GR

ORIGINAL SUBMISSION

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Novo Nordisk

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16 November, 1999

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,

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 aspartic proteinase enzyme preparation, produced by *Aspergillus oryzae* expressing the gene encoding an aspartic proteinase from *Rhizomucor miehei*. The proteinase enzyme preparation is to be used in the meat industry as a tenderizing agent.

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)

1999 NOV 17 P 2:39

November 16, 1999

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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 aspartic proteinase preparations produced by submerged fermentation of *Aspergillus oryzae* carrying the gene coding for aspartic proteinase from *Rhizomucor miehei* 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.*

Aspartic proteinase enzyme preparation from *Aspergillus oryzae* carrying the gene coding for aspartic proteinase from *Rhizomucor miehei.*

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

The proteinase preparation is to be used in the meat industry as a tenderizing agent. 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. A published article on the safety of *A. oryzae* is 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.

John Carroll
Director, Regulatory Affairs

11-16-99
Date

Novo Nordisk

NovoCarne™ Tender

an aspartic proteinase preparation produced by

Aspergillus oryzae expressing the gene encoding

an aspartic proteinase from *Rhizomucor miehei*

Lori Gregg, Enzyme Regulatory Affairs, NNBNA, USA
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1. GENERAL INTRODUCTION

NovoCarne Tender is the Novo Nordisk A/S trade name used for an aspartic proteinase preparation produced by submerged fermentation of *Aspergillus oryzae* expressing the gene encoding an aspartic proteinase from *Rhizomucor miehei*[1].

The proteinase preparation is to be used in the meat industry as a tenderizing agent. The enzyme is applied in liquid form by injection into the meat followed by tumbling to enhance distribution. It can also be mixed with the marinade for tumbling. NovoCarne Tender has a narrow specificity acting only on the myofibrillar proteins, not the connective tissue proteins[2]. NovoCarne Tender also expresses self-limiting hydrolysis of myosin[2]. These factors prevent NovoCarne Tender from overtenderizing the meat.

The information provided in the following sections is the basis for our determination of general recognition of safety of a proteinase enzyme preparation produced by *A. oryzae* expressing the gene encoding a proteinase from *Rhizomucor miehei*. 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 a published article reviewing the safety of *A. oryzae*[3]. A summary of the safety studies conducted on the enzyme preparation is also included in Appendix E.

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 this proteinase, *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.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The microbial production strain was constructed by plasmid transformation of the industrial strain, *A. oryzae* strain IFO 4177. 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].

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 well-characterized *Escherichia coli* vectors, and synthetic oligonucleotide fragments. The specific DNA sequences include: a gene encoding a *Rhizomucor miehei* proteinase enzyme; an *Aspergillus nidulans* selectable marker gene, *amd*S (acetamidase)[12] ; well-characterized noncoding regulatory sequences from *Aspergillus niger*[13] and *Aspergillus oryzae*[14] ; known sequences from *Escherichia coli* plasmids pUC19[15], pBR322[16] and pIC19R[17], and synthetic oligonucleotide adaptor fragments.

2.2 Recipient Organism

The recipient microorganism is the well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn designated strain IFO 4177 obtained from the Institute for Fermentation, Osaka, Japan (IFO). This strain was used as the host organism without further genetic manipulation. The classification of the *A. oryzae* IFO 4177 strain has been confirmed by the Centraalbureau voor Schimmelcultures, Baarn, Holland[18].

2.3 Proteinase Expression Plasmid

The 5.9 kb proteinase expression plasmid 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 *R. miehei* proteinase gene
0.7 kb DNA from the *A. niger* glucoamylase gene terminator sequence
2.7 kb DNA from the *E. coli* plasmid pUC19
0.05kb DNA from the *E. coli* plasmid pIC19R
0.02kb DNA from the *E. coli* plasmid pBR322

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2.4 Selectable Marker Plasmid

The 8.8 kb selectable marker plasmid used in the construction of the production strain contains the following genetic material (see Appendix A, Figure 2):

5.0 kb DNA from *A. nidulans* encoding the *amdS* gene
3.8 kb DNA from the *E. coli* plasmid pBR322

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 proteinase enzyme preparation have been analyzed by dot blot hybridization and Southern analysis 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]. Faint positives indicating a DNA content of 0.1 - 1 ng/g were found in some batches. The detection limit for pure plasmid DNA is 1 pg plasmid DNA/dot. The DNA was low molecular weight DNA and no distinct bands were present.

No transformation capability was detected in the samples. Analyses were done on 1 mg. Sensitivity can be stated as 0.1-0.2ng DNA (pUC19)/g.

2.7 Antibiotic Resistance Gene

The selectable marker plasmid 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.

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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 proteinase enzyme preparation.

Finally, tests of *A. oryzae* production strains containing the *bla* gene integrated as part of an expression vector have not shown any evidence of β-lactamase expression.

2.8 Absence of Production Organism in the Product

The absence of the production organism is an established specification for the commercial product, NovoCarne Tender. The production organism will not be present 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 NovoCarne Tender which follows standard industry practices[24-26]. The quality management system used in the manufacturing process for NovoCarne Tender complies with the requirements of ISO 9001. It is also manufactured in accordance with current good manufacturing practices.

3.1 Raw Materials

The raw materials used in the manufacturing process for NovoCarne Tender are standard ingredients used in the enzyme industry[24-26]. The raw materials 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 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 NovoCarne Tender product is less than 1%.

3.2 Fermentation Process

NovoCarne Tender is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *A. oryzae* described in Section 2. 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 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism, *A. oryzae,* described in section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

3.2.2 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.

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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.

3.3.1 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. Heat treatment
5. Treatment to reduce thermostability
6. pH adjustment
7. Blank filtration-remove amylase precipitation
8. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation
9. Formulation and Standardization

3.4 Quality Control of Finished Product

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

4. ENZYME IDENTITY

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

Classification:	Aspartic proteinase (generic name)
IUB nomenclature:	Mucor pepsin
IUB No.:	3.4.23.23
CAS No.:	78169-47-8
Specificity:	Hydrolyzes peptide bonds in proteins
Molecular weight:	44.7 kDa
Amino acid sequence:	The total nucleotide and amino acid sequences have been determined

5. COMPOSITION AND SPECIFICATIONS

5.1 Formulation

NovoCarne Tender will be available in a liquid formulation with an activity of 0.2 AU/ml and the following typical composition:

Enzyme solids (E-TOS)	2.6%
Carbohydrates (mainly sucrose)	32.0%
Ash (mainly sodium chloride)	5.0%
Acetic acid	2.0%
Sodium acetate	3.0%
Methionine	1.0%
Potassium sorbate	0.1%
Water	54.4%

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

5.2 Specifications

NovoCarne Tender complies with the purity criteria recommended for enzyme preparations as described in Food Chemicals Codex, 4th edition 1996[19]. In addition, NovoCarne Tender 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[20].

The following specifications have been established for NovoCarne Tender:

Proteinase activity, AU/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 5×10^4
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

Proteinases hydrolyze proteins. The major meat proteins responsible for meat tenderness are the myofibrillar proteins and the connective tissue proteins. NovoCarne Tender has a narrow specificity and hydrolyzes only the myofibrillar proteins (mainly actin and myosin) not the connective tissue proteins[2]. NovoCarne Tender expresses self-limiting hydrolysis of myosin[2]. Also, NovoCarne Tender is thermolabile and is easily inactivated by cooking temperatures (see Appendix C). These characteristics prevent NovoCarne Tender from overtenderizing the meat which can result in a mushy texture.

Other enzymes used for tenderizing meat have very broad specificity and breakdown both myofibrillar and connective tissue proteins resulting in a mushy texture. Papain, which is the most widely used enzyme for tenderization, is very heat stable and is not easily inactivated by cooking. This can also lead to overtenderizing.

6.2 Application

NovoCarne Tender is applied in liquid form by injection into meat followed by tumbling to facilitate distribution. It can also be mixed with the marinade for tumbling. Injection, tumbling or marinade incorporation should be done at low temperatures (5 -10°C) where the

enzyme has little or no activity. The enzyme is active during cooking, but is inactivated by the end of the cooking process.

Further information is given in the enclosed application sheet, "NovoCarne Tender for Meat Tenderization". (Appendix C)

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 NovoCarne Tender is 0.05-0.1 AU/kg meat.

6.4 Enzyme Residues in the Final Food

NovoCarne Tender is applied to the uncooked meat and is not removed before consumption. However, the enzyme has been shown to be inactivated at the temperatures used to cook meat. (See Appendix C)

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[21].

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[3]. *A. oryzae* is accepted as a constituent of foods[21]. *A. oryzae* has been used to produce soy sauce in the United States since before 1958[3,5]. 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]. A GRAS petition, 3G0016 submitted by the Ad Hoc Enzyme Technical Committee (now the Enzyme Technical Association), proposing affirmation that enzyme preparations from *A. oryzae* are GRAS for use in food was submitted to FDA and accepted for filing in 1973[22]. Enzyme preparations from *A. oryzae* have been marketed in the US as GRAS by Novo Nordisk and other companies since that time. Therefore, enzyme preparations from *A. oryzae* are also considered GRAS[4,5,21].

An evaluation of the genetically modified production microorganism for NovoCarne Tender, 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 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 Aspartic Proteinase 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 proteinase enzyme preparation derived from the genetically modified *A.oryzae* is considered safe[5,23]. To confirm the safety of the enzyme, safety studies were performed and are described in Section 7.4. A summary of the safety data is included in Appendix E.

In addition, the proteinase enzyme from the genetically modified *A. oryzae* is essentially identical to a proteinase from *R. miehei*. The *R. miehei* proteinase coding sequence incorporated into the *A. oryzae* production strain has not been altered. The strain of *R. miehei* used as the donor for the aspartic proteinase of this notification has been used to produce milk clotting enzyme since 1969 without reported incidences of human pathogenesis. Milk clotting enzyme from *R. miehei* has been approved for use in cheese production since 1972 (21CFR 173.150). In 1997, the enzyme preparation which is the subject of this notification

was approved for milk clotting (21CFR 173.150). Based on these facts, the proteinase enzyme from the genetically modified *A. oryzae* is considered safe for use in food applications[4,23].

7.3 Safety of the Manufacturing Process

NovoCarne Tender 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 proteinase 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[24-26].

7.4 Safety Studies

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

7.4.1 Description of Test Material

The safety studies described below were all conducted on a test batch of the proteinase preparation which was obtained by the mixing of six 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) Pathogenicity of *A. oryzae*

2) Short term and subchronic toxicity
   -4 week oral in rats
   -Max. Dose in dogs
   -13 week oral in rats
   -13 week oral in dogs

3) Teratogenicity in rats

4) Genetic toxicity
   -Gene mutation (Ames test with *S. typhimurium*).
   -Gene mutation (mammalian cells)

-Chromosome aberrations (*in vitro* cytogenetics with human lymphocytes).

7.4.3 Mycotoxin analyses

The final product 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.

Some strains of *A. oryzae* may produce one or more of the mycotoxins: beta-nitropropionic acid, cyclopiazonic acid, and kojic acid[3]. Mycotoxin analyses have been done on NovoCarne Tender. These *A. oryzae* specific mycotoxins were either below the limit of detection or were well below levels that would be considered toxic.

7.5 Estimates of Human Consumption and Safety Margin

This section discusses the potential human exposure to NovoCarne Tender from meat. The enzyme is inactivated at temperatures used to cook meat. However, in order to demonstrate the "worst case" situation, the following calculations are made assuming that all enzyme activity is retained in the food.

According to the Agriculture FACT BOOK 98 (http://www.usda.gov/news/pubs/fbook98/ch1a.htm), Americans consumed 192 pounds of meat per capita in 1997(total meat including beef, pork, veal, lamb, poultry and fish). This corresponds to 239 g of meat per day. The minimum dose level of NovoCarne Tender is 0.02 AU/kg meat. However, doses of 0.05-0.1 AU/kg meat are recommended to achieve the desired effect.

As a precautionary measure, a maximum application dosage of 0.1 AU/kg meat is assumed. This corresponds to 0.5 ml of NovoCarne Tender per kg meat. NovoCarne Tender contains 2.58% TOS. Therefore, the maximum dose contains 0.0129 g TOS/kg meat.

Estimate of human consumption:

0.239 kg meat x 0.0129 g TOS/kg meat = **0.003 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 an 81.7% TOS content. The batch has a proteinase activity of 5.2 AU/ml.

The dose level in the 13 weeks oral toxicity study in rats, which could be given without any toxic effects, was 138.1 mg/kg bw/day or 0.11 g TOS/kg bw/day, which is the no-observed-adverse-effect level (NOAEL).

The safety margin for the tenderizing application indicated above can thus be calculated assuming an adult body weight of 70 kg as follows:

[0.11 g TOS/kgbw/day ÷ (0.003g TOS/person/day ÷ 70 kg)] = **2750**

7.6 Results and Conclusion

The results of the tests described in section 7.2.2 show that the proteinase 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 proteinase can be safely manufactured and used as a tenderizing agent in meat.

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8. LIST OF APPENDICES

A. Protease expression plasmid map (Figure 1), Selectable marker plasmid map (Figure 2).

B. Novo Nordisk Preliminary Product Sheet for NovoCarne Tender.

C. Preliminary Application Sheet: NovoCarne Tender for Meat Tenderization

D. On the Safety of *Aspergillus oryzae:* a Review, Peder Barbesgaard, Hans Peter Heldt-Hansen, and Børge Diderichsen, Appl. Microbiol. Biotechnol., 36:569-572, 1992.

E. Summary of Safety Data (File: 1999-07439-02)

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Figure 1. Protease expression plasmid map

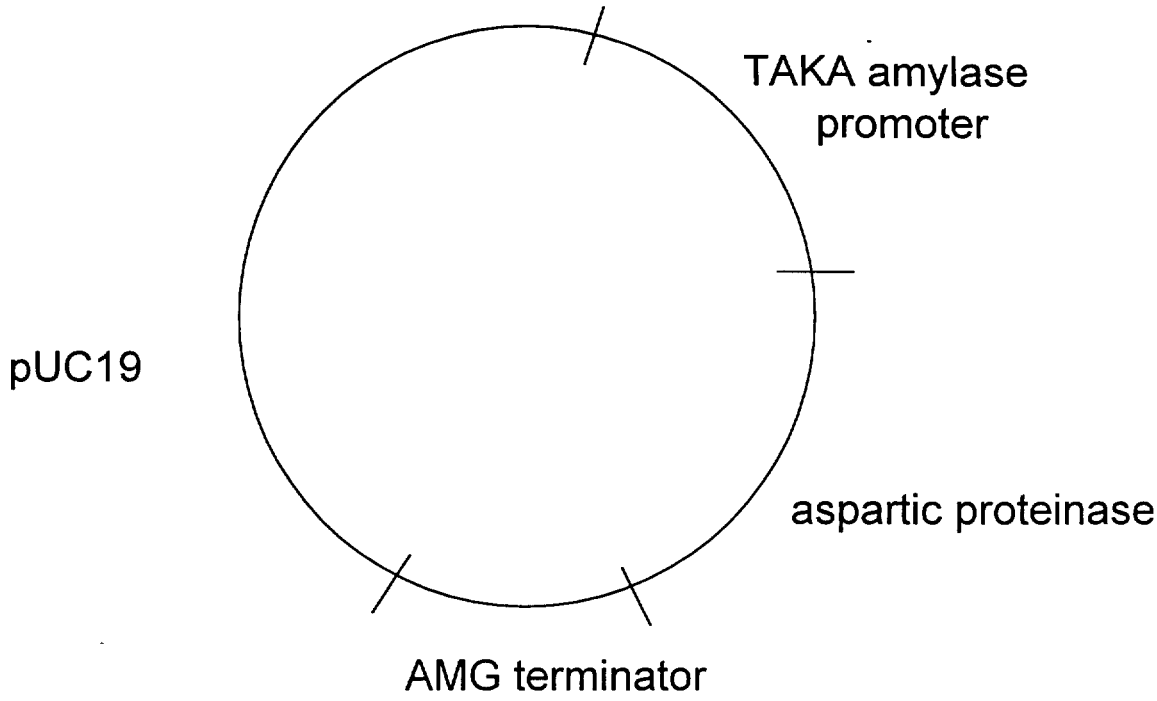
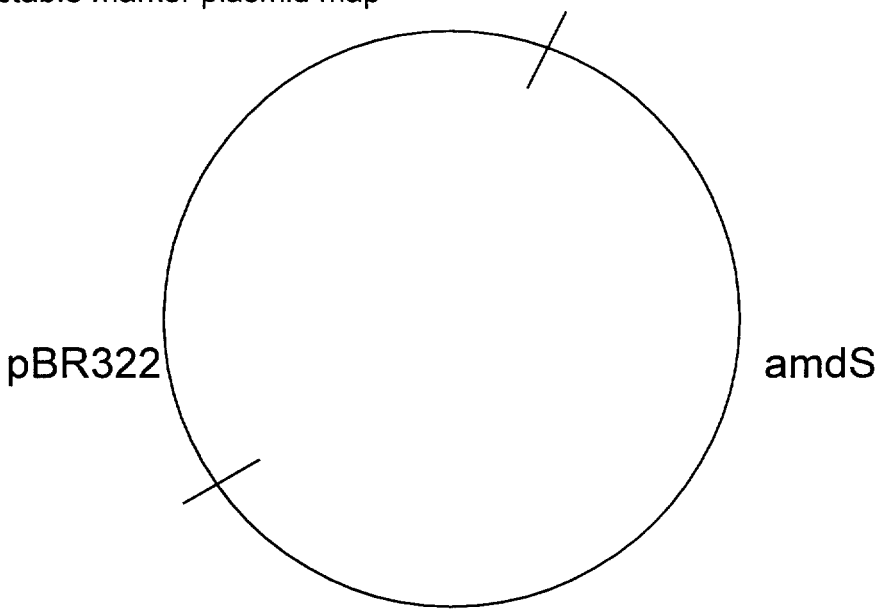


Figure 2. Selectable marker plasmid map



Appendix B

NovoCarne Tender

Application

NovoCarne Tender cause selective and limited hydrolysis of proteins. A more detailed description of the application can be found in the application sheet B-1217.

Description

NovoCarne Tender is a specific endo-protease preparation. NovoCarne Tender is produced by submerged fermentation of a genetically modified *Aspergillus oryzae* strain.
NovoCarne Tender is a brown liquid; it is readily soluble in water at all concentrations. The color may vary from batch to batch and color intensity is not an indication of product strength.

Specifications

NovoCarne Tender is standardized in Anson Units per g (AU/g).

	NovoCarne Tender
Declared activity	0.20 AU/g

Activity
The analytical method (AF 4), which is based on denatured haemoglobin in a 0.02 M Ca^{++} buffer at pH 5.5 at 37 °C, is available on request.

Other Characteristics
The product complies with the recommended purity specifications for food grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC).

Enzyme Characteristics

NovoCarne Tender retains full activity under normal conditions at temperatures of 30-50°C (86-122°F) and pH of 4-6. NovoCarne Tender is not active at low temperatures (e.g. under refrigeration).

Safety

Enzymes are proteins and inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact.
The product may create easily inhaled aerosols if splashed or vigorously stirred. Spilled product may dry out and create dust.
Spilled material should be flushed away with water. Avoid splashing.
Leftover material may dry out and create dust.
Material Safety Data Sheets and separate material describing how to handle the product safely are available upon request.
A separate Novo Nordisk leaflet, "How to handle liquid Novo Nordisk enzymes safely" (B 144), is available on request.

Novo Nordisk

Storage

Enzymes gradually lose activity over time depending on storage temperature. Cool conditions are recommended.
When stored at 5°C (41°F), the product will maintain the declared activity for at least 3 months.
Extended storage and/or adverse conditions, including higher temperature, may lead to higher dosage requirement.

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Appendix C

NovoCarne Tender for Meat Tenderization

Background

Tenderness is a major quality attribute that affects market price and consumer acceptance of meat products. The lack of consistency of beef tenderness is a major reason for decreased beef consumption in the U.S. There are several methods including mechanical tenderization, elevated temperature storage, calcium chloride injection, electrical stimulation, muscle stretching, shock-wave pressure, dry and wet ageing, and enzyme tenderization available for improving meat tenderness. The most widely used is the enzymatic procedure which involves papain, bromelain, and ficin. These enzymes, however, have very broad specificities and therefore indiscriminately breakdown the major muscle proteins (connective tissue/collagen and myofibrillar proteins) resulting in a mushy textured product i.e., over-tenderization. Furthermore, papain which is the most widely used, is a very heat-stable enzyme and therefore not easily inactivated allowing continued product texture deterioration after cooking.

Hence an effective control/improvement of meat tenderness would require an enzyme or enzymes which, unlike papain, would

1) have very narrow specificity
2) express self-limiting hydrolysis of meat proteins
3) break down either of the two major proteins, but not both
4) be thermolabile or readily inactivated at cooking temperatures
5) have no adverse effect on flavour.

NovoCarne Tender meets these requirements making it a very potent tenderising tool for the meat processor.

NovoCarne Tender

The enzyme recommended for this application is NovoCarne Tender, a food grade endoprotease generally regarded as safe (GRAS).

Application

The enzyme may be applied in liquid form by injection into meat followed by tumbling to enhance distribution or mixed with the marinade for tumbling (Fig. 1). Like most fresh meat operations, injection, tumbling, or marinade incorporation should be done at low temperatures (5-10°C) where the enzymes have little or no activity.

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NovoCarne Tender may be injected or tumbled to tenderize meat

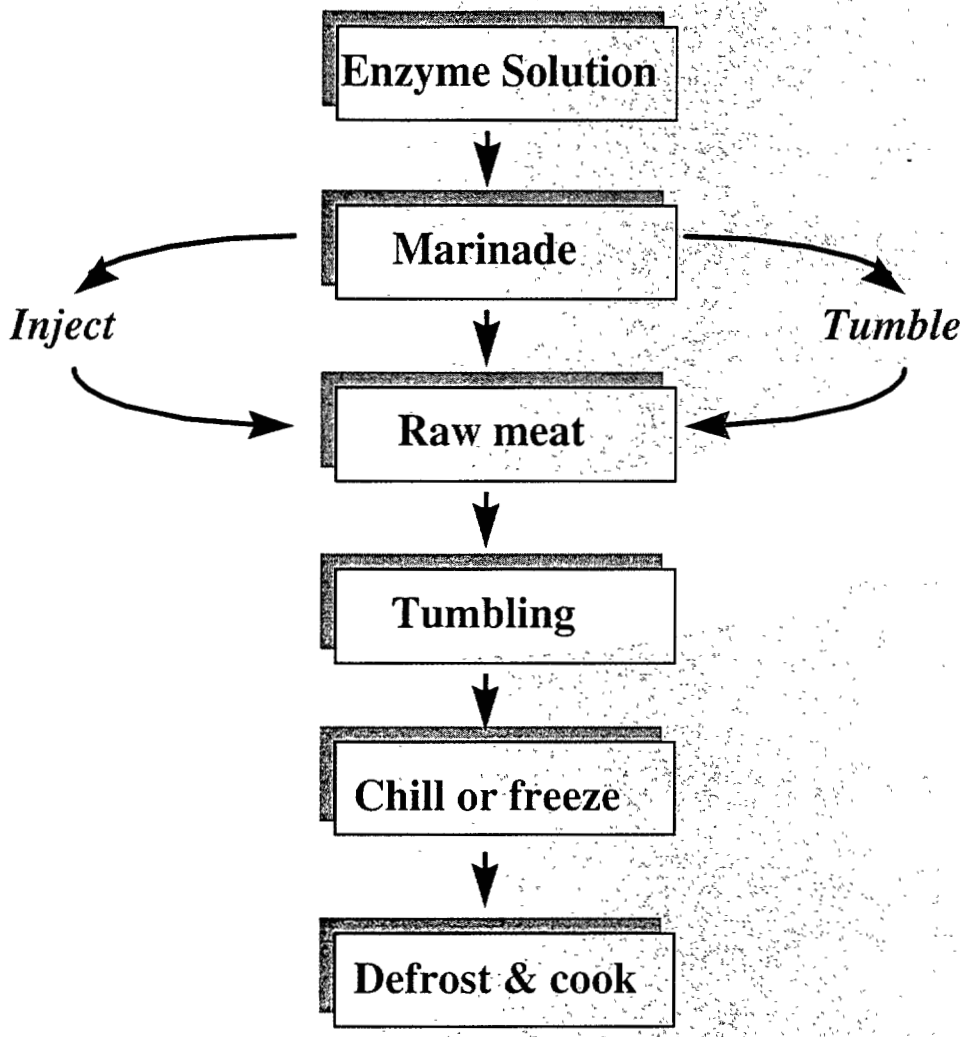

Fig. 1: Mode of application of NovoCarne Tender for meat tenderization

Recommended Dosages

For injection applications, minimum enzyme concentration to effect adequate improvement in meat tenderness is about 0.002 AU (Anson units)/100 g meat at 5% pump gain. It is recommended, however, that concentrations between 0.005-0.01 AU/100 g meat be used (see Fig. 2).

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NovoCarne Tender will not over-tenderize meat

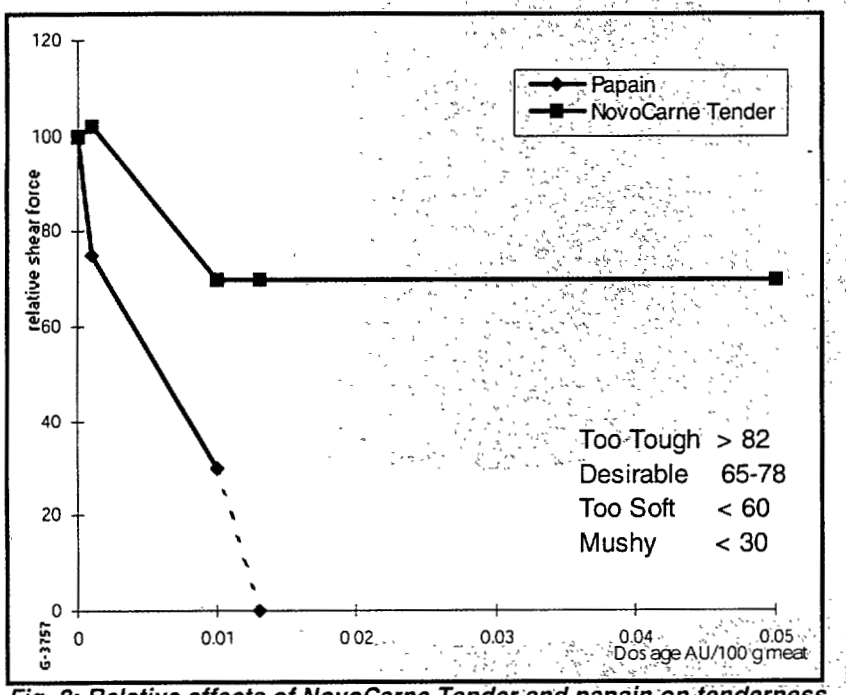

Fig. 2: Relative effects of NovoCarne Tender and papain on tenderness.

Process Description

The enzyme is mixed with water (or selected solution e.g., marinade) to obtain the desired dosage at the chosen pump level. Please note that the pump level may be changed to meet different national standards while still maintaining enzyme dosage. No pH adjustment is necessary for the injecting solution or marinade as the enzyme works very well at the meat pH. To ensure uniform enzyme distribution within the meat portion, it is recommended that injection should be followed by tumbling at refrigeration temperatures for about 2 hr prior to storage. The injected products may be refrigerated or frozen until use with minimal changes in tenderness since the tenderizing effect is mainly observed during cooking (Fig. 3).

NovoCarne Tender has the unique advantage of retaining no activity after cooking (Fig. 4).

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NovoCarne Tender does not affect tenderness during refrigerated storage

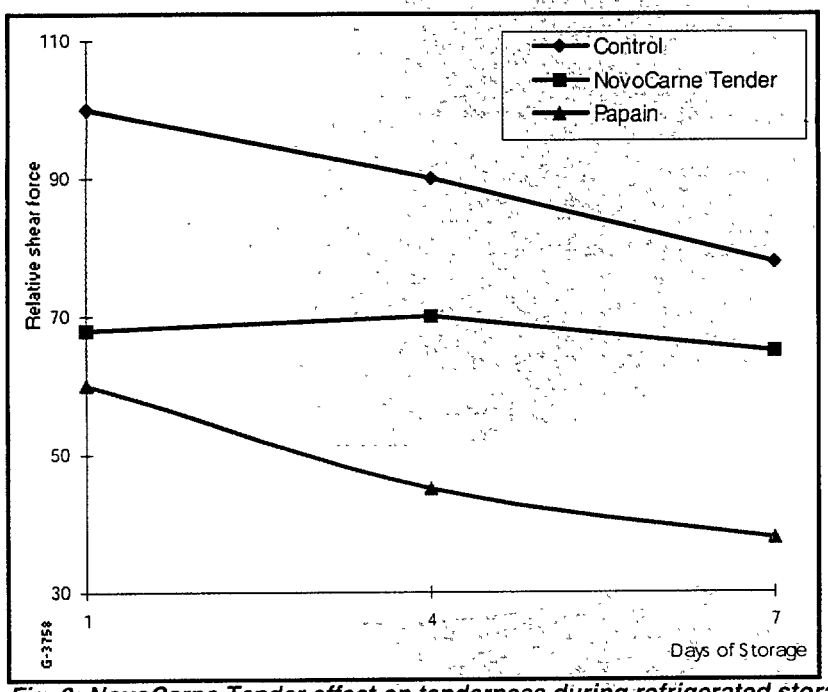

Fig. 3: NovoCarne Tender effect on tenderness during refrigerated storage.

NovoCarne Tender is readily inactivated during cooking

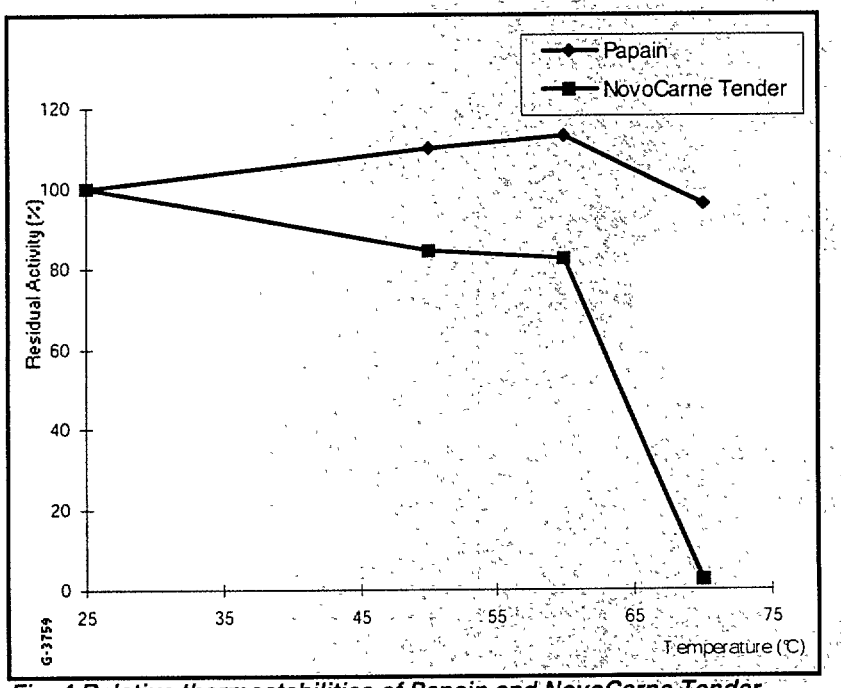

Fig. 4 Relative thermostabilities of Papain and NovoCarne Tender

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Appendix D

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Appendix E

Enzyme Business Toxicology

Date : 1999-08-25
Ref.: PBjP/DSc
File : 1999-07439-02
(Based on QR-9417814)

SUMMARY OF SAFETY DATA

Aspartic proteinase from *Rhizomucor miehei* expressed by *Aspergillus oryzae*

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1. INTRODUCTION

This summary covers the toxicological investigations, that have been carried out on a test batch of an aspartic proteinase from *Rhizomucor miehei* expressed by *Aspergillus oryzae*.

The studies were carried out at Life Science Research (LSR), England; Hazleton Microtest, Harrogate, England and Novo Nordisk A/S, Bagsvaerd, Denmark. All studies were conducted in accordance with current guidelines of OECD and EEC and with the principles of Good Laboratory Practice (GLP) according to OECD principles of GLP, May 1981.

All studies referred to in this summary were performed on the same batch designated to toxicological investigations and having the batch no.: PPR 3583. It has been produced by submerged fermentation and recovered by purification of the fermented culture broth. The appearance of PPR 3583 was a light brown lumpy powder.

The enzyme activity of this batch is 5.18 AU/g . It contains 89.8% dry matter, 8.1% ash and 81.7% Total Organic Solids (TOS), calculated as dry matter % - ash %.

2. PATHOGENICITY

One of the aims of this study was to elucidate the pathogenic potential of the production strain *Aspergillus oryzae* RET-1. The study was performed as a comparative study, and a total of four genetically engineered *Aspergillus oryzae* strains were investigated together with the recipient strain. The results referred in this summary are only the ones dealing with the recipient strain *Aspergillus oryzae* A 1560 and the recombinant strain Aspergillus oryzae RET-1.

2.1 Report: A Study on the Relative Pathogenicity of Five Strains of the Fungus *Aspergillus oryzae* by Intravenous Administration to Mice. Novo Nordisk ref.: F-891593. Study No.: 0589, July 1989.

The pathogenic potential of *Aspergillus oryzae* strains was investigated by a single intravenous injection of spores to groups of 5 NMRI male mice, followed by a 21 day observation period during which clinical symptoms, mortality and abnormal behaviour were registered. At the end of the study all surviving animals were killed and a macroscopic post mortem examination was performed. Lung, spleen, heart, liver, kidneys and brain were examined histopathologically.

No mice died in the low dose group of both strains (5x104 CFU pr kg). The intermediate dose level (5x106 CFU pr kg) caused death of three mice dosed either of the strains. All mice dosed 108 CFU pr kg died. The target organs were the lung and kidney for both strains.
It is concluded that the pathogenicity of these strains are equal. The genetic manipulation has not added any pathogenic abilities to the organism.

3. GENERAL TOXICITY

3.1. Investigations on short-term oral toxicity

The objective of the tests including repeated daily dosing was to determine the dose level for the following subchronic studies and to disclose target organs.

3.1.1. PPR 3583: Preliminary toxicity study by dietary administration to CD rats for four weeks.

LSR Report No.: 91/NLE030/0940, February 1992.
(Novo Nordisk Study No.: 91705, F-9201038).

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The study was performed as a dietary study. PPR 3583 was incorporated in the diet in three concentrations, i.e. 200 ppm, 1400 ppm, or 10000 ppm. Each preparation was offered to a group of ten male and ten female rats. A similarly constituted control group received untreated diet.

The achieved dosages averaged 19.0, 135.7 and 926.1 mg/kg/day for males and 19.0, 132.1 and 947.4 mg/kg/day for females treated at 200, 1400 and 10000 ppm respectively.

During the four week observation period no signs of toxicity was observed. Food consumption was unaffected by treatment. The overall body weight gain of females in the high dose group was lower than that of the controls.

The composition of blood and urine was unaffected by treatment, and no organ weight changes or macroscopic/microscopic abnormalities, which were considered to be related to treatment, were found.

It was considered that administration of the aspartic proteinase preparation to CD rats at dietary levels of 10000 ppm was associated with only minor evidence of toxicity in females. The no effect level determined during this study was 1400 ppm.

3.1.2. PPR 3583: A maximum tolerated dose study by dietary administration to Beagle dogs.
LSR Report No.: 91/NLE032/1005, February 1992.
(Novo Nordisk study no.:91707, F-9201042)

Two male and two female beagle dogs received PPR 3583 via the diet according to the following schedule:

Day 1-3: 10000 ppm; day 4-7: 20000 ppm; day 8-17: 25000 ppm.

There were no signs of reaction to treatment.

The achieved dosages averaged 342, 670 and 823 mg/kg/day during treatment at 10000, 20000 and 25000 ppm respectively. Food consumption and body weights were unaffected by treatment.

Haematological changes after 15 days of treatment when compared to pre-treatment values comprised marginally high packed cell volumes, haemoglobin concentrations and erythrocyte counts in both males and one female.

Biochemical changes in the plasma, that were attributed to treatment comprised slightly high alkaline phosphatase activities and total plasma protein concentrations in all animals, and slightly high alanine amino-transferase activity in one male.

The composition of urine was unaffected by treatment. High absolute and body weight-relative spleen weights were recorded for all animals. Necropsy indicated swollen spleens in all animals and large spleens in both males.

It was concluded that the maximum dietary concentration of the aspartic proteinase preparation tolerated by beagle dogs was greater than 25000 ppm.

3.2. Investigations on subchronic toxicity

These studies were initiated to determine the no-observed-effect level (NOEL) of the tox batch and to determine the safety factor when used in food for human consumption.

These studies were conducted according to Toxicological Principles for the Safety Assessment of Direct Food Additives and Colour Additives Used in Food. US Food and Drug Administration Bureau of Foods, 1982.

3.2.1. PPR 3583: Toxicity study by dietary administration to CD rats for 13 weeks.

LSR report No.: 92/NLE031/0310, September 1992.
Amendment to final report, March 1994.
(Novo Nordisk Study No.: 91706, F-9203938)

Three groups of 20 male and 20 female CD rats received PPR 3583 in the diet at concentrations of 400, 2000 or 10000 ppm for 13 weeks. A similarly constituted control group received untreated diet.

The achieved dosages averaged 27.8, 138.1 and 680.2 mg/kg/day for males and 32.6, 154.0 and 774.0 mg/kg/day for females treated at 400, 2000 and 10000 ppm respectively.

There were no deaths and no signs related to the treatment. Body weight gains, food consumption and the efficiency of food utilisation were unaffected by treatment.
The composition of the blood and urine was unaffected by treatment. There were no organ weight changes or macroscopic alterations which could be related to treatment. Chronic myocarditis was seen at a greater incidence and severity than in controls in males treated at 10000 ppm.

It is concluded that administration of the aspartic proteinase preparation to CD rats at a dietary level of 10000 ppm was associated with only minor toxicity. The no-observed-effect level (NOEL) in this study was 2000 ppm, equivalent to a dosage of 138.1 mg/kg/day in males and 154.0 mg/kg/day in females.

3.2.2. PPR 3583: Toxicity study by dietary administration to beagle dogs for 13 weeks.

LSR report No.: 92/NLE033/0598, December 1992.
(Novo Nordisk Study No.: 91708, F-9300343)

Three groups of four male and four female beagle dogs received PPR 3583 in the diet at concentrations of 1000 ppm, 5000 ppm or 25000 ppm for 13 weeks. A similarly constituted control group received untreated diet. The achieved dosages averaged 35.3, 175.3 and 771.9 mg/kg/day for males and 39.7, 197.7 and 964.3 mg/kg/day for females treated at 1000, 5000 and 25000 ppm respectively.

There were no deaths and no signs of reaction to treatment.
Food consumption and body weight gains were unaffected to treatment.
Organ weights as well as the post mortem macroscopic and histopathological examination revealed no reaction to treatment with the aspartic proteinase preparation.

Haematological changes after 12 weeks of treatment comprised low packed cell volumes, haemoglobin concentrations and erythrocyte counts in females receiving 25000 ppm. Biochemical examinations of the plasma did not indicate any changes attributable to treatment. Urinary composition was also unaffected by treatment.

It is concluded that the no-observed-effect level (NOEL) in this study was 25000 ppm for males and 5000 ppm for females, equivalent to a dosage of 771.9 mg/kg/day in males and 197.7 mg/kg/day in females.

4. TERATOGENICITY

The objective of this study was to investigate the influence of PPR 3583 upon the progress and outcome of pregnancy in sexually mature rats of the CD strain.

4.1. PPR 3583: Teratology Study in the Rat.

LSR report No.: 92/NLE034/0104, April 1992.
(Novo Nordisk Study No.: 91709, F-9201965).
PPR 3583 was administered in the diet at concentrations of 200, 1400 or 10000 ppm to groups of 22 pregnant rats from Day 6 to Day 15 of gestation inclusive. Control animals received untreated diet throughout.

All females were killed on Day 20 of gestation for examination of their uterine contents.

The achieved dosage of PPR 3583 averaged 19, 136, and 972 mg/kg/day for females receiving 200, 1400 and 10000 ppm respectively.

The general condition of the females were unaffected by treatment with PPR 3583. Body weight gain of females showed no effects that could be attributed to treatment with PPR 3583.

At necropsy females showed no effects of treatment with PPR 3583. Litter parameters and foetal survival, growth and morphological development were unaffected by treatment.

It was concluded from this investigation that dietary administration of the aspartic proteinase preparation to pregnant rats at dietary concentrations up to 10000 ppm had no adverse effect upon maternal condition, weight gain or food intake. Implantation, survival, growth and morphological development of the foetuses showed no effects that were attributable to treatment.

5. INVESTIGATIONS ON THE GENETIC TOXICITY

The primary purpose of these short term investigations of the genetic toxicity, is to reveal the potential of a substance to induce mutations in the human DNA, which is considered an indicator for the carcinogenic potential of the test substance.

5.1. Gene mutation assays

5.1.1. PPR 3583: Testing for mutagenic activity with *Salmonella typhimurium* TA 1535, TA 1537, TA 98 and TA 100 in a liquid culture assay. November 1991, Study No.: 91548, F-914311.

PPR 3583 was examined for mutagenic activity using *Salmonella typhimurium* strain TA 1535, TA 100, TA 1537 and TA 98.
The principle of the study was based upon OECD guidelines No. 471.

A liquid culture assay was applied. Bacteria were exposed to 6 doses of the test substance in a phosphate buffered nutrient broth for 3 hours. The highest concentration being 10000 mg/ml. After incubation the test substance was removed by centrifugation prior to plating. The number of revertants to prototrophy and viable cells were estimated.

The test was conducted in the presence and absence of metabolic activation by a rat liver post mitocondrial fraction (S-9) from aroclor 1254 induced animals.

The sensitivity of the individual bacterial strains was confirmed by significant increases in the number of revertant colonies induced under similar conditions by diagnostic mutagens (2-Aminoantracene, 9-Aminoacridine, N-Methyl-N'-Nitro-Nitrosoguanidine or 2-Nitrofluorene). All results were confirmed by conducting two independent experiments.No dose related and reproducible increases in revertants to prototrophy were obtained with any of the bacterial strains exposed to PPR 3583 either in the presence or absence of S-9.

It is concluded, that the results of the experiments described give no indication of mutagenic activity of the aspartic proteinase preparation.

5.1.2. Study to determine the ability of PPR 3583 to induce mutations at the thymidine kinase (tk) locus in mouse lymphoma L5178Y cells using a fluctuation assay.
Hazleton Microtest report No.: 2TKRENOD.026, December 1991. (Novo Nordisk Study No.: 91719, F-915087).

The methodology in this study complies with OECD Test Guideline 476.
PPR 3583 was assayed for its ability to induce mutation at the tk locus (5-trifluorothymidine resistance) in mouse lymphoma cells using a fluctuation protocol.

The study consisted of two independent experiments.

Following a wide range of treatments, separated by two fold intervals and reaching 5000 mg/ml, cells survived all doses of PPR 3583 in experiment 1. Survival in the absence and presence of S-9 was tested in all dose levels employed. Accordingly, the top 5 doses were plated for viability and 5-trifluorothymidine resistance 2 days after treatment. In the second experiment the same dose range was selected.

Negative (solvent) and positive control treatments were included in each experiment in the absence and presence of S-9. Mutant frequencies in negative control cultures fell within normal ranges, and statistically significant increases in mutation were induced by the positive control chemicals 4-nitroquinoline 1-oxide (without S-9) and benzo(a)-pYrene (with S-9).

In the absence of S-9, no significant increases in mutant frequency were observed following treatment with PPR 3583 at any dose level in either experiment. Similarly, in the presence of S-9 no statistical significant increases were obtained in Experiment 1 or 2 following PPR 3583 treatment. Although there were no significant increases in mutant frequency at any dose level in Experiment 1, a significant linear trend was indicated in this experiment. However in Experiment 2 PPR 3583 was tested over the same dose range and no linear trend was obtained. Therefore the dose-relationship indicated in Experiment 1 was not reproducible and is attributed to a chance effect of no biological significance.

It is concluded that the aspartic proteinase preparation, under the conditions employed in this study, has no mutagenic activity.

5.2. Chromosome aberration assays

5.2.1. Study to evaluate the chromosome damaging potential of PPR 3583 by its effects on cultured human lymphocytes using an in vitro cytogenetic assay.

Hazleton Microtest report No.: 2HLRENOD.026, December 1991. (Novo Nordisk study No.: 91720, F-9200189).

The methodology in this study complies with OECD Test Guideline 473, UKEMS Test Guideline and EEC Annex V B 10.

PPR 3583 was tested in an in vitro cytogenetics assay using duplicate human lymphocyte cultures from one male and one female donor in 2 independent experiments.

The highest dose level was 5000 mg/ml. Treatments covering a broad range of doses, separated by narrow intervals were performed both in the presence and absence of S-9. The test compound dose levels for chromosome analysis were selected by evaluating the effect of PPR 3583 on mitotic index. Chromosome aberrations were analysed at three consecutive dose levels. In experiment 1, treatment in the absence of S-9 was continuous for 20 hours. Treatment in the presence of S-9 was for three hours only followed by a 17 hour recovery period prior to harvest.
Experiment 2 included a delayed sampling time. Treatment in the absence of S-9 was continuous for 20 or 44 hours. Treatment in the presence of S-9 was for 3 hours followed by a 17 or 41 hour recovery period.

Appropriate negative (solvent) control cultures were included in the test system in both experiments at both sampling times. The proportion of cells with structural aberrations in these cultures fell within historical solvent control ranges. 4-Nitroquinoline 1-oxide (NQO) and cyclophosphamide (CPA) were employed as positive control chemicals in the absence and presence of S-9 respectively. Cells receiving these were sampled in each experiment 20 hours after the start of treatment; both compounds induced statistically significant increases in the proportion of cells with structural aberrations.

Treatment of cultures with PPR 3583 in both the absence and presence of S-9 resulted in frequencies of cells with aberrations which were similar to and not significantly different from those observed in concurrent negative control cultures. Numbers of cells with aberrations in all PPR 3583 treated cultures fell within normal historical ranges.

It is concluded that the aspartic proteinase preparation was unable to induce chromosome aberrations in cultured human peripheral blood lymphocytes when tested to 5000 mg/ml in both the absence and presence of S-9.

SUBMISSION END

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March 15, 2000

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

RE: GRAS Notice No. 000034

Dear Dr. Kahl,

We are submitting the following information to clarify several points in our GRAS notice No. 000034 regarding an aspartic proteinase preparation produced by *Aspergillus oryzae* expressing the gene encoding an aspartic proteinase from *Rhizomucor miehei.*

We would like to further describe the concept of "self limiting hydrolysis of myosin". This aspartic proteinase, NovoCarne Tender, is more specific than other proteinases and will only hydrolyze myosin to a certain degree. NovoCarne Tender will hydrolyze myosin down to large proteins (130-140 kDa) that are resistant to further hydrolysis by NovoCarne Tender. Other proteinases such as papain will completely degrade myosin leaving the meat with a "mushy" texture.

The recipient microorganism is the well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn designated strain IFO 4177 obtained from the Institute for Fermentation, Osaka, Japan (IFO) also known as A1560. Reference 18 is a report from Centraalbureau voor Schimmelcultures, Baarn, Holland that confirms that A1560 is *A. oryzae.*

We inadvertently left off the date in reference 15. The complete citation is:

Yanisch-Perron, C., Vieira, J. and Messing, J.. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors, Gene, 33:103-119, 1985.

In section 2.7, the antibiotic resistance gene is discussed. The source of the β-lactamase gene is the *E. coli* plasmid pBR322. The discussion in section 2.7 is a basic principle of molecular cell biology and genetics and is generally recognized within the scientific community. Prokaryotic and

eukaryotic sites and signals for effective and functional gene expression and gene product synthesis are different.[1,2,3,4]

In section 3.3.1, the purification process is described. Step 8 is a germ filtration step which is done to remove residual production organisms and to reduce the total viable count to prevent the possible loss of enzyme activity caused by protease enzymes produced by any microbial contaminants present in the product.

In section 2.6, we describe the results of two tests for the presence of rDNA sequences in the enzyme preparation. The dot blot analysis is used to determine if there is any rDNA present. The size of the rDNA cannot be determined by the dot blot analysis so a Southern analysis is conducted. The detection limit for the dot blot is 1 pg plasmid DNA/dot. The dot blot showed faint positives indicating a DNA content of 0.1- 1 ng/g. The Southern analysis showed that the DNA was low molecular weight and no distinct bands were present.

The activity of this enzyme is measured in Anson Units (AU) and is determined by the "Modified Anson-Hemoglobin Method for the Determination of Proteolytic Activity". In this method, denatured hemoglobin is digested under standard conditions. The digested hemoglobin is precipitated with trichloroacetic acid (TCA) and the amount of TCA soluble product is determined with phenol reagent. One Anson Unit is the amount of enzyme which under the given reaction conditions digests hemoglobin at an initial rate such that there is liberated an amount of TCA soluble product per minute which gives the same color with phenol reagent as one milliequivalent of tyrosine.

As stated in Section 7.3, NovoCarne Tender meets the general and additional requirements for enzyme preparations as outlined in the monograph on enzyme preparations in Food Chemicals Codex. This product also meets the general specifications for enzyme preparations used in food processing outlined by FAO/WHO JECFA. The specifications presented in section 5.2 of the notification have been established by Novo Nordisk for NovoCarne Tender. The heavy metals and lead specs meet FCC and exceed JECFA requirements. The arsenic, total viable count and *E.coli* specs meet JECFA requirements and are not included in FCC. The

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[1] World of the Cell, W.M. Becker, L.J. Kleinsmith, J.Hardin, Benjamin Cummings Science, 2000

[2] Molecular Cell Biology; H. Lodish, A. Berk, L. Zipursky, P. Matsudaira, D. Baltimore, J. Darnell; W.H. Freeman and Company, 1999.

[3] Genes VI, Benjamin Lewin, Oxford University Press, 1997

[4] Molecular Biology of the Gene, J.D. Watson, N.H. Hopkins, J.W. Roberts, J.A.Steitz, A.M. Weiner, Benjamin Cummings Science, 1987

total coliforms and *Salmonella* specs meet both FCC and JECFA. The antibiotic activity and mycotoxins specs meet JECFA and are not included in FCC (although FCC mentions mycotoxins but has not established tolerances). The production microorganism spec is a Novo Nordisk specification and is not mentioned in FCC or JECFA.

In section 7.5 of the notification the safety margin was calculated. The safety margin was calculated assuming that an adult person weighs 70 kg. The NOAEL from the 13 week oral toxicity study is 0.11 g TOS/kgbw/day. The estimated human consumption is 0.003 TOS/person/day. This is divided by 70 kg to get 0.00004 g TOS/kgbw/day. The safety margin is 0.11 g TOS/kgbw/day divided by 0.00004 g TOS/kgbw/day or **2750.**

Finally, we would like to restate that this enzyme has been used in food since 1969. This enzyme comes from *Rhizomucor miehei* which was previously known as *Mucor miehei*. The proteinase enzyme from the genetically modified *A. oryzae* is essentially identical to a proteinase from *R. miehei*. The *R. miehei* proteinase coding sequence incorporated into the *A.* oryzae production strain has not been altered. In 1972, milk clotting enzyme from *M. miehei* (now known as *R. miehei*) was approved for use in cheese production (21CFR 173.150). The enzyme and production organism discussed in this notification is the same enzyme and production organism that was approved in 1997 for milk clotting (21 CFR 173.150). The 1997 Federal Register notice final rule for milk clotting enzymes from *A. oryzae* carrying the gene from *R. miehei* discusses the nomenclature change from *M. miehei* to *R. miehei* (62 FR 59282).

If you have any questions or require additional information about our notification, please contact me by direct telephone at 919 494-3151 or direct fax at 919 494-3420.

Sincerely,

Lori Gregg
Regulatory Specialist

Reference List for Industry Submission, GRN 000034

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000034 - 000037	Barbesgaard, Peder; Heldt-Hansen, Hans Peter; Diderichsen, Berge	On the safey of Aspergillus oryzae: a review	1992	Appl Microbiol Biotechnol	Volume 36, pgs 569-572

NA- Not applicable