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Novo Nordisk

Novo Nordisk BioChem
North America, Inc.

77 Perry Chapel Church Ro
Box 576
Franklinton, NC 27525-057

Tel., 919-494-3000
FAX 919-494-3450

October 12, 1998

Dr. Nega Beru
Office of Premarket Approval (HFS-206)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, D.C. 20204

Dear Dr. Beru,

We are hereby submitting, in triplicate, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novo Nordisk's pectin esterase enzyme preparation, produced by *Aspergillus oryzae* carrying the gene coding for pectin esterase from *Aspergillus aculeatus*. The pectin esterase preparation is to be used in the food industry, primarily in fruit and vegetable applications, as a processing aid which catalyzes the conversion of high methoxylated pectin to low methoxylated pectin. The properties of low methoxylated pectin can be utilized, for example, to improve rheological properties of the treated product.

Please contact me by direct telephone at 919 494-3151 or direct fax at 919 494-3420 if you have any questions or require additional information.

Sincerely,

Lori Gregg
Regulatory Associate

Enclosures (3 binders)

1998 OCT 14 A 9:56

Novo Nordisk

Novo Nordisk BioChem North America, Inc.

77 Perry Chapel Church Roa
Box 576
Franklinton, NC 27525-0576

Tel. 919-494-3000
FAX 919-494-3450

October 12, 1998

RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R.§ 170.36 (c)(1) Novo Nordisk BioChem North America Inc. hereby claims that pectin esterase preparations produced by submerged fermentation of *Aspergillus oryzae* carrying the gene coding for pectin esterase from *Aspergillus aculeatus* are Generally Recognized as Safe; therefore, they are exempt from statuatory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36 (c)(1)(i) *The name and address of the notifier.*

Novo Nordisk BioChem North America Inc.
77 Perry Chapel Church Rd., Box 576
Franklinton, NC 27525

Proposed § 170.36 (c)(1)(ii) *The common or usual name of notified substance.*

Pectin esterase enzyme preparation from *Aspergillus oryzae* carrying the gene coding for pectin esterase from *Aspergillus aculeatus*.

Proposed § 170.36 (c)(1)(iii) *Applicable conditions of use.*

The above described pectin esterase preparation is to be used in the food industry, primarily in fruit and vegetable applications, as a processing aid which catalyzes the conversion of high methoxylated pectin to low methoxylated pectin. The properties of low methoxylated pectin can be utilized, for example, to improve rheological properties of the treated product. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

Proposed §170.36 (c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures.

Proposed § 170.36 (c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Published safety studies on the production strain and the enzyme preparation are also included in the notification package. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

12-Oct-98

John Carroll — Date
Director, Regulatory Affairs

1998 OCT 14 A 9:56

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Novo Nordisk

Rheozyme™

a pectin esterase preparation produced by

***Aspergillus oryzae* expressing the gene encoding**

a pectin esterase from *Aspergillus aculeatus*

Birger Rostgaard Jensen, Enzyme Regulatory Affairs, NNA/S, Denmark
Lori Gregg, Enzyme Regulatory Affairs, NNBNA, USA
Scott Shore, Enzyme Regulatory Affairs, NNBNA, USA
Annette Gøtzsche-Larsen, Enzyme Regulatory Affairs, NNBNA, USA

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1. GENERAL INTRODUCTION

Rheozyme™ is the Novo Nordisk A/S trade name used for a pectin esterase preparation produced by submerged fermentation of *Aspergillus oryzae* carrying the gene coding for pectin esterase from *Aspergillus aculeatus*[1].

The pectin esterase preparation is to be used in the food industry, primarily the fruit and vegetable industries, as a processing aid. The enzyme may be used alone to modify the viscosity of fruit and vegetable based products, which will eliminate the need for adding exogenous pectin or other thickeners. It can also be used in combination with the traditional multi-component pectinase products, which are comprised of polygalacturonases, pectin lyases and pectin esterase, as an efficient booster in clarification of fruit juice or wine.

The information provided in the following sections is the basis for our determination of general recognition of safety of a pectin esterase enzyme preparation (commercial product trade name, Rheozyme) produced by *A. oryzae* expressing the gene encoding a pectin esterase from *A. aculeatus*. Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation. This includes published studies on the production strain[2] and the enzyme preparation[3].

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food[4]. The production organism for Rheozyme, *A. oryzae*, is discussed in Sections 2 and 7. An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material[5-10]. The genetic modifications used to construct the production microorganism are well defined and are described in Section 2. The safety studies performed and described in Section 7 show no evidence to indicate that any of the cloned DNA sequences and incorporated plasmid DNA encode or express a harmful or toxic substance.

It should be noted that within some of the attachments, the terms "Novozym 572", "Pectin Esterase", "Pectin methyl esterase SP 572" are used to describe Rheozyme. These three names are internal names used before a decision concerning the trade name was made.

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2. PRODUCTION MICROORGANISM

2.1 Production Strain

The microbial production strain, designated Km-1-1, was constructed by plasmid transformation of a derivative of the industrial strain, *A. oryzae* strain IFO 4177 (synonym A1560). This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms[11]. It also meets the criteria for a safe production microorganism as described by Pariza and Foster and several expert groups[5-10]. This production strain is identified as an *A. oryzae* fungal strain. The classification and morphological characteristics are described[12].

Two plasmids were used in the strain construction, one an expression plasmid and the other a selectable marker plasmid. These plasmids contain strictly defined fungal chromosomal DNA fragments, DNA from a well-characterized *Escherichia coli* vector, and synthetic oligonucleotide fragments. The specific DNA sequences include: a gene encoding an *Aspergillus aculeatus* pectin esterase enzyme; an *Aspergillus nidulans* selectable marker gene, *amd*S (acetamidase)[13] ; well-characterized noncoding regulatory sequences from *Aspergillus niger*[14] and *Aspergillus oryzae*[15] ; known sequences from *Escherichia coli* plasmid pUC19[16], and synthetic oligonucleotide adaptor fragments.

The recipient strain as well as the expression plasmid and selectable marker plasmid are the same as those used for Novo Nordisk's enzyme preparations Novozym 677 BG and Pentopan Mono. Novozym 677 BG is a lipase preparation produced by *A. oryzae* expressing the lipase gene from *Thermomyces lanuginosus* and Pentopan Mono is a xylanase produced by *A. oryzae* expressing the xylanase gene from *T. lanuginosus*.

2.2 Recipient Organism

The recipient microorganism, designated HowB 104, used in the construction of the pectin esterase production strain is an amylase negative derivative of the fully characterized, well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn designated strain IFO 4177 (synonym A1560) obtained from the Institute for Fermentation, Osaka, Japan (IFO). The classification of the original *A. oryzae* IFO 4177 strain has been confirmed by the Centraalbureau voor Schimmelcultures, Baarn, Holland[17].

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2.3 Pectin esterase Expression Plasmid

The 5.4 kb pectin esterase expression plasmid, pA2PEI, used in the construction of the production strain, contains the following genetic material (see Appendix A, Figure 1):

1.1 kb DNA from the *A. oryzae* TAKA amylase gene promoter
1.3 kb DNA from the *A. aculeatus* pectin esterase gene
0.7 kb DNA from the *A. niger* glucoamylase gene terminator sequence
2.3 kb DNA from the *E. coli* plasmid pUC19

2.4 Selectable Marker Plasmid

The 5.4 kb selectable marker plasmid, pToC90, used in the construction of the production strain contains the following genetic material (see Appendix A, Figure 2):

2.7 kb DNA from *A. nidulans* encoding the *amdS* gene
2.7 kb DNA from the *E. coli* plasmid pUC19

2.5 Stability of the Transformed Plasmid Genetic Sequences

The presence and configuration of the introduced DNA sequences was determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a component of the safety evaluation of the production microorganism[5-10]. The transforming plasmid DNA is stably integrated into the *A. oryzae* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable[11].

2.6 Absence of rDNA Sequences in the Enzyme Preparation

Samples of the pectin esterase enzyme preparation have been analyzed by dot blot hybridization for the presence of rDNA sequences used in the production strain construction as an additional component of the safety evaluation of the production microorganism[5,6]. No rDNA sequences were detected. The detection limit is 20 pg plasmid DNA/g sample. Therefore, there is no potential for transfer of rDNA sequences to other organisms which ingest foods processed with the pectin esterase enzyme preparation.

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2.7 Antibiotic Resistance Gene

Plasmid pToC90 contains the β-lactamase gene, *bla*, encoding resistance to ampicillin. These genes are prokaryotic in origin and lack the appropriate sites and signals (promoter, ribosome binding site, etc.) to be functionally expressed when integrated in an eukaryotic chromosome. Tests of *A. oryzae* production strains containing the pToC90 plasmid have not shown any evidence of β-lactamase expression.

In addition, the prokaryotic *bla* gene lacks appropriate eukaryotic signal sequences and processing signals necessary for secretion and export. Therefore, any β-lactamase potentially produced by expression of the *bla* gene would be intracellularly localized and not be present in the final product, the pectin esterase enzyme preparation.

2.8 Absence of Production Organism in the Product

The absence of the production organism is an established specification for the commercial product, Rheozyme. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC[5] is satisfactorily addressed.

3. MANUFACTURING PROCESS

This section describes the manufacturing process for Rheozyme. The quality management system used in the manufacturing process for Rheozyme complies with the requirements of ISO 9002. Rheozyme is also manufactured in accordance with current good manufacturing practices.

3.1 Raw Materials

Raw materials used for fermentation and for recovery conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novo Nordisk A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

The antifoams used in fermentation and recovery and listed below in sections 3.2.1 and 3.3.1 are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of these antifoams in the Rheozyme product is less than 1%.

3.2 Fermentation Process

Rheozyme is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *A. oryzae* described in Section 2. A flow sheet of the fermentation process is shown in Appendix B, Figure 1. All 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 confirm strain identity.

3.2.1 Raw Materials for the Fermentation Process

The raw materials used in the fermentation process are listed below. The list includes both the raw materials used for the seed fermenter as well as those used for the main fermenter.

- Sucrose*, glucose*, maltose, starch and/or starch hydrolysates*, glycerol*
- Yeast extract*
- Vegetable protein*
- Ammonia*, urea* or other nitrogen sources
- Minerals (phosphates, chlorides, sulfates, carbonates)
- Trace metals
- Alkali and acid (ammonia*, citric acid*, sodium hydroxide*, sodium carbonate* and phosphoric acid*) for pH adjustments
- Polymeric antifoam compounds- Pluronic PE 6100 (polyoxypropylene-polyoxyethylene copolymer), and P2000 (polypropylene glycol)*
- Potable water

*Conforms to FCC

3.2.2 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism *A. oryzae*, Km-1-1, described in section 2. Each new batch of the stock culture is

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thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

3.2.3 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermenter and the main fermenter before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48 hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions are fulfilled:

1. Infection is observed in 2 or more samples by microscopy

2. Infection is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

3.3 The Recovery Process

The recovery process is a multistep operation which starts immediately after the fermentation process and consists of both the purification and the formulation processes. A flow sheet of the recovery process is shown in Appendix B, Figure 2.

3.3.1 Raw Materials in the Recovery Process

- Alkali and acids for pH adjustment (sodium hydroxide*, ammonia*, acetic acid*, phosphoric acid*)
- Filter aids (diatomaceous earth*/Celite, Dicalite, Perlite or similar)
- Antifoaming agents- Antifoam FD, (polydimethylsiloxane)*
- Precipitation/flocculating agent (calcium chloride)*
- Stabilizers and formulation aids (potassium chloride*, glycerol*)
- Water

*Conforms to FCC

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3.3.2 Purification Process

The enzyme is recovered from the culture broth by the following series of operations:

1. Pretreatment - pH adjustment
2. Primary Separation - vacuum drum filtration
3. Concentration - ultrafiltration and evaporation
4. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation
5. Stabilization - potassium chloride addition
6. Final Concentration - evaporation, if enzyme concentration is too low to reach the target yield

3.3.3 Formulation and Standardization Processes

The stabilized concentrate is blended with water, glycerol, potassium chloride and the product is standardized according to the product specification.

3.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in section 5.

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4. ENZYME IDENTITY

Key enzyme and protein chemical characteristics of the pectin esterase are given below:

Classification:	Pectinase (generic name)
IUB nomenclature:	Pectin esterase
IUB No.:	3.1.1.11
CAS No.:	9025-98-3
EINECS No.:	232-807-0
Specificity:	Ester linkages between methanol and polygalacturonic acid in high methoxylated pectin
Molecular weight:	36.2 kDa
Amino acid sequence:	The total nucleotide and amino acid sequences have been determined

5. COMPOSITION AND SPECIFICATIONS

5.1 Formulation

Rheozyme will be available in a liquid formulation with an activity of 10 PEU/ml and with the following typical composition:

Water	approx. 66.6%
Salts	approx. 17.0%
Glycerol	approx. 15.0%
Enzyme solids (E-TOS)	approx. 1.4%

Further information on the product is given in the enclosed Product Sheet (Appendix C).

5.2 Specifications

Rheozyme complies with the purity criteria recommended for enzyme preparations as described in Food Chemicals Codex, 4th edition 1996[18]. In addition, Rheozyme also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications[19].

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The following specifications have been established for Rheozyme:

Pectin esterase activity, PEU/ml	according to declaration
Heavy metals	not more than 30 ppm
Lead	not more than 5 ppm
Arsenic	not more than 3 ppm
Total viable count/g	not more than 5 x 10^4
Total coliforms/g	not more than 30
Enteropathogenic *E. coli*/25 g	negative by test
Salmonella/25 g	negative by test
Production organism	negative by test
Antibiotic activity	negative by test
Mycotoxins	negative by test

6. APPLICATION

6.1 Mode of Action

Pectin esterases (EC 3.1.1.11) hydrolyze the ester linkage between methanol and galacturonic acid in esterified pectin. Pectin esterases occur both in plants and in some plant cell-wall degrading micro-organisms.

Pectic substances are the major components of the primary cell wall and middle lamella of the major crops cultured by man, such as fruit and vegetables. The polygalacturonic acid residues can be methylesterified to a varying degree. The enzymatic de-esterification results in a low methoxylated pectin, which in the presence of calcium ions forms a strong gel.

The enzymatic conversion of high methoxylated (HM) pectin to low methoxylated (LM) pectin makes an *in situ* viscosity increase or gel formation possible and may, in products such as jam and ketchup, render further addition of thickening agents unnecessary.

Pectin esterase may also find an application for firming of fruit and vegetables. Pectin esterase is either by means of passive infusion or by vacuum infusion transported into the tissue and allows the conversion of HM pectin to LM pectin to take place.

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6.2 Application

Rheozyme is generally used to modify the functional properties of pectin containing materials by reducing the number of methoxyl groups in the pectic part resulting in:

- in situ de-esterification and gelation of plant materials
- viscosity increase (thickening) of plant preparations
- hardening of fruits and vegetables
- controlled demethylation of HM pectins

Further information is given in the enclosed application sheets for Fruit Preparations, Compote, Cider, Jam, Tomatoes (Appendix D) and a Report: Pectin esterase (PE). Traditional Jam Preparation vs. Enzymatic Gelification. Heat Inactivation of PE. Measurement of Methanol Formation, ED-9710066 (Appendix E).

6.3 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices (GMP).

The recommended dosage for Rheozyme in most applications is 1-15 ml/kg fruit or 10-150 pectin esterase units (PEU)/kg fruit. For cider treatment, the usage is much lower (0.5-1 PEU/kg fruit).

6.4 Enzyme Residues in the Final Food

Rheozyme is added to the fruit or vegetables and is active during the controlled demethylation of pectin. The enzyme is inactivated by heat treatment when the desired rheological properties are achieved.

6.5 Methanol Content in Rheozyme Treated Products

The hydrolysis of HM pectin by pectin esterase results in the release of methanol. This does not result in greater intake of methanol compared to fruits or vegetables not treated with pectin esterase, because the total pool of methanol (free plus ester bound in pectin) is constant.

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Application trials have shown that addition of approximately 0.4 % pectin can be omitted if the jam is treated with pectin esterase (Appendix E). Therefore, the total pool of methanol can be decreased by using Rheozyme.

7. GENERAL RECOGNITION OF SAFETY

7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food[4]. If the organism is nontoxigenic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume[5]. Pariza and Foster (1983) define a nontoxigenic organism 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". *A. oryzae* meets these criteria for nontoxigenicity and nonpathogenicity. In addition, *A. oryzae* is not considered pathogenic by JECFA[20].

Barbesgaard et al. reviewed the safety of *A. oryzae* and describe it as having a very long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food grade enzymes[2]. *A. oryzae* is accepted as a constituent of foods[20]. *A. oryzae* has been used to produce soy sauce in the United States since before 1958[2,5]. Therefore, *A. oryzae* meets the criterion of "common use in foods in the US before 1958" and can be considered "generally recognized as safe", GRAS[5]. Enzyme preparations from *A. oryzae* are also considered GRAS[4,5,20]. A GRAS petition, 3G0016, proposing affirmation that enzyme preparations from *A. oryzae* are GRAS for use in food was submitted to FDA and accepted for filing in 1973[21]. Enzyme preparations from *A. oryzae* have been marketed in the US as GRAS since that time.

An evaluation of the genetically modified production microorganism for Rheozyme, embodying the concepts initially outlined by Pariza and Foster, 1983 and further developed by IFBC in 1990, the EU SCF in 1991, the OECD in 1992, ILSI Europe Novel Food Task Force in 1996, and FAO/WHO in 1996, demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host strain, a description of the plasmids used, the sources and functions of the introduced genetic material, an

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outline of the genetic construction of the production strain, and some charactertistics of the production strain and the enzyme derived from it are given in Section 2.

7.2 Safety of the Pectin Esterase Enzyme

Because the genetic modifications are well characterized and specific, and the incorporated plasmid DNA does not encode and express any known harmful or toxic substances, the pectin esterase enzyme preparation derived from the genetically modified *A.oryzae* is considered safe[5,22]. To confirm the safety of the enzyme, safety studies[3] were performed on the enzyme preparation and are described in Section 7.4.

In addition, the pectin esterase enzyme from the genetically modified *A. oryzae* is essentially identical to a pectin esterase from *A. aculeatus*. The *A. aculeatus* pectin esterase coding sequence incorporated into the *A. oryzae* production strain has not been altered. Fungal pectic enzymes have been used in the fruit juice industry since 1930[23]. In 1985, GRAS petition 5G0297, which proposed the affirmation of the GRAS status of mixed carbohydrases (including pectinases) from *A. aculeatus*, was accepted for filing by the FDA[24]. Pectinases from *A. aculeatus* have been marketed in the US as GRAS since that time. Carbohydrases from *A. aculeatus* are approved for use in food applications such as juice and brewing in 37 countries including Australia, Canada, Great Britain, Denmark, Italy, and Sweden. The European Commission, Scientific Committee on Animal Nutrition found that carbohydrases from *A. aculeatus* do not pose any risks to human or animal health or to the environment (http://europa.eu.int/comm/dg24/health/sc/scan/out10_en.html). Therefore, the pectin esterase enzyme from the genetically modified *A. oryzae* is considered safe for use in food applications[4,22].

7.3 Safety of the Manufacturing Process

Rheozyme meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 4, the pectin esterase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes[25-27].

7.4 Safety Studies

This section describes the studies and analyses performed to evaluate the safety of the use of Rheozyme.

7.4.1 Description of Test Material

The safety studies described below were all conducted on a test batch of the pectin esterase preparation which was obtained by the mixing of five sub-batches each of which were produced according to the description given in section 3, except that stabilization and standardization were omitted.

7.4.2 Studies

The following studies were performed:

1) Gene mutation (Ames test with *S. typhimurium*).
2) Chromosome aberrations (*in vitro* cytogenetics with human lymphocytes).
3) Subchronic toxicity (Rats - 13 weeks)

7.4.3 Mycotoxin analyses

Some strains of *A. oryzae* may produce one or more of the mycotoxins beta-nitropropionic acid, kojic acid and cyclopiazonic acid[2]. Mycotoxin analyses have been done on the enzyme concentrate for Rheozyme. These *A. oryzae* specific mycotoxins were either below the limit of detection or were well below levels that would be considered toxic.

The final product, Rheozyme, must meet the specifications for food grade enzymes established by the Food Chemicals Codex and FAO/WHO JECFA. This includes testing to ensure that mycotoxins are not present in the final product.

7.5 Estimates of Human Consumption and Safety Margin

This section discusses the potential human exposure to Rheozyme from food. All enzyme activity is inactivated during the final heat treatment. However, in order to demonstrate the "worst case" situation, the following calculations are made assuming that all enzyme activity is retained in the food.

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The recommended dose level for general applications is 5 ml Rheozyme/kg product. Rheozyme has a declared activity of 10 PEU/ml. The highest intake conceivable is related to consumption of pectin esterase treated products consumed "as is" (i.e. fruits and vegetables). Firming of fruits and vegetables may require the application of pectin esterase at higher dosage levels such as 10-12.5 ml/kg.

As a precautionary measure, a maximum application dosage of 25 ml/kg is assumed. Rheozyme contains 1.4% TOS. Therefore, the maximum dose contains 0.35 g TOS/kg fruit.

Adults:
In 21 CFR § 101.12, Table 2 lists reference amounts of food items customarily consumed per eating occasion. The largest relevant amount indicated for products possibly consumed "as is" is 140 g per eating occasion (fruit/vegetables). Assuming three servings per day this amounts to a daily intake of 420 g product. This corresponds to a consumption of

0.42 kg fruit x .35 g TOS/kg fruit = 0.147 g TOS/person/day

Infants:
In 21 CFR § 101.12, table 1 the relevant figure for fruit or vegetable intake per eating occasion is 15 g dry mix. Infants initiated to vegetable or fruit feeding will usually receive one serving of these food items per day. Assuming that two servings may occasionally take place, this corresponds to a daily intake of 30 g product/infant/day. This corresponds to a consumption of

0.03 kg fruit x .35 g TOS/kg fruit = 0.0105 g TOS/infant/day

Pectin esterase intake in general:
The estimated maximum pectin esterase intakes stated above do, to the best of our knowledge, represent worst cases. The major anticipated pectin esterase applications will in general involve lower pectin esterase dosages, consumption of pectin esterase treated products in multi-component food items (not on an "as is" basis) and/or lower consumed quantities of the food products. By way of examples and with reference to the above mentioned tables the daily intake of pectin esterase related to two major anticipated applications is indicated below.

Jams:
For products such as jams, where fruits are primarily used as ingredients, the enzyme dosage level would be 5 ml Rheozyme/kg jam. The indicated daily consumption for adults in three servings is 155 g. This corresponds to a consumption of

0.155 kg x 0.07 g TOS/kg jam = 0.0108 g TOS/person/day

Ketchup
The consumption of ketchup or tomato sauce in three servings will typically amount to 180 g. At the recommended enzyme dosage of 5 ml Rheozyme/kg ketchup, this will correspond to a consumption of

0.180 kg x 0.07 g TOS/kg ketchup = 0.0126 g TOS/person/day

Safety Margin:

The safety margin is calculated as the dose level with no observed adverse effect (NOAEL) in rats divided by the estimated human consumption.

The test batch used for the 13 week rat study has a 11.9% TOS content. The batch has a pectin esterase activity of 88.8 PEU/g.

The dose level in the 13 weeks oral toxicity study in rats, which could be given without any toxic effects, was 10.0 g/kg bw/day or 888 PEU/kg bw/day or 1.19 g TOS/kg bw/day, which is the no-observed-adverse-effect level (NOAEL).

The safety margin for the above indicated applications can thus be calculated assuming an adult body weight of 70 kg and an infant body weight of 5 kg. to be:

Food item	Daily intake TOS/person	Daily intake TOS/kg bw	Safety Margin
Fruit, adults	0.147	0.0021	566
Fruit, infants	0.0105	0.0021	566
Jam	0.0108	0.00015	7933
Ketchup	0.0126	0.00018	6611

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[redacted]

7.6 Results and Conclusion

The results of the tests described in section 7.2.2 show that the pectin esterase preparation does not exhibit any mutagenic activity, clastogenic activity or toxic effect under the conditions of each specific test. On the basis of the evaluation contained in this section, a review of the published literature, the history of use of *A.oryzae*, and the limited and well defined nature of the genetic modifications, the pectin esterase can be safely manufactured and used as a processing aid in the fruit and vegetable industry as well as in other food applications.

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[redacted]

8. LIST OF APPENDICES

A. pA2PEI plasmid map (Figure 1), pToC90 plasmid map (Figure 2).

B. Fermentation Flow Diagram (Figure 1), Recovery Flow Diagram (Figure 2).

C. Novo Nordisk Product Sheet for Rheozyme.

D. Application Sheets on Rheozyme: General, Test of applicability, Fruit Preparations, Compote, Cider, Jam, Tomatoes.

E. Pectin esterase (PE). Traditional Jam Preparation vs. Enzymatic Gelification. Heat Inactivation of PE. Measurement of Methanol formation. ED-9710066.

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Appendix A

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Figure 1. pA2PEI plasmid map

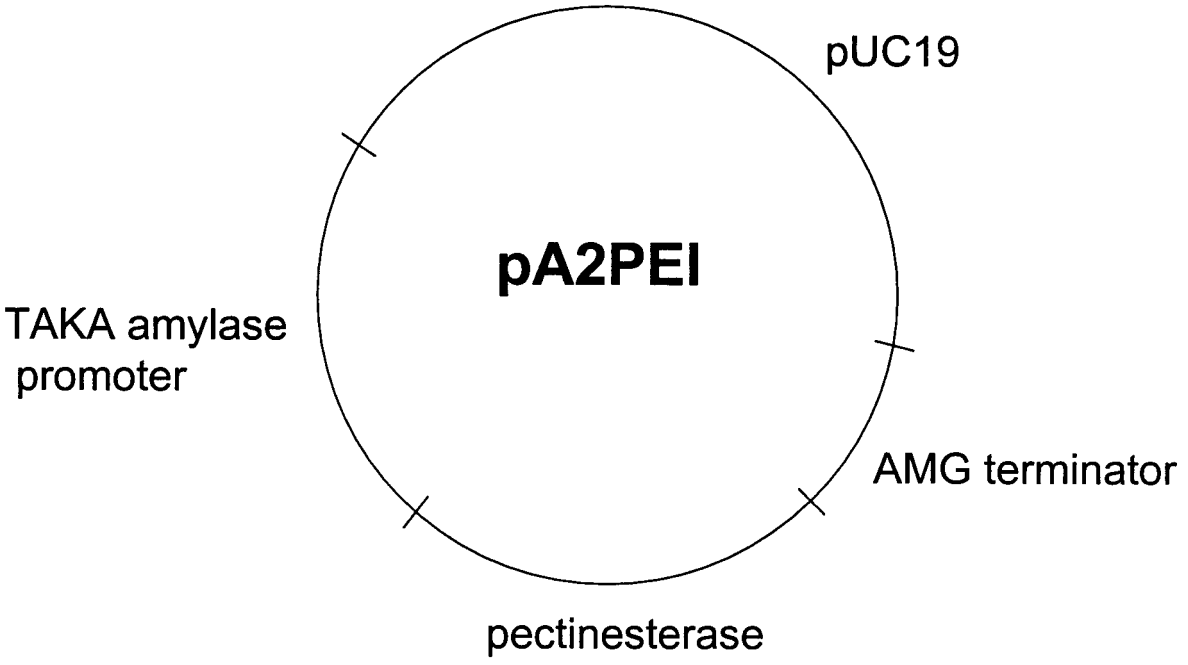
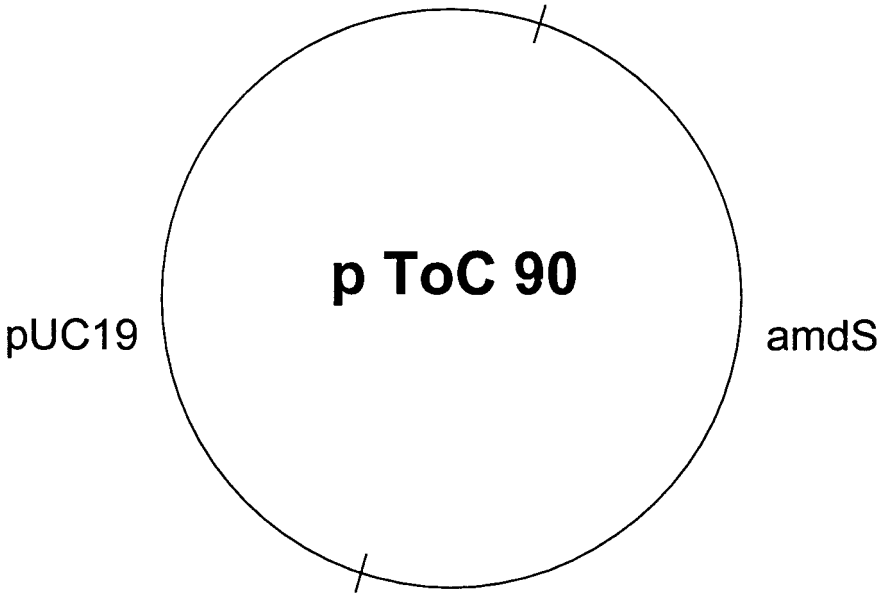


Figure 2. pToC 90 plasmid map



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Appendix B

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FERMENTATION

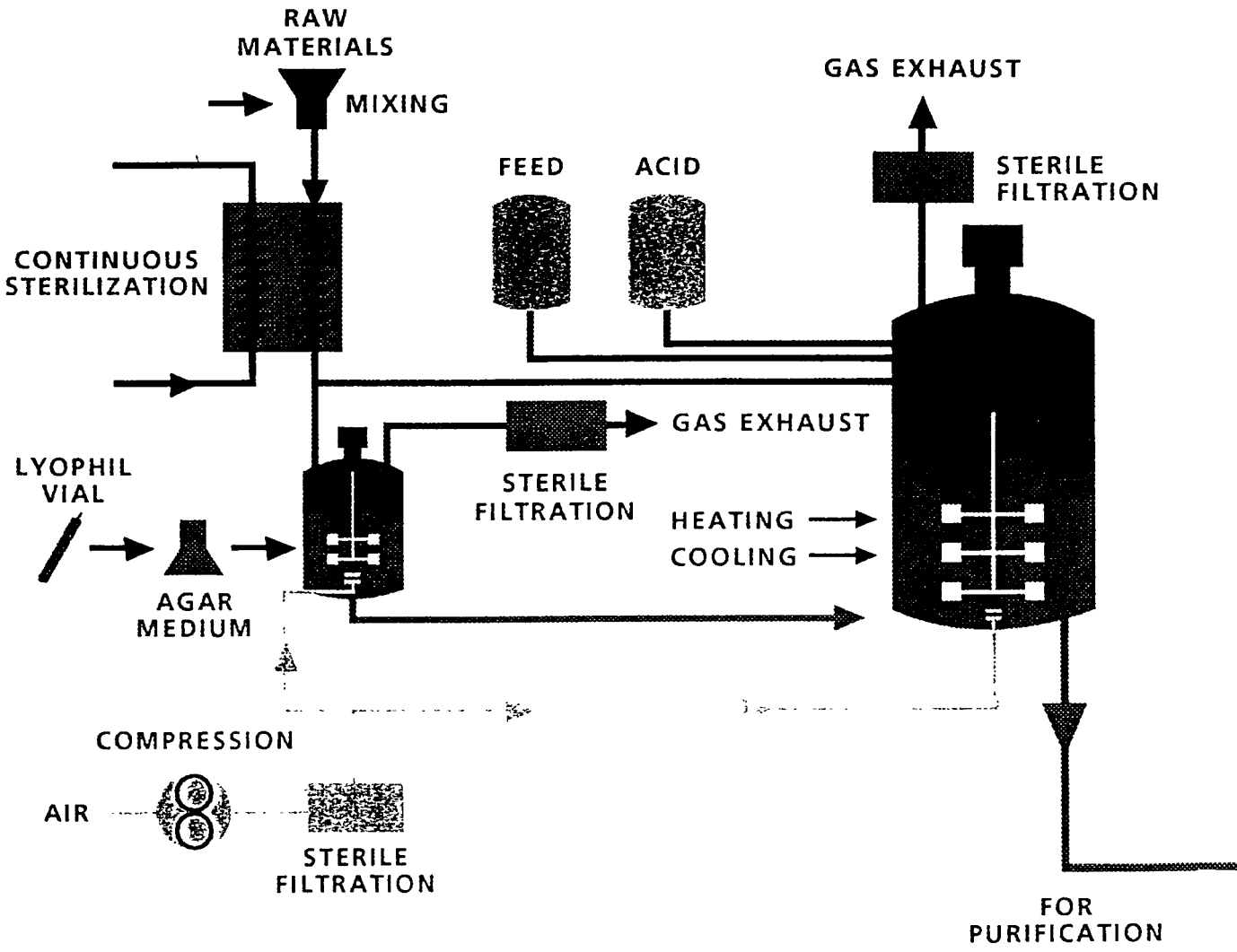

FIGURE 1

000237

RECOVERY

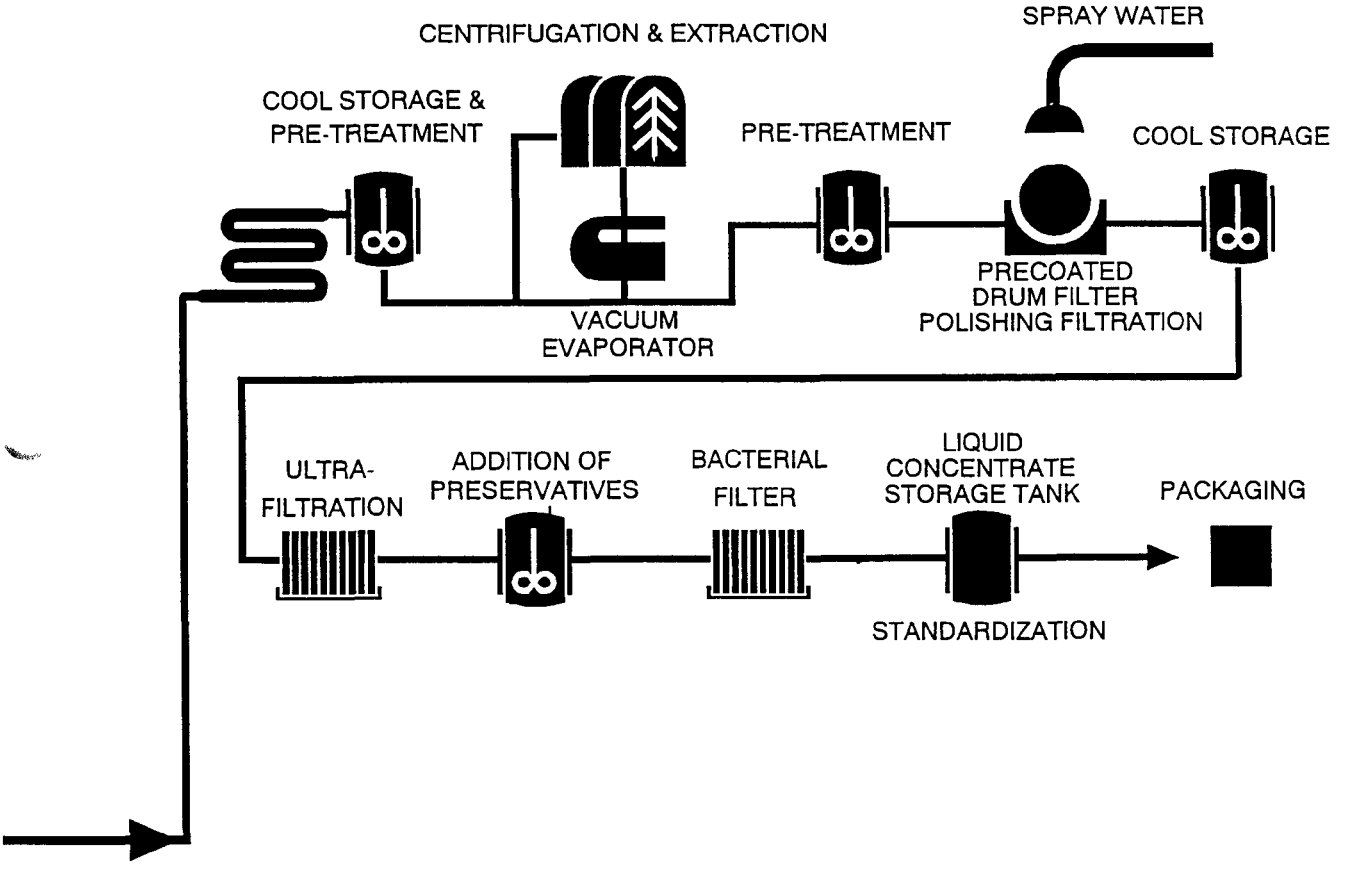

FIGURE 2

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Appendix C

000239

Rheozyme™

Description

Rheozyme is a selected enzyme preparation of a microbial pectinesterase (E.C.3.1.1.11, pectin pectylhydrolase) from *Aspergillus aculeatus* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism.
Rheozyme hydrolyzes the methylesterified galacturonic acid residues in pectins.

Application

Rheozyme is generally used for the modification of the functional properties of pectin-containing materials by modifying the distribution of the methoxyl groups in the pectic part:

- in situ de-esterification and gelation of plant materials
- viscosity increase (thickening) of plant preparations
- hardening of fruits and vegetables
- controlled demethylation of HM pectins

Product Specifications

Activity
Rheozyme is available as a liquid with a declared activity of 10 PEU/ml.
The product is standardized in Pectin Esterase Units (PEU).
The analytical method is available on request.

Safety

Rheozyme complies with the FAO/WHO JECFA and FCC recommended specifications for food-grade enzyme preparations.

Storage

It is recommended that Rheozyme is stored at 0-10°C. At this temperature the product will maintain the declared activity for at least one year and at 25°C for at least three months.

Handling Precautions

Detailed handling recommendations are described in a separate leaflet, "How to handle liquid Novo Nordisk enzymes - safely" (B144), available on request.

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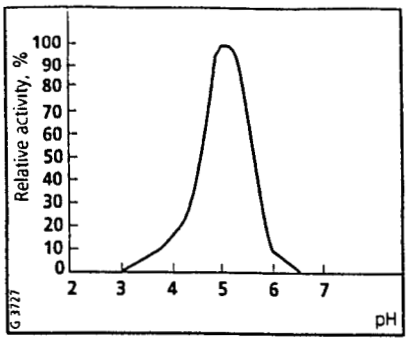

Fig. 1. Rheozyme - influence of pH on activity.
Substrate: 0.5% citrus pectin, DE 72%
Temperature: 20°C
Novo Nordisk method used at various pH values

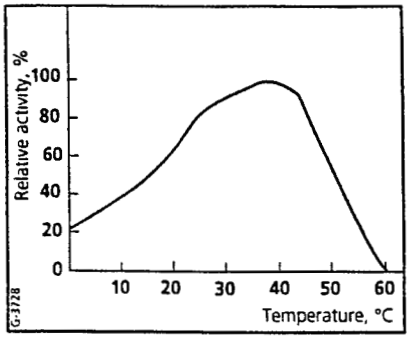

Fig. 2. Rheozyme - influence of temperature on activity.
Substrate: 0.5% citrus pectin, DE 72%
pH: 4.8
Novo Nordisk method used at various temperatures

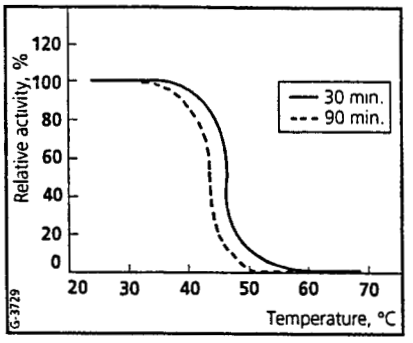

Fig. 3. Rheozyme - influence of temperature on stability.
Substrate: 0.5% citrus pectin, DE 72%
pH: 4.8
Reaction times: 30 min. and 90 min.
Novo Nordisk method used at various temperatures

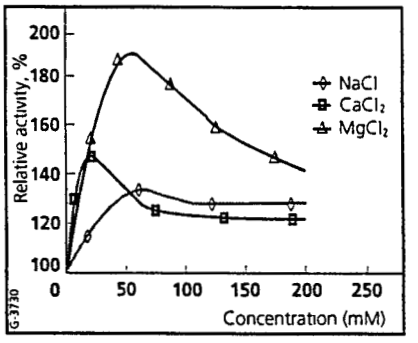

Fig. 4. Rheozyme - influence of cations on activity.
Substrate: 0.5% citrus pectin, DE 72%
Temperature: 20°C
pH: 4.8
Cations: Na^+, Ca^{++}, Mg^{++} used as salts in chloride form
Novo Nordisk method used at various cation concentrations

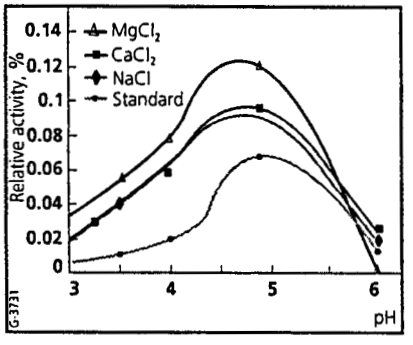

Fig. 5. Rheozyme - influence of cations at different pHs on activity.
Substrate: 0.5% citrus pectin, DE 72%
Temperature: 20°C. pH: 4.8
Concentration of cations:
Na^+: 70 mM, Ca^{++}:10 mM;
Mg^{++}: 50 mM
Novo Nordisk method used at various pH values

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Enzyme Business

Novo Nordisk Ferment Ltd.
Neumatt
4243 Dittingen
Switzerland

Tel. +41 61 7656111
Fax +41 61 7656333
Telex 962970

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Appendix D

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Rheozyme™

Application: General

Description

The enzyme preparation Rheozyme can generally be used for the modification of pectin-containing plant materials: modification of physicochemical parameters related to pectic substances like viscosity, gelation, syneresis, flow behaviour, appearance (e.g. colour, taste, flavour), flocculation, cloud stability, and so on.

Application

Industrial applications are:

- in situ gelation of plant materials (e.g. jam)
- viscosity increase (thickening) of fruit and vegetable preparations (e.g. compote, purée, paste, fillings for bakery and dairy products, ketchup, sauces)
- reducing syneresis
- juice treatment (cloud stability, clarification: e.g. cider)
- hardening of fruits and vegetables
- tailored modification of pectins (e.g. controlled demethylation of HM pectins to LM pectins)
- etc.

Storage

It is recommended that Rheozyme is stored at 0-10°C. At this temperature the product will maintain the declared activity for at least one year and at 25°C for at least three months.

Handling Precautions

Detailed handling recommendations are described in a separate leaflet, "How to handle liquid Novo Nordisk enzymes - safely" (B144), available on request.

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Enzyme Business

Novo Nordisk Ferment Ltd.
Neumatt
4243 Dittingen
Switzerland

Tel. +41 61 7656111
Fax +41 61 7656333
Telex 962970

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Rheozyme™

General test of applicability to plant products

Plant materials contain different amounts of pectins with different degrees of esterification (due to the type of material, the harvesting time - fresh, stored, unripe, ripe - seasonal changes, and so on). To check whether the pectinesterase is applicable at all, the following initial test procedure is recommended:

- for Rheozyme the pH range of the raw material should be within pH 4-5
- take the relevant raw material
- mince the raw material to a kind of purée
- pasteurize the purée (inactivate the plant's own enzymes, increase the accessibility of the pectin)
- ensure that the temperature is between 20-40°C
- divide into three portions:
  - sample 1: for blank
  - sample 2: for Rheozyme treatment alone
  - sample 3: for Rheozyme treatment after addition of $CaCl_2$
  - enzyme dosage recommended: 20,000 ppm ~ 20 ml Rheozyme/kg
  - $CaCl_2$ dosage recommended: 50 mM $CaCl_2$ = 7.5 g $CaCl_2$ x $2H_2O$/litre
- observe the modification of the physico-chemical parameters that are of interest (e.g. viscosity, gelation, syneresis, flow behaviour, appearance, etc.) for at least 30-60 minutes and compare to the blank.

This test procedure gives an indication of what it will be possible to achieve with Rheozyme on a given raw material. Calcium is added to one sample in order to see what the effect of changing the cation sensitivity of the pectin fraction during Rheozyme treatment will be.
These results can be used to decide on further tests (target definition, optimization, etc.).

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Storage

It is recommended that Rheozyme is stored at 0-10°C. At this temperature the product will maintain the declared activity for at least one year and at 25°C for at least three months.

Handling Precautions

Detailed handling recommendations are described in a separate leaflet, "How to handle liquid Novo Nordisk enzymes - safely" (B144), available on request.

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Enzyme Business

Novo Nordisk Ferment Ltd.
Neumatt
4243 Dittingen
Switzerland
[redacted]

Tel. +41 61 7656111
Fax +41 61 7656333
Telex 962970

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Rheozyme™

Application: Fruit Preparations

Background

Fruit preparations are used industrially as the fruit basis for dairy products (e.g. yoghurt, ice-cream). Beside flavours and colorants, different texturizers are added for mainly two purposes: to achieve a pumpable texture and to avoid water exudation in the final product.
LM pectin is generally used to ensure a soft and firm stucture and so obtain a homogeneous distribution of fruit in the end-product.
The structure of the fruit pieces themselves is of great importance for the desired mouthfeel and appearance of the end-product.
Fruit variety and ripeness play an important role in achieving a desirable texture.
Rheozyme is a selected enzyme preparation of a microbial pectinesterase, which hydrolyzes the methylesterified galacturonic acid residues in pectins.
Rheozyme modifies the distribution of the methoxyl groups in the pectic substances of the fruits to be processed and enables in situ de-esterification, thickening and hardening of fruit pieces.

Rheozyme will yield the following advantages:

- reduced or no need for food additives
- improved texture of fruit pieces
- improved distribution of fruit pieces within the matrix
- reduced syneresis
- improved flovour

The use of Rheozyme is limited to the natural content of the pectic substances available in the plant material and to a more soft type gel structure. To test the applicability of Rheozyme for a particular product, a general test procedure is recommended, which is available on request (Rheozyme: General test of applicability to plant products).

Product Specifications

Rheozyme is available as a liquid with a declared activity of 10 PEU/ml.
Further information is available in the Product Sheet (available on request: Rheozyme, Product Sheet).

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Application

Rheozyme: a process aid for manufacturing fruit preparations
General process

- thawing of frozen fruit pieces
- mixing and heating of fruit and sugar if wanted
- heating to 40°C
- enzyme treatment
  addition and mixing of Rheozyme:
  - dosage: 1-5 ml/kg fruit
  - reaction time: 10-60 minutes

  (the actual dosage and reaction time needed are dependent on the fruits used (type, pectin content, ripeness, Ca content) and the desired final consistency)
- mixing of desired additives
- pasteurization / sterilization
- cooling to filling temperature
- filling

Storage

It is recommended that Rheozyme is stored at 0-10°C. At this temperature the product will maintain the declared activity for at least one year and at 25°C for at least three months.

Handling Precautions

Detailed handling recommendations are described in a separate leaflet, "How to handle liquid Novo Nordisk enzymes - safely" (B144), available on request.

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Enzyme Business

Novo Nordisk Ferment Ltd.
Neumatt
4243 Dittingen
Switzerland

Tel. +41 61 7656111
Fax +41 61 7656333
Telex 962970

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Rheozyme™

Application: Compote

Background

Fruit compotes are made industrially by grinding selected fruits after several pretreatments, mixing them with sugar and heat-treating this mixture to the desired consistency. Normally no gelling agents are allowed as additives.
To reach the desired consistency the fruit variety and ripeness play an important role in avoiding texture problems.

Rheozyme

Rheozyme is a selected enzyme preparation of a microbial pectin-esterase, which hydrolyzes the methylesterified galacturonic acid residues in pectins.
Rheozyme modifies the distribution of the methoxyl groups in the pectic substances of the plant materials to be processed and enables in situ de-esterification and thickening.

Rheozyme will yield the following advantages:

- improved texture
- reduced syneresis
- less heat treatment to achieve a certain texture
- no use of food additives

The use of Rheozyme is limited to the natural content of the pectin substances available in the fruits.
To test the applicability of Rheozyme for a particular product, a general test procedure is recommended, which is available on request (Rheozyme: General test of applicability to plant products).

Product Specifications

Rheozyme is available as a liquid with a declared activity of 10 PEU/ml.
Further information is available in the Product Sheet (available on request: Rheozyme, Product Sheet).

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Application

Rheozyme: a process aid for manufacturing compote
General process

- washing + sorting of fruits
- removal of peels and cores
- grinding
- heat treatment
  - fruit pasteurization
  - inactivation of native enzymes
  - solubilization of pectins
  - achieving the desired consistency

  (the actual dosage and reaction time needed are dependent on the fruits used (pectin content, ripeness) and the desired final consistency)
- addition of sugar
- sterilization
- filling

Storage

It is recommended that Rheozyme is stored at 0-10°C. At this temperature the product will maintain the declared activity for at least one year and at 25°C for at least three months.

Handling Precautions

Detailed handling recommendations are described in a separate leaflet, "How to handle liquid Novo Nordisk enzymes - safely" (B144), available on request.

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Enzyme Business

Novo Nordisk Ferment Ltd.
Neumatt
4243 Dittingen
Switzerland

Tel. +41 61 7656111
Fax +41 61 7656333
Telex 962970

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Application: Cider

Background

Ciders are produced by first processing an apple must, which is traditionally clarified before the first fermentation in order to eliminate impurities, avoid side-fermentations giving off-flavours and slow down the main fermentation.
Traditionally this fining is done by flotation using either the endogenous pectin-esterase (dependent on fruit variety and ripeness) or commercial enzyme preparations.

Rheozyme

Rheozyme is a selected enzyme preparation of a microbial pectin-esterase, which hydrolyzes the methylesterified galacturonic acid residues in pectins.
Rheozyme modifies the distribution of the methoxyl groups in the pectic substances in the must, enabling a better flotation with quicker and more compact building of Ca pectate.
Rheozyme will yield the following advantages:

- improved clarification (better, faster)
- delayed start of fermentation
- slowing down the main fermentation
- improved taste and flavour
- reduced methanol content in the cider

Product Specifications

Rheozyme is available as a liquid with a declared activity of 10 PEU/ml.
Further information is available in the Product Sheet (available on request: Rheozyme Product Sheet).

Application

Rheozyme: a process aid for manufacturing cider
General process

- washing and sorting of apples
- grinding
- maceration
- pressing
- must clarfication:
  - enzyme treatment
  - addition and mixing of Rheozyme
  - dosage: 50-100 ml/1000 l must
  - reaction time: 5-15 minutes
  - ambient temperature (5-20°C)

  (the actual enzyme dosage and reaction time needed are dependent on the content of the pectic substances (apple variety, ripeness) in

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the must and the ambient temperature)
- first fermentation (alcoholic fermentation)
- second fermentation (development of flavours)
- filtration
- flash pasteurization
- bottling

Storage

It is recommended that Rheozyme is stored at 0-10°C. At this temperature the product will maintain the declared activity for at least one year and at 25°C for at least three months.

Handling Precautions

Detailed handling recommendations are described in a separate leaflet, " How to handle liquid Novo Nordisk enzymes - safely" (B144), available on request.

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Enzyme Business

Novo Nordisk Ferment Ltd.
Neumatt
4243 Dittingen
Switzerland

Tel. +41 61 7656111
Fax +41 61 7656333
Telex 962970

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Rheozyme™

Application: Jam

Background

Traditionally industrial jams are made from fruit, sugar, water, pectin and edible acid. Pectin is added in various forms as a gelling agent to obtain the desired texture in this multicomponent system.
High-methylated pectins (HM pectins) are used in products with a soluble solids content of >55% and pH values below 3.5 (average pH 2.8-3.2), resulting in a non heat-reversible strong texture. These conditions are necessary in order to achieve a 3D network gel via hydrogen bonds.
Preparations with lower sugar content or higher pH values (pH range from 1-5 or higher) are manufactured with low-methylated pectins (LM pectins), which have the advantage of forming heat-reversible gels with a soft structure via calcium bonds independently of sugar and acids.
The pectins used are produced by extraction from fruit waste materials, e.g. citrus or apples.
Pectins in general are structural polysaccharides occurring in plant cell walls. The tissues of the edible parts of fruits and vegetables consist of parenchymatic cells with a middle lamella mainly consisting of pectins. The primary cell wall is a firm gel of pectin, cellulose, hemicellulose and protein. Technologically these pectic substances play the greatest role. They account for 0.5-4% of the weight of fresh material.

Rheozyme is a selected enzyme preparation of a microbial pectinesterase, which hydrolyzes the methylesterified galacturonic acid residues in pectins.
Rheozyme modifies the distribution of the methoxyl groups in the pectic part of the plant materials to be gelated and enables in situ de-esterification and gelation without any further addition of pectins as gelling agents (HM or LM pectins).
Rheozyme will induce a type of structure different from that of added pectins, which will yield the following advantages:

- soft texture
- increased spreadability
- enhanced flavour (not masked by added pectins)
- reduced syneresis
- reduced need for water evaporation
- optimized use of fruits
- no use of food additives

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The use of Rheozyme is limited to the natural content of the pectic substances available in the plant material and to a more soft type gel structure. To test the applicability of Rheozyme for a desired product, a general test procedure is recommended, which is available on request (Rheozyme: General test of applicability to plant products).

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Product Specifications

Rheozyme is available as a liquid with a declared activity of 10 PEU/ml.
Further information is available in the Product Sheet (available on request: Rheozyme Product Sheet).

Application

Rheozyme: In situ gelation of jam
General process

- mixing of fruits and sugar
- pre-cooking
  - fruit pasteurization
  - inactivation of native enzymes
  - sugar inversion
  - solubilization of pectins
- concentration
  - adjustment to the required soluble solids content (°Brix)
  - deaeration
- cooling to 40°C
- enzyme treatment
  addition and mixing of Rheozyme:
  - dosage: 1-5 ml/kg fruit
  - reaction time: 10-60 minutes

  (the actual dosage and reaction time needed are dependent on the fruits used (pectin content, ripeness) and desired final consistency)
- sterilization
- filling

Storage

It is recommended that Rheozyme is stored at 0-10°C. At this temperature the product will maintain the declared activity for at least one year and at 25°C for at least three months.

Handling Precautions

Detailed handling recommendations are described in a separate leaflet, "How to handle liquid Novo Nordisk enzymes - safely" (B144), available on request.

000254

Enzyme Business

Novo Nordisk Ferment Ltd.
Neumatt
4243 Dittingen
Switzerland

Tel. +41 61 7656111
Fax +41 61 7656333
Telex 962970

[redacted]

Laws, regulations and third party rights may prevent customers from importing, processing, applying and/or reselling certain products in a given manner. It is the responsibility of the customers that their specific use of products from Novo Nordisk does not infringe relevant laws and regulations and, furthermore, does not infringe patents or other third party rights.

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Rheozyme™

Application: Tomatoes

Background

Tomatoes are processed industrially into a variety of different end-products: peeled tomatoes, paste, ketchup and sauces.
According to their needs, each of these products may be differently formulated, e.g. sauces for serving either cold or hot have to be formulated differently with texturizing agents (pectins, starch, alginates, carrageenans or guar gum).
Rheozyme is a selected enzyme preparation of a microbial pectinesterase, which hydrolyzes the methylesterified galacturonic acid residues in pectins.
Rheozyme modifies the distribution of the methoxyl groups in the pectic part of the tomatoes and enables in situ de-esterification and thickening without any further addition of texturizers.

Rheozyme will yield the following advantages:

- improved, stabilized texture and flavour
- improved flow behaviour
- increased viscosity
- reduced syneresis
- no use of food additive

The use of Rheozyme is limited to the natural content of pectins available in the processed tomatoes. The product is intended for use only in hot-break tomato pastes, where the endogenous enzymes are destroyed before interfering with the pectins available. The texture produced with Rheozyme is heat-reversible and calcium-sensitive. The texture is best suited to products served cold.

Product Specifications

Rheozyme is available as a liquid with a declared activity of 10 PEU/ml.
Further information is available in the Product Sheet (available on request: Rheozyme Product Sheet).

Application

Rheozyme: a process aid for manufacturing tomato sauces and ketchup without adding thickeners.

Novo Nordisk

General process

- hot-break paste
- homogenization
- enzyme treatment
  addition and mixing of Rheozyme:
  - dosage: 1-5 ml/kg tomato paste with 22°Brix
  - reaction time: 10-60 minutes

  (the actual dosage and reaction time needed are dependent on the tomatoes used (type, pectin content, ripeness, efficiency of the hot-break process) and the desired final consistency)
- formulation of end-product
- heat treatment
- filling

Storage

It is recommended that Rheozyme is stored at 0-10°C. At this temperature the product will maintain the declared activity for at least one year and at 25°C for at least three months.

Handling Precautions

Detailed handling recommendations are described in a separate leaflet, "How to handle liquid Novo Nordisk enzymes - safely" (B144), available on request.

000256

Enzyme Business

Novo Nordisk Ferment Ltd.
Neumatt
4243 Dittingen
Switzerland

Tel. +41 61 7656111
Fax +41 61 7656333
Telex 962970

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Appendix E

[redacted]

To: TEMa, BRP, KDO, CaBo, BRJ, HFPe

Cc.: Hans

From: GiBu

ED-9710066

1997-01-28

PECTINESTERASE (PE)

Traditional Jam Preparation vs. Enzymatic Gelification
Heat Inactivation of PE
Measurement of Methanol Formation

1. Objective:

The purpose of this work has been to carry out a comparison of pectinesterase treated jam and traditional pectin added jam. The test was carried out at various levels of soluble solids - sugar/berry ratio.

An other objective has been to analyse the condition for the minimum time/temperature level for the inactivation of pectinesterase.

Finally the study also includes quantification of the formed methanol, being one of the products from the PE action.

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2. Conclusion:

2.1 It can be concluded that PE-strawberry jam is comparable, at the given conditions, to traditional jam added approx. 0.4 % pectin. For traditional jams the recommended level of added low methoxylated pectin is 0,5-0,7 %.

2.2 A temperature level of 80°C and a holding time of 1 min is adequate and secures a complete inactivation of the added enzyme and thus the heat inactivation allows a stringent control of the enzyme reaction.

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2.3 Evaporation is a possible way to reduce the content of free methanol. The content of free methanol is dependent on the dosage. The content of free methanol

[redacted]

is, irrespective the processing method , in the same level as seen in more traditional, non alcoholic, vegetable products as e.g. tomato juice (Wucherpfennig,K. et al, 1983). The present observations is, however, of minor importance as more articles point out that the total methanol pool should be the area of concern.

3. Key words:

Pectinesterase, functionality, strawberry, jam, gel, inactivation, methanol

4. Experimental:

4.1 Traditional Jam Preparation vs. Enzymatic Gelification

In order to test the functionality of a PE-jam versus traditional jam, traditional jams with increasing content of pectin were compared to a PE-jam.

Typically low sugar jam contains from 35-40 % soluble solids (SS) or approx. 30-35 % sugar while the most common jam types contain approx. 60 % soluble solids or 55 % sugar or even higher. In the present study a 38 % SS and a 57% SS jam have been tested.

4.1.1 Materials and methods:

Pectinesterase, batch PPJ 4300, Activity 425 PEU/g
Pectin, Genu pectin LM 102, Copenhagen Pectin
Pectin, Genu pectin LM 104, Copenhagen Pectin
Strawberries, frozen, sort : Senga sengana
SMS Texture Analyser TA-XT2

Texture Analysis:
The gel strength/hardness of the slurries was measured by the Texture Analyzer by compression analysis. The compression was carried out by using a flat cylinder, diameter: 20 mm and with a speed of 2.0 mm/s. The compression make up a total of 20 % of the sample height. From the record of the force-time curve, the gel strength, measured as the peak force, is directly obtained. The gel strength was measured as an average of four measurements and is given in N.

Spread Meter Test:
The Spread Meter Test determines the fluidity of a 100 g sample of jam, adjusted to room temperature, and placed in the centre of an acrylic plate with printed concentric circles. The spreading out of the jam is measured after 15 min. The spread-value is given in cm and is an average of 8 points on the circle.

The activity of the pectinesterase is given in Pectin-Esterase-Units (PEU) defined as the amount of enzyme which under standardised conditions liberates 1 mmol carboxyl groups per minute (ABT-SM-0005.02.1)

The preparation of the jam was carried out according to standard procedure for jam preparation (SOP ABF-SP-4002.02).

Two different jam types were prepared:

- Low sugar jam, 38 % soluble solids (SS), (berries 51 %, sugar 33 %, water 16 %)

- Medium sugar jam, 57 % (SS), (berries 41 %, sugar 53 %, water 6 %)

The water was added in order to dissolve the pectin. Additional water was also added to the PE-jam only to secure equal evaluation conditions. LM pectin 104 was used in the preparation of SS 38 %, and LM 102 for the preparation of SS 57% (Annon., 1993).

Jam-samples based on low sugar jams (38% SS) and on medium sugar jam, 57 %SS, were prepared as follows:

	38 % SS	57% SS
Pectin added jam	1. 0,0 % LM 104 *) 2. 0,2 % LM 104 3. 0,4 % LM 104 4. 0,8 % LM 104	1. 0,0 % LM 102 * 2. 0.1 % LM 102 3. 0,3 % LM 102 4. 0,5 % LM 102 5. 0,8 % LM 102
Pectin esterase jam	5. 10 PEU /kg fruit	6. 10 PEU /kg fruit

*) The pectin is added in percentage of total jam mass

After the enzyme reaction, 60 min at 40°C, the jams were heat treated to inactivate PE at 85°C for 3 min, and then distributed into blue-cap bottles, 50 g in each. The samples were placed at 5°C until analysis took place.

4.1.2 Results:

The results from the analysis appear from below:

Fig. 4.1.1 Gel strength, effect of PE vs LM Pectin in a strawberry jam , SS 38 %

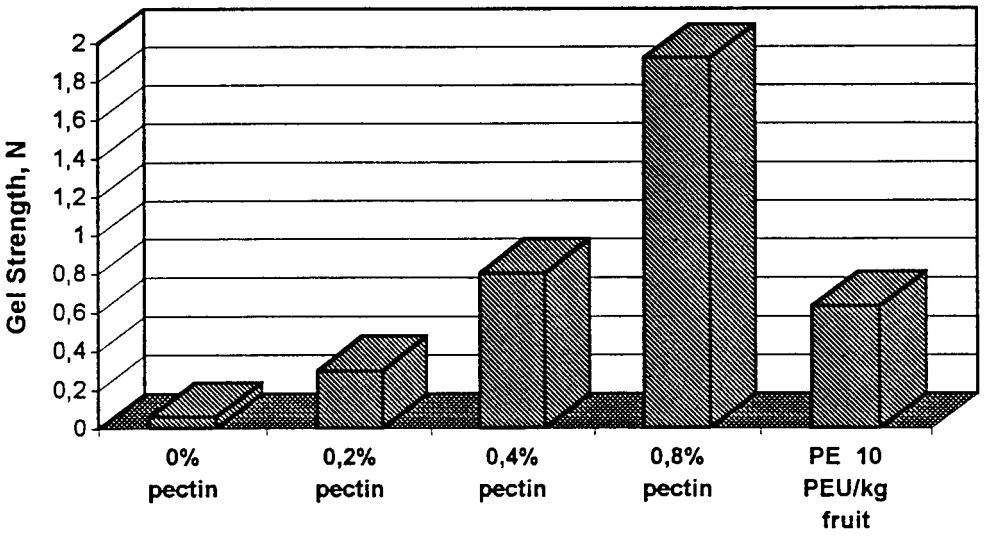

Fig. 4.1.2 Spread Meter Test, effect of PE vs LM Pectin in strawberry jam , SS 38 %

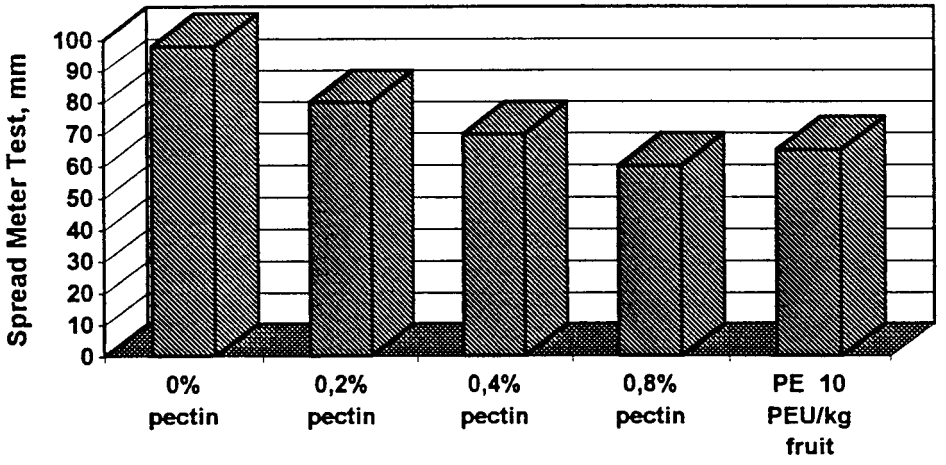

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Fig. 4.1.3 Gel strength, effect of PE vs LM Pectin in a strawberry jam , SS 57%

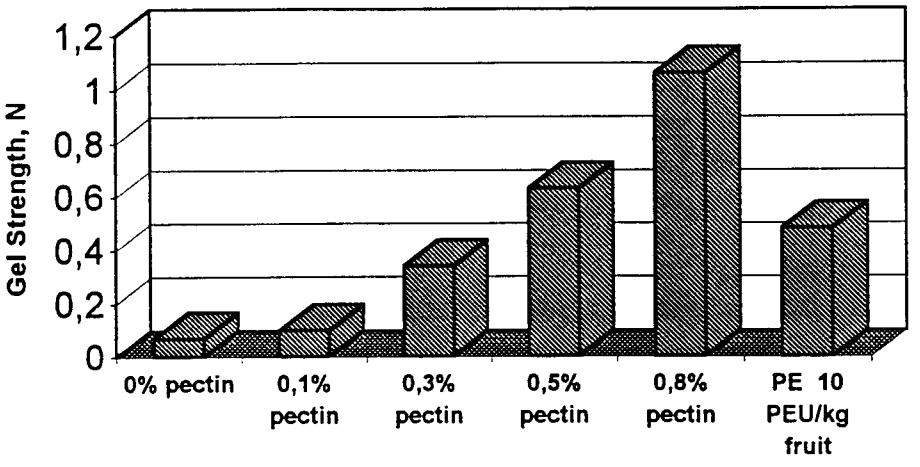
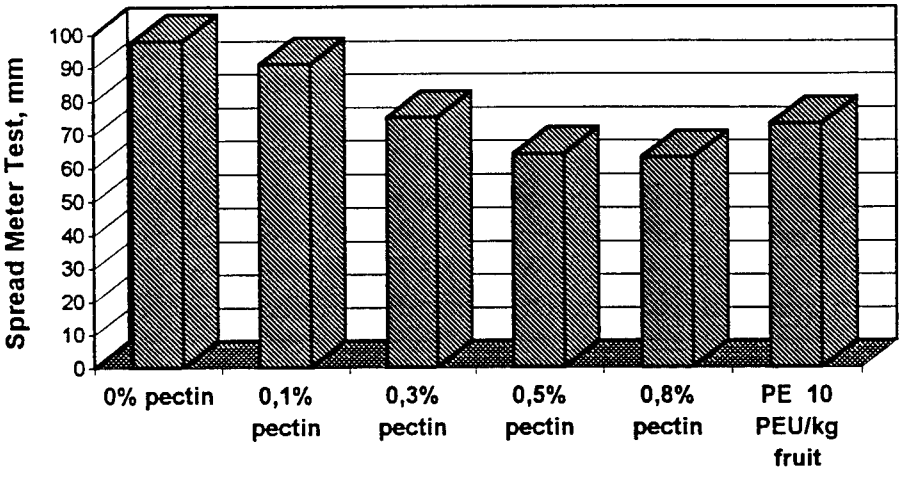


Fig 4.1.4 Spread Meter Test, effect of PE vs LM Pectin in strawberry jam , SS 57 %



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[redacted]

4.1.3 Conclusion:

Pectin is traditionally added in the level of 0.2 - 1.0 %. For low to medium sugar jams only LM pectins are relevant gelifying agents, the recommended level is approx. 0,5-0,7 % (Annon., 1993).

Based on the spread meter test as well as the gel strength it can be concluded that at both levels of soluble solids the PE-jam is comparative to jam added approx. 0.4 % pectin. The results is in accordance with the contents given in the literature e.g. Fishman, M.L. and Jen, J.J., 1986 and Vidal-Valverde, C. et al., 1982 as an approximate level of pectin of strawberries is analysed to be in the range of 0.4-0.6 %.

4.2 Heat Inactivation of PE - Measurement of Residual Activity:

In order to specify the conditions for the minimum time/temperature level for inactivation of PE the following experiment was carried out.

4.2.1 Material and methods:

Pectinesterase, batch PPJ 4300

The PE residual activity was measured according to ED 9710067. The principle of the assay is to allow a PE containing sample to de-esterify high methoxylated pectin (HM-pectin) . A given sample is placed in wells in an agarose plate containing 2 % HM pectin. The activity of the enzyme is after a certain incubation time visualised by precipitation of the demethylated pectin by adding quaternary ammonium ions. Demethoxylated pectin results in a turbid circle around the application well.

The following standard-jams were prepared: 0, 5, 10, 20, 30, 50, 70, 100 PEU/kg fruit. Jams added PE at the level of 0, 50 and 100 PEU/kg fruit were also prepared. The enzyme was allowed to react for 60 min at 40°C. The test samples were heat inactivated at 3 different temperatures: 80, 85, 92 °C for 0, 1, 3, 8 min, respectively. The temperature was raised to the specific temperature in the microwave oven and then kept for the mentioned time ranges. The samples were placed in the refrigerator until analysis could take place. The jam preparations were carried out in dublicate and the order of the individual sample treatments were chosen randomly. Then both the test samples and the standards were diluted 1+1 and 10 µl of each were added to the wells. The plates were incubated over night at 30°C and then treated according to the "PE residual method".

4.2.2 Results and discussion:

The results appear from enclosure. When analysing the results the diameters of the zones were directly used as a measure of activity. The standard curve was tested in other calculation models to possibly obtain a linearity of the dosage as function of the response (area, ln). However no linearity was obtained and thus the diameter was used as is.

As no residual enzyme activity was observed in the test samples, the standard curve has not as such been used. However, the standard curve contributes with information about the detection limit. Under the given conditions the detection limit is 5 PEU/ kg analysed product or 2,5 PEU/kg fruit.

It is seen from the results, that no residual activity could be detected in any of the heat treated samples, the conclusion is thus straight forward: A temperature level of 80°C and a holding time of 1 min is adequate and secures a complete inactivation of the added enzyme and thus the heat inactivation allows a stringent control of the enzyme reaction.

4.3 Methanol content as a function of PE dosage and evaporation temperature:

The major part of the industrial production of jam is carried out in semi-continuous plants. Such plants includes vacuum evaporation in order to adjust the final content of dry matter. The evaporation temperature has from two customers been informed to be in the range of 70-75°C, that is the absolute pressure has been in the range of 310 -380 mbar. The evaporator is most often placed immediately after mixing of fruit and sugar. The industry indicates a possible holding time at that stage and thus a way to introduce the PE induced gelification.

In the present trials the effect of evaporation was tested in lab scale in order to quantify the methanol formed and to evaluate the possible reduction of methanol. GC-head space was used to test the methanol formation as function of various combinations of PE dosages.

The initial content of fruit in this jam is 70 % meaning that fruit and thus methanol containing pectin is present at a rather high level.

[redacted]

4.3.1 Materials and method:

Pectinesterase batch PPJ 4300

Vacuum rotary evaporator, Rotavapor RE 140

Perkin Elmer Gaschromatograph, GC 8700 with a DB 624 column
Head space sampler, Perkin Elmer HS 101
GC setup:

Oven temp.: 50 C
Injektor: 220 C
Detector: 250 C, FID
Start time: 2 min.
Rate 30 C/min
End temp. 200 C
Final time: 10 min
Sample temp 80 C
Needle temp: 100 C
Transfer line 100 C
Thermo. time 60 min.
Pressur. time: 2 min.
Injec. time 0.05 min.
Withdrawel time 0.2 min.
Column press. 23 psi.
Needle vent. 9.6 ml/min.
Vial press. 40 psi

Vacuum rotary evaporator
A vacuum rotary evaporator with adjustable rotation speed and destillation pressure (model Rotavapor RE-140 produced by Bucher) was used for the vaccum evaporation. A glass container of 500 ml was used.

The preparation of samples were carried out according to 4.1.1 Materials and methods. However, in the present test the inactivation was carried by evaporation to a final dry matter of 50°brix, and not by microwave oven as described in SOP ABF-SP-4002.02/01. The ratio of berries and sugar was 70 % berries : 30 % sugar. After pre-cooking the temperature was adjusted to 40°C. Samples with three different enzyme levels: 0, 10, 100 PEU/kg fruit were prepared. The samples were then allowed to react for 60 min at 40°C. Afterwards the samples were evaporated at 350 mbar while the temperature of the water bath was 70 - 75°C. Samples were run, in doublicate. The order of preparation was randomly chosen.

The jam samples were further prepared as described below and then the samples were analysed for free methanol by means of head space gas liquid chromatography. The gaschromatograph was a Perkin Elmer Gaschromatograph, GC 8700 fitted with a DB 624 column. The samples were injected by a head space sampler, Perkin Elmer HS 101. The analysis were carried out at CC2, Søborg.

Sample preparation for GC-head space:
A volume of 2.5 ml of water was added to 2.5 ml of sample in a head space vial. The concentration of methanol was calculated by comparing the detector response to a standard curve of methanol in water. Recovery solutions were prepared by mixing 2.5 ml jam sample and 2.5 ml of a suitable standard solution of methanol (in water) in a head space vial.

4.3.2 Results and discussion:

The results appears from below:

Table 4.3.1 Methanol formation as function of dosage

	Sample no. 1	Sample no. 2	Sample no. 3	Sample no. 4
0 PEU/kg fruit	26 ppm	26 ppm	20 ppm	20 ppm
10 PEU/kg fruit	118 ppm	104 ppm	110 ppm	124 ppm
100 PEU/kg fruit	170 ppm	134 ppm	126 ppm	160 ppm

The data were analysed by means of Statgraphics. The ANOVA test demonstrated a significant effect at the 99,9 % level of the concentration of PE on the final concentration of methanol. The values are in general in accordance with earlier analysis (ED 9613559). The latter experiment was also carried out according to SOP ABF-SP-4002.02.01. The strawberry/sugar ratio was lower: 60/40 compared to 70/30 and no vacuum evaporation was applied. PE concentration was 10 PEU/kg fruit. In this latter case the methanol analysis was carried out by water distillation of the sample and then determination by using GC MS. The methanol content of the jam was analysed to 200 ppm. Even though the content of strawberries were lower compared to the trials described above and even though no evaporation took place and thus no reduction of the mass, this 200 ppm-level is higher than the mean value of the present trials of 114 ppm. In conclusion the evaporation does have an impact on the content of free methanol.

of the present trials of 114 ppm. In conclusion the evaporation does have an impact on the content of free methanol.

The total pool of methanol in e.g. strawberries can be estimated to 700-1000 ppm if the pectin content is estimated to 0,4 - 0,6 % and the degree of esterification (DE) is 75 %. It is worth emphasising that this total pool of methanol is of course not increased by the PE activity. In a traditional low sugar jam the total pool can be estimated to 1000-1300 ppm (on the assumption that 0,6 % low methoxylated pectin was added, with a DE of 30-40 %). This level is obviously higher than the level obtained when jam is prepared with pectinesterase.

Wucherpfennig,K. et al, 1983 has analysed bound as well as free methanol and claims that from a nutritional and a physiological point of view, the free as well as the bound methanol should be considered. Further Wucherpfennig, K. et al has listed average contents of free and bound methanol in various vegetable products, found by a number of workers. The free methanol is in the range of 15-171 ppm and thus in the same range as seen above. Grassin, C. and Fauquembergue, P. , 1996, states that pectin is digested by the human body and that methanol is released as a results of pectin esterase derived from intestinal bacteria. Seen in the light of the above and several references dealing with the aspartame -case and -approval, the human absorption of methanol from vegetables should be considered independent of its free and bound status.

In conclusion, evaporation is a possible way to reduce the content of free methanol. Further, the content of free methanol is dependent of the dosage. The content of free methanol is, irrespective the processing method , at the same level as seen in more traditional, non alcoholic, vegetable products as e.g. tomato juice (Wucherpfennig,K. et al, 1983). The present observations is however of minor importance as more workers point out that the total methanol pool should be the area of concern.

5. References:

1. Annon. 1993, Handbook for the Fruit Industry, Copenhagen Pectin A/S
2. Fishman, M.L. and Jen, J.J., 1986, Chemistry and Function of Pectins
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8. Skott, W. P. *Die* Industrielle Obst- und Gemüseverwertung, 1970 **55** 229-23
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10. Wucherpfennig, K. Dietrich, H. and Bechtel, J. Flüssiges Obst, 8, 348-354, 1983

6. Initials/signature/date:

– 1997-28 c)

[redacted]

Enclosure - Results from the heat inactivating test (4.2)

Temp °C	Time, min.	PEU/ kg	Test result, mm
85	1	20	0
80	3	20	0
85	3	20	0
92	3	20	0
85	3	20	0
40	0	20	10
80	1	20	failed
92	1	20	0
92	8	20	0
80	8	20	0
80	3	20	0
80	8	20	0
80	1	20	0
92	8	20	0
85	8	20	0
85	1	20	0
40	0	20	6
85	8	20	0
92	1	20	0
92	3	20	0
85	1	0	0
80	3	0	0
85	3	0	0
92	3	0	0
85	3	0	0
40	0	0	0
80	1	0	0
92	1	0	0
80	8	0	0
80	3	0	0
80	8	0	0
80	1	0	0
92	8	0	0
85	8	0	0
85	1	0	0
40	0	0	0
92	3	0	0
92	1	0	0
85	8	0	0
40	0	100	6
40	0	100	16
92	1	100	0
85	1	100	0
92	1	100	0
80	8	100	0
80	1	100	0

[redacted]

	Time, min.	PEU/ kg	Test re sult, mm
85	8	100	0
92	8	100	0
92	3	100	0
80	8	100	0
85	8	100	0
80	1	100	0
85	3	100	0
85	3	100	0
85	1	100	0
92	8	100	0
80	3	100	0
92	3	100	0
80	3	100	0
85	8	50	0
92	3	50	0
80	1	50	0
92	1	50	0
80	8	50	0
92	8	50	0
85	8	50	0
80	3	50	0
92	8	50	0
80	8	50	0
85	1	50	0
85	3	50	0
92	1	50	0
80	1	50	0
40	0	50	1
40	0	50	8
92	3	50	0
85	3	50	0
80	3	50	0
85	1	50	0

Conc of standards	1 set of standards Diameter, mm	2. set of standards Diameter, mm
100 PEU/kg fruit	16,0 mm	18,0 mm
70 PEU/kg fruit	18,0 mm	15,0 mm
50 PEU/kg fruit	11,0 mm	16,0 mm
30 PEU/kg fruit	16,0 mm	16,0 mm
20 PEU/kg fruit	12,5 mm	15,0 mm
10 PEU/kg fruit	15,0 mm	13,0 mm
5 PEU/kg fruit	10,0 mm	8,0 mm
0 PEU/kg fruit	0 mm	0 mm

Appendix F

000270

Pages 000271 - 000274 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix G

[redacted]

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SUBMISSION END

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AM

Novo Nordisk

Novo Nordisk BioChem
North America, Inc.

77 Perry Chapel Church Roa
Box 576
Franklinton, NC 27525-0576

Tel. 919-494-3000
FAX 919-494-3450

October 30, 1998

Linda S. Kahl, Ph.D.
Regulatory Policy Branch, HFS-206
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, D.C. 20204

Dear Dr. Kahl,

In response to your request, please find enclosed a diskette which contains Word document files for Novo Nordisk's GRAS Notice No. GRN 000008, for a pectin esterase preparation from *Aspergillus oryzae* expressing the gene encoding a pectin esterase from *Aspergillus aculeatus*. The body of the notice is contained in the file "GRN00008" and the GRAS exemption claim letter is contained in the file "GRASexempclaim".

We are providing these word processing files for internal FDA use only. The files should not be distributed to the public.

Please contact me at (919) 494-3151 if you have any questions or require further information.

Sincerely,

Lori Gregg
Regulatory Associate

1998 NOV -3 P 3:08

000290

[redacted]

Reference List for Industry Submission, GRN 000008

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000271 - 000274	Barbesgaard, Peder; Heldt-Hansen, Hans Peter; Diderichsen, Berge	On the safety of Aspergillus oryzae: a review	1992	Applied Microbiology and Biotechnology	Volume 36, pgs 569-572
000276 - 000285	Lissau, Bodil G.; Pedersen, Peder Bjarne; Petersen, Bent Riber; Budolfsen, Gitte	Safety evaluation of a fungal pectinesterase enzyme preparation and its use in food	1998	Food Additives and Contaminants	Volume 15, Number 6, pgs 627-636

NA- Not applicable