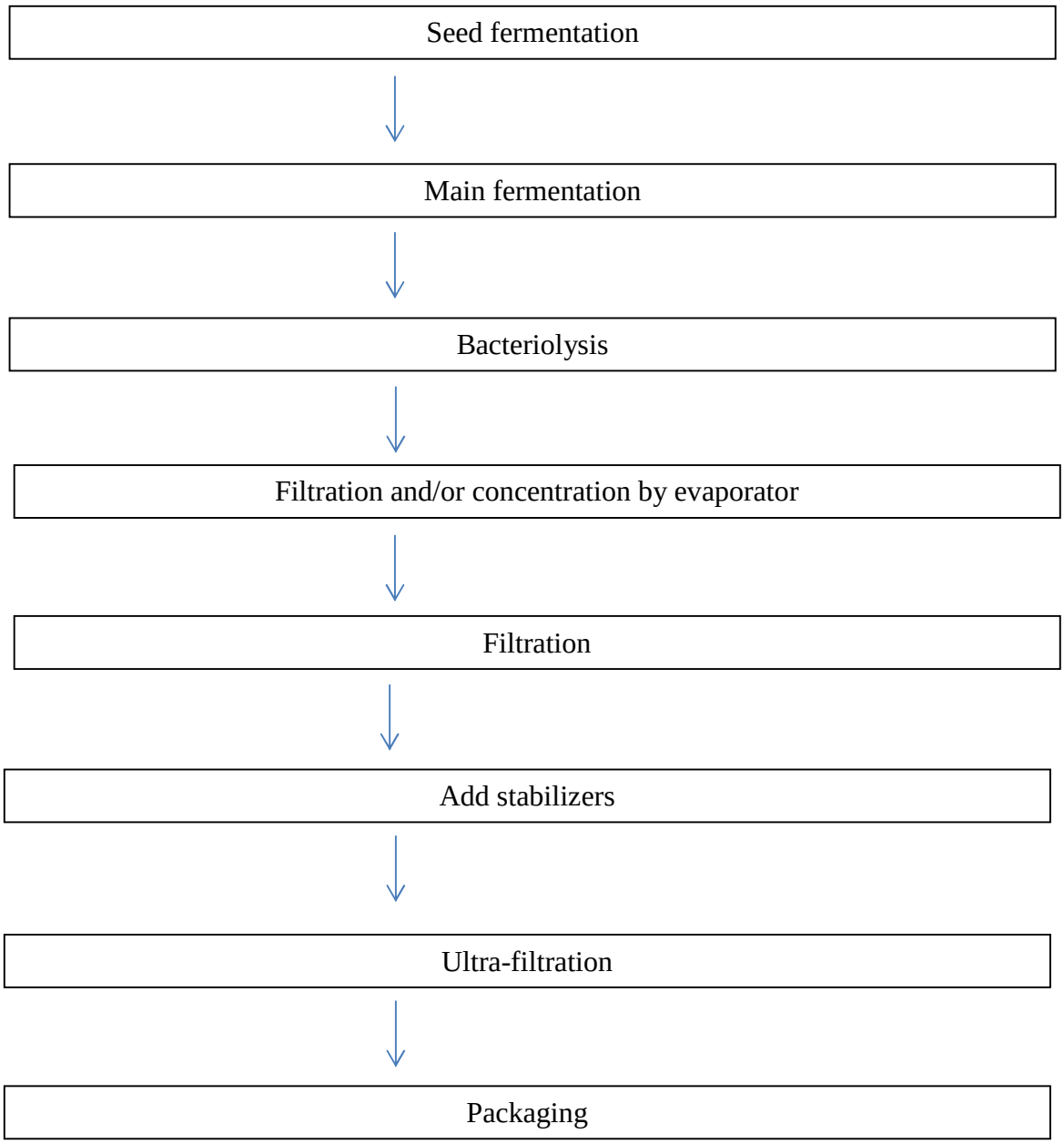
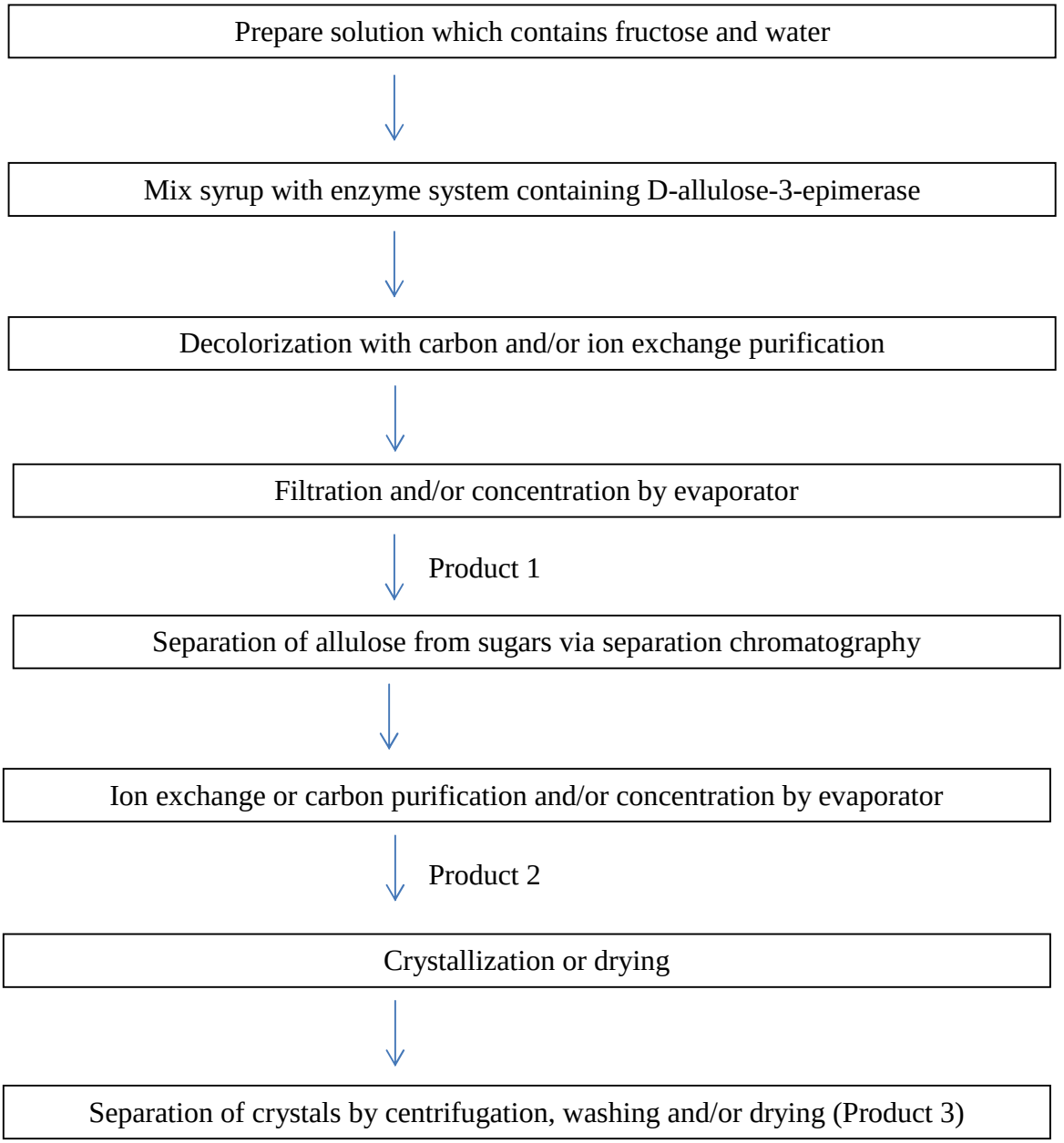
Annex 1

Flow Chart of Matsurase FE Manufacturing Process



Flow Chart of Allulose Manufacturing Process



Annex 2

Amino acid sequence of the Matsurase FE from *Escherichia coli* K-12 W3110

MKIGCHGLVWTGHFDAEGIRYSVQKTREAGFDLVEFPLMDPFSFDVQTAKSALAEHGL

AASASLGLSDATDVSSEDPAVVKAGEELLNRAVDVLAELGATDFCGVIYSAMKKYMEP

ATAAGLANSKAAVGRVADRASDLGINVSLEVVNRYETNVLNTGRQALAYLEELNRPNL

GIHLDTYHMNIEESDMFSPILDTAEALRYVHIGESHRGYLGTGSVDFDTFFKALGRIGYD

GPVVFESFSSSVVAPDLSRMLGIWRNLWADNEELGAHANAFIRDKLTAIKTIELH

Annex 3

Determination of Matsurase FE Activity

Method :

Reagents:

A) 50 mM Sodium Phosphate buffer (pH 8.0)

B) 0.2 M D-allulose solution with distilled water

C) 10mg of Matsurase FE diluted with 990 Micro litter 50mM Sodium Phosphate buffer (pH 8.0)

D) 1 M MgCl solution with distilled water

Procedure:

1. Pipette 2 μl of Reagent D, 100 uL of Reagent C into a screw capped test tube.
2. Add 398 μl Reagent A into the test tube.
3. Add 500 μl of Reagent B into the test tube to mix and start its reaction at 50 degree C for 10 min.
4. Put the test tube into boiled water bath immediately for deactivation.
5. Desalting reaction mixture with ion exchange resins.
6. Analyze with HPLC to measure D-Fructose conversion.

Calculation:

Activity can be calculated by using the following formula:

Enzyme activity (U/g) = S x **ΔF** /100 / t / Vs

S : Substrate in the reaction mixture (100 μmole)*

ΔF : Amount D-fructose formed (%)

t : Reaction time (10 minutes)

Vs : Sample volume (0.001 g)

For example, if D-Fructose were made 6.1%,
0.2M D-allulose/500 μl contains 100 μmole of D-allulose
100 x 6.1 / 100 / 10 / 0.001 = 610 U/g

1 unit (U) is the amount of enzyme that catalyzes the reaction of 1μmole of substrate per minute.

Annex 4

12/15/2015

Matsutani Chemical Industry Co. Ltd.

R&D

Analysis of Matsurase FE at ExPASy Peptide Cutter

Objective: Analyze Matsurase FE by ExPASy Peptide Cutter (URL: http://web.expasy.org/peptide_cutter/) to show its digestibility.

Method:

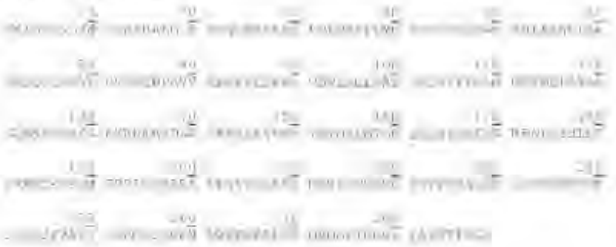
Search with Matsurase FE's amino acid sequence to check digestibility by chymotrypsin and trypsin. The search was held on 15th day of December, 2015.

Result:

As a result (See Figure below), there were many places that chymotrypsin and trypsin can cut to digest Matsurase FE.

PeptideCutter

The sequence to investigate:



The sequence is 289 amino acids long

Available enzymes

The enzyme(s) that you have chosen:

- Chymotrypsin-low specificity (C-term to [FYWML], not before P)
- Pepsin (pH1.3)
- Trypsin

You have chosen to display all possible cleaving enzymes.

These enzymes cleave the sequence:

Name of enzyme	No. of cleavages	Positions of cleavage sites
Chymotrypsin-low specificity (C-term to [FYWML], not before P)	69	1 6 8 10 13 14 21 31 33 38 39 42 44 53 56 58 64 66 86 87 94 97 102 107 110 113 114 122 139 145 151 156 163 165 168 169 174 177 178 181 183 190 191 195 201 203 205 210 213 214 221 224 225 228 233 239 242 251 254 255 258 261 262 268 271 275 280 288 289
Pepsin (pH1.3)	68	7 13 14 30 31 32 33 36 37 41 43 44 53 57 63 64 65 66 85 86 87 93 94 96 97 101 102 121 122 138 139 144 145 155 156 163 165 166 168 169 174 177 178 190 195 200 201 214 220 221 223 224 225 227 238 239 241 242 251 254 260 267 268 274 275 280 287 288
Trypsin	23	2 20 25 27 50 81 89 111 112 126 131 135 150 160 202 211 226 230 253 259 277 279 284

These are the cleavage sites of the chosen enzymes and chemicals mapped onto the entered protein sequence:

Annex 5

WORKING GROUP ON CONSUMER ALLERGY RISK FROM ENZYME RESIDUES IN FOOD

AMFEP	*Members*
Thierry Dauvrin	**Frimond**
Gert Groot	**Gist-brocades**
Karl-Heinz Maurer	**HenkelCognis**
David de Rijke	**Quest International**
Henning Ryssov- Nielsen	**Danisco Ingredients**
Merete Simonsen	**Novo Nordisk**
Torben B. Sorensen (chairman)	**TBS Safety Consulting ApS**

Copenhagen, August 1998

Contents

Summary

In recent years, claims have been made by the media and some consumer organisations that enzyme residues in bread and other foods can result in allergic responses in the consumers of that food.

AMFEP established an Expert Group to evaluate whether residual enzymes in foods are an allergy risk for consumers. The Expert Group was asked to investigate existing scientific data and to report the results of the findings.

The main questions were whether enzymes in, for example, bread can sensitise a consumer of the bread, and subsequently if the presence of the enzyme residue could induce symptoms of allergy.

A further question was if a person with existing allergy to common allergens could develop allergy symptoms upon eating foods containing residual enzymes by cross reaction. This is not uncommon in the case of food allergy.

The literature survey was made to search for general food allergy, epidemiology and to find cases of food related enzyme allergy. In addition a survey of enzyme producers' files was carried out to look for adverse reactions to food enzymes.

High daily doses of industrial enzymes in are prescribed for patients with insufficient function of the pancreas. The literature on adverse events was reviewed and telephone interviews were undertaken with authorities and university hospital departments to check if experience of enzyme related gastrointestinal allergy were observed but not published.

Studies of common food allergy indicate a relatively low prevalence of about 2% of populations in Europe and the United States. There is however, a significant discrepancy between the perception of being allergic to foods (15%) and those that can be verified as food allergy (2%).

Yet, there are no firm data of the doses required to sensitise a person via the gastrointestinal tract, but the doses required to induce sensitisation seem to be very high. Indeed, patients with insufficient enzyme production of the pancreas need to take industrial enzymes in doses 100.000 - I million times higher than the amounts found in food.

There are no published cases of people that have been sensitised by the ingestion of food with residual enzymes, and even people who ingest high daily doses of enzymes as digestive aids are not reported to have gastrointestinal allergy to enzymes, even after many years of daily intake.

There are a few case histories of people who had reactions to papain, extracted from the papaya fruit. Papain in powder form is used as a meat tenderiser in some countries. It is unclear if the sensitisation in these cases occurred by inhalation of the powder or by ingestion of the meat with the papain.

One case history described a person who reacted with hay-fever upon eating a lactase tablet. This case was incomplete in describing the possible source of sensitisation.

There are 2 cases of people with baker's asthma and allergy to α-amylase, and wheat flour who developed symptoms after the ingestion of bread. The symptoms were somewhat more pronounced after bread prepared with α-amylase than bread without. One case with occupational allergy to α-amylase reacted upon ingestion of a very high test-dose of pure α-amylase, but not at lower doses. Four other persons with occupational α-amylase allergy did not react at any dose.

The question of cross reactions between common moulds and enzymes produced in related moulds was described in a double blind placebo controlled food challenge study of asthma patients with allergy to Aspergillus fumigatus. This mould is closely related to *Aspergillus oryzae* and - *niger* which are used for the production of industrial α-amylase. None of the test persons could be challenged to elicited symptoms by eating bread prepared with enzymes.

The expert group concludes that there are no scientific indications that the small amounts of enzymes in bread and other foods can sensitise or induce allergy reactions in consumers.

Employees with respiratory occupational enzyme allergy should be informed that in rare cases, symptoms may be induced by ingestion of food with residual enzymes. Enzyme residues in bread or other foods do not represent any unacceptable risk to consumers.

1.0. Introduction

Since the late 80'ies, and particularly since 1992 it has been repeatedly claimed that enzyme residues in foods may represent a hazard to consumers in the form of allergies, and that a certain percentage of the population are at risk of having allergicy reactions to enzymes in bread and other foods.

In particular it has been claimed that consumers were at risk of developing severe allergy symptoms caused by α-amylase. The public was somewhat alarmed and there have been complaints, questions and other reactions of concern to bakers and other suppliers.

The media's interest was based on results from a study by Schata[1], published only as a 1/2-page abstract which does not allow for scientific evaluation.

However the issue was effectively raised within the public, and industry had no data with which to make a response.

Since 1992, the issue of allergy risk in consumers have emerged from time to time on television in the TV and the printed media. The general issue as it has emerged over these years is that there is a concern in the public that enzymes are unsafe, and as far as the bakers and the flour improvers are concerned, require and request data to oppose the allegations.

An additional concern is the possible cross reaction between enzymes produced by fermentation of certain moulds which may be related to common moulds. In theory, a person with a preexisting allergy to *Aspergillus sp.* might react to enzymes from e.g. *Aspergillus niger or A. oryzae*.

2.0 Background

2.1 General

In the public mind there is some confusion about the frequency of allergy, and in particular on food allergy. However, in the scientific community there seem to be consensus of the following:

- The frequency of common allergy (all allergies included) is 20 - 30%, in most populations around the world. The figure is increasing. Part of the increase may be due to higher awareness and improved diagnostic methods, however, a true increase cannot be ruled out.
- The frequency of occupational allergy in bakers is 8 - 27%. About 30 - 35%, of the bakers with occupational allergy to flour have an additional respiratory allergy to α-amylase and/or other baking enzymes.
- There is a reasonably good documentation of the frequency of food allergy in the general population at 1 - 2%. However, the frequency of perceived food allergy allergy in the general population is 12 - 16%
- Food allergy does not differ from inhalation allergies with regard to the biological mechanisms taking place in the immune system. Any 'true' allergy is based on allergy antibodies (IgE). Allergy antibodies are produced by the white blood cells called lymphocytes after the allergen has been introduced to these cells by inhalation or by ingestion. This process is called 'sensitisation'.
- Sensitisation then, is merely the event of the body recognising the foreign aller genic protein and reacting to it by producing allergy antibodies specifically recognising the particular allergen.
- Sensitisation is not a disease.
- It only becomes an allergic disease if the person develop symptoms related to exposure to the particular allergen.
- Not all sensitised people exhibit symptoms of allergy have allergy-symptoms.

2.2 Occupational respiratory allergy

allergy caused by inhalation of airborne particles of proteins, incl. Enzymes

Fungal enzymes, bacterial enzymes and extracted plant and animal enzymes are equally capable of inducing respiratory allergy - Papain and Bromelain[2-4], Trypsin[5], protease's from the skin yeast Candida albicans[6], from bacteria/ subtilisins[7,8], fungal amylases[9,10], bacterial amylases[11], fungal hemicellulases[12], lipases[13], xylanases and cellulases14,15 are all examples of industrial enzymes known to induce allergic sensitisation and respiratory occupational allergy. This is a feature characterised by highly purified enzyme protein products rather than the origin or the methods of production.

They all share the structural and biological properties that may cause sensitisation when inhaled.

The classical food allergens are also capable of inducing respiratory allergy when they are brought into a dust- or aerosol form and inhaled. Soya[16], eggs[17,18], milk[19] and fish[20] are just examples. Soya may be one of the best described examples of epidemic inhalation allergy to an allergen also well recognised as a food allergen[21].

3.0. Food allergy

3.1. Allergy caused by ingestion of proteins in foods

Eight percent of children under 3 years of age are allergic to food[22]. In, and in this age group, milk, egg, fish and soya are examples of common allergens. Many of these allergies disappear with age, but food allergy is seen also in older children and in adults. The overall frequency of verified food allergy is 1 - 2% of the population[22-25].

Food allergy is the adverse reaction to food characterised by allergic sensitisation to food proteins and elicitation of symptoms by ingestion of the same food proteins.

Symptoms

The symptoms of food allergy are gastrointestinal with vomiting and diarrhoea, sometimes accompanied by urticaria, asthma or hay-fever. Generalised very severe reactions occur in rare cases.

Many food allergies are very mild, with symptoms of itching and burning sensation in the mouth. This is also a feature of most of the well known cross-reactions between common inhalation allergens and foods. An example can be found in patients with a birch pollen allergy who also react to e.g. fresh apples, without having a specific allergy to apples. Another well known cross reaction is that of latex and bananas. There are a number of such cross reactions between common pollen allergens and certain foods.

Types of food allergens

Examples of 'true food allergens' are proteins in milk, egg, soya, wheat, fish, nuts and, peanuts and a few more. There are others, but only about 10 food allergens account for more than 95% of severe cases. However the list of food allergens is extremely long and a large number of food allergens only give rise to allergy in sporadic cases.

The common features of food allergens are largely shared by those of respiratory allergens. However, foods are very often treated by cooking and other physico-chemical means that may destroy part of the protein structure and thereby its allergenic properties.

Properties of food allergens

The molecular weights of allergens are typically in the range of 10 -70 (90) kDa.

They have a number of 'epitopes', i.e. sequences of 8 - 16 amino acids. These are the structural 'units' which can be identified by the immune system and lead to production of specific IgE (sensitisation). In the sensitised individual the specific IgE readily recognises the epitopes on the par-ticular protein, resulting in allergy symptoms. Some of these epitopes are described in literature26-28.

Food allergens are stable to digestion and most also to heating by cooking, and in most cases, food allergens can represent a very large proportion of the food itself Enzymes are not well described with regard to neither their fate after ingestion nor their allergenic properties after cooking.

The TNO Institute performed a study[58] on native α-amylase from *Aspergillus oryzae* in a gastrointestinal model simulating the physiological events in the stomach.

The results indicate that about 92%, of the epitopes of the α-amylase are destroyed and about 8%, of the epitopes on the α-amylase are intact at the delivery from the stomach to the duodenum.

However, it can be expected that the proteolytic pancreatic enzymes will reduce even further, the remaining 7 – 8%, of the α-amylase during the passage through the duodenum.

Doses at which food allergy occurs

The doses and other conditions necessary to sensitise an individual are not well known. It is believed that the sensitising doses must be considerably higher than doses required for elicitation of symptoms in patients already sensitised. There are many examples of sensitised people reacting to trace amounts of allergens in the food - some of them with fatal outcomes.

It is therefore understandable that there is some focus on hidden allergens like traces of milk, nuts and peanuts in other foods.

Steinman[29] wrote a leading article in the August 1996 issue of J. Allergy Clin. Immunol. regarding hidden allergens in food. It is representative of the concern in the medical profession and in the public. He suggested a number of preventive measures including labelling in clear language. His article does not mention enzymes.

Food produced by GMO's

Genetically Modified Organisms (GMO's), and enzymes produced by GMO's have raised concern in general and also specifically for enzymes used in food processing.

Scientists in the fields of gene technology[30-33] and allergy seem to agree that gene technology and the results thereof expressed in foods should not cause concern with regard to allergy risk. However, gene technology does bring about new proteins, and it is important to be aware that some of these new proteins may be allergenic.

Genetically modified proteins may, or may not share allergenic properties with traditional allergens. This would relate to the nature of the protein as it does in all other circumstances, and there are no examples of involuntary (or voluntary) changes of allergenicity of proteins in food.

A possibility may be that in the future, gene technology may be used as a tool to produce less allergenic proteins. This might be a future example of voluntary change of allergenicity.

Enzymes produced by GMO's have been on the market in some countries for many years. Enzyme producers have not experienced any difference in allergenicity of these enzymes as compared to traditional extracted or fermented enzymes. They appear to have the same sensitising potential as are capable of sensitising exposed employees at the same rate as traditional enzymes.

3.2. Epidemiology of Food Allergy

In a survey of 5000 households in the USA carried out in 1989, 1992 and again in 1993[25] it was found that 13.9 -16.2% of the households reported at least one member to be allergic to foods.

A study of food allergy in a random sample of 1483 adults in Holland[23] showed that 12.4% reported allergy to foods, but by controlled tests only 2.4% could be confirmed by Double Blind Placebo Controlled Food Challenge (DBPCFC).

In Spain, 3034 patients from the outpatient allergy clinics at two hospitals were tested for food allergy[24]. The patients were tested by skin prick, RAST and open food challenge. They found 0.98% positive to one or more foods.

When looking at food additives, the same pattern emerges. In a survey of a population sample in the UK, 7% claimed to have reactions to food additives. Double blind challenge tests could verify only 0.01 - 0.23% to be true reactions to food additives[34].

The frequencies of confirmed food allergy in different countries in Europe and the USA are quite uniform at 1 - 2.5% of the populations.

A number of explanations to the discrepancy of perception and verified cases has been offered. There are indications that the public attribute a number of conditions to 'something in the food' and consider themselves allergic without ever having it tested.

A certain number of perceived food allergy may be induced by members of the medical profession, conducting less efficiently controlled test programs. In some cases, patients are declared food allergic solely based on skin prick tests -which may well over-diagnose food-reactions. High focus on food allergy in the media combined with personal and psychological conditions may also play a role. Actually some specialists in food allergy consider the psychological disorders the most important differential- diagnosis from food allergy.

A diagnosis must rest upon a combination of a medical history and objective tests to confirm or reject the tentative diagnosis. In the field of food-related allergies, the diagnostic test systems have been difficult to establish. However, the Double Blind Placebo Controlled Food Challenge (DBPCFC)[35,36], is the method of choice to confirm or reject indications of food allergy that may derive from the patient's perception and in many cases also from skin prick testing.

The experience from food allergy centres is that objective test programs to confirm or reject a suspected 'food allergy', requires skin- and blood tests and up to 6 placebo controlled challenges to be reliable.

Therefore a diagnosis of food-related allergy, based solely on medical history and a skin prick test is not good clinical practice and must be regarded un-ethical

3.3. Enzymes in food

In theory, enzyme sensitisation and allergy symptoms may be induced by direct ingestion of consumer products containing enzyme residues may occur

The tendency in recent years to focus on allergy and food allergy in particular may explain part of the marked discrepancy between the public perception of allergy to food - and the relatively few cases that can be verified in controlled clinical tests.

Papain is relatively widely used as a meat tenderiser, often supplied in a powder form to apply to the meat before cooking.

In 1983 Mansfield and co-workers[37] published a case story of a person who had allergicy symptoms after ingestion of papain used as a meat tenderiser. - Later, in 1985 they reported a study of 475 patients[38] with allergy of which 5 had a positive skin prick test to Papain.

The 5 papain positive were subjected to oral challenge with papain and all had positive reactions to the challenge.

Unfortunately, the challenge was only single blinded, and there is no report of occupational exposure or the use of powdered meat tenderisers that may have caused respiratory sensitisation.

In one other case story by Binkley[39], described below in the section 3.6.2, it can't be totally excluded that sensitisation took place by ingestion of a food product containing relatively high amounts of industrial produced enzymes.

A recent review by Wûthrichl[40] of enzymes in food concluded that orally ingested enzymes are not potent allergens and that sensitisation to ingested enzymes is rare as is also the case of reactions to bread in bakers with occupational allergy to enzymes.

The member companies of AMFEP have not registered, experienced or heard of consumers that have become sensitised to enzymes or enzyme residues in consumer products by ingestion.

It has not been possible to verify the claims in the media of such cases, and they seem as yet un-substantiated as examples of enzyme allergies in consumers. The patients presented and the symptoms and tests described are not documented, merely describing sensations and feelings,however presented as facts.

A large proportion of adverse reactions to food must be ascribed to digestive disorders such as intolerance to for example gluten and lactose, which are not allergic reactions.

3.4. The Theory of cross reactions

people sensitised with common moulds might react to enzymes produced in related moulds

The theory that people with allergy to common moulds which are related to those used for the fermentation of enzymes might react to enzyme residues in food was one of Schata's[1] claims and was given relatively high coverage in the media.

The theory could not be readily rejected as cross-reactions are relatively common in allergy. A number of food allergy reactions are merely cross reactions than caused by primary sensitisation.

The most commonly used moulds for fermenting enzymes are *Aspergillus oryzae* or *A. niger*.

According to the theory, people with allergy to *Aspergillus*-moulds would be a high risk population. *Aspergillus* allergy occurs in less than 0.5%, of the population.

A study by Cullinan[41] was conducted with the objective of testing if patients with a well-documented allergy to the widely distributed common mould *Aspergillus fumigatus* reacted upon the ingestion of bread prepared with enzymes of *Aspergillus* origin. The study was a double blind placebo controlled food challenge study on 17 *Aspergillus* allergic people.

The 17 test persons all had allergy antibodies to *Aspergillus fumigatus,* but in addition, 6 also reacted at the skin prick test to the enzymes produced in *A. oryzae* or *A. niger*.

Each patient was challenged with bread baked with the 2 enzymes in standard doses and with placebo bread baked without enzymes. Allergy symptoms and a number of general physiological parameters were monitored before, during and for 24 hours after the challenge.

No allergicy reactions were seen upon ingestion of enzyme containing bread as compared to placebo bread.

This study clearly demonstrates that patients who must be considered at the highest risk for cross reactions to baking enzymes do not react with clinical symptoms when they eat enzyme containing bread containing enzymes.

It is a general experience that once a person is sensitised, even very small amounts of the allergen can elicit allergy symptoms.

In the case of baking enzymes it seems well documented that even patients with severe asthma caused by *Aspergillus fumigatus* did not react to the baking enzymes produced in *A. oryzae* and *A. niger.*

3.5. Food related reactions in occupationally sensitised people

The situation of possible reactions to enzymes in bread in patients with occupational allergy to enzymes

There are a few papers describing cases of allergy symptoms elicited by the ingestion of enzymes in people who have occupational allergy to enzymes:

Kanny & Moneret-Vautrin,.[42] and Baur & Czuppon[43] each describes one patient who since late childhood, has had asthma and occupational asthma with allergy to flour and enzymes for several years. Both patients were tested for elicitation of symptoms by ingestion of bread baked with and without enzymes. Kanny & Moneret-Vautrin's patient was tested in a blinded design, Baur's patient in an open, non-controlled programme. In both cases the result was elicitation of respiratory symptoms after challenge with bread baked with enzymes. Baur's patient also had a slight reaction to bread without enzymes, however not as pronounced as the reaction after the enzyme containing bread.

Losada et al[44] investigated occupational allergy to α-amylase in a pharmaceutical plant and found a number of employees sensitised to α-amylase. None reported reactions related to ingestion of bread. Five patients, all positive to α-amylase were given oral doses of native α-amylase in doses up to 10 mg.

At this dosage, one of the 5 test persons reacted with respiratory- and generalised allergy symptoms. Four did not react.

Baur et al[45] described the possible background for consumer sensitisation to α-amylases in bread. 138 subjects, of which 98 were allergic, and 11 bakers with occupational allergy were tested. The bakers reacted to α-amylase as may be expected. None of the atopics and none of the control persons reacted to skin prick test with α-amylase. Two atopics had weak RAST to native α-amylase and one reacted also to heated ce-amylase. Reactions to other related compounds, for example *Aspergillus* was not tested.

Tarlo and co-workers[46] reported results of testing for papain allergy in 330 allergy patients. - Seven had positive RAST and Skin prick test but none of them had any gastrointestinal or other allergic symptoms to papain.

The elicitation of gastrointestinal symptoms upon respiratory sensitisation is also reported for flours. One example is reported by Vidal et al[47] and describes a man with occupational asthma after exposure to flours and other grain dusts. He was sensitised to barley, and experienced gastrointestinal reaction upon ingestion of foods and beverages made from barley.

Enzyme producers and other companies handling concentrated enzymes do see cases of employees being sensitised to baking enzymes. These would be the people at the highest risk of reacting to enzyme residues in bread.

However, none of the members of AMFEP had any reports of sensitised employees who had experienced allergy symptoms in connection to ingestion of bread, and there are no reports of α-amylase sensitised employees avoiding bread.

Cases of people with occupational allergy to flours and food-related reactions to ingestion of flours/bread do occur. One case report describes a person with asthma to barley dust and also with reaction to beverages and foods produced from barley.

The conclusion from these reports of people with pre-existing occup. allergy to α-amylase is:

- Allergic reactions after ingestion of enzyme containing foods are described in 3 individuals.
- The 3 cases are people with definite occupational respiratory allergy to flour and an additional sensitisation to α-amylase. It means they are most probably sensitised by inhalation of flour dust and enzyme dust and not by eating bread or other foods with enzyme residues in it.

3.6 The consumption of enzymes for medical purposes and as digestive aids:

Many people around the world eat enzymes for medical purposes or for convenience as digestive aids.

In many countries enzymes are used routinely as digestive aids by healthy people. The number of people in the world, frequently eating enzyme preparations must be counted in millions.

A number of diseases require the daily addition of enzyme preparation to the food to compensate the patient's insufficient production of digestive enzymes.

3.6.1. Medical uses:

Medical use of enzyme preparations are subject to clinical trials, the results of which are normally reported to the health authorities, and such adverse effects are described in the pharmacopoeia/registry of drugs.

Patients with chronic pancreatitis suffer from insufficient production of digestive enzymes from the pancreas. They are dependent on daily intake of enzymes, some of these produced from *Aspergillus* and other moulds, some extracted from animal glands. The doses of these enzymes are in the order of gram's a day. - we have not been able to identify published documentation of allergy to enzymes in these patients, and the drug registry's does not even mention allergy as an adverse effect.

Proteolytic enzymes and mixtures of different enzymes are commonly used for treatment of a number of physical lesions and also for a number of more special conditions[48-50].

The enzymes are administered in the form of tablets with mixtures of enzymes and in doses of 6 to 600 mg per day, in some cases several times more.

We have not been able to find any evidence of sensitisation or allergy symptoms caused by the ingestion of enzymes from these enzyme preparations. One example is the use of enzymes given as tablets for the treatment of non-articular rheumatism. Uffelmann[51] describes a double blind study of 424 patients, of which 211 received enzyme treatment. The daily doses of the mixed enzyme preparations was 240 mg Lipase, 240 mg Amylase, 1,44 g Papain, 1,08 g Bromelain and 2.4 g Pancreatin,. This dosage was given for 8 weeks and no serious adverse effects and no allergy reactions were reported.

Patients with Cystic Fibrosis suffer a hereditary disease characterised by severe lung symptoms and insufficient production of digestive pancreatic enzymes. They too are dependent of daily intake of grain-doses of enzymes. - There are a few reports of parents

and hospital staff who have become sensitised by inhalation of dust from these enzyme preparations[52-54]. This of course might also happen to the Cystic fibrosis patients when they handle the enzyme preparations themselves. However no cases of enzyme allergy in Cystic Fibrosis patients have been described, but there are reports of allergy to common food allergens[55].

An informal telephone survey on unpublished cases of enzyme allergy to European Cystic fibrosis Centres, resulted in only one possible case. The patient was a boy who reacted with vomiting after administration of the enzyme preparation containing amylase, protease and lipase. - The enzyme treatment had been stopped because of suspected allergy to the enzymes. However, testing for specific allergy antibodies by Maxisorp RAST[56] did not confirm sensitisation to any of the enzymes. Challenge tests have not been performed[57]

3.6.2. Digestive aids one possible case of allergy to digestive aid enzymes

In some cultures the use of digestive enzymes after large meals is very common. Enzymes for this purpose are 'over the counter' (OTC) drugs. We have found no studies of possible allergy to enzymes in these populations. That may be irrelevant if no-one ever thought of the possibility that enzymes might be the cause of allergicy symptoms had not been considered. - However, with millions of people using enzymes frequently, some cases of adverse effects in the form of allergic symptoms would be expected to emerge and be described in the literature. In most patients with allergic reactions, symptoms would appear immediately or very shortly after the intake.

Binkley[39], described a case of allergic reaction to ingested lactase. This patient had a respiratory allergy with positive skin prick test reaction to *Aspergillus sp*.

He had had two incidents with allergic reactions in the form of swelling and burning s tion of Lactaid tablets. The lactase was produced from fermentation of Aspergillus oryzae. Skin prick test with extracts of Lactase tablets gave a very strong positive reaction. He had not taken Lactaid tablets previous to the first experience of symptoms, but he had taken milk products containing lactase from *Saccharomyces fragilis* and from *Kluyveromyces lactis*. Although highly unlikely, it may be speculated if these may cross react with Lactaid. In this case it seems unlikely that sensitisation was caused by the Lactaid tablets as the symptoms appeared the first time he ever took Lactaid. It could be a 'cross reaction' based on sensitisation to yeast-produced lactase and symptoms elicited by the ingestion of Lactaid. Another possibility may be a cross reaction from his pre-existing *Aspergillus sp.* allergy.

This case may be regarded a possible but not verified case of oral sensitisation to enzymes in food.

A few other consumers haves claimed allergy to these OTC drugs but thorough testing has not verified allergy to enzymes in any of these cases.

With the background of the very high awareness of food related allergy in the populations, the widespread use of digestive aid and medical uses of enzymes should have attracted interest if allergy to ingested enzymes were of importance. However, up to now, only the single case mentioned above have been described.

To evaluate the risk of sensitisation from ingestion of enzymes and eventually experience of symptoms, we are aware of only the one case that may have become sensitised by ingestion.

This has to be related to the total number of people world-wide who ingest enzymes for short periods of time as part of a medical treatment, and to those who are dependent of daily intake of high amounts of digestive enzymes.

4.0. Conclusion

The working group has studied the available literature on these subjects and came to the conclusion that from a scientific point of view there is no indication that enzyme residues in bread or in other foods may represent an unacceptable risk for consumers.

Lack of scientific data is not evidence of lack of risk, and the working group realises that evidence of 'no risk' is extremely difficult or impossible to generate.

The group wish to stress that a ‘zero-risk’ can never be proved by science, and it must be anticipated that even an extremely low risk (e.g. 1 in 50 or 100 millions) of verified allergy to enzymes in food may well be perceived as a significant and unacceptable risk by the public in which more than 10% believe they are allergic to food.

Scientific data are of high value as the credible background for promotion to the public, to trade organisations and individual customers and for an ongoing dialogue with opinion leaders and consumer organisations.

It is the opinion of the group that many cases of perceived allergy to enzymes may be attributed to insufficient diagnostic procedures employed by members of the medical profession.

A minimum requirement for establishing a diagnosis of food related enzyme allergy should be a well conducted DBPCFC.

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AMFEP, Av. De Roodebeck, 30, B-1030 Bruxelles
Tel. +32 2 743 8730 /1 Fax. +32 2 736 8175

Annex 6

ENZYME TECHNICAL ASSOCIATION

1800 Massachusetts Avenue, NW, 2nd Floor
Washington, DC 20036-1800

Telephone (202) 778-9335
Fax (202) 778-9100
www.enzymetechnicalassoc.org

POSITION PAPER

ETA Position On Food Allergen Labeling of Microbially Derived Enzymes Under FALCPA as it Applies to Fermentation Media Raw Materials

It is the position of the Enzyme Technical Association (ETA) that microbially derived enzymes do not fall within the scope of the Food Allergy Labeling and Consumer Protection Act (FALCPA) and that labeling for food allergens is not triggered by the use of a microbially derived enzyme preparation. There may be other reasons why a manufacturer labels a food product with regard to allergen content, but the use of a microbially derived enzyme preparation is not a reason for such labeling.

Enzymes are not one of the eight major allergenic foods, often referred to as the big 8, so they do not fit within the first requirement of FALCPA. In addition, microbial enzymes are not byproducts of nor are they derived from the major food allergens. Although enzymes are not major food allergens,[1] many enzymes are produced with microorganisms and the nutrient media used to feed these microorganisms may contain protein from one or more of the major food allergens. The enzymes are not derived from raw materials containing major food allergens, but rather are obtained from the microorganisms which are used to produce the enzyme proteins. In other words, enzymes obtained from fermentation are directly derived from microorganisms fed on media that may include protein obtained from one or more of the major food allergens. Proteins and other nitrogenous material are consumed by the microorganisms for cell growth, cell maintenance, and production of enzyme protein. It is the intent of the enzyme manufacturer to supply enzymes, therefore it is critical that the ratio of nutrient to enzyme yield is carefully controlled. It is also the intent of the manufacturer that these raw materials are added to the fermentation as food to be consumed by the microorganism and are not added as formulation ingredients.

In arriving at its position ETA also considered that:

- The regulatory agencies in the EU and Japan have determined that enzyme preparations are not required to have allergen labeling for the raw materials used in the fermentation process. Indeed, the European Commission's Health & Consumer Protection Directorate General has clearly stated that enzymes

[1] To the extent the enzyme producer uses an allergenic material, such as wheat flour diluent in the final product formulation, labeling may be required.

are outside the scope of the Directive 2003/89/EC which amended the EU Food Labelling Regulations.

- Enzyme broths are normally processed to separate biomass and fermentation materials from the enzyme, to concentrate the enzymatic activity, and formulated to achieve a uniform and stable enzyme product.

- The unique role of enzymes in food processing is as a catalyst. Due to the specific nature of enzymes, only small amounts are required to make desired modifications to the property of a food.

- Many enzymes do not become a component of the food ingredient or final food. Some enzymes are used in an immobilized form or are denatured during processing. Further, processing of the food ingredient after the enzyme catalyst has performed the expected function often reduces or eliminates the enzyme from the product.

- ETA has made an extensive review of the published scientific literature and has found no reports that even suggest there has been an allergenic reaction to a component of the fermentation media which was used to feed the microorganism that produced the enzyme.

The above position paper and accompanying report were provided to FDA on September 12, 2005 and to date ETA has received no comment.

Annex 7

University of Nebraska Lincoln

INSTITUTE OF AGRICULTURE AND NATURAL RESOURCES
FOOD ALLERGY RESEARCH AND RESOURCE PROGRAM

EXPERT OPINION STATEMENT
FOOD ALLERGY RESEARCH & RESOURCE PROGRAM
UNIVERSITY OF NEBRASKA

Testing of Microbially Derived Enzymes for Potential Allergens from Fermentation Media Raw Materials

August 13, 2013

Prepared by: Steve L. Taylor, Ph.D., Co-Director
and
Joe L. Baumert, Ph.D., Co-Director

with assistance from Enzyme Technical Association

Microbially derived enzymes are used by food processors as additives and processing aids in a wide variety of foods. Enzymes obtained from microbial fermentation are directly derived from microorganisms fed on sterilized media[1] that may include protein sources obtained from one or more of the recognized commonly allergenic foods (e.g., milk, soybean) or from a cereal source of gluten (e.g., wheat, barley). This paper addresses the relevance of testing microbial enzymes for allergenic material from the fermentation growth media.[2]

It has been the long-standing position of the Food Allergy Research & Resource Program (FARRP) at the University of Nebraska that testing of the products of fermentation (with limited exceptions), including microbially derived enzymes is unreliable using enzyme-linked immunosorbent assays (ELISAs).

While various fermentation media may contain one or more of the major food allergens, the biochemical reactions that occur during fermentation result in the breakdown of the fermentation media proteins. The extent of proteolysis is dependent upon the fermentation culture and the resultant enzyme (e.g., some enzymes are proteases). As proteins are digested, the resulting amino acids, along with other

[1] Aunstrup, K., O. Andresen, E.A. Falch, and T.K. Nielsen (1979) *Microbial Technology*. (Perlman and Peppler, eds.) Academic Press, pp. 281-309.

[2] For this paper, FARRP's analysis is limited to microbially derived enzymes that are intended for additive and processing aid applications in food.

143 Food Industry Building / P.O. Box 830955 / Lincoln, NE 68583-0955
Co-Director Phone (ST) (402) 472-2833 / Co-Director (SH) (402) 472-4430
Lab Phone (402) 472-4484 / FAX (402) 472-1693

nitrogenous material, are consumed by the microorganisms for cell growth, cell maintenance, and production of enzyme protein.

Upon completion of fermentation, remaining fermentation media that are not consumed by the microorganism are typically separated and/or purified from the enzyme in the recovery process. Enzymes are recovered from the fermentation broth by standard chemical engineering operations, such as filtration and centrifugation, broadly used in enzyme production.[3,4] (See Appendices for further information.) The recovery steps result in separation of microbial biomass and other fermentation solids from the enzyme, concentration of the enzyme, and removal of impurities prior to final formulation with food-grade ingredients.

Any potential residual fragments from the food allergen would be difficult to measure as there is no reliable assay. Commercial ELISAs are able to detect only intact proteins in most cases. Any peptides, even larger ones, would not likely be detected, although this possibility has not been well investigated. Results would typically be reported as below the limit of quantitation for the enzyme preparation. Further, if any residual but undetected fragments of the food allergen remain, the relevance of any such residual material to food allergenicity is unproven. Accordingly, testing of fermented product does not result in reliable or useful data.

In addition, due to the specific catalytic nature of enzymes, only very small amounts of enzymes are generally required and used by food processors to make the desired modifications to the property of a food, and therefore any *de minimis* amount of fermentation media protein that may survive the fermentation process will not pose a significant public health risk to the consumer.[5]

FARRP also notes that regulatory agencies in the European Union and Japan do not require allergen labeling of enzyme preparations for the raw materials used in the fermentation process.

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[3] Atkinson, B. and F. Mavituna (1991) *Biochemical Engineering and Biotechnology Handbook.* (Atkinson, B. and Mavituna, F., eds.) Stockton Press, Hampshire, pp. 1146-1158.

[4] Kroschwitz, J.I. (1994) *Enzyme Applications in Encyclopedia of Chemical Technology.* 4th edition, Volume 9. (Kroschwitz, J.I., ed.), pp. 567-620.

[5] To the extent the enzyme producer uses an allergen as diluent to formulate the final product, labeling for such allergen is appropriate and required under Food Allergen Labeling and Consumer Protection Act.

SUBMISSION END