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October 13, 1999

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VIA HAND DELIVERY

Dr. Alan Rulis
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
200 C Street, S.W.
Washington, DC 20204

Re: GRAS Notification for Pectin Lyase Enzyme Preparation Produced with *Trichoderma reesei* Containing a Gene from *Aspergillus niger*

Dear Dr. Rulis:

I am enclosing, on behalf of Röhm Enzyme GmbH, the above referenced GRAS Notification in accordance with proposed 21 C.F.R. § 170.36 (62 Fed. Reg. 18938 (April 17, 1997)). This submission is being made in triplicate.

Please contact me at (202) 496-7645 if you have any questions or require additional information.

Very truly yours,

Gary L. Yingling

GLY/so
Enclosure(s)
cc: Rudaina H. Alrefai, Ph.D., FDA Consumer Safety Officer
Röhm Enzyme GmbH (w/o enclosures)

1999 OCT 14 A 10:00

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Dr. Alan M. Rulis
Office of Premarket Approval (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street. SW
Washington, DC 20204
USA

Darmstadt, 29. September 1999

Re: GRAS notification for pectin lyase enzyme preparation produced with *Trichoderma reesei* containing a gene from *Aspergillus niger*

Dear Dr. Rulis,

Pursuant to proposed 21 C.F.R. §170.36 (c), Röhm Enzyme GmbH hereby claims that the pectin lyase enzyme preparation derived from *Trichoderma reesei* (formerly classified as: *Trichoderma longibrachiatum*) carrying a gene coding for pectin lyase (also called pectin transeliminase) from *Aspergillus niger* is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act for use as a processing aid, because we have determined, based on scientific procedures, that such use is Generally Recognized As Safe (GRAS) under the conditions of its intended use.

The pectin lyase enzyme preparation is intended to be used as a processing aid, at minimum levels necessary to achieve the desired effect in accordance with Good Manufacturing Practice, for the preparation of fruit and vegetables juices, purees and concentrates by various different production processes, including the pulp wash processes and in-line pulp wash processes in the processing of citrus fruit. The purpose for using the pectin lyase enzyme preparation is the degradation of the pectin in order to lower the viscosity or, also, to clarify juices, and the recommended dosage is 5 to 100 ppm of enzyme preparation. The enzyme is inactivated during the pasteurization process of the juices, purees and concentrates.

A summary of the data and information supporting this GRAS notification is attached.

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The complete data and information that are the basis for this determination are available for review by the Food and Drug Administration at Röhm Enzyme GmbH, Kirschenallee 45, D-64293 Darmstadt, Germany. Alternatively, the documents will be made available for review at the law office of Mr. Gary L. Yingling at McKenna & Cuneo, L.L.P., 1900 K Street NW, Washington, DC 20006-1108, USA.

Sincerely,

Dr. Wolfgang Braun
President
Röhm Enzyme GmbH

Katja Thomas
Regulatory Affairs
Röhm Enzyme GmbH

Enclosures

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GRAS NOTIFICATION FOR PECTIN LYASE ENZYME PREPARATION (PECTIN TRANSELIMINASE ENZYME PREPARATION) PRODUCED WITH *TRICHODERMA REESEI* CARRYING A GENE FROM *ASPERGILLUS NIGER*

29. September 1999

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I. Introduction

The pectin lyase enzyme preparation is a special pectolytic enzyme preparation for the processing of fruits and vegetables containing the highly specific pectin transeliminase or endo pectin lyase activity.

The pectin degradation occurs by transelimination steps without previous deesterification.
The pectin lyase enzyme preparation is used for applications in the processing of fruit and vegetables where the fast reduction of the viscosity is needed without the formation of methanol.

The enzyme preparation derives from a microbial source.
The donor strain for the pectin lyase gene is a nontoxic, nonpathogenic strain of *Aspergillus niger*.
The recipient strain for the pectin lyase gene is a nontoxic, nonpathogenic strain of *Trichoderma reesei*.
The safety of the genetic modification is based on the use of defined gene sequences coding for defined proteins.

The GRAS determination is based on scientific procedures. The enzyme has been used in the beverage industry for several decades and the host strain and its products are safe.

II. Information about the identity of the substance

A. Systematic name

Poly(methoxygalacturonide)lyase

B. Common name

pectin lyase or pectin transeliminase
Both names are used in the literature and will be used interchangeably in this summary.

C. Enzyme commission number

4.2.2.10

D. CAS number

9033-35-6

E. Quantitative composition

Percent of	protein	0.5 - 3 %
	ash	13 - 18 %
	TOS: Glycerol	30 %
	other organic compounds	2 - 3 %

F. Characteristic properties

The pectin lyase enzyme preparation has the following characteristics:

a) liquid product
b) brown colored with aromatic smell
c) specific weight: ~ 1.15 g ml^{-1}.
d) pH value: 4.2 - 4.8
e) enzyme activity: 15 - 100 PTF mg^{-1}.

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The amount of enzyme is not determined on the basis of its chemical constitution but on the basis of its catalytic activity, with the rate of substrate conversion or the rate of product formation serving as a measure of the activity. (Böing, J.T.P.)

Activity of the enzyme preparation:
The primary enzymatic activity is pectin lyase. In the final enzyme preparation the level of activity is between 15 and 100 U PTF mg^{-1}.

Enzyme units: PTF mg^{-1}
The enzyme cleaves the methylated polygalacturonic acid backbone of esterified pectin by a transelimination reaction. The cleavage products (oligomers) containing a 4,5-dehydrogalacturonic acid group at the non-reducing end are determined by their UV absorbance at 235 nm.
1 U PTF corresponds to the amount of enzyme, which leads to an increase in absorbance of 0.01 AU min^{-1} at 235 nm at 30°C under standardized conditions.
Method of analysis: Determination of PTF-Activity (For method see Appendix 1)

Secondary enzymatic activity
- Cellulase activity: 7 ECU per 1000 PTF Units per gram (For method see Appendix 2).

The enzyme is a protein. It denaturates irreversibly and becomes inactivated at the conditions used in fruit juice pasteurization.

Degradation of pectins:
Pectic substances are important structural constituents of the middle lamella and the primary cell walls of higher plants, in particular fruits and vegetables. Pectins are the main components of the middle lamella which leads to the view that they function as intercellular adhesive. The primary cell walls are a firm-gel composed of cellulose microfibrils embedded in a matrix of pectins, hemi-cellulose and protein.The extent and mode of their degradation affect many aspects of the processing of fruits and vegetables.

They are acid polysaccharides of high molecular weight.

Usually, the following nomenclatures for pectic substances are used:

Pectic acid:	Polygalacturonic acid
Pectin:	Pectic acid partially esterified with methanol and containing some neutral sugars
Protopectin:	Pectic substances fixed in the plant tissue

Pectin is a heteropolysaccharide with galacturonic acid and methanol as the main components, and some neutral sugars attached, such as D-galactose, L-arabinose and D-xylose. Pectin molecules are formed by α-1,4-glycosidic linkages between the pyranose rings of D-galacturonic acid units. These galacturonan chains are interrupted at intervals by the insertion of 1,2-linked α-L-rhamnopyranosyl residues. The carboxyl groups of pectin are partially esterified with methanol, and the hydroxyl groups are sometimes partially acetylated.

There are three types of pectic enzymes that degrade pectic substances:

Protopectinase:	degrades protopectin to form soluble pectin
Pectin esterase:	splits off methoxyl groups from pectin
Pectin-depolymerases:	split the glycosidic α-1,4-linkage in the galacturonan backbone

Three groups of pectin depolymerases exist:

Polygalacturonases:	split glycosidic linkages next to free carboxyl groups by hydrolysis; preferred substrate: pectic acid and low methoxyl pectins
Pectate lyases:	degrade glycosidic linkages next to free carboxyl groups by β-elimination; preferred substrate: pectic acid and low methoxyl pectins
Pectin lyases:	eliminative splitting enzymes, specific for highly methylated pectins

Naturally occurring pectins can be efficiently degraded by the combination of pectin esterase and polygalacturonase or pectate lyase, or else, if they are sufficiently highly methylated by pectin lyase alone.

Pectin lyase is a highly specific endo-enzyme. These endo-enzymes have the ability to cleave highly esterified polygalacturonic acid. One proton is transferred by transelimination from the carbon-(5) atom to the oxygen of the glycosidic group, with simultaneous splitting of this α-(1,4) glycosidic

bond. However, only those glycosidic bonds adjacent to a methyl ester group are split. Pectin lyase therefore shows high activity only towards highly esterified pectins. The degree of esterification of the water-soluble pectin is 65 – 98%. The cleavage of only a small number of glycosidic linkages already causes a sharp decrease in viscosity. The decrease in viscosity has a favorable influence both on the rate of filtration and on the heat transmission coefficient during concentrate manufacture.
Literature: Schobinger, 1987; Voragen, 1989; Sakai, 1992

G. Method of manufacture

1. Source of the enzyme

The enzyme preparation derives from a microbial source.
The donor strain for the pectin lyase gene is a nontoxic, nonpathogenic strain of *Aspergillus niger*.
The recipient strain for the pectin lyase gene is a nontoxic, nonpathogenic strain of *Trichoderma reesei*.

2. Enzyme production

The enzyme is produced as described in the attached flow chart (see Appendix 3). The production process is a procedure described frequently in the literature, see: Wang et al., 1979.

Strain:	genetically modified *Trichoderma reesei* containing the *Aspergillus niger* gene for pectin lyase
Genetics:	GMO
Media:	Lactose Processed grain material Salts KH_2PO_4 and $(NH_4)_2SO_4$ Ammonia/sulfuric acid for pH adjustment Tap water
Fermentation:	suitable pH: 4.7 to 5.5 suitable temperature: 25°C to 31°C suitable time: 200 to 220 hours suitable feeding conditions: as needed by the strain

Downstream process:

suitable aids for filtration: Kieselgur (diatomaceous earth)

others (e.g. for pH-adjustment: Sulfuric acid or Caustic soda)

Process description:

Fermentation Process

The plant consists of a series of bioreactors used for upstream processing and several production scale bioreactors. The first bioreactor is inoculated with a suspension of cells grown in shake flasks. When vigorous growth has developed the culture is transferred aseptically to the first bioreactor and finally to the production bioreactor.
The strain used for the fermentation is a nonpathogenic, nontoxic strain of *Trichoderma reesei* carrying a gene coded for pectin lyase from a nontoxic, nonpathogenic strain of *Aspergillus niger.*
The criteria for the control and, if necessary, rejection of a culture batch are performed as follows:

Fungal strains are normally characterized and identified by their morphology, i.e. appearance, and metabolic efficiency. We perform precisely these analyses to ensure that our strains remain unchanged up to and during fermentation. All strains are passed on to production in the form of tested dry preserves. From these, production preserves are prepared and stored at -150°C in the following manner: A petri dish is inoculated from the dry preserve in such way, that individual colonies can be selected upon germination. 30 individual colonies are inoculated into shake flasks. Care is taken to select only those colonies which present the familiar picture (same phenotype). The shake flasks constitute the culture stage. Mutation does not normally occur, and if so, only in the vegetative state during cell division. Due to the above-described procedure, this vegetative state of the cultures is reduced to a minimum during production.

From each shake flask, one oblique tube and another shake flask are inoculated. The latter is subjected to a so-called productivity test, i.e. after the shake flask cultivation is being completed, we measure the enzymatic activity, which must correspond to a given value. If this value

is not reached, the culture is discarded. This test determines the characteristic metabolic efficiency of each strain, i.e. to establish its identity. The tubes whose parallel shake flasks show the highest results are then flushed with glycerol solution. The suspensions obtained this way are deep frozen in portions of 0.5 ml at -150°C (50 productivity preserves each).
The annual production starts from these productivity preserves.

Raw materials used for the fermentation process are water, lactose and processed grain material. All raw materials used are food-grade.
The pH-control is made with ammonia water and sulfuric acid.
Foaming during fermentation is prevented by using an antifoam agent . The use of the antifoam agent used by Röhm Enzyme GmbH is safe in accordance with 21 CFR Part 173 Secondary Direct Food Additives Permitted in Food for Human Consumption, §173.340 Defoaming agents.
The medium is always sterilized at 121°C for 60 minutes before being used for production. The sterilization kills all organisms that might be present in the medium. The absence of toxins in the medium is ensured by the analysis of the final product.
During fermentation, samples are taken frequently and analyzed for enzyme activity and microbiological purity.

Processing: Harvesting, Purification Process

During the down stream processing, the broth is cooled below 10°C with ice water. The production strain is removed and the solution containing the enzyme is purified by several filtration steps:
Solids removal
Clarification 1
Ultrafiltration
Diafiltration
Clarification 2
Sterile Filtration (if necessary according to results of a microbiological testing)

The filter aid used in the steps is food-grade Kieselgur (diatomaceous earth). The use of Kieselgur is considered safe since it has been used in the production of food for decades (clarification of beer and fruit juices, for example) without rising doubts about its safety.

The microbiological quality is assured by using a separator and plate and frame filtrations.

During the down stream process, samples are taken frequently and analyzed for enzyme activity and microbiological quality.

Stabilization and Standardization

The pectin lyase enzyme concentrate is stabilized with 30% glycerol and 13% potassium chloride.

Standardization:
The pectin lyase enzyme preparation is standardized with tap water.
The pH value is adjusted to 4.2 - 4.8 with either H_2SO_4 or NaOH.

Before packaging, the solution is filtered with plate-and frame filter using asbestos free filter sheets and filter aid Kieselgur (diatomaceous earth).

Process control

The whole fermentation process is controlled with computer control systems. In addition to the normal measurements (temperature, pH value, etc.), and control circuits, the systems also have sequences for sterilization and CIP, which reduces the risk of human errors in critical process steps.

Product control

Each batch is analyzed to assure the high quality of the pectin lyase enzyme preparation.

The criteria for the product control are
- specific weight
- pH
- enzymatic activity
- microbial purity

H. Potential human toxicants

The tests performed with the pectin lyase enzyme preparation to determine the content of mycotoxins according to the method of Patterson and Roberts, Journal AOAC, 62, 1265 (1979), show that no naturally occurring toxicants in the enzyme preparation can be detected.

I. Specifications for food-grade material

The food-grade quality of the enzyme preparation has been established by meeting the following specifications:

Identification of the strain:
The microbial strains are identified according to generally recognized specifications for identification. Only nonpathogenic, nontoxigenic strains are used for production.

With regard to the purity specifications for the enzyme preparations, the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee of Food Additives (JECFA) of the FAO/WHO and the specifications in the Food Chemicals Codex are met.

The maximum permissible levels are:

Arsenic	< 3 ppm
Lead	< 5 ppm
Heavy Metals	< 30 ppm
Antibacterial activity	not present
Microbial purity:	
Coliforms*	< 30 per gram
*E. coli***	none in 25 grams
*Salmonella****	none in 25 grams
Total viable count	< 5 x 10^4 per gram

*Coliforms are determined according to the method L-01.00-2 of the collection of approved methods according to §35 of the German Food Law (LMBG).

**E. coli* is determined according to the method L-01.00-25 of the collection of approved methods according to §35 of the German Food Law (LMBG).

***Salmonella* is determined according to the method L-00.00-20 of the collection of approved methods according to §35 of the German Food Law (LMBG).

The determination of the mycotoxins is done according to the method of Patterson and Roberts, Journal AOAC, 62, 1265 (1979).

Mycotoxins:	
Aflatoxin B2	< 1 µg/kg
Ochratoxin A	< 10 µg/kg
Sterigmatocystin	< 10 µg/kg
Zearalenone	< 50 µg/kg
T2 Toxin	< 20 µg/kg

III. Applications for the pectin lyase enzyme preparation

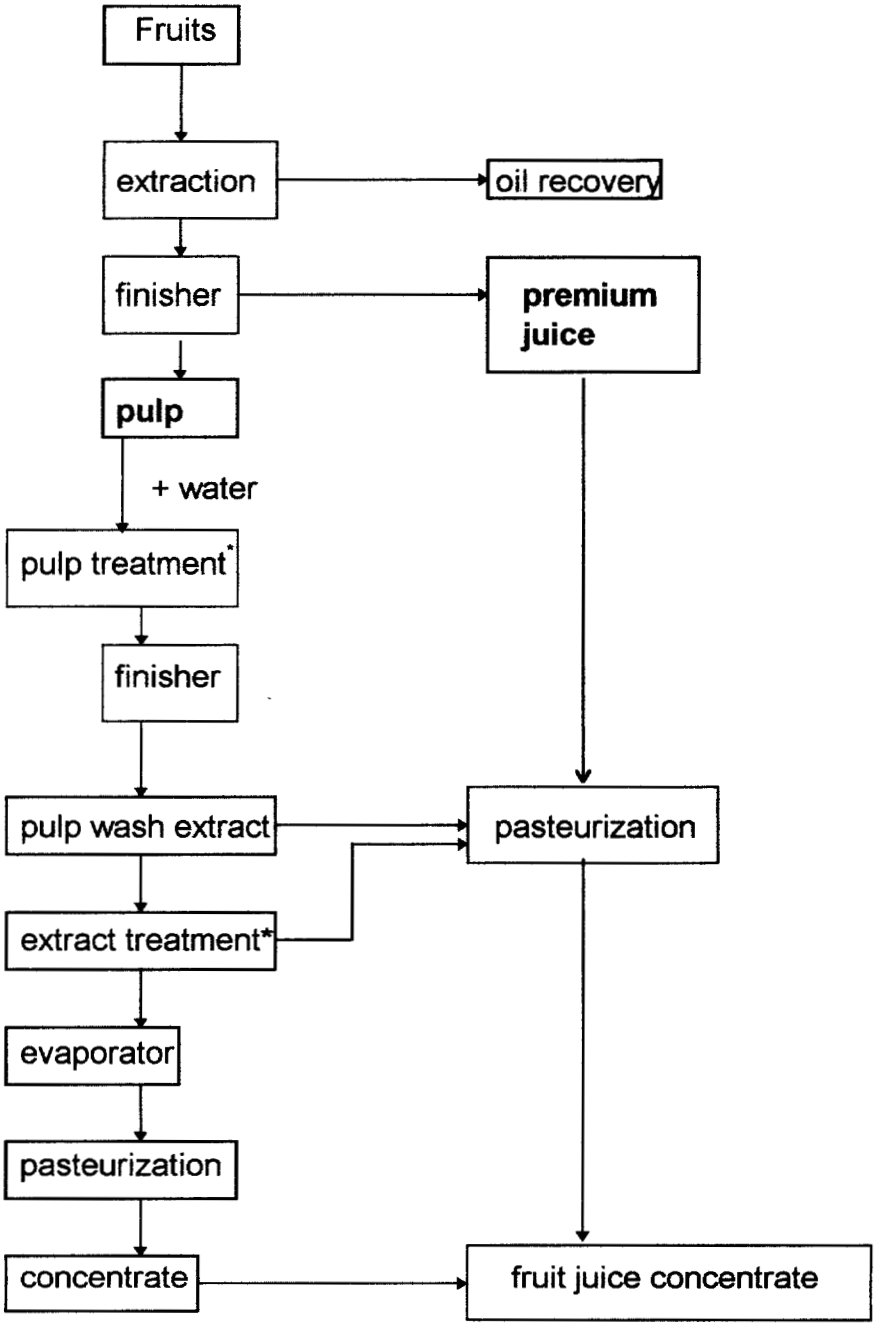
The pectin lyase enzyme preparation is used for applications in the processing of fruits and vegetables where a fast reduction of the viscosity is needed.

Additionally, for the processing of carrot juice, the treatment with pectin lyase increases the yield on juice and coloring matter.

Application	Reaction conditions	Recommended Use level
Citrus juice (orange, grape fruit)	10 - 25 ° C, 10-20 min	5 - 10 ppm
Citrus pulp wash (in line; off line)	30 - 45° C, 10 - 30 min	20 - 60 ppm
Carrot juice and other vegetable juices	mash: 45 - 50 °C, 1 - 2 hrs (in combination with polygalacturonase)	100 ppm
Puree concentrates, i.e. mango, banana, maracuja, apricot, peaches etc.	35 - 45° C, 10 - 20 min	20 - 40 ppm

A typical application for the use of the pectin lyase enzyme preparation is the production of citrus juices. The use of the pectin lyase enzyme preparation in the production of fruit juices is a long known and well-known process described frequently in the literature, for example by Schobinger, 1987.
A typical production process for fruit juice, the pulp wash processes (in line or off line), therefore is only described briefly with a flow chart.

Method of fruit juice production (flow chart of Pulp wash treatment)



* The pulp treatment and the extract treatment with the pectin lyase enzyme preparation can be done as an in line process or as an off line process.

Self-limiting level of use for the applications:
There is a self-limiting level of use for the application in cloudy juices and purees only. If the pectin lyase enzyme preparation is added to cloudy juices and purees in overdoses, an unwanted separation of supernatant and cloudy matter will occur, the cloud stability is lost and, for example, juices will become clear. This is an unacceptable, unappealing property for purees and cloudy juices, such as, for example, orange juices, banana juices, peach juices or grapefruit juices and purees, and thus makes them unappealing for consumption.

However, for all applications in which the pectin lyase enzyme preparation is used as a processing aid, the enzyme will become irreversibly denaturated by heat during the pasteurization step in the manufacturing process of the respective food. Therefore, the enzyme preparation is only present in the final food in a manner that prevents it from having any function. The only residue in the final product will be very small amounts of protein. (With a protein content of 0.5 % in the enzyme and a maximum recommended dosage of 100 ppm, there will be about 0.5 ppm protein in the final juice, for example. The naturally occurring protein content of orange juice, for example, is about 5000 ppm. So, the protein amount added to orange juice by the inactivated enzyme would be only 0.01 %.)

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Detailed summary of the basis for the determination that the use of the pectin lyase enzyme preparation is GRAS under the intended conditions of use

IV. Safety of the Production Process

The pectin lyase/pectin transeliminase enzyme preparation is produced by cultivating the genetically modified *Trichoderma reesei* in shake flasks in the laboratory first and then transferred to large scale bioreactors.
At a constant temperature with ample supply of nutrients and oxygen, the fungus grows and secretes pectin lyase into the nutrient medium. After 5 – 10 days, the fermentation is terminated. The microorganism is separated from the culture supernatant containing the enzyme by filtration steps. After concentration of the liquid by ultrafiltration the enzyme fraction is then standardized to a constant enzyme activity.

The manufacturing process used for the fermentation, processing, and formulation of the pectin lyase enzyme preparation is a standard method. (See attached flow chart; Wang et al., 1979)

The pectin lyase production is part of a DIN/ISO 9002 certified production process. Each production includes control steps comprising strain identification, monitoring of the production process for the absence of infections, and product safety in all production stages.

Röhm Enzyme GmbH, Darmstadt has established and applies a HACCP System for its enzyme products for the food industry. The General Principles of Food Hygiene and Hazard Analysis Critical Control Point System and Guidelines for its Application of the FAO/WHO Codex Alimentarius Commission are fulfilled. (see Appendix 4: HACCP Certificate for Röhm Enzyme GmbH)

Plant Hygiene

All bioreactors, vessels and pipelines are washed thoroughly after use with a CIP system. Hot caustic soda and nitric acid are used as cleaning agents. After cleaning, the vessels are inspected visually. Critical pieces of the downstream equipment are sanitized with disinfectants that are approved for the food industry.

A maintenance system for all relevant equipment is installed. The equipment is checked regularly, and parts are exchanged as necessary.

All media are sterilized with steam injection. The production bioreactor is filled with sterile medium after the sterilization.

The fermentation process is controlled and contamination is prevented by the controlled growth conditions and sterile air for the aeration.

V. Safety of the Production Organism *Trichoderma reesei* (formerly classified as *T. longibrachiatum*)

Röhm Enzyme GmbH, using the following studies and publications which provide no evidence that the production organism produces toxins, has concluded that the production strain belongs to a class of microorganisms, *Trichoderma reesei,* that is non-toxigenic and non-pathogenic.

Trichoderma is a haploid organism which grows as mycelium and in suitable inducing conditions generates haploid spores (for more detailed information on the life cycle, see Rifai, 1969). The species belonging to the genus *Trichoderma* are common in soil as well as on vegetable debris and they seem to be widespread all over the world (Rifai, 1969).

T. reesei strains are widely used as production organisms for enzymes and they have a long history in fermentation industry. Like many other organisms with long history in industrial use, *T. reesei* strains are nowadays used as hosts for construction of production strains by genetic engineering. A *T. reesei* strain was used as a production organism for cellulases at Alko/Primalco Ltd´s enzyme factories at Koskenkorva from 1982 and at Rajamäki Alko/ROAL factories from 1987.

T. reesei is considered a safe production organism for enzymes for food processing and other industrial applications. A review of the industrial use, non-pathogenicity studies, production of mycotoxins and antibiotics, safety in food use and allergenicity was published in 1994. (Nevalainen et al, 1994)

In the AMFEP´s (The Association of Microbial Food Enzyme Producers) brochures, *T. reesei* is recognized as a safe source of microbial enzymes based on a documented history of use in food or food additives production and on scientific evidence from toxicological testing.

Literature: AMFEP brochures "Regulatory aspects of food enzymes produced by recombinant microorganisms" and "Regulatory aspects of microbial food enzymes" are attached (see Appendix 5 and Appendix 6).

Safety evaluations conducted on two other enzymes, cellulase and β-glucanase, also produced by *Trichoderma reesei* raised no safety questions which confirms the non-toxigenic, non-pathogenic nature of the organism.

Cellulase preparations

Extensive testing has been performed and published on the safety evaluation of an acid cellulase enzyme preparation derived from *Trichoderma reesei* (Hjortkjaer et al., 1986) (Nevalainen et al., 1994). The testing includes:

Acute oral toxicity in mice and rats, acute oral toxicity in dogs, 13 weeks oral toxicity in rats and dogs, studies on reproduction and teratogenicity, Ames-test, chromosome aberration in vivo, dominant lethal assay, 14 days - inhalation toxicity in rats, skin irritation and eye irritation in rabbits, skin irritation in man, sensitization in guinea pigs and in humans, pathogenicity studies in mice, guinea-pigs and rabbits. Mycotoxin and antibiotic screenings were performed as well.

The overall data demonstrate the absence of toxic, mutagenic and pathogenic properties for the enzyme produced by *Trichoderma reesei*. While a large inoculum given to immunosuppressed mice was pathogenic, this effect has been is observed as well on other well known microorganisms used for food production (e.g. *Mucor meihei* and *Aspergillus* spp.) and is not considered of toxicological significance. (Hjortkjaer et al., 1986)

In the light of the published experimental evidence, *Trichoderma reesei* is considered a safe production organism for enzymes (especially cellulase and hemicellulase) for food processing and other industrial applications, as well as a safe host for other harmless gene products. (Nevalainen et al., 1994)

A cellulase enzyme preparation from *Trichoderma longibrachiatum* has also been affirmed as GRAS by the FDA for use in processing food. 21 C.F.R. § 184.1250 (see 61 Fed. Reg. 28359 (May 26, 1999)). The studies relied upon in support of the cellulase GRAS affirmation were conducted on strains of *T. reesei* QM 9414, the same strain Röhm Enzyme GmbH uses in the production of its pectin lyase enzyme preparation.

ß-Glucanase preparations

Other enzymes derived from *Trichoderma reesei* have also been tested for their safety. All of these results help to establish the overall safety of *Trichoderma reesei* as a production strain for enzymes.

For example, in a safety evaluation of β-Glucanase derived from *Trichoderma reesei*, the following tests were performed:

Ames-Test, chromosome aberration in vitro, acute oral toxicity (rat), acute inhalation toxicity (rat), skin irritation (rabbit), eye irritation (rabbit), skin sensitization (guinea pig), 14 day range-finding study (rat, oral) and 90 day oral toxicity (rat). (Coenen et al., 1995)

There was no evidence for oral or inhalation toxicity, mutagenic potential, eye or skin irritancy. No mycotoxins have been detected and the test for antimicrobial activity was negative.

An extensive literature search performed by the authors confirmed the non-pathogenic and non-toxic properties of the production organism *Trichoderma reesei*.

The enzyme revealed a strong sensitizing potential when tested in the guinea pig maximization test. This adverse effect, which is known for enzyme preparations in general, has to be taken in account for occupational health precaution measures (skin contact; inhalation). (Coenen et al., 1995)

Finally, the toxicology studies done by Coenen et al., and Hjortkjaer et al., in addition to the studies used for the GRAS affirmation of *Trichoderma reesei* (formerly also known as *Trichoderma longibrachiatum*), were done with the strain *Trichoderma reesei* QM9414 (personal communications with Roostgard), the same strain used by Röhm Enzyme GmbH.
From the above mentioned studies and the study performed by Huuskonen (see Appendix 7: Study Report: Pathogenicity of the Genetically Modified *Trichoderma reesei* strain ALKO 2224 in Mice after a single intravenous Administration), Röhm Enzyme GmbH concludes that the strain *Trichoderma reesei* is non-toxigenic and non-pathogenic. The strains used in these studies are all from the same parent

strain and cultivated under different conditions. In none of the cases have any toxic effects been observed.

Therefore, Röhm Enzyme GmbH regards its strain used for the production of pectin lyase as safe and non-toxigenic.

There is literature that suggests questions about the safety of *Trichoderma reesei:*

The production of mycotoxins by *Trichoderma* strains is mentioned in 3 publications: Eveleigh, D., 1985, Goldman et al., 1988, and Hao, 1987.

The publication from Eveleigh, 1985, describes the production of different mycotoxins by *Trichoderma*. There is no evidence given about the production of mycotoxins by *Trichoderma reesei*. Also, no conclusions are made about a hazardous potential of the use of enzymes derived from *Trichoderma reesei* in the food industry. It is mentioned, however, that cellulase preparations from *Trichoderma viride* are used as digestive aid in Japan for several years, and *Trichoderma polysporum* preparations are registered as fungicides, and that both strains have a negligible toxicity to rats.

In the publication from Goldman et al, 1988, the statement is made that „*Trichoderma* ssp are suspicious to be used in the food industries because they produce mycotoxins". However, there is no specification of the strains that are suspicious of mycotoxin production, there is no detailed information on the kind of mycotoxins that are supposedly produced by *Trichoderma*, and there is no reference given to any literature supporting this statement. Thus, there is no objective support for the statement in the Goldman article.

The publication from Hao, 1987 could not be evaluated, since only a short, rather unspecific abstract is available. The complete article, which is written in Chinese could not be obtained by us.

Specific information about or evidence for the mycotoxin production by the strain *Trichoderma reesei/longibrachiatum* or other information questioning the safety of *Trichoderma reesei/longibrachiatum* could not be found in the literature reviewed under the search parameters noted on pages 42 - 43.

As an additional step to assure the absence of mycotoxins and monitor the production process, Röhm Enzyme GmbH tests the final pectin transeliminase/pectin lyase enzyme preparation for mycotoxins according to the method of Patterson and Roberts, Journal AOAC, 62, 1265 (1979) and meets the specifications of JECFA. No mycotoxins have been detected.

VI. Safety of the Donor Organism *Aspergillus niger*

Aspergillus niger has a long history of safe use in the fermentation industry: it became an industrially used organism when citric acid was first produced by fermentation in 1919 (Zidwick, 1992). The FDA has listed *Aspergillus niger* as a source of citric acid (21 CFR § 173.280).
In addition to citric acid, *Aspergillus niger* is a rich source of enzymes: pectinases, proteinase and amyloglucosidase were the first ones produced industrially in surface cultures before 1950 (Frost & Moss, 1987).
The use of pectin lyase and other pectolytic enzymes from *Aspergillus niger* in the wine and fruit juice production to reduce viscosity before pressing and to improve clarification is common practice (Berka et al., 1992).
FAO/WHO experts have repeatedly reviewed and accepted enzyme preparations from *Aspergillus niger*, including the microorganism itself, (FAO/WHO 1987, 1981, 1978, 1972), listing them with an Acceptable Daily Intake (ADI) of not specified.

Aspergillus niger is generally regarded as a nonpathogenic fungi widely distributed in nature. Humans are exposed to its spores daily.
Several experimental studies aimed at demonstrating the pathogenic potential of *Aspergillus niger* have come to the conclusion that neither the ingestion of high doses of spores (Nyiredy et al., 1975), nor the inhalation of spores (Bhatia et al., 1969) induces mycosis in the test animals: one day after ingestion, *Aspergillus niger* was no longer detected in the digestive tract. It also showed no significant effect on the animals in the inhalation study.

In the long history and intensive research, only few cases of toxin formation by *Aspergillus niger* have been reported. However, many reports have been subsequently disproven and the toxic effect has been attributed to secondary metabolites.
Two reports according to which *Aspergillus niger* cultures produce aflatoxin B1 (Hanssen, 1968, Kulik and Holaday, 1966) have been disproved (Wilson et al., 1968, Mislivec et al., 1968) by studies showing clearly that none of the *Aspergillus niger* strains in question produces any aflatoxin.
In few cases only, suspected toxins have been purified from cultures. Nigrillin has been isolated from cultures of *Aspergillus niger* (Caesau et al., 1969, Cole and Cox, 1981).
Malformins, a group of closely related cyclic peptides, generate deformations in plants and have been isolated from cultures of *Aspergillus niger* (Steyn, 1977).

A series of naphto-γ-pyrones produced by some strains of *Aspergillus niger* have been reported to be toxic for the vertebrate central nervous system (Ghosal et al., 1979, DeLucca et al., 1983, Ehrlich et al., 1984). However, these secondary metabolites cannot be considered mycotoxins (Bennett, 1989), as they were not shown to be toxic when administered by natural route.

After reviewing the available scientific literature on *A. niger*, it is apparent that there is consensus among the experts that *Aspergillus niger* has never been identified to be the primary cause of any disease in man. Sporadic toxin formation under undefined conditions has not been observed under controlled fermentation conditions. So it is concluded that *Aspergillus niger* is a safe production organism for industrial use when the rules of good manufacturing practice are observed.

VII. Safety of the genetically modified microorganism *Trichoderma reesei* producing *Aspergillus niger* pectin lyase

The genetically modified *Trichoderma reesei* strain produces *Aspergillus niger* pectin lyase.
It is very unlikely that the pectin lyase production would render a nonpathogenic fungus to a pathogenic one. Pathogenity consists and requires several traits that are controlled by many genes.
Insertions have been made. They do not raise any safety questions.
In our fermentation studies the proportional amounts of different cellulase and hemicellulase activities can be changed by varying the culture conditions. Differences in proportional amounts of different enzymes produced can be regarded as a natural trait.

The pectin lyase gene (*pelD*) in the recombinant *T. reesei* strain is under the control of the *cbh*1 promoter. The donor strain *Aspergillus niger* is used for the production of food enzymes for more than 3 decades.

Because *T. reesei* has been shown to be non-pathogenic and the genetically modified strain does not contain any new unknown activities it can be regarded as safe as the parent organism. In the studies performed by Huuskonen (see Appendix 7: Study Report: Pathogenicity of the Genetically Modified *Trichoderma reesei* strain ALKO 2224 in Mice after a single intravenous Administration), it was shown that a genetically modified *T. reesei* strain is non-pathogenic, as is the host.

- Stability of the production strain:

The strain has proved to be stable since its construction in 1998. Fungal strains are normally characterized and identified by their morphology, i.e. appearance, and metabolic efficiency. We perform precisely these analyses to ensure that our strains remain unchanged up to and during fermentation. All strains are passed on to production in the form of tested dry preserves. From these, production preserves are prepared and stored at -150°C in the following manner: A petri dish is inoculated from the dry preserve in such way, that individual colonies can be selected upon germination. 30 individual colonies are inoculated into shake flasks. Care is taken to select only those colonies which present the familiar picture (same phenotype). The shake flasks constitute the culture stage.

Mutation does not normally occur, and if so, only in the vegetative state during cell division. Due to the above-described procedure, this vegetative state of the cultures is reduced to a minimum during production.

From each shake flask, one oblique tube and another shake flask are inoculated. The latter is subjected to a so-called productivity test, i.e. after the shake flask cultivation is being completed, we measure the enzymatic activity, which must correspond to a given value. If this value is not reached, the culture is discarded. This test determines the characteristic metabolic efficiency of each strain, i.e. to establish its identity. The tubes whose parallel shake flasks show the highest results are then flushed with glycerol solution. The suspensions obtained this way are deep frozen in portions of 0.5 ml at -150°C (50 productivity preserves each).

The annual production starts from these productivity preserves. Six of them are thawed for inoculation of six shake flasks and subsequent inoculation of the first process bioreactor from these.

VIII. Safety of Pectin Lyase

A. Safety of enzymes in general:

Enzymes are large proteins that catalyze chemical reactions. They are natural substances that can be found through living nature. Like most natural substances, they are subject to degradation and metabolism. Therefore, cumulative effects are virtually unknown for enzymes.
Enzymes have never been reported to be mutagenic or carcinogenic. Given the current understanding of carcinogenesis and mutagenesis, it is unlikely that an enzyme with such activity will ever be discovered.
No teratogenic or other reproductive effects resulting from enzymes have ever been established.
When the folded configuration of the enzyme is disturbed, the activity of the enzyme disappears leaving a protein composed of the same amino acid building blocks found in all other proteins present in nature.
Exhaustive literature reviews support the proposition that enzyme preparations from nontoxigenic, nonpathogenic organisms are safe to consume. Under the usual conditions of use, enzymes do not pose a hazard for consumers. For example, ingesting active proteases at low levels could not affect the human gastrointestinal tract where many proteases are already are present at levels to digest food. Active proteases are widely distributed in fresh fruits, vegetables, cheeses and other uncooked foods. Many enzymes are inactivated in the gastrointestinal tract and digested as protein.

(Pariza and Foster, 1982)
(Noordervliet and Toet, 1987)

B. Safety of pectolytic enzymes in general:

Many bacteria and fungi have long been known to produce pectolytic enzymes. Pectolytic enzymes are essential in the decay of dead plant material by nonpathogenic microorganisms, and thus assist in recycling carbon compounds in

the biosphere. They also play a decisive role in the microbial spoilage of fruits and vegetables.
Several types of enzymes are involved in the degradation of pectin materials. The two main groups are the pectin esterases and the pectin depolymerases. The depolymerizing enzymes are further classified by the following criteria: preference for pectic acid or pectin as substrate, hydrolytic or transeliminative cleavage of the glycosidic linkages, and endo- or exo-types of the action mechanism.
Occurrence of pectolytic enzymes has been reported in a large number of bacteria and fungi. In the majority of cases, microorganisms produce a variety of pectolytic enzymes and, for this reason many commercially available enzyme preparations are mixtures of these enzymes.
Extensive use of pectolytic enzymes is made in processing fruit juices for increasing yields on pressing, as aid in clarification of juices, and for depectinizing in order to obtain high density fruit juice concentrates. Fungal enzymes are widely used in producing, for example, apple juice, grape juice, and wine. (Böing, J.T.P.)
Commercial pectinase enzyme preparations must contain a high pectin lyase activity to break down apple pectin (Grassin, C. et al.)
So, it can be concluded that the wide and long use of pectolytic enzyme preparations which, in most cases, naturally and also purposely include pectin lyase activity, gives no indication for pectin lyase to be unsafe.

C. Safety of pectin lyase:

To confirm the safety of the enzyme preparation, safety studies were performed.

Summary of Toxicological Studies with Pectin lyase:

Pectin lyase produced with *Aspergillus niger* var. *awamori* was tested thoroughly for its toxicological safety. The gene producing the pectin lyase with the *Aspergillus niger* var. *awamori* was the same gene as the one used to produce the pectin lyase with *Trichoderma reesei*, both gene codings derived from the same strain of *Aspergillus niger.* Three toxicological studies were performed as described below:

The final purified and concentrated fermentation product of a pectin lyase enzyme preparation derived from a biotechnological process using an *Aspergillus niger* var. *awamori* strain was tested for genotoxicity in two in vitro assays and for subchronic toxicity to rodents in a 90 day oral gavage study in rats.

The results of the toxicological tests were as follows:

(a) Wollny, H.-E. et al. (1997): Salmonella typhimurium Reverse Mutation Assay with RH 7001. CCR Cytotest Cell Research GmbH, Project No. 543501

The product was non-mutagenic in the Ames *S. typhimurium* assay with and without metabolic activation. See Appendix 8: Summary of toxicological studies with Pectin Lyase.

(b) Czich, A. et al. (1997): Chromosome Aberration Assay with Human Lymphocytes in Vitro with RH 7001. CCR Cytotest Cell Research GmbH, Project No. 543502

The pectin lyase enzyme preparation did not show any clastogenic activity in an in vitro cytogenetic assay using human peripheral lymphocytes with and without metabolic activation. See Appendix 8: Summary of toxicological studies with Pectin Lyase

(c) Schmid., H. et al. (1998): Subchronic 13-Week Oral Toxicity (Gavage) Study with RH 7001. RCC Research and Consulting Company LTD, Itingen, Switzerland, Project No. 610918
In a study for subchronic toxicity in rats, a NOAEL ≥ 1000 mg/kg body weight /day was established for the pectin lyase enzyme preparation. No treatment-related effects were seen up to the highest administered dose. See Appendix 8: Summary of toxicological studies with Pectin Lyase

All tests were performed in compliance with GLP in accordance to OECD, EU and FDA guidelines.

Material tested :

The studies were performed on batch no. RPT 05088 of the final purified and concentrated fermentation product. A test material with 5.93 % of dry matter and an enzymatic activity of 377.7 PTF units was used.

The stability of the material at test conditions for about four hours has been demonstrated.

Reference:
Sponsor: Röhm GmbH
Investigator: K. Biedermann
Laboratory: RCC Umweltchemie AG, CH-4452 Itingen / Switzerland
Analytical report to 13 w oral toxicity study, RCC Project No. 610918
Date: July 28, 1998

The toxicology study of pectin lyase produced by a recombinant *Aspergillus* sp. strain demonstrates, that the recombinant host strain does not produce any toxic compounds, and that the pectin lyase protein in its native form does not cause toxic effects.

The toxicology study on the recombinant ALKO strain ALKO 2224 is relevant for the product of this application, as it was performed with a recombinant strain which used the same host strain from which the recombinant *Trichoderma reesei* production strain for the pectin lyase is derived. The test showed that the *Trichoderma reesei* strain does not produce toxic compounds even when alterations in the genome are done concerning the pattern of secreted proteins (see Appendix 7: Study Report: Pathogenicity of the Genetically Modified *Trichoderma reesei* strain ALKO 2224 in Mice after a single intravenous Administration).

Therefore, we concluded that the genetic modifications Röhm Enzyme GmbH uses to obtain the *Trichoderma reesei* production strain for pectin lyase will not cause the strain to produce toxic compounds, and that the pectin lyase protein will not be toxic, thus resulting in a safe product.

IX. Consideration of the probable consumption of the substance

The processing aid pectin lyase will be used for the manufacturing process of vegetables and fruit juices, purees and concentrates, including, for example, orange juice and concentrate, grapefruit juice and concentrate, banana puree, peach puree, apricot puree and others. See Section III, Applications for the Pectin Lyase Enzyme Preparation.

The maximum recommended use of the enzyme preparation is 100 ppm (= 100 mg enzyme preparation per kg) and is sometimes used in carrot juice processing. The usual amount used will almost always be much lower.

To estimate daily intake, dietary exposure to the enzyme preparation was calculated using orange juice as the beverage and the maximum use level derived from carrot juice.
Based on information from the Florida Department of Citrus, the average per capita consumption in the USA for orange juice in the years 1996-1997, was 5.38 gallons, which equals 20.34 liters.

The estimated daily intake (EDI), calculated for the pectin lyase enzyme preparation with the highest recommended use for the average consumer is 5.57 mg pectin lyase enzyme preparation per day.

The no effect level NOAEL established in a 90 day oral toxicity study with rats is 1000 mg enzyme dry matter per kg body weight per day.

Assuming an average weight of 60 kg per person and the maximum enzyme content of 3 % in the enzyme preparation, the safety factor for the use of the pectin lyase enzyme preparation for the intended use with a dosage of 100 ppm is more than 300 000.

Based on known differences between average consumption and 'higher'-consumption covered by the 90 % - percentile, an additional safety factor between three and four has to be taken in account.

Considering a factor of four for the 'higher' consumption results in a safety factor of more than 75 000 for the use with a dosage of 100 ppm.

Additionally, it is important to note that the enzyme in the 90 day oral toxicity study was not inactivated, whereas the enzyme used in the intended applications will be inactivated by heat during the pasteurization process. So even if the pasteurization step in the food manufacturing process should fail for some reason, consuming the active enzyme with the final product will not harm the consumer. The inactivation of the enzyme further increases the safety profile of the pectin lyase enzyme preparation.

X. Consideration of the exposure to substances that are a product of the enzyme reaction

Properties of the pectin lyase enzyme preparation:

The enzyme preparation possesses the ability to cleave soluble methylated pectin.
The reaction products are oligomers of highly esterified pectin with a 4,5-dehydrogalacturonic acid group at the non-reducing end.

Potentially hazardous degradation products:

There are no degradation products expected other than those occurring naturally in the organism during digestion. Thus, there is no evidence for non-physiological properties of these products.

XI. Basis for concluding expert consensus

The conclusion that the use of the pectin lyase enzyme preparation derived from *Trichoderma reesei* (formerly classified as: *Trichoderma longibrachiatum*) carrying a gene coding for pectin lyase from *Aspergillus niger* for use as a processing aid, primarily in fruit and vegetable products, is safe is based on:

- the safety of the production process established through GMP, food-grade materials used in the process, DIN/ISO 9002 and HACCP certification
- the safety of the host organism *Trichoderma reesei* established through numerous references in published literature and a long history of safe use
- the safety of the donor organism *Aspergillus niger* established through numerous references in published literature and a long history of safe use
- the safety of the genetically modified microorganism established through generally available scientific information and data
- the safety of pectin lyase established through toxicity studies

From a review of the publicly available scientific literature and information and corroborative evidence about the safety of the pectin lyase enzyme preparation for its intended use as a processing aid, Röhm Enzyme GmbH concludes that there is the necessary expert consensus about the safety of the pectin lyase enzyme preparation derived from a nontoxic, nonpathogenic *Trichoderma reesei* microorganism carrying the gene coded for pectin lyase from a nontoxic, nonpathogenic strain of *Aspergillus niger*.
In the light of the data and information described in the notice, including both favorable and unfavorable information, there is reasonable certainty that the pectin lyase enzyme preparation is generally recognized as safe under the intended conditions of use.

XII. References

Bennett, J.W., 1989. Mycotoxins, mycotoxicosis, mycotoxicology and mycopathologia. Mycopathologia 100 (1989) : 3 - 5

Berka, R.M., Dunn-Coleman, N.S., Ward, M., 1992. Industrial enzymes from *Aspergilli.* In: Bennett, J.W. and Klich, M.A. (eds), *Aspergillus*, biology and industrial applications. Butterworth-Heinemann, London, 1992, p. 155-202

Bhatia, Y.N., Mohapatra L.N., 1969. Experimental aspergillosis in mice. Part I: Pathogenic potential of *Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus niger.* Mykosen 12 (1969): 651-654

Böing, J.T.P., 1982. Chapter 15: Enzyme Production. In: Prescott & Dunn's Industrial Microbiology, 4th Edition, Edited by Gerald Reed. The AVI Publishing Company, Westport, CT, USA

Caesau F., Jansson, K. and Mutschler E., 1969. Nigragillin, a new alkaloid from the *Aspergillus niger* group. I. Isolation and structural elucidation of nigragillin and a dioxopiperazine. Helv Physiol Pharmacol Acta 44 (1969): 676-690

Coenen, T. M. M., Schoenmakers, A. C. M. and Verhagen, H., 1995. Safety evaluation of β-glucanase derived from *Trichoderma reesei*: Summary of toxicological data. Fd Chem. Toxic. Vol. 33, No. 10, pp 859 – 866, 1995

DeLucca A.J., Ehrlich K.B. and Ciegler, A., 1983. Toxicity of extracts of *Aspergillus niger* isolated from stored cottonseed. J Food Safety 5 (1983): 95-101

Ehrlich K.B., DeLucca, A.J., Ciegler, A., 1984. Naphto-γ-pyrone production by *Aspergillus niger* isolated from stored cottonseed. Appl Environ Microbiol 48 (1984): 1-4

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Eveleigh, D. E., 1985, *Trichoderma*, In: Demain, AL and Solomon, N.A. (Eds.). Biology of Industrial Microorganisms, Biotechnology Series, The Benjamin/Cummings Publishing Company Inc., Menlo Park, CA, pp 487 - 509

FAO/WHO 1972. Specifications for identity and purity of some enzymes and certain other substances. 15th report of the Joint FAO/WHO Expert Committee on Food Additives, Food and Agricultural Organization of the United Nations, World Health Organization, Rome

FAO/WHO 1978. Specifications for identity and purity. The 22th meeting of the Joint FAO/WHO Expert Committee on Food Additives, Food and Agricultural Organization of the United Nations, Rome

FAO/WHO 1981. Specifications for identity and purity. The 25th meeting of the Joint FAO/WHO Expert Committee on Food Additives, Food and Agricultural Organization of the United Nations, Rome

FAO/WHO 1987. Toxicological evaluation of certain food additives. The 31st meeting of the Joint FAO/WHO Expert Committee on Food Additives, World Health Organization, Geneva

Frost, G.M., Moss, D.A., 1987. Production of enzymes by fermentation. In: Rehm, H.J. and Reed G. (eds). Biotechnology, Vol 7a. VCH; Weinheim, p. 65-102

Ghosal, S., Biswas, K. and Chakrabarti D.K., 1979. Toxic naphto-γ-pyrones from *Aspergillus niger*. J Agric Food Chem 27 (1979): 1347-1351

Goldman, G. H., Pelegrinelli Fungaro, M. H. and de Azevedo, J. L., 1988. The evaluation of the cellulolytic activity in some strains of *Aspergillus niger* group. Rev. Microbiol., Sao Paulo, 19(4): 438 - 441

Grassin, C. and Fauquembergue, 1996. Chapter 2.13: Fruit juices. In: Godfrey, T. and West S. (eds), Industrial Enzymology, Macmillan Press Ltd, London

Hanssen, E., 1969. Schädigung von Lebensmitteln durch Aflatoxin B1. Naturwissenschaften 56 (1969): 90

Hao, J. and Zhang, F.,1987. Problems with trichodermal toxin contamination in application of cellulase, Zhongguo Tiaoweipin (1987), 4, 1 - 5

Hjortkjaer, R. K., Bille-Hansen, V., Hazelden, K. P., McConville, M., McGregor, D. B., Cuthbeert, J. A., Greenough, R. J., Chapman, E., Gardener, J. R. and Ashby R., 1986. Safety evaluation of Celluclast®, an acid cellulase derived from *Trichoderma reesei*. Fd Chem. Toxic. Vol. 24, No 1, pp 55 – 63, 1986

Kulik M.M. and Holaday C.E., 1966. Aflatoxin: A metabolic product of several fungi. Mycopathologia et Mycologia Aplicata 30 (1966): 137-140

Mislivec, P.B., Hunter J.H. and Tuite, J., 1968. Assay for aflatoxin production by the genera *Aspergillus* and *Penicillium*. Appl Microbiol 16 (1968): 1053-1055

Nevalainen, H., Suominen, P. and Taimisto, K. 1994. On the safety of *Trichoderma reesei*. J. Biotechnol. 37: 193 - 200.

Noordervliet, P.F. and Toet, D.A., 1987. Safety in Enzyme Technology. In: Biotechnology, edited by H.J. Rehm and G. Reed, Volume 7a Enzyme Technology, Volume Editor: John F. Kennedy. VCH

Nyiredy, I., Etter, L., Fesüs, I. and Mayer G., 1975. The fate of mould „spores" in the digestive tract of chicks. Acta Vet Acad Scient Hung 25 (1975): 123-128

Pariza, M.W. and Foster, E.M., 1983. Determining the Safety of Enzymes Used in Food Processing. Journal of Food Protection, Vol. 46, May 1983

Rifai, M.A. 1969. A revision of the genus *Trichoderma*. Mycological Papers no 116, Commonwealth Mycological Institute, Kew, Surrey, England.

Sakai; T. 1992. Degradation of pectins. In: Microbial degradation of natural products, edited by G. Winkelmann, chapter 3. VCH, Weinheim, Germany

Schobinger, U., 1987. Handbuch der Lebensmitteltechnologie: Frucht- und Gemüsesäfte. Verlag Eugen Ulmer, Stuttgart

Steyn, P.S., 1977. Some new mycotoxins. Pure & Appl Chem 49 (1977): 1771-1778

Voragen, A.G.J. and Pilnik, W., 1989. Pectin-degrading enzymes in fruit and vegetable processing. In: Biocatalysis in agricultural biotechnology, chapter 7. American Chemical Society: 94 - 115

Wang, Daniel I. C., Cooney, Charles L., Demain, Arnold L., Dunnhill, Peter, Humphrey, Arthur E., and Lilly, Malcolm, D., 1979. Fermentation & Enzyme Technology, A Wiley-Interscience Publication, John Wiley & Sons, New York

Wilson B.J., Campbell T.C., Hayes A.W. and Hanlin R.T., 1968. Investigation of reported aflatoxin production by fungi outside the *Aspergillus flavus* group. Appl Microbiol 16 (1968): 819-821

Zidwick M.J., 1992. Organic acids. In: Finkelstein D.B. and Ball C. (eds). Biotechnology of the filamentous fungi. Butterworth-Heinemann, Boston, p. 303-338

Literature Search:

Evaluation of Potential Hazards of Trichoderma in Food Contact Applications

In order to evaluate potential hazards of trichoderma in food contact applications, a literature search was performed.

The data files used were REGISTRY, CA, BIOSIS, BIOTECHDS, DIALOG, MEDLINE, IPA

The key words used were ALIMENTATION? OR JUICE? OR WINE? OR BEVERAGE? OR FOOD?)
AND (TOX? OR LD50 OR ALLERG? OR DERMAT? OR HAZARD? OR IRRIT?)

94 citations were found. Most of them described potential hazards of moulds and mycotoxins in food as a result of food contamination.

Only seven (7) articles address *Trichoderma* as a production organism for food contact applications.

Literature Search:

Pectin Lyase

In order to evaluate the safety of pectin lyase, a literature search was performed.

The search profile was as follows:

Enzyme: PECTIN LYASE or PECTIN TRANSELIMINASE
IUB-Nr.: 4.2.2.10
CAS-Nr.: 9033-35-6

Data files:

REGISTRY, 'AGRICOLA, BIOSIS, CA, EMBASE, TOXLINE, TOXLIT'

Key words:

ALIMENTATION? OR JUICE? OR WINE? OR BEVERAGE? OR FOOD?)
AND (TOX? OR LD50 OR ALLERG? OR DERMAT? OR HAZARD? OR IRRIT?)

As a result of the search, 20 citations were found.

Most of the citations found were in the field of fungi and enzymes involved in plant decay.

None of the citations identified dealt with potential hazards of pectin lyase as an enzyme in food contact applications.

XIII. List of Appendices

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Appendix 2: Method of analysis: Assay of Endo-1,4-β-Glucanase Activity

Appendix 3: Flow chart of the enzyme production

Appendix 4: HACCP Certificate for Röhm Enzyme GmbH

Appendix 5: Regulatory aspects of microbial food enzymes by AMFEP

Appendix 6: Safety aspects of Enzymes produced by Genetically Modified Micro-Organisms by AMFEP

Appendix 7: Study Report: Pathogenicity of the Genetically Modified *Trichoderma reesei* strain ALKO 2224 in Mice after a single intravenous Administration

Appendix 8: Summary of toxicological studies with Pectin Lyase

TAB 1

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Appendix 1: Method of analysis: Determination of PTF-Activity

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1. Purpose

Determination of the enzyme activity Pectinetranseliminase as a single activity out of the Pectinase activity complex.

2. Area of application

RÖHM, department PEM 38
ROAL
ALKO laboratories

3. References

"Qualitätssicherheitshandbuch QSH" of department ET 38 (enzyme technology)
"ROAL / RÖHM Quality Assurance Manual"
"ROAL / RÖHM" Analytical procedure 0 / 011 / 001:
"Use of Shewhart charts in enzyme analysis".

4. Terms

PTF Unit of Pectinetranseliminase activity.

Definition

One unit PTF causes an increase of Extinction " E_{235nm} of 0,01 per minute under conditions mentioned below (0,5 % Pectine solution, pH = 5,8, 30 °C) . So :

1 PTF = 0,01 * "E_{235nm} / min.

PTF/mg PTF activity concentration.

Rohapect D5L RÖHM final product, used as internal standard.

5. Responsibilities

Staff list of RÖHM department PEM 38 applies.
Staff list of ALKO laboratory, Rajamäki applies.

Editor of original and revisions: Head of PEM 38-Q or substitute.

Procedure of analysis (time, observance of the parameters of the working procedure, evaluation): staff.

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6. Procedure

6.1 Equipment

Spectral photometer, if possible double beam type, preferably with reference automation and cuvett changer.
Ultraviolett range of determination.
Cuvett stand resp. cuvett holder temperature controlled.

6.2 Solutions

6.2.1 0,1 m Citrate buffer, pH 5,8, according to Mc Ilvaine.

6.2.1.1 35,6 g Bisodiumhydrogenphosphate (Na2HPO4 * 2 H2O) p.a. are dissolved in 1 000 ml of distilled water.

6.2.1.2 21 g of Citric acid, monohydrate are dissolved in 1 000 ml of distilled water.

6.2.1.3 57 ml of solution 6.2.1.1 and 43 ml of solution 6.2.1.2 add to 100 ml buffer solution. pH can be adjusted to 5,8 by additionally adding one of aforesaid solutions.
Before measurement the pH-probe has to be calibrated to pH 6.

6.2.2 0,5 % substrate solution

0,5 g of Pectine ("Copenhagen Pectine X, 2955 dated 18.5.93) are mashed with 2 ml alcohol (absolute or 96 %) in a beaker and stirred to homogenity with a magnetic stirrer.
Slowly (allow 2 minutes for this step) add 80 ml buffer solution 6.2.1.3 and continue stirring, avoiding foam formation.
Measure pH and if necessarry adjust to pH = 5,8 with solutions 6.2.1.1 or 6.2.1.2 respectively.
Transfer solution to a volumetric flask and add buffer solution 6.2.1.3 up to 100 ml.
Keep substrate solution cool over night (refrigerator).
Next day centrifuge solution 10 minutes at 12 000 g.

6.2.3 enzyme solutions

6.2.3.1 Standard procedure for sample dilution.
A distinct mass of sample m_S (preferably 0,5 g to 2 g) is weight into a volumetric flask. Fill up to 100 ml with buffer solution. Transfer 3 ml of this solution to a next volumetric flask and fill up with buffer to 25 ml.

6.2.3.2 Sample solutions have to be prepared immediatelly before analysis.

6.2.3.3 Internal standard

0,0012 g of ROHAPECT D5L, batch nr. 90072, resemble to 10,5 PTF/mg. Dilute 1 g of ROHAPECT D5L as descibed above. The concentration of the solution thus is c_S = (1g / 100 ml) * (3 ml / 25 ml) = 0,0012 g / ml.

6.3 Working procedure

6.3.1 Reference is distilled water

6.3.2 Sample (applies for enzyme sample as well as for internal standard):

0,1 ml of enzyme solution is added to 3 ml of substrate solution (pre-thermostated to 30 °C).

Analysis sample volume thus is V_{sa} = 3,1 ml.

Analysis sample concentration is c_{sa} = 0,1 ml / 3,1 ml * c_S.

After short mixing the extinction E at 235 nm is registered over 8 minutes (one figure each minute).

Determination is done in triplicate, using one single sample concentration.

When using a simple monochromator the width of the slit should not exceed 1 mm, to ensure a spectral accuracy of 3 nm.

If slit width is larger than 1 mm the gain must be increased.

These rules do not apply for photometers with double monochromator or slit width automation.

6.4 Evaluation

6.4.1 General formula

Extinction data are plotted over time and the slope of the extinction, "E / "t, is determined in the linear section of the function. The linear part should encompass at least 5 minutes (6 data points). Delays of the enzyme reaction before the linear part (lag-phase) are not taken into account.

PTF concentration is calculated as $PTF/mg = \frac{\Delta E_{235} / \Delta t}{0,01 * V_{sa} * c_{sa}}$ (formula 6.1)

The extinction difference "E_{235} should not exceed 0,03 per minute.

Best values are within a range of 0,02 to 0,03.

Each analysis is accompanied by a triple determination of the internal standard. Sample analysis is only valid if these figures fit into the allowed range of the internal standard Shewhart control chart.

000205

6.4.2 Simplified formula

If sample preparation is done exactly according to the procedure descibed above (see 6.2.3.1) all the dilution factors can be lumped together. Thus in the denominator of formula (6.1) sample mass m_s instead of $V_{sa} * c_{sa}$ can be used.

In this case formula simplifies to $$PTF/mg = \frac{\Delta E_{235} / \Delta t}{ms * 1{,}2 * E-6}$$ (formula 6.2)

Example

Sample size: 0,4 g.

Sample is diluted as follows:

0,4 g in 100 ml, out of this 3 ml in 25 ml.

c_s = (0,4 g / 100 ml) * (3 ml / 25 ml) = 0,00048 g / ml.

Analysis sample concentration c_{sa} = 0,1 ml / 3,1 ml * c_s = 15,4839 * 10^{-6} g / ml.

analysis sample volume V_{sa} = 3,1 ml.

Determined slope of extinction rise "E_{235} / "t = 0,015 min^{-1}.

According to formula (6.1)

$$PTF/mg = \frac{0{,}015}{0{,}01 * 3{,}1 * 15{,}4839 * E-6} = 31{,}25.$$

As in this case dilution has been done as described, formula (6.2) can be used as well.

m_s = 0,4 g, thus:

$$PTF/mg = \frac{0{,}015}{0{,}4 * 1{,}2 * E-6} = 31{,}25.$$

7. General remarks

- / -

000206

8. Appendix

8.1 Symbols

c_s	[g / ml]	Sample concentration
c_{sa}	[g / ml]	Analysis sample concentration
E_{235}	[1]	Extinction at 235 nm
"E_{235}	[1]	Extinction difference
m_s	[g]	Sample mass
t	[min]	Time
"t	[min]	Time difference
V_{sa}	[ml]	Analysis sample Volume

000207

TAB 2

Appendix 2: Method of analysis: Assay of Endo-1,4-β-Glucanase Activity

ASSAY OF ENDO-1,4-β-GLUCANASE ACTIVITY
Method B-030, Rev. C

Principle

Endo-1,4-β-glucanase in the sample hydrolyzes the substrate, hydroxyethylcellulose, and the reducing sugars thus produced are assayed spectrophotometrically using dinitrosalicylic acid.

Unit of activity

One endo-1,4-β-glucanase unit (ECU) is defined as the amount of enzyme producing one nmole of reducing sugars as glucose in one second (1 ECU = 1 nkat).

Field of application

The method is suitable for measurement of enzyme samples containing *Trichoderma* originated endo-1,4-β-glucanase. The method is not specific for endo-1,4-β-glucanase. Other hydroxyethyl cellulose degrading enzymes (e.g. β-glucosidase) may have an effect on the result.

Safety

DNS-reagent is harmful by inhalation, in contact with skin and eyes and if swallowed.

Assay conditions

Substrate	hydroxyethyl cellulose
pH	4.8
Incubation temperature	50 °C ± 0.5 °C
Incubation time	10 min

Equipment

Water bath	50 °C
Water bath	100 °C
Test tube mixer (vortex)	
Spectrophotometer	

Reagents

All solutions are prepared in deionized water, Milli-Q or equivalent.

1. Citrate Buffer (0.05 M, pH 4.8)
Prepare 0.05 M solutions of both citric acid ($C_6H_8O_7 \cdot H_2O$, 10.51 g/l) and sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$, 14.71 g/l) in water. Adjust the pH of the 0.05 M citrate solution to 4.8 with the 0.05 M citric acid solution (should require about 667 ml of citric acid solution per 1 l of sodium citrate solution).

000210

2. Substrate
Dissolve 1.00 g hydroxyethylcellulose (mittelviskos, Fluka AG, 54290) in citrate buffer and make the volume up to 100 ml. The powder is dissolved with magnetic stirring for at least one hour, after which it must stand for a further 1 h to clarify. Store at + 4 °C for a maximum of 2 weeks.

3. DNS reagent
Dissolve 50.0 g of 3,5-dinitrosalicylic acid (Sigma D-0550) in about 4 l of water. With continuous magnetic stirring, gradually add 80.0 g of NaOH and let it dissolve. Add 1500 g of Rochelle Salt (K-Na-tartrate, Merck 8087) in small portions with continuous stirring. The solution may be cautiously warmed to a maximum temperature of 45 °C. Cool to room temperature and dilute with water to 5000 ml in a volumetric flask. If the solution is not clear, filter through Whatman 1 filter paper. Store in a dark bottle at room temperature.

Sample

The sample is diluted in citrate buffer. A suitable dilution will yield an absorbance of 0.20 - 0.25.

Assay

Add 1.8 ml of substrate solution to two test tubes and equilibrate at 50 °C for 5 min. Add 200 µl of suitably diluted sample solution to one of the tubes and mix with a vortex mixer. After exactly 10 min incubation, add 3.0 ml of DNS reagent to both tubes and mix. Add 200 µl of sample solution to that tube (blank) which was incubated without sample. Place both tubes in a boiling water bath one at a time. After boiling for exactly 5 min, remove the tubes and cool to room temperature. Measure the sample absorbance against the blank at 540 nm. Read the activity from the standard line and multiply the result by the dilution factor.

Standard

Prepare 0.1 M stock solution of glucose. Dissolve 1.802 g glucose (Merck, 8337)[2] in citrate buffer and make the volume up to 100 ml. Make the following dilutions from the stock solution in citrate buffer:

Dilution	Glucose concentration µmol/ml	Activity ECU/ml[1]
1:25	4.0	6.67
1:15	6.67	11.11
1:10	10.00	16.67

Do triplicate assays of each standard dilution. Pipette to test tubes 1.8 ml of substrate, 200 µl of standard dilution and 3.0 ml of DNS reagent. Boil for exactly 5 min, cool and measure the absorbances against the reagent blank at 540 nm. The reagent blank is prepared by adding 200 µl of citrate buffer instead of the standard dilution.

1. The corresponding endo-1,4-β-glucanase activity (nkat/ml) is obtained by multiplying the glucose concentration (µmol/ml) by 1000 and dividing by the time of the hydrolysis, 600 s.

000211

2. Store in a desiccator.

Reference

Bailey, M.J., Nevalainen, K.M.H., Enzyme Microb. Technol. 3, 1981, 153-157.

Author: Marja Turunen
Revised: 11.7.97
Approved: 11.7.97

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Appendix 3: Flow chart of the enzyme production

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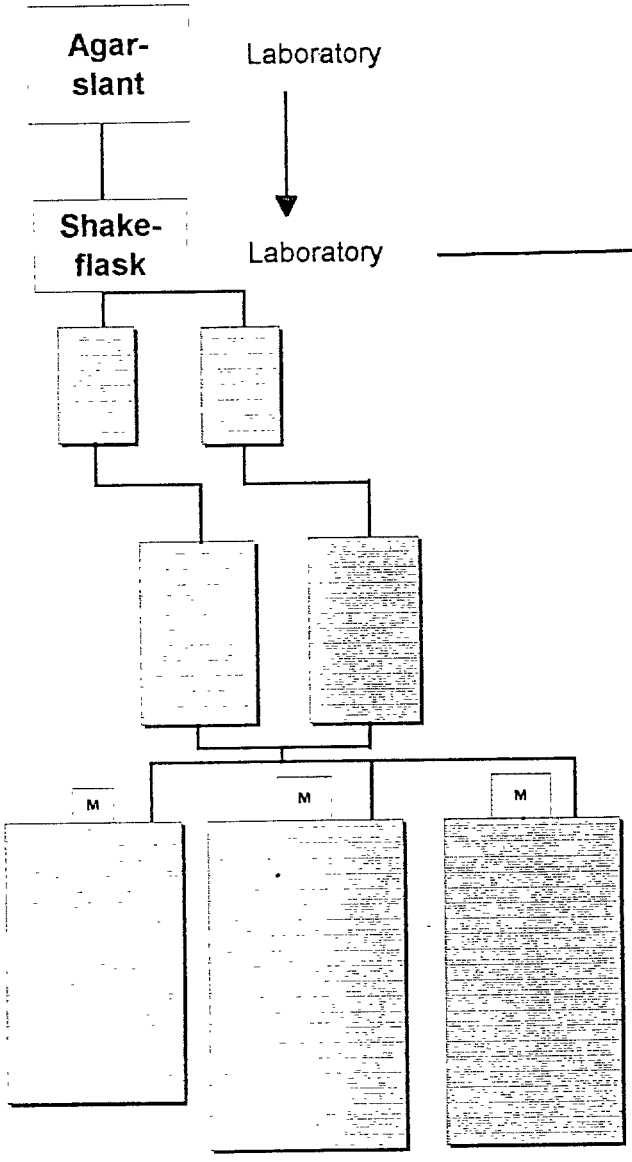
ENZYME PRODUCTION

1. UPSTREAM



PRINCIPAL STEPS

Production

Pre fermentation I

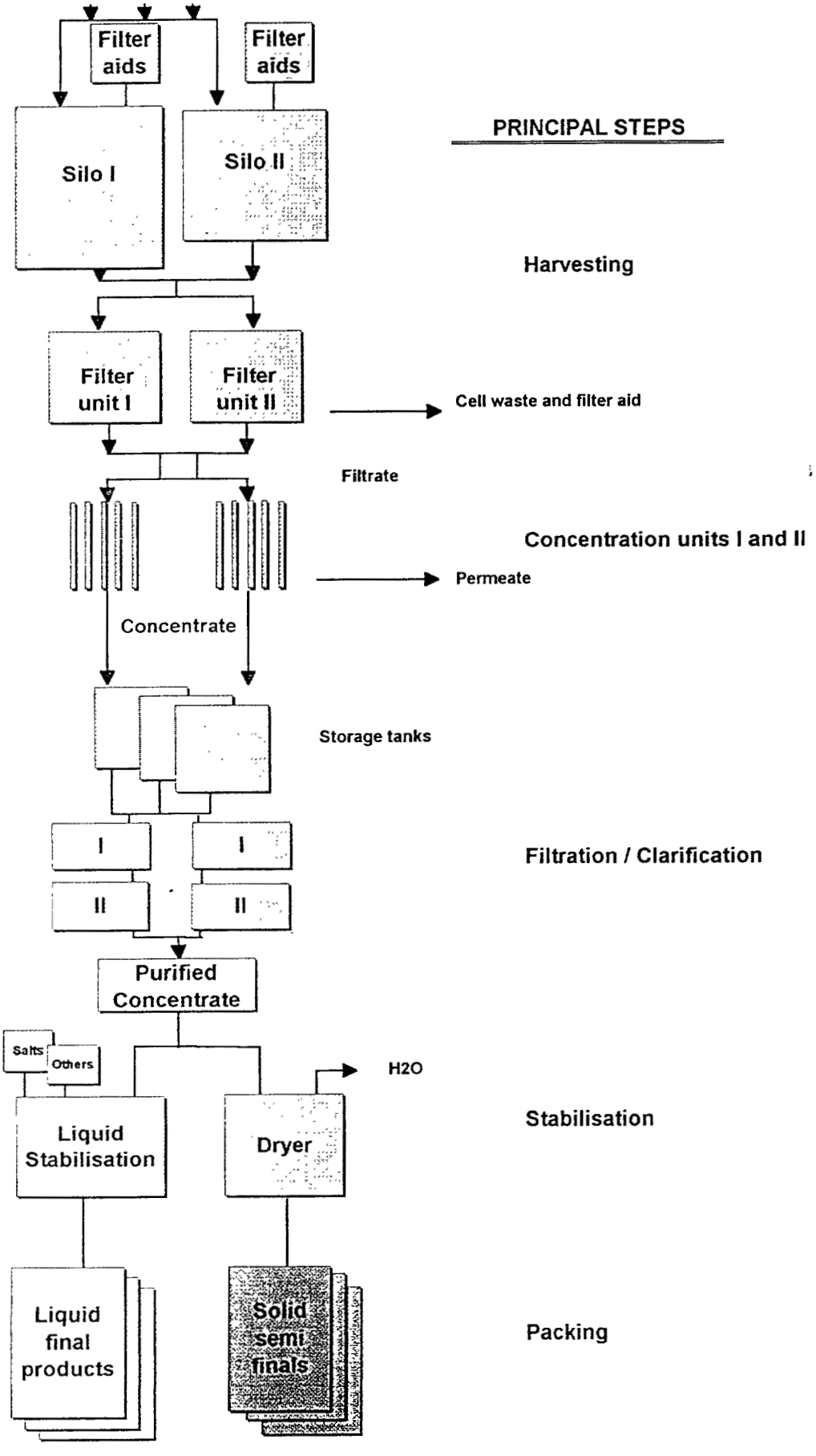
Pre fermentation II

Main fermentation

CSTR: control of °C; rpm; pH; time
Supply with O2; energy
Media with carbon, nitrogen, salts
and others

000215

2. DOWN STREAM



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Appendix 4: HACCP Certificate for Röhm Enzyme GmbH

000218

TÜV Rheinland/
Berlin-Brandenburg

TÜV

HACCP Certificate

The Certification Body for HACCP Systems of TÜV Anlagentechnik GmbH
hereby certifies that the Enterprise

Röhm Enzyme GmbH

Kirschenallee 45, D-64293 Darmstadt

has established and applies a HACCP System for

Enzymatic Products for the Food Industry

An audit was performed, Report No. 9801
Proof has been furnished that the requirements according to ALINORM 97/13
General Principles of Food Hygiene and Hazard Analysis Critical Control Point (HACCP) System and Guidelines for its Application of the FAO/WHO Codex Alimentarius Commission
are fulfilled.

The certificate is valid until **July 2001**

Certificate Registration No. 09 150 9801

000219

Cologne, 24.09.1998

Dr. Wilfried Rabe

Certification Body for HACCP Systems of TÜV Anlagentechnik GmbH

Dr. oec. troph. Angela Moritz

Rev. 0

Appendix 5: Regulatory aspects of microbial food enzymes by AMFEP

000221

Pages 000222 - 000231 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

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000232

Appendix 6: Safety aspects of Enzymes produced by Genetically Modified Micro-Organisms by AMFEP

Pages 000234 - 000242 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 7: Study Report: Pathogenicity of the Genetically Modified *Trichoderma reesei* strain ALKO 2224 in Mice after a single intravenous Administration

4-90

STUDY REPORT

PATHOGENICITY OF THE GENETICALLY MODIFIED TRICHODERMA REESEI-STRAIN ALKO 2224 IN MICE AFTER A SINGLE INTRAVENOUS ADMINISTRATION

Prepared by Hannele Huuskonen

National Public Health Institute
Department of Environmental Hygiene
and Toxicology
P.O.Box 95
SF-70701 Kuopio
FINLAND

Date: October 2, 1990

Study number 4-90

The study "Pathogenicity of the Genetically Modified Trichoderma Reesei-strain ALKO 2224 in Mice after a Single Intravenous Administration" has been carried out in National Public Health Institute, Department of Environmental Hygiene and Toxicology, in cooperation with the National Laboratory Animal Center, University of Kuopio, Finland.

The study was started on April 11 (the study protocol finally approved), 1990. Administration of the test article to animals was initiated on April 25, 1990 and the observation of the animals was finished on June 13, 1990.

The raw data and the specimens analyzed by the testing facilities and the final report have been archived in the archives of the testing facilities, Kuopio, Finland.

It is certified that this report provides a true and complete report of data generated and that the study was carried out according to the GLP procedures stated.

Kuopio October 2, 1990

Hannele Huuskonen, M.Sc,
Study Director

Kuopio October 2, 1990

Hannu Komulainen, Ph.D., Manager, Docent in Toxicology

National Public Health Institute
Department of Environmental Hygiene and Toxicology
P.O.Box 95
SF-70701 Kuopio, FINLAND

GLP compliance.

The study was conducted in compliance with the following guidelines and Good Laboratory Practice regulations:

OECD Principles of Good Laboratory Practice, Decision of the Council Concerning Mutual Acceptance of Data in the Assessment of Chemicals, Annex 2, Adopted May 12, 1981.

Good Laboratory Practice for Nonclinical Laboratory Studies, Food and Drug Administration. 21 CFR 58 (4-1-88 Edition).

Standard Operation Procedures of the testing facilities were followed where relevant and these are available for examination.

NATIONAL PUBLIC HEALTH INSTITUTE
DEPARTMENT OF ENVIRONMENTAL HYGIENE AND TOXICOLOGY
LABORATORY OF TOXICOLOGY/TOXICITY STUDY UNIT

Title of the study: PATHOGENICITY OF THE GENETICALLY MODIFIED TRICHODERMA REESEI -STRAIN ALKO 2224 IN MICE AFTER A SINGLE INTRAVENOUS ADMINISTRATION

Study number: 4-90

QUALITY ASSURANCE AUDIT STATEMENT

The conduct of this study has been subjected to periodic inspections and this report has been audited by the Quality Assurance Unit of National Public Health Institute. The dates of inspections are given below.

Date of Q.A. Inspection	Date of Report to Study Director and Management
April 11, 1990	April 11, 1990
April 17, 1990	April 17, 1990
April 25, 1990	April 25, 1990
June 4, 1990	June 4, 1990
September 20, 1990	September 20, 1990

This report accurately describes the methods and procedures used in the study. The collection of the raw data of this study has been performed according to the GLP procedures stated and the reported results are an accurate reflection of the raw data of the study.

Signed: _____ Date: October 2, 1990

Head of the Quality Assurance Unit

Testing facilities and personnel responsible principally for the study

The study was conducted in cooperation with the following institutes and departments:

National Public Health Institute
Department of Environmental Hygiene and Toxicology
P.O.Box 95, 70701 Kuopio, Finland

Hannele Huuskonen, M.Sc, Study Director

Hannu Komulainen, Ph.D., Manager, Docent in Toxicology

Coordination of the study and reporting

National Laboratory Animal Center
University of Kuopio
P.O.Box 6, 70211 Kuopio, Finland

Timo Nevalainen, Ph.D., DVM, Director

Eila Kaliste-Korhonen, Junior Scientist
Heikki Karhunen, Laboratory animal technician

Animal care

Department of Pathology
University of Kuopio
P.O.Box 6, 70211 Kuopio, Finland

Veli-Matti Kosma, Ph.D., MD, Docent in Pathology

Histological analysis of tissue samples in health monitoring program

Alko Ltd.
Biotechnology
05200 Rajamäki, Finland

Kaarina Taimisto, M.Sc. (Chem. Eng.), Manager
Product Safety and Regulatory Affairs

Sponsor; identification, viability and purity of the test article

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3.4.2 Administration of the test article
3.4.3 Body weights
3.4.4 Clinical signs

4. COLLECTION OF DATA AND ITS STATISTICAL ANALYSIS

5. RESULTS
5.1 Body weights
5.2 Clinical signs

6. DISCUSSION

7. CONCLUSION

Tables 1 and 2
Appendixes 1 - 11

1. SUMMARY

Pathogenicity of genetically modified Trichoderma reesei-strain ALKO 2224 was studied in NMRI-strain mice. The host strain (ALKO 233) of this modified test microorganism was used as a reference. Male mice (7-9 weeks old, 15 animals/group) were inoculated with the test article or the reference article as a single dose of 5 x 10^6 living spores intravenously. The microorganism spore suspension was prepared in 0.9% sterile physiological NaCl-solution and inoculated in a volume of 0.1 ml into the tail vein of mice. Other groups of animals were made immunosuppressed with a subcutaneous dose of 5 mg cortisone acetate 2 days before inoculation of these spore strains or their vehicle. Control animals received 0.9% sterile physiological NaCl-solution intravenously instead of spores. The animals were observed daily for morbidity or mortality for the next 40 days.

Neither Trichoderma reesei-strain ALKO 2224 nor its host strain ALKO 233 were pathogenic to NMRI. Pretreatment of the animals with cortisone acetate did not affect this response of mice. The test and the reference articles did not affect significantly body weights of the animals nor caused any clinical signs during the observation period.

2. OBJECTIVE OF THE STUDY

The aim of the study was to determine the pathogenic potency of the microorganism Trichoderma reesei-strain ALKO 2224 in mice after a single intravenous dose. The host strain ALKO 233 was used as a reference.

The mouse is considered to be an appropriate species for this type of study.

The test microorganism was inoculated as viable spores intravenously to obtain maximal possible pathogenicity.

3. MATERIALS AND METHODS

3.1 Test article

The test microorganism was Trichoderma reesei ALKO 2224 which was genetically modified from the host strain ALKO 233. The date of preparation of the spore suspension was 23.4.1990 and that of its delivery 24.4.1990. The host strain ALKO 233, a reference strain, was suspended and delivered on 3.5.90. Certificate of analysis is

as Appendix 11.

The sponsor provided the test article as a spore suspension in sterile 0.9% NaCl-solution. The consentration of the suspension was 5 x 10^7 spores/ml. The suspension was packed in 1,5 ml plastic tubes. The host strain was suspended and packed in the same way.

Test article suspension was stored at +4 °C temperature in the dark before administration.

3.2 Animals

88 SPF (specific pathogen free) male Bom:NMRI-mice (Bomholtgård Breeding and Research Centre Ltd., Denmark) were used.

The animals were at the age of 7 - 9 weeks at the time of the administration of the test article. At that time the average body weight of the males was 37 g.

Health status of the animals was evaluated after their arrival to the Laboratory Animal Center. 8 randomly selected animals were sacrificed for the health monitoring program. The animals appeared outwardly normal and both the microbial analysis (Appendix 1) and the histopathological analysis (Appendix 2) suggest them to have been free from major rodent diseases in the beginning of the study.

The animals were reobserved at the end of the quarantine period (14 days long) in the context of randomization to experimental groups. The acclimatization period before start of the study was 8 days after randomization.

3.3 Animal facilities and care

The study was carried out in the biohazard unit of the animal facilities of the National Laboratory Animal Center, University of Kuopio, Kuopio (room no 2267). The unit was maintained as biohazard after administration of the microorganism. Entry was limited only to responsible people.

The racks located in cubicles, separated from the room space with gliding doors with windows. Inoculated and control animals were housed in separate cubicles (controls in cubicle 1, others in cubicle 2).

000252

The animal rooms were cleaned regularly. The floor and the walls were wet-wiped with a solution containing Hytox 21-detergent (Leverindus, Turku, Finland) weekly. The floor was cleaned daily.

The animal room and cubicles were airconditioned to maintain the following atmosphere during the study: the mean (± SD) temperature, minimum 21.3 ± 0.4 °C (cubicle 1), 20.8 ± 0.4 °C (cubicle 2) and maximum 21.7 ± 0.4 °C (cubicle 1), 21.4 ± 0.4 (cubicle 2) and the mean relative humidity 47.6 ± 5.1 % (cubicle 1) and 44.3 ± 4.6 % (cubicle 2).

The animal room was artificially lighted the light cycle being 14 hours light/10 hours darkness (9 p.m. - 7 a.m.).

The animals were housed in groups of 5 in stainless steel cages (42.0 cm x 24.5 cm x 15.0 cm) containing autoclaved aspen chip bedding (Finn Tapvei Ky, Kaavi, Finland). The cages were changed to clean ones once a week. The racks were washed weekly with Hytox 21-detergent.

The cages were washed after autoclaving in a tunnel-type washing machine (Electrolux D-165-S, Oy Electrolux-Teollisuus Ab, Espoo, Finland) using Sumazon Special-detergent (Leverindus, Turku, Finland).

The animals had free access to commercial fodder (R3 - EWOS-ALAB Brood Stock Feed for Rats and Mice, Ewos Ab, Sweden, Appendix 3). Typical results from random analysis of the selected contaminants in the type of fodder used are presented in Appendix 4.

The drinking water was tap water. A typical certificate of analysis of the water by the Waterworks of the City of Kuopio is presented as an Appendix 5 and other analyses of impurities in the water in Appendixes 6, 7 and 8. Both the fodder and the drinking water fulfilled the GLP-criteria set by the Laboratory Animal Center.

The drinking water was provided in plastic bottles with metal cabs. The bottles and drinking water were changed twice a week. The bottles were autoclaved out of biohazard and prewashed in a tunnel-type washer and finally washed in a bottle-washing machine (HAKA-100/DEKO 421, Hackman Oy, Naarajärvi, Finland) using Sumatox DEKO-detergent (Leverindus, Turku, Finland).

3.4 Experimental procedures

3.4.1 Identification of the animals and allocation to groups

In the beginning of the acclimatization period, the animals were allocated to groups (15 animals/group) by means of the sequenced randomization procedure, 5 animals/cage.

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After allocation the animals were individually identified by ear punching. Grouping of animals, their identification numbers and treatments were as follows:

Study design

Group	Treatment	Dose (spores/ mouse	Number of animals	Identification numbers
1	Control	0	15	1 - 15
2	Control, pretreated with cortisone	0	15	16 - 30
3	Test microorganism (ALKO 2224)	5×10^6	15	31 - 32, 85, 34 - 45
4	Test microorganism, (ALKO 2224) pretreated with cortisone	5×10^6	15	46 - 55, 82, 57, 86, 59 - 60
5	Host microorganism (ALKO 233), pretreated with cortisone	5×10^6	15	61 - 75
6	Control, pretreated with cortisone	0	13	87 - 99

The animals 33, 56 and 58 were replaced with the animals 85, 86 and 82 after the initial randomization due to change in health condition.

Group 6 (13 animals) was formed after the initial randomization of the animals to obtain a control group for Group 5 animals which were inoculated a week later than animals in other groups.

Each cage of animals was labelled with a cage card colour coded by the dose group showing: number of animals, animal identification numbers, sex, study number, group number, treatment and start date.

3.4.2 Administration of the test article

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The test microorganism suspension was administered as a single intravenous dose (0.1 ml/mouse) into the tail vein to pretreated or non-pretreated animals. As a pretreatment cortisone acetate (No. C-3130, Lot 77F 0475, Sigma Chemical Company, St Louis, Mo, USA) was injected subcutaneously 5 mg/mouse as a suspension of 25 mg/ml in 10 % acacia (ACAC. GUMM 90 µm Ph. Eur. 2nd Ed II 164786,

Pharmacy of the University, Helsinki, Finland) two days before the inoculation of the living spores. The host microorganism was administered by the same way as the test microorganism.

The day the test article was administered is denoted Day 0.

3.4.3 Body weights

The animals were weighed at the start of the study and on the last observation day, Day 40.

3.4.4 Clinical signs

The animals were observed for clinical signs once a week in detail and daily for mortality for the next 40 days after fungus inoculation.

4. COLLECTION OF DATA AND ITS STATISTICAL ANALYSIS

The data on body weights were analyzed statistically with Student's t-test (SPSS/PC+™ V 3.0, SPSS Inc., Chicago, Illinois). The significance of the difference from control values was tested and $P < 0.05$ was considered to be statistically significant.

5. RESULTS

5.1 Body weights (Table 1, Appendix 9)

Test microorganism did not affect weight gain of the animals, even after a cortisone treatment. The reference microorganism ALKO 233 did neither affect body weight of the animals.

The mean body weight of Group 5 animals was significantly higher at the start and end of the study compared to Group 6 but the weight gain did not differ between these groups.

5.2 Clinical signs (Table 2, Appendix 10)

No test or reference organism-related clinical signs was observed in animals in any group.

Due to fighting of animals scabs and wounds were in several animals in all groups. After subcutaneous cortisone treatment small nodules in the neck were recorded in several animals.

6. DISCUSSION

The results indicate that neither the test microorganism, Trichoderma reesei-strain ALKO 2224, nor the host microorganism, strain ALKO 233, were lethal in mice after a single dose of 5 x 10^6 spores. They did not even affect the body weight gain or general health status of cortisone immunosuppressed mice.

The wounds, scabs, oedemas and hairloss recorded in all groups resulted from fighting between cage mates and were by no way related to the test or the reference substance.

7. CONCLUSION

The genetically modified Trichoderma reesei-strain ALKO 2224 is not lethal to mice nor cause clinical signs in 40 days after a single i.v. dose of 5 x 10^6 living spores. Immunosuppression by cortisone acetate does not modify this nonlethal response. The reference host strain ALKO 233 is equally nonlethal at the same dose.

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

Table 1.

SUMMARY OF BODY WEIGHTS (grams)

STUDY ID: 4-90 SEX: MALE PAGE: 1

Group and treatment	Body weights (MEAN, SD, N)		Weight gain (MEAN, SD, N)
	Initial	Day 40	
1. Control	37.1 3.2 15	42.0 4.8 15	4.9 2.7 15
2. Cortisone	35.9 3.3 15	41.3 4.3 15	5.4 3.0 15
3. ALKO 2224	37.9 2.0 15	44.4 2.3 15	6.6 1.7 15
4. Cortisone, ALKO 2224	35.2 2.7 15	40.5 2.7 15	5.2 1.4 15
5. Cortisone, ALKO 233	39.7* 3.2 15	45.9** 3.7 15	6.3 1.7 15
6. Cortisone	36.8 2.3 13	41.5 3.4 13	4.7 2.7 13

* P less than .05 Student's t-test compared to Group 6
** P less than .01 Student's t-test compared to Group 6

Group 5 and 6 individual body weights are integers.

(END OF REPORT)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

Table 2.

SUMMARY OF OBSERVATION INCIDENCE

STUDY ID: 4-90 SEX: MALE PAGE: 1

PERIOD	GROUP: 1	2	3	4	5	6
WEEK 1						
No. observed	15	15	15	15	15	13
Normal	13	6	13	7	15	9
Wound/Scab	2	1	2	4		4
Hairloss						
Nodule		9		7		
WEEK 2						
No. observed	15	15	15	15	15	13
Normal	13	6	13	6	11	8
Wound/Scab	2	3	2	4	1	4
Oedema					1	
Rough fur					1	
Emaciated						1
Nodule		7		6	2	1
WEEK 3						
No. observed	15	15	15	15	15	13
Normal	11	5	12	6	11	8
Wound/Scab	3	1	3	6	1	4
Hairloss	1					
Oedema					1	
Emaciated						1
Nodule		9		5	3	1
WEEK 4						
No. observed	15	15	15	15	15	13
Normal	7	3	10	6	9	7
Wound/Scab	8	2	5	7	6	5
Hairloss		1			3	1
Oedema	1				2	1
Rough fur						1
Emaciated						1
Piloerection					1	2
Nodule		11		3		1

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

SUMMARY OF OBSERVATION INCIDENCE

STUDY ID: 4-90 SEX: MALE PAGE: 2

PERIOD GROUP:	1	2	3	4	5	6
WEEK 5						
No. observed	15	15	15	15	15	13
Normal	7	3	10	4	10	8
Wound/Scab	8	2	5	8	3	4
Hairloss	3	3	2	6	3	
Oedema	2					
Rough fur					1	
Piloerection				1		
Nodule		10		4		1
WEEK 6						
No. observed	15	15	15	15	15	13
Normal	6	7	10	3	8	7
Wound/Scab	5	1	4	8	3	4
Hairloss	7	3	2	5	4	
Oedema	4	1		2	3	
Rough fur	1				2	
Emaciated	1					
Suppurative discharge					1	
Reddish, suppurative area						1
Nodule		6		3		1

(END OF REPORT)

National Laboratory Animal Center
University of Kuopio

Health monitoring report

Project: 4-90

Sampling day: 9 April 1990

The following NMRI-mice were taken to health monitoring:

Males	9x	31 g	10x	29 g
	11x	26 g	12x	25 g
	13x	28 g	14x	29 g
	15x	27 g	16x	24 g

Serum samples were taken from all the animals. Serum samples were sent to Abtek biologicals in UK. The mice were all negative for Polyoma, Reo-3, LCM, Mouse adeno, Sendai, MHV, GD VII, PVM, Ectromelia and Mycoplasma pulmonis, but 4 sera out of 8 were positive for MVM.

Throat, lungs and caecum of all the animals were cultured for following pathogens:

- Bordetella bronchiseptica
- Corynebacterium murium
- Listeria monocytogenes
- Pasteurella multocida
- Pasteurella pneumotropica
- Salmonella sp.
- Streptobacillus moniliformis
- Streptococcus pneumoniae
- Streptococcus β-haemolytica
- Yersinia pseudotuberculosis

All the cultures were negative. These cultures were done by National Public Health Institute in Kuopio.

The presence of ectoparasites was screened with stereomicroscope and of endoparasites with wet preparation and flotation. All the samples were negative.

Kuopio 7 May 1990

000260

Timo Nevalainen DVM, MS, PhD

Appendix 2.

HEALTH CONTROL GROUP (MOUSE)
(4-90)

HISTOLOGICAL ANALYSIS OF TISSUE SAMPLES

Liver
Lung

Health control animals

Mice 9-16, male

Histopathology: liver

Mouse 9

Large nuclei in hepatocytes can be seen. Hepatocytes with mitoses are seen as well. These changes correspond to regeneration. They are normally seen in young animals.

Mouse 10

Many hepatocytes with mitoses and large nuclei are seen.

Mouse 11

Many hepatocytes with mitoses and large nuclei are seen.

Mouse 12

Many hepatocytes with mitoses and large nuclei are seen.

Mouse 13

Many hepatocytes with mitoses and large nuclei are seen.

Mouse 14

Many hepatocytes with mitoses and large nuclei are seen.

Mouse 15

Many hepatocytes with mitoses and large nuclei are seen.

Mouse 16

Many hepatocytes with mitoses and large nuclei are seen.

Conclusion (liver)

Increased number of mitotic figures and large nuclei in hepatocytes were seen in all animals. This is a normal finding and due to the young age of these animals.

Histopathology: lung

Mouse 9

No abnormality.

Mouse 10

No abnormality.

Mouse 11

No abnormality.

Mouse 12

No abnormality.

Mouse 13

No abnormality.

Mouse 14

No abnormality.

Mouse 15

No abnormality.

Mouse 16

No abnormality.

Conclusion: (lung)

No abnormalities were seen.

Kuopio 11.6.1990

Veli-Matti Kosma, docent
Department of Pathology
University of Kuopio

Appendix 3.

R3 - EWOS-ALAB Brood Stock Feed for Rats and Mice

- A thoroughly tested complete feed
- Works also autoclaved on SPF-animals - contains e.g. vitamin K_1
- Composed according to actual international specifications (NIH)

Raw materials: Cereals, wheat germs, wheat middlings, soya meal (toasted), fish protein concentrate, fodder yeast, minerals, animal and vegetable fat, vitamin concentrate, trace element concentrate

Typical analysis

Crude protein	22,0 %
Pepsin dig.protein	20,5 %
Crude fat	5,0 %
Crude ash	6,5 %
Moisture	11,0 %
Fibre	4,0 %
Carbohydrates	51,5 %
Calcium	1,1 %
Phosphorus	0,8 %
Magnesium	0,2 %
Sodium	0,3 %
Potassium	0,8 %
Sodium chloride	0,7 %

Trace elements

Iron	195 ppm
Coppar	30 ppm
Cobalt	1 ppm
Manganese	115 ppm
Zinc	120 ppm
Iodine	2 ppm

Amino acids	g/16 g N
Lysine	6,0
Histidine	3,5
Methionine	2,0
Arginine	6,5
Aspartic acid	8,5
Threonine	3,5
Serine	4,5
Glutamic acid	18,5
Proline	6,0
Glycine	5,0
Alanine	5,5
Valine	5,0
Isoleucine	4,0
Tyrosine	4,0
Phenylalanine	4,5
Cystine	1,5
Leucine	7,0
Ammonia (NH_3)	2,0

Fatty acids % of TEA

Palmitic acid	C16:0	21
Palmitoleic acid	C16:1	2
Stearic acid	C18:0	4
Oleic acid	C18:1	22
Linoleic acid	C18:2	42
Linolenic acid	C18:3	7
Others		2

Special analysis	mg/kg
Aflatoxin	not detectable
Ochratoxin	not detectable
Dieldrine	≤ 0,01
DDT	≤ 0,1
PCB	≤ 0,2
Lead	≤ 1,25
Cadmium	≤ 0,25
Mercury	≤ 0,2
Nitrite	≤ 10

Vitamins added

Vitamin A	12 000 IU/kg
Vitamin D_3	1 500 IU/kg
Vitamin E	20 mg/kg
Vitamin K_1	0,25 mg/kg
Vitamin K_3	10 mg/kg
Vitamin B_1	3 mg/kg
Vitamin B_2	12 mg/kg
Vitamin B_6	5 mg/kg
Vitamin B_{12}	0,02 mg/kg
Ca pantothenate	10 mg/kg
Niacin	40 mg/kg
Choline chloride	1 000 mg/kg

Feed consumption

Rats 10 - 15 g/animal day
Mice 3 - 5 g/animal day

Free of salmonella
Contains no antibiotics

Metabolizable energy: 13,0 MJ/kg
Pellet size: 13 x 25 mm
Packing: 25 kg paper bag

Appendix 4a. Analysis of the selected contaminants in the type of fodder used. (Translated from the enclosed analysis report written in Swedish, Appendix 4b)

1/2

EWOS
Hannele Suominen
Finnewos Aqua Ltd
P.O.Box 113
SF-00241 HELSINKI
FINLAND

Date 1990-06-12

Quarter-analysis of laboratory animal fodder for GLP-analysis Quarter 1, 1990 includes following batches:

	Quarter 1
K1	900207
	900321
R3	900201
	900222
	900313
	900405
	900409
	900430
R36	900123
	900129
	900223
	900316
	900404

SLL

Statens lantbrukskemiska laboratorium

Date 1990-06-06

Analysis protocol F 4764-4770/89-90

mg/kg sample

Sample	Analysis	Pb	Cd	Hg	Nitrite	PCB	DDT	Dieldrin
K1 Quarter 1-90	F 4764	<0.3	0.05	0.010	2.8	<0.1	<0.05	<0.01
K2 Quarter 1-90	F 4765	<0.3	0.05	0.008	2.0	<0.1	<0.05	<0.01
K3 Quarter 1-90	F 4766	0.3	0.06	0.008	7.5	<0.1	<0.05	<0.01
R3 Quarter 1-90	F 4767	0.4	0.05	0.008	1.2	<0.1	<0.05	<0.01
R34 Quarter 1-90	F 4768	0.4	0.04	0.003	1.3	<0.1	<0.05	<0.01
R36 Quarter 1-90	F 4769	<0.3	0.04	0.004	1.0	<0.1	<0.05	<0.01
H1 March -90 ID 752	F 4770	0.4	0.06	0.003	1.9	<0.1	<0.05	<0.01

In these samples no aflatoxin or ochratoxin could be indicated.
Aflatoxin was determined following AOAC Methods 1984, 26.026 - 26.031.
Ochratoxin was determined following AOAC Methods 1984, 26.110 - 26.117.

Caisa Nyberg

FWOS

A CULTOR COMPANY

1990-06-12 UN/ST

Hannele Suominen
Finnewos Aqua Oy
Box 113
SF-00241 HELSINGFORS
FINLAND

Tertialproven på labdjursfoder för GLP-analyser tertial 1, 1990 innehåller följande batcher:

	Tertial 1
K1	900207
	900321
R3	900201
	900222
	900313
	900405
	900409
	900430
R36	900123
	900129
	900223
	900316
	900404

SLL
STATENS LANTBRUKSKEMISKA
LABORATORIUM

1990-06-06

ANALYSPROTOKOLL F 4764-4770/89-90

mg/kg prov

Provmärkning	SLL:s nr	Pb	Cd	Hg	Nitril	ΣPCB	ΣDDT	Dieldrin
K1 tertial 1-90	F 4764	<0,3	0,05	0,010	2,8	<0,1	<0,05	<0,01
K2 tertial 1-90	F 4765	<0,3	0,05	0,008	2,0	<0,1	<0,05	<0,01
K3 tertial 1-90	F 4766	0,3	0,06	0,008	7,5	<0,1	<0,05	<0,01
R3 tertial 1-90	F 4767	0,4	0,05	0,008	1,2	<0,1	<0,05	<0,01
R34 tertial 1-90	F 4768	0,4	0,04	0,003	1,3	<0,1	<0,05	<0,01
R36 tertial 1-90	F 4769	<0,3	0,04	0,004	1,0	<0,1	<0,05	<0,01
III mars-90 ID 752	F 4770	0,4	0,06	0,003	1,9	<0,1	<0,05	<0,01

I de insända proven har aflatoxin och ochratoxin ej påvisats.

Aflatoxin har bestämts enl. AOAC Methods 1984, 26.026 - 26.031.

Ochratoxin har bestämts enl. AOAC Methods 1984, 26.110 - 26.117.

Caisa Nyberg

STATENS LANTBRUKSKEMISKA LABORATORIUM
NATIONAL LABORATORY FOR AGRICULTURAL CHEMISTRY SWEDEN

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000269

Appendix 5a. (Translated from the enclosed analysis report written in Finnish, Appendix 5b) 1/2

THE WATERWORKS OF THE CITY OF KUOPIO

in Reposaari

Date: 1990-08-30

SUMMARY REPORT during 90-04-01 ... 90-04-30

Analysis		Untreated water		Treated water			
		1	2	Mean conc.	Lower limit	Upper limit	N.of cases
Temperature	°C						
Turbidity	NTU		1.80	0.34	0.10	0.44	19
Colour	mgPt/l			2.00	1.00	5.00	18
pH	pH-unit		6.10	8.49	8.20	8.80	19
Alkalinity	mmol/l						0
Electrical conductivity	mS/m		23.30	23.94	23.10	24.60	13
$KMnO_4$	mgO_2/l		11.00	3.73	2.40	4.70	16
Fluoride	mgF/l			0.99	0.88	1.04	19
Aluminum	mgAl/l						0
Total chlorine	$mgCl_2/l$			0.20	0.13	0.26	19
Free chlorine	$mgCl_2/l$			0.14	0.07	0.20	19
Iron	mgFe/l		6.00	0.03	0.02	0.04	5
Manganese	mgMn/l		0.36	0.01	0.01	0.01	5
Ammonium	$mgNH_4/l$						0
Nitrite	$mgNO_2/l$						0
Nitrate	$mgNO_3/l$						0
Total hardness	mmol/l			0.55	0.53	0.57	3
Calcium hardness	mmol/l						0
Bicarbonate hardness	mmol/l						0
Chloride	mgCl/l						0
Free CO_2	$mgCO_2/l$						0
Oxygen	mgO_2/l						0
Coliform bacteria	N/100ml			0.00	0.00	0.00	4
Fecal coliform bacteria	N/100ml			0.00	0.00	0.00	4
Fecal streptococci	N/100ml			0.00	0.00	0.00	4
Total colonies	N/ml						0
Total number of samples		0.00	1.00	19.00			

Untreated water 2 : well 2

THE WATERWORKS OF THE CITY OF KUOPIO

in Itkonniemi

Date: 1990-08-30

SUMMARY REPORT during 90-04-01 ... 90-04-30

Analysis		Untreated water 1	Untreated water 2	Treated water Mean conc.	Treated water Lower limit	Treated water Upper limit	Treated water N.of cases
Temperature	°C	2.98		3.49	3.30	3.80	30
Turbidity	NTU	6.62		0.14	0.08	0.41	19
Colour	mgPt/l	42.00		1.61	1.00	4.00	18
pH	pH-unit	6.03		8.20	8.00	8.40	20
Alkalinity	mmol/l	0.13		0.63	0.57	0.80	8
Electrical conductivity	mS/m	6.74		13.62	13.40	13.90	13
$KMnO_4$	mgO_2/l	14.81		6.81	5.10	7.90	16
Fluoride	mgF/l	0.08		1.03	0.86	1.15	18
Aluminum	mgAl/l			0.03	0.02	0.03	8
Total chlorine	$mgCl_2/l$			0.34	0.27	0.42	19
Free chlorine	$mgCl_2/l$			0.00	0.00	0.01	19
Iron	mgFe/l	1.08		0.01	0.01	0.01	5
Manganese	mgMn/l	0.11		0.03	0.02	0.04	5
Ammonium	$mgNH_4/l$						0
Nitrite	$mgNO_2/l$						0
Nitrate	$mgNO_3/l$						0
Total hardness	mmol/l	0.14		0.47	0.46	0.50	3
Calcium hardness	mmol/l	0.09		0.43	0.43	0.43	3
Bicarbonate hardness	mmol/l						0
Chloride	mgCl/l						0
Free CO_2	$mgCO_2/l$						0
Oxygen	mgO_2/l						0
Coliform bacteria	N/100ml	0.00		0.00	0.00	0.00	4
Fecal coliform bacteria	N/100ml	0.00		0.00	0.00	0.00	4
Fecal streptococci	N/100ml	8.60		0.00	0.00	0.00	4
Total colonies	N/ml						0
Total number of samples		29.00	0.00	31.00			

Untreated water 1 is from Hietasalo

Appendix 5b, (The original analysis report) 1/2

Kuopion kaupungin vesilaitos
Itkonniemenkatu 81
70500 KUOPIO
90-08-30 10:12:21

puh. 971-185364
tai 971-185361

KUOPION KAUPUNKI VSL Pvm : 1990-04-01 sunnuntai

VESILAITOS - KOONTARAPORTTI - Reposaari

kuukausikeskiarvot 90-04-01 ... 90-04-30

		Raaka-vesi 1	Raaka-vesi 2	Puhdas vesi keskia	min	max	an-lkm
lämpöt	aste-C						0
sameus	FTU		1.80	0.34	0.10	0.44	19
väri	mgPt/l			2.00	1.00	5.00	18
pH	pH-yks		6.10	8.49	8.20	8.80	19
alkalit	mmol/l						0
johtok	mS/m		23.30	23.94	23.10	24.60	13
KMnO4	mg/l		11.00	3.73	2.40	4.70	16
F	mgF/l			0.99	0.88	1.04	19
Al	mgAl/l						0
Cl2-kok	mgCl2/l			0.20	0.13	0.26	19
Cl2-vap	mgCl2/l			0.14	0.07	0.20	19
Fe	mgFe/l		6.00	0.03	0.02	0.04	5
Mn	mgMn/l		0.36	0.01	0.01	0.01	5
NH4	mgNH4/l						0
NO2	mgNO2/l						0
NO3	mgNO3/l						0
kok-kov	mmol/l			0.55	0.53	0.57	3
Ca-kov	mmol/l						0
bik-kov	mmol/l						0
Cl	mgCl/l						0
CO2	mgCO2/l						0
O2	mgO2/l						0
kolif.b	N/100ml			0.00	0.00	0.00	4
fek.kol	N/100ml			0.00	0.00	0.00	4
fek.str	N/100ml			0.00	0.00	0.00	4
kok.pes	kpl/ml						0
näytteitä yht		0.00	1.00	19.00			

000272

Raakavesi 2 : kaivo 2

Kuopion kaupungin vesilaitos
Itkonniemenkatu 81
70500 KUOPIO
puh. 971-185364
tai 971-185361

90-08-30 10:07:40

KUOPION KAUPUNKI VSL Pvm : 1990-04-01 sunnuntai

VESILAITOS - KOONTARAPORTTI - Itkonniemi

kuukausikeskiarvot 90-04-01 ... 90-04-30

		Raaka-vesi 1	Raaka-vesi 2	Puhdas vesi keskia	min	max	an-lkm
lämpöt	aste-C	2.98		3.49	3.30	3.80	30
sameus	FTU	6.62		0.14	0.08	0.41	19
väri	mgPt/l	42.00		1.61	1.00	4.00	18
[illegible]	pH-yks	6.03		8.20	8.00	8.40	20
[illegible]kalit	mmol/l	0.13		0.63	0.57	0.80	8
johtok	mS/m	6.74		13.62	13.40	13.90	13
KMnO4	mg/l	14.81		6.81	5.10	7.90	16
F	mgF/l	0.08		1.03	0.86	1.15	18
Al	mgAl/l			0.03	0.02	0.03	8
Cl2-kok	mgCl2/l			0.34	0.27	0.42	19
Cl2-vap	mgCl2/l			0.00	0.00	0.01	19
Fe	mgFe/l	1.08		0.01	0.01	0.01	5
Mn	mgMn/l	0.11		0.03	0.02	0.04	5
NH4	mgNH4/l						0
NO2	mgNO2/l						0
NO3	mgNO3/l						0
kok-kov	mmol/l	0.14		0.47	0.46	0.50	3
Ca-kov	mmol/l	0.09		0.43	0.43	0.43	3
bik-kov	mmol/l						0
Cl	mgCl/l						0
CO2	mgCO2/l						0
O2	mgO2/l						0
kolif.b	N/100ml	0.00		0.00	0.00	0.00	4
fek.kol	N/100ml	0.00		0.00	0.00	0.00	4
fek.str	N/100ml	0.00		0.00	0.00	0.00	4
kok.pes	kpl/ml						0
[illegible]ytteitä yht		29.00	0.00	31.00			

000273

Raakavesi 1 Hietasalosta

VTT
Valtion teknillinen tutkimuskeskus
Food Research Laboratory

Tutkimusselostus nro ELI01018/90

1

Requested by	University of Kuopio National Laboratory of Animal Centre Timo Nevalainen PL 6 70211 KUOPIO
Order	4.6.1990/Eila Kaliste-Korhonen
Sample	Drinking water in open animal room, June 4, 1990 (1-90)
Object	To determine the lead, cadmium, mercury and arsenic content of the sample
Results	The metal contents of the sample were all less than the limits of determinations:

Lead	< 0,002	mg/l
Cadmium	< 0,0005	"
Mercury	< 0,0005	"
Arsenic	< 0,002	"

Lead, cadmium and arsenic concentrations were measured by atomic absorption spectrometry using the graphite furnace technique (PE 5000/Zeeman, HGA-400, AS-40). Mercury was determined by atomic absorption spectrometry using the cold vapour technique (Coleman MAS-50).

Espoo 25.7.1990

TECHNICAL RESEARCH CENTRE OF FINLAND
FOOD RESEARCH LABORATORY

Senior Research Officer Kimmo Himberg

Research Officer Helena Liukkonen-Lilja

KH/HL-L/hl-l
59/989b

The laboratory is by virtue of the State Council decree No. 347/59 the official leading food research institute.

000274

tional Public Health Institute
Department of Environmental Hygiene and Toxicology
P.O.Box 95
SF-70701 Kuopio, Finland

1/2

Study report

This report contains results concerning analysis of chlorophenols in drinking water of the National Laboratory Animal Center, University of Kuopio. The analyses were made at the Department of Chemistry, University of Kuopio and the Department of Environmental Hygiene and Toxicology, National Public Health Institute, Kuopio.

Methods

Chlorophenols were extracted from acidic water (conc. H_2SO_4) into diethyl ether by shaking the sample after which they were acylated (1). 3,5-Dichlorophenol was added for an internal standard into the water at the beginning of the analysis. 2,3,4,6-Tetrachlorophenol standard was from University of rväskylä (J. Knuutinen), the other chlorophenol standards re from Fluka AG.

The analysis was performed with GC (Hewlett Packard 5890) equipped with DB-5 quartz capillar column and EC-detector. The detection limit of the analysis was 0.005 µg/l.

Results

Results has been presented in Table 1.

(1) J. Knuutinen. Analysis of Chlorinated Quaiacols in Spent Bleach Liquor from a Pulp Mill. Journal of Chromatogr. 248, 1982:289-295

2/2

Table 1. Concentrations of chlorophenols (µg/l) in drinking water of conventional animal rooms (the sample July 2, 1990).

2,4,6-tri	2,4,5-tri	3,4,5-tri	2,3,5,6-tetra	2,3,4,6-tetra	2,3,4,5-tetra	penta
<0.005	<0.005	<0.005	<0.005	0.019	<0.005	0.005

Kuopio September 20, 1990

rttu Vartiainen
Special researcher, Ph.D.

National Public Health Institute
Department of Environmental Hygiene and Toxicology
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National Public Health Institute
Department of Environmental Hygiene and Toxicology
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1/2

Study report

This report contains results concerning analysis of PCB-compounds and pesticides in drinking water of the National Laboratory Animal Center, University of Kuopio. The analyses were made at the Department of Environmental Hygiene and Toxicology, National Public Health Institute, Kuopio.

Methods

1. Pesticides

Analysis of the pesticides were made by the method based on hexan extraction. Pentachlorobenzen (1.00 µg) was added for an internal standard into the water. The water samples were extracted with 5 ml hexan and 2 ml aliquots of hexan were taken for analysis.

The analysis was performed with GC (Hewlett Packard 5890A) equipped with DB-5 capillar column and EC-detector.

2. PCB-compounds

PCB-compounds were extracted from water by shaking the sample three times with hexan (3x 50 ml). The combined extracts were concentrated into 2 ml by evaporation and cleaned up three times with conc. H_2SO_4 and washed with H_2O. A sample from the hexan phase was injected into GC (Perkin Elmer) equipped with DB-5 quartz capillar column and EC detector.

The PCB standards were ten different di-, tri-, tetra-, penta-, hexa-, and heptachlorobiphenyl isomers (Analabs, RCS-120 and RCS 122, USA).

The detection limit of the analysis was 0.05 µg/l.

Results

Results has been presented in Table 1.

Table 1. Concentrations of pesticides and PCB-compounds in drinking water of conventional animal rooms (the sample July 2, 1990).

Compound	Concentration µg/l
Pesticides	
Hexachlorobenzen	< 0.05
Heptachlor	< 0.05
Aldrin	< 0.05
Heptachlorepoxide	< 0.05
o,p'-DDE	< 0.05
alfa-Chlordane	< 0.05
p,p'-DDE	< 0.05
Dieldrin	< 0.05
o,p'-DDD	< 0.05
Endrin	< 0.05
p,p'-DDD	< 0.05
p,p'-DDT	< 0.05
PCB-compounds	
2,4'-dichlorobiphenyl	< 0.05
2,4,6-trichlorobiphenyl	< 0.05
2,2',5-trichlorobiphenyl	< 0.05
2,4',5-trichlorobiphenyl	< 0.05
2,4,4'-trichlorobiphenyl	< 0.05
2,2',5,5'-tetrachlorobiphenyl	< 0.05
2,2'4,5,5'-pentachlorobiphenyl	< 0.05
2,2',4,4',5,5'-hexachlorobiphenyl	< 0.05
2,2',3,4,4',5'-hexachlorobiphenyl	< 0.05
2,2',3,4,4',5,6-heptachlorobiphenyl	< 0.05

Kuopio July 16, 1990

Terttu Vartiainen
Special researcher, Ph.D.

National Public Health Institute
Department of Environmental Hygiene and Toxicology
P.O.Box 95
SF-70701 Kuopio, Finland

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

Appendix 9.

INDIVIDUAL BODY WEIGHTS (grams)

STUDY ID: 4-90 GROUP: 1 SEX: MALE PAGE: 1
TREATMENT: 0.9% NaCl iv (control)

Animal no	Initial weight	Day 40	Weight gain
1	42.8	44.4	1.6
2	35.8	40.6	4.8
3	37.4	44.9	7.5
4	37.4	45.5	8.1
5	36.0	40.1	4.1
6	38.2	44.1	5.9
7	36.9	42.1	5.2
8	42.8	49.1	6.3
9	36.9	39.9	3.0
10	33.1	32.6	-0.5
11	34.5	39.8	5.3
12	32.1	32.9	0.8
13	33.0	40.2	7.2
14	39.7	47.9	8.2
15	39.4	46.0	6.6
MEAN	37.1	42.0	4.9
SD	3.2	4.8	2.6
N	15	15	15

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL BODY WEIGHTS (grams)

STUDY ID: 4-90 GROUP: 2 SEX: MALE PAGE: 2
TREATMENT: Cortisone sc
0.9% NaCl iv

Animal no	Initial weight	Day 40	Weight gain
16	36.8	45.4	8.6
17	35.4	42.4	7.0
18	41.8	44.3	2.5
19	36.0	46.4	10.4
20	32.5	35.9	3.4
21	33.8	40.5	6.7
22	36.0	40.5	4.5
23	36.3	44.2	7.9
24	42.0	43.0	1.0
25	30.9	32.6	1.7
26	38.6	41.8	3.2
27	38.3	47.1	8.8
28	34.6	38.0	3.4
29	30.9	34.7	3.8
30	35.1	43.4	8.3
MEAN	35.9	41.3	5.4
SD	3.3	4.3	2.9
N	15	15	15

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL BODY WEIGHTS (grams)

STUDY ID: 4-90 GROUP: 3 SEX: MALE PAGE: 3
TREATMENT: ALKO 2224 iv

Animal no	Initial weight	Day 40	Weight gain
31	39.2	45.7	6.5
32	37.7	44.7	7.0
85	36.5	41.2	4.7
34	35.5	39.7	4.2
35	36.5	45.1	8.6
36	37.1	42.0	4.9
37	38.5	45.6	7.1
38	41.5	46.8	5.3
39	40.6	46.7	6.1
40	38.4	45.9	7.5
41	39.3	43.4	4.1
42	35.8	44.1	8.3
43	34.6	42.0	7.4
44	36.8	46.9	10.1
45	40.2	46.9	6.7
MEAN	37.9	44.4	6.5
SD	2.0	2.3	1.7
N	15	15	15

(REPORT CONTINUED)

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4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL BODY WEIGHTS (grams)

STUDY ID: 4-90 GROUP: 4 SEX: MALE PAGE: 4
TREATMENT: Cortisone sc
ALKO 2224 iv

Animal no	Initial weight	Day 40	Weight gain
46	37.5	40.7	3.2
47	32.0	35.7	3.7
48	32.9	41.1	8.2
49	34.4	39.7	5.3
50	34.8	41.3	6.5
51	35.7	41.6	5.9
52	35.9	41.3	5.4
53	33.7	39.0	5.3
54	37.8	43.8	6.0
55	33.5	38.5	5.0
82	41.5	43.9	2.4
57	37.2	43.0	5.8
86	37.4	43.0	5.6
59	31.1	35.1	4.0
60	32.8	39.2	6.4
MEAN	35.2	40.5	5.2
SD	2.7	2.7	1.4
N	15	15	15

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL BODY WEIGHTS (grams)

STUDY ID: 4-90 GROUP: 5 SEX: MALE PAGE: 5
TREATMENT: Cortisone sc
ALKO 233 iv

Animal no	Initial weight	Day 40	Weight gain
61	41[a)]	51.1	10.1
62	46	53.9	7.9
63	40	44.6	4.6
64	39	46.1	7.1
65	41	46.9	5.9
66	33	40.7	7.7
67	44	48.5	4.5
68	36	41.0	5.0
69	37	40.7	3.7
70	41	48.5	7.5
71	42	46.4	4.4
72	39	44.8	5.8
73	39	43.6	4.6
74	38	44.0	6.0
75	39	45.5	6.5
MEAN	39.7	45.8	6.1
SD	3.2	3.7	1.7
N	15	15	15

a) Accuracy in weighing was integer

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL BODY WEIGHTS (grams)

STUDY ID: 4-90 GROUP: 6 SEX: MALE PAGE: 6
TREATMENT: Cortisone sc

Animal no	Initial weight	Day 40	Weight gain
87	35[a)]	40.6	5.6
88	38	44.4	6.4
89	37	43.1	6.1
90	35	38.8	3.8
91	36	43.2	7.2
92	41	41.4	0.4
93	37	45.2	8.2
94	40	42.8	2.8
95	40	46.0	6.0
96	37	41.7	4.7
97	35	42.3	7.3
98	34	33.0	-1.0
99	34	37.9	3.9
MEAN	36.8	41.6	4.7
SD	2.3	3.5	2.7
N	13	13	13

a) Accuracy in weighing was integer

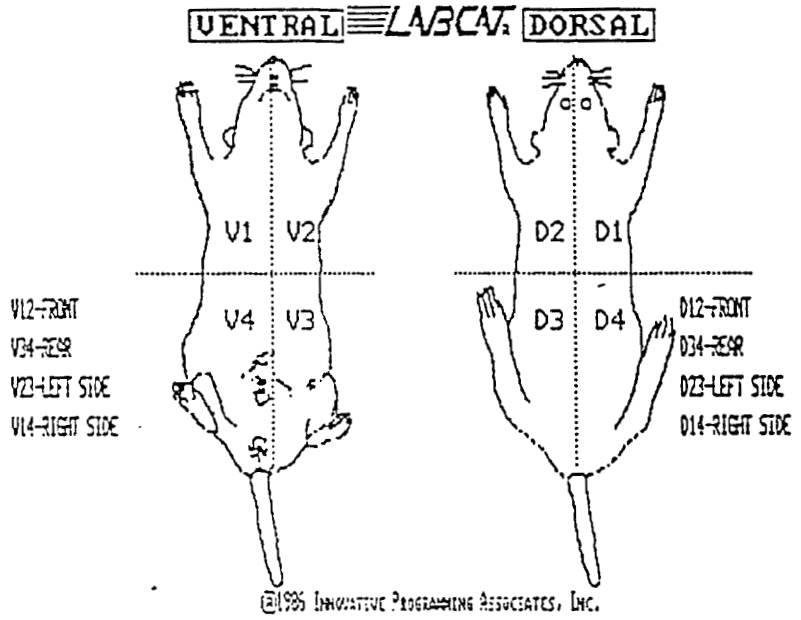
(END OF REPORT)

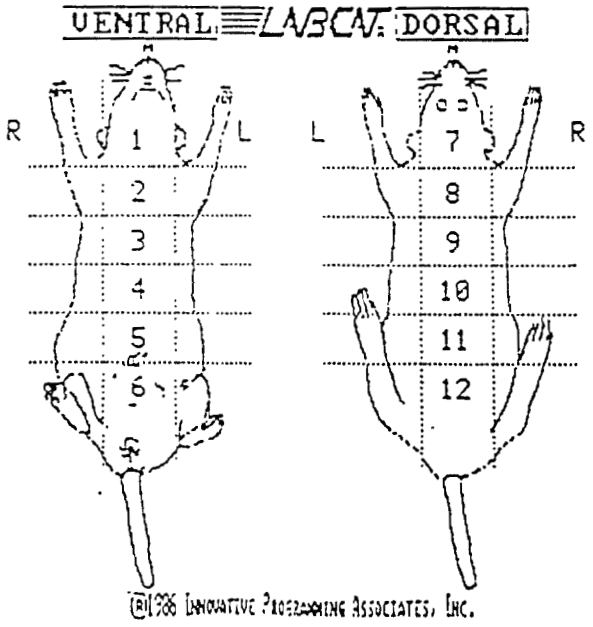
4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

Appendix 10.

INDIVIDUAL OBSERVATIONS

LOCATION MAP





4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 1 SEX: MALE PAGE: 1
WEEK 1 - WEEK 6 TREATMENT: 0.9% NaCl iv (control)

Animal no	Observations	Location	Time occurred
1	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Wound	M6	WEEK 5
	Oedema	M6	WEEK 5
	Wound	M6	WEEK 6
	Oedema	M6	WEEK 6
2	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Scab	M12	WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
3	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
4	Normal		WEEK 1
	Normal		WEEK 2
	Hairloss	M12	WEEK 3
	Wound	M6, M12	WEEK 4
	Oedema	M6	WEEK 4
	Hairloss	M6, M12	WEEK 5
	Scab	M6, M12	WEEK 5
	Hairloss	M12	WEEK 6
	Oedema	M6	WEEK 6
5	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Scab	M12	WEEK 4
	Wound	M6	WEEK 5

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 1 SEX: MALE PAGE: 2
WEEK 1 - WEEK 6 TREATMENT: 0.9% NaCl iv (control)

Animal no	Observations	Location	Time occurred
5	Oedema	M6	WEEK 5
	Hairloss	M6	WEEK 6
	Oedema	M6	WEEK 6
6	Normal		WEEK 1
	Normal		WEEK 2
	Wound	R7	WEEK 3
	Wound	R7, M12	WEEK 4
	Scab	M12	WEEK 4
	Hairloss	R7, M12	WEEK 5
	Wound	M12	WEEK 5
	Scab	R7	WEEK 5
	Hairloss	R7	WEEK 6
	Scab	M12	WEEK 6
	Wound, suppurative	M12	WEEK 6
7	Wound	M12	WEEK 1
	Wound	M12	WEEK 2
	Wound	M12	WEEK 3
	Wound	M12	WEEK 4
	Wound, suppurative	M12	WEEK 5
	Hairloss	M12	WEEK 6
	Scab	M12	WEEK 6
	Wound, suppurative	M12	WEEK 6
	Oedema	M6	WEEK 6
	Wound	M6	WEEK 6
8	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Wound	M10	WEEK 4
	Wound	M10	WEEK 5
	Hairloss	M10	WEEK 6
	Wound, suppurative	M10	WEEK 6
9	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3

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(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 1 SEX: MALE PAGE: 3
WEEK 1 - WEEK 6 TREATMENT: 0.9% NaCl iv (control)

Animal no	Observations	Location	Time occurred
9	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
10	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Scab	M10-M12	WEEK 4
	Wound	M6, M12	WEEK 5
	Rough fur		WEEK 6
	Scab	M12	WEEK 6
	Wound	M6	WEEK 6
	Emaciated		WEEK 6
11	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Hairloss		WEEK 6
12	Wound	M6	WEEK 1
	Wound	M6	WEEK 2
	Wound	M6	WEEK 3
	Scab	M12	WEEK 4
	Wound	M6	WEEK 4
	Wound	M6	WEEK 5
	Scab	M12	WEEK 5
	Hairloss	M12	WEEK 5
	Hairloss		WEEK 6
13	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 1 SEX: MALE PAGE: 4
WEEK 1 - WEEK 6 TREATMENT: 0.9% NaCl iv (control)

Animal no	Observations	Location	Time occurred
14	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
15	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 2 SEX: MALE PAGE: 5
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
0.9% NaCl iv

Animal no	Observations	Location	Time occurred
16	Nodule	M8	WEEK 1
	Nodule	M8	WEEK 2
	Scab	M12	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Hairloss	R7	WEEK 5
	Nodule	M8	WEEK 5
	Nodule	M8	WEEK 6
17	Normal		WEEK 1
	Normal		WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Nodule	M8	WEEK 6
18	Nodule	M8	WEEK 1
	Wound	M12	WEEK 2
	Normal		WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Nodule	M8	WEEK 6
19	Nodule	M8	WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Nodule	M8	WEEK 6
	Hairloss	L7,R7,L12,R12	WEEK 6
20	Nodule	M8	WEEK 1
	Normal		WEEK 2
	Wound	M12	WEEK 3
	Wound	M12	WEEK 4
	Scab	M12	WEEK 5
	Hairloss	M12	WEEK 5

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(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 2 SEX: MALE PAGE: 6
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
0.9% NaCl iv

Animal no	Observations	Location	Time occurred
20	Wound	M12	WEEK 6
21	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
22	Normal		WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Nodule	M8	WEEK 6
23	Nodule	M8	WEEK 1
	Wound, suppurative	R7	WEEK 1
	Wound, suppurative	R7	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Hairloss	R7	WEEK 4
	Scab	R7	WEEK 4
	Hairloss	R7	WEEK 5
	Nodule	M8	WEEK 5
	Hairloss	R7	WEEK 6
	Oedema	R7	WEEK 6
	Nodule	M8	WEEK 6
24	Nodule	M8	WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
25	Nodule	M8	WEEK 1

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(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 2 SEX: MALE PAGE: 7
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
0.9% NaCl iv

Animal no	Observations	Location	Time occurred
25	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Scab	M6	WEEK 5
	Hairloss	M6	WEEK 6
26	Nodule	M8	WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Normal		WEEK 6
27	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Normal		WEEK 6
28	Normal		WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Normal		WEEK 6
29	Nodule	M8	WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Normal		WEEK 6
30	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3

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(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 2 SEX: MALE PAGE: 8
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
0.9% NaCl iv

Animal no	Observations	Location	Time occurred
30	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 3 SEX: MALE PAGE: 9
WEEK 1 - WEEK 6 TREATMENT: ALKO 2224 iv

Animal no	Observations	Location	Time occurred
31	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
32	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
85	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
34	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
35	Wound	M12	WEEK 1
	Wound	M12	WEEK 2
	Wound	M12	WEEK 3
	Wound	M12	WEEK 4
	Scab	M12	WEEK 4
	Hairloss	M12	WEEK 5
	Scab	M12	WEEK 5
	Hairloss	M12	WEEK 6
	Wound	M12	WEEK 6

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 3 SEX: MALE PAGE: 10
WEEK 1 - WEEK 6 TREATMENT: ALKO 2224 iv

Animal no	Observations	Location	Time occurred
36	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
37	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
38	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
39	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
40	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
41	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3

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(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 3 SEX: MALE PAGE: 11
WEEK 1 - WEEK 6 TREATMENT: ALKO 2224 iv

Animal no	Observations	Location	Time occurred
41	Wound	M6	WEEK 4
	Scab	M6,M12	WEEK 4
	Scab	M6,M12	WEEK 5
	Scab	M6,M12	WEEK 6
42	Wound	M6	WEEK 1
	Scab	M6	WEEK 1
	Scab	M6	WEEK 2
	Scab	M6	WEEK 3
	Scab	M6,M12	WEEK 4
	Scab	M6	WEEK 5
	Scab	M6	WEEK 6
43	Normal		WEEK 1
	Normal		WEEK 2
	Scab	M6	WEEK 3
	Scab	M6,M12	WEEK 4
	Scab	M6,M12	WEEK 5
	Hairloss	M12	WEEK 5
	Hairloss	M6	WEEK 6
44	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
45	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Scab	M6,M12	WEEK 4
	Scab	M6,M12	WEEK 5
	Scab	M6	WEEK 6

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 4 SEX: MALE PAGE: 12
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
ALKO 2224 iv

Animal no	Observations	Location	Time occurred
46	Nodule	M8	WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Wound	M12	WEEK 6
47	Nodule	M8	WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Scab	M12	WEEK 4
	Nodule	M8	WEEK 5
	Nodule	M8	WEEK 6
48	Wound	M12	WEEK 1
	Scab	M12	WEEK 1
	Wound	M12	WEEK 2
	Scab	M12	WEEK 2
	Wound	M12	WEEK 3
	Scab	M12	WEEK 3
	Scab	M12	WEEK 4
	Wound	M6	WEEK 5
	Scab	M6,M12	WEEK 5
	Hairloss	M12	WEEK 5
	Scab	M12	WEEK 6
	Hairloss	M12	WEEK 6
	Wound	M12	WEEK 6
49	Nodule	M8	WEEK 1
	Scab	M12	WEEK 1
	Scab	M12	WEEK 2
	Nodule	M8	WEEK 2
	Scab	M12	WEEK 3
	Nodule	M8	WEEK 3
	Scab	M12	WEEK 4
	Hairloss	M12	WEEK 5

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(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 4 SEX: MALE PAGE: 13
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
ALKO 2224 iv

Animal no	Observations	Location	Time occurred
49	Scab	M12	WEEK 5
	Hairloss	M12	WEEK 6
	Wound	M12	WEEK 6
50	Scab	M10	WEEK 1
	Nodule	M8	WEEK 1
	Wound	M12	WEEK 1
	Scab	M12	WEEK 1
	Wound	M12	WEEK 2
	Scab	M12	WEEK 2
	Wound	M12	WEEK 3
	Wound	M12	WEEK 4
	Scab	M10,M12	WEEK 4
	Hairloss	M12	WEEK 5
	Scab	R12, M12	WEEK 5
	Piloerection		WEEK 5
	Hairloss	M12	WEEK 6
	Scab	M12	WEEK 6
51	Normal		WEEK 1
	Normal		WEEK 2
	Wound	M12	WEEK 3
	Wound	M6,M12	WEEK 4
	Hairloss	M12	WEEK 5
	Wound	M12	WEEK 5
	Hairloss	M12	WEEK 6
	Wound, suppurative	M12	WEEK 6
	Oedema	M6	WEEK 6
52	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
53	Normal		WEEK 1

000298

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 4 SEX: MALE PAGE: 14
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
ALKO 2224 iv

Animal no	Observations	Location	Time occurred
53	Wound	M6	WEEK 2
	Wound	M6	WEEK 3
	Wound, suppurative	M6	WEEK 4
	Wound	M12	WEEK 4
	Wound	L12	WEEK 5
	Wound, suppurative	M6	WEEK 5
	Hairloss	R12	WEEK 5
	Scab	M6	WEEK 5
	Wound	M6	WEEK 6
	Scab	L12	WEEK 6
	Oedema	M6	WEEK 6
54	Nodule	M8	WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Nodule	M8	WEEK 6
55	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Wound	M12	WEEK 5
	Normal		WEEK 6
82	Nodule	M8	WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Wound	M12	WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Scab	M12	WEEK 6
57	Normal		WEEK 1
	Nodule	D1	WEEK 2
	Nodule	D1	WEEK 3

000299

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 4 SEX: MALE PAGE: 15
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
ALKO 2224 iv

Animal no	Observations	Location	Time occurred
57	Nodule	D1	WEEK 4
	Nodule	D1	WEEK 5
	Nodule	D1	WEEK 6
86	Nodule	M8	WEEK 1
	Nodule	M8	WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Scab	M6	WEEK 5
	Nodule	M6	WEEK 5
	Normal		WEEK 6
59	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Hairloss	M12	WEEK 5
	Scab	M12	WEEK 5
	Scab	M12	WEEK 6
60	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Scab	M12	WEEK 4
	Normal		WEEK 5
	Hairloss	M12	WEEK 6

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 5 SEX: MALE PAGE: 16
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
ALKO 233 iv

Animal no	Observations	Location	Time occurred
61	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
62	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
63	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
64	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
65	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
66	Normal		WEEK 1
	Rough fur		WEEK 2

000301

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 5 SEX: MALE PAGE: 17
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
ALKO 233 iv

Animal no	Observations	Location	Time occurred
66	Nodule	R7	WEEK 3
	Scab	M12,L12,R12	WEEK 4
	Oedema	L12,R12	WEEK 4
	Hairloss	M12	WEEK 5
	Rough fur		WEEK 5
	Hairloss	M12	WEEK 6
67	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Scab	M12	WEEK 4
	Normal		WEEK 5
	Hairloss	M12	WEEK 6
68	Normal		WEEK 1
	Scab	R7	WEEK 2
	Oedema	R7	WEEK 2
	Scab	R7,L7	WEEK 3
	Oedema	L7	WEEK 3
	Scab	L7	WEEK 4
	Hairloss	L7	WEEK 4
	Oedema	R7, L7	WEEK 4
	Scab	L7	WEEK 5
	Hairloss	R7, L7	WEEK 6
69	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Scab	M12	WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
70	Normal		WEEK 1
	Nodule	M8	WEEK 2
	Nodule	R7	WEEK 3
	Scab	M6,M10	WEEK 4
	Hairloss	R7	WEEK 4

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(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 5 SEX: MALE PAGE: 18
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
ALKO 233 iv

Animal no	Observations	Location	Time occurred
70	Piloerection		WEEK 4
	Scab	M10	WEEK 5
	Wound	M10,R7	WEEK 6
	Oedema	M6	WEEK 6
	Suppurative discharge	M6	WEEK 6
71	Normal		WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
72	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Hairloss		WEEK 5
	Scab	R7	WEEK 5
	Rough fur		WEEK 6
	Hairloss		WEEK 6
	Wound	R7	WEEK 6
	Scab	R7	WEEK 6
	Oedema	R7,L7	WEEK 6
73	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
74	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 5 SEX: MALE PAGE: 19
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
ALKO 233 iv

Animal no	Observations	Location	Time occurred
74	Rough fur		WEEK 6
75	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Hairloss	M6,L7	WEEK 4
	Scab	L7	WEEK 4
	Hairloss		WEEK 5
	Scab	R7,L7	WEEK 6
	Oedema	R7,L7	WEEK 6

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 6 SEX: MALE PAGE: 20
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
0.9% NaCl iv

Animal no	Observations	Location	Time occurred
87	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
88	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
89	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
90	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
91	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
92	Normal		WEEK 1
	Normal		WEEK 2

(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 6 SEX: MALE PAGE: 21
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
0.9% NaCl iv

Animal no	Observations	Location	Time occurred
92	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
93	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Normal		WEEK 4
	Normal		WEEK 5
	Normal		WEEK 6
94	Normal		WEEK 1
	Normal		WEEK 2
	Normal		WEEK 3
	Scab	M6	WEEK 4
	Normal		WEEK 5
	Reddish, suppurative area	M6	WEEK 6
95	Wound	M12	WEEK 1
	Wound	M12	WEEK 2
	Wound, suppurative	M12	WEEK 3
	Wound, suppurative	M12	WEEK 4
	Wound, suppurative	M12	WEEK 5
	Wound, suppurative	M12	WEEK 6
96	Wound	M12	WEEK 1
	Wound	M12	WEEK 2
	Emaciated		WEEK 2
	Wound, suppurative	M12	WEEK 3
	Scab	M12	WEEK 3
	Emaciated		WEEK 3
	Hairloss	D1-D4	WEEK 4
	Wound	M12	WEEK 4
	Emaciated		WEEK 4
	Piloerection		WEEK 4
	Wound	D3-D4	WEEK 5

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(REPORT CONTINUED)

4-90
PATHOGENICITY IN MICE
TRICHODERMA REESEI-STRAIN ALKO 2224
NATIONAL PUBLIC HEALTH INSTITUTE

INDIVIDUAL OBSERVATIONS

STUDY ID: 4-90 GROUP: 6 SEX: MALE PAGE: 22
WEEK 1 - WEEK 6 TREATMENT: Cortisone sc
0.9% NaCl iv

Animal no	Observations	Location	Time occurred
96	Scab	D3-D4	WEEK 5
	Wound	D3-D4	WEEK 6
97	Wound	M12	WEEK 1
	Wound	M12	WEEK 2
	Wound, suppurative	M12	WEEK 3
	Wound, suppurative	M12	WEEK 4
	Wound, suppurative	M12	WEEK 5
	Wound	M12	WEEK 6
	Scab	M12	WEEK 6
	Wound, suppurative	M6	WEEK 6
98	Wound	M12	WEEK 1
	Scab	M12	WEEK 1
	Wound	M12	WEEK 2
	Wound, suppurative	M12	WEEK 3
	Wound, suppurative	M12	WEEK 4
	Oedema	R12,L12	WEEK 4
	Rough fur		WEEK 4
	Piloerection		WEEK 4
	Wound, suppurative	M12	WEEK 5
	Scab	M12	WEEK 5
	Wound, suppurative	M12	WEEK 6
99	Normal		WEEK 1
	Nodule	M8	WEEK 2
	Nodule	M8	WEEK 3
	Nodule	M8	WEEK 4
	Nodule	M8	WEEK 5
	Nodule	M8	WEEK 6

(END OF REPORT)

CERTIFICATE OF ANALYSIS

Sample: *Trichoderma reesei* ALKO 2224 (recombinant strain, CBHI$^-$)

Content: 5 x 10^7 conidia/ml 0.9 % NaCl

Batch: 23.4.1990

Sample: *Trichoderma reesei* ALKO 223 (control strain)

Content: 5 x 10^7 conidia/ml 0.9 % NaCl

Batch: 3.5.1990

Helena Nevalainen, Ph.D.
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Appendix 8: Summary of toxicological studies with Pectin Lyase

(a) Genetic toxicity – Wollny, H.E. et al. (1997)

S. typhimurium reverse mutation assay

Method:

The method conforms to OECD guideline No. 471. The standard plate incorporation method has been slightly modified to overcome the problem of histidine content of the product. Materials containing histidine may result in difficulties in the interpretation of test results if the standard plate incorporation method is used as the number of his^+ revertant colonies is directly related to the amount of histidine added to the plates. Therefore, the treat and plate method was used in this study: Tubes containing bacteria, nutrient, S9 mix or phosphate buffer, solvent or test solution, were incubated with shaking at 37 ^{o}C for 3 h. After the incubation period the tubes were centrifuged and the resulting pellets resuspended in phosphate buffer (pH 7.4, 0.05 M). Samples of this suspension were mixed with top agar and then poured onto the medium plates.
The assay was performed in three independent experiments. The third experiment was performed using the strains TA 1535 and TA 1537 only.
Triplicate plates were prepared for each bacterial strain and concentration of test substance both in the presence and absence of S9 mix.

Test material and concentration:

The test material with 5.93 % (batch no. RPT 05088) of dry matter and a enzymatic activity of about 377.7 PTF units was used.

The material was tested at the following concentrations in presence as well as in absence of S9 mix:

1%; 10%; 40%; 60%; 80%; 100% dilution of stock solution

Strains of Salmonella typhimurium:

TA 1535, TA 1537, TA 98, TA 100.

000310

Metabolic activation system:

S9 mix from liver preparations of Aroclor 1254 induced male Wistar rats plus appropriate cofactors.

Positive control substances:

2-Aminoanthracene (2-AAN), 4-Nitro-o-phenylene-diamine (4-NOPD), Sodium acide (NaN_3)
Solvent for positive control substances: dimethylsulphoxide; aqua bidest. .

Vehicle control and diluent for test substance:

aqua bidest.

Results:

Concurrent positive controls demonstrated the sensitivity of the assay and the metabolizing activity of the S9 mix.

In the first of three experiments, there was evidence for some dose-dependent increase of the number of revertant colonies for the strains TA 1535 and TA 1537, both in presence and in absence of S9 mix. Since this effect could not be reproduced in the second and third experiment, the first experiment was judged to be biologically irrelevant. For the strains TA 98 and TA 100, there was no evidence for an increase of revertant numbers.

Results of the Ames/Salmonella mutation assay show a certain intrinsic degree of stochastic variation based on a random distribution of reversion mutants over the total number of bacteria used in the test. Therefore, a repeat experiment is always performed in order to minimize the number of coincidential findings. A single positive test result which is not reproduced in the repeat experiment should therefore be interpreted as an overall negative based on this internal logic. In order to eliminate any remaining uncertainty, a third confirmatory experiment has been performed which, in this case, also gave a negative result.

This confirms that the result of the first experiment was a coincidential finding which was biologically not significant. As a consequence, the overall interpretation of the test results of the Ames/Salmonelly mutation assay is that the test shows no mutagenicity of the pectin lyase enzyme preparation.

In conclusion, the pectin lyase enzyme preparation was non-mutagenic to Salmonella typhimurium under test conditions.

Reference:

Sponsor: Röhm GmbH
Investigator: Wollny, H. E.
CCR Project no. 543501
Laboratory: CCR Cytotest Cell Research GmbH & Co. KG, Roßdorf, Germany
Date: Jan. 10, 1997

(b) In vitro chromosomal aberration assay – Czich, A. et al (1997)

Method:

The test was performed in compliance with EC (annex 5) and OECD 473 guidelines.

Two independent experiments were conducted. In the first and second test the respective duplicate cultures were treated for 4 h in the presence and for 22 h or 46 h in the absence of S 9 mix.
The chromosomes were prepared 22 h (experiment I and II) and 46 h (experiment II) after treatment.
The highest concentration tested was 10 % (v/v).

Test material and concentration

The test material with 5.93 % (batch no. RPT 05088) of dry matter and an enzymatic activity of about 377.7 PTF units was used (stability cf. 9.2.1).

Evaluated concentrations of the test material in experiment I were 0.3, 1.0 and 3.0 % (v/v) without S9 mix and 0.5, 3.0 and 5.0 % with S9 mix.
In the second experiment, the test concentrations evaluated were 0.3, 1.0 and 3.0 % in presence of S9 mix and 0.5, 2.5 and 5.0 % without S9 mix.

Test system:

Human peripheral lymphocytes.

Blood samples:

Whole peripheral blood obtained from a healthy 44 year-old female donor was used throughout both experiments.

Tissue culture medium

Basic culture medium: Dulbecco's modified Eagle medium/Ham's F12 medium (1:1) with 10 % fetal calf serum

Mitotic stimulant

Phytohaemagglutinin (PHA)

Cell culture

Whole blood (9 %) was added to tissue culture medium containing PHA. Antibiotic solution (10,000 U/ml penicillin, 10,000 µg/ml streptomycin) and heparin were added. Cells were incubated at 37 °C.

Metabolic activation system:

S9 mix was prepared from livers of Aroclor 1254 treated male Wistar rats of 8-12 weeks age. The S9 mix together with appropriate cofactors was added to the cell cultures to result in a final protein content of 0.75 mg/ml.

Positive control substances:

In presence of S9 mix: Cyclophosphamide (CPH) at concentrations of 30-37.5 µg/ml
In absence of S9 mix: Ethylmethane sulfonate (EMS) at concentrations of 330-440 µg/ml

Vehicle control:

Cell culture medium

Harvesting cultures:

Colcemid was added to all cultures at a final concentration of 0.2 µg/ml 3 hours before harvest. Cultures were treated with hypotonic solution (0.0375 M KCl) at 37 °C for 20 min. After that, the cells were fixed with methanol/acetic acid (3 : 1 v/v).

Stain:

Giemsa

Examination of cells

At least 100 cells from each culture were examined and scored for structural and numerical chromosomal abnormalities. Only metaphases with 46 centromer regions were included in the analysis. The mitotic index was determined to characterize cytotoxicity. In addition, the number of polyploid cells was evaluated.

Results:

The pectin lyase enzyme preparation was tested up to a concentration of 10 % of the culture medium.
In experiment I, there was no reduction of mitotic indices neither with or without presence of S9 mix. In experiment II, mitotic indices were reduced to values ≤ 50 % with and without metabolic activation.
No biologically relevant increase in the frequencies of polyploid metaphases was found.
In all experiments performed, there was no evidence that the pectin lyase enzyme preparation induced structural chromosomal aberrations.

Therefore, the pectin lyase enzyme preparation is considered to be non-mutagenic in this chromosome aberration assay.

Reference:

Sponsor: Röhm GmbH
Investigator: Czich, A.
CCR Report No. 543502
Laboratory: CCR Cytotest Cell Research GmbH & Co. KG, Roßdorf, Germany
Date: Jan. 08, 1997

(c) 90 day (13 week) oral toxicity studies in rats – Schmid, H. et al. (1998)

Method:

The test was performed according to guideline OECD No. 408.

Animals:

Wistar rats (Hannover-derived, outbread, SPF), 80 male, 80 female per group, ca. 4 weeks of age at delivery (body weight: males ca. 71 g , females ca. 59 g); 7 days acclimatization period; computer-generated random allocation to treatment and control groups.

Diet: Pelleted standard Kliba 343 rat maintenance diet (Kliba Mühlen AG, Kaiseraugst/Switzerland) ad libitum.

Compound preparation and administration:

Dosing solutions were prepared fresh daily using distilled water as vehicle. A daily aliquot of the test material was thawed overnight in a refrigerator. The dose levels were reported as mg dry matter of the enzyme preparation per kg body weight based on stock solution with 5.93 % dry matter (batch no. RPT 05088) of the final purified and concentrated fermentation product. The stability of the material under test conditions has been proven.

Route of administration:

Oral gavage

Frequency and duration of treatment:

Once daily, 7 days per week, 13 weeks

Volume of dosing solution:

20 ml/kg bw (body weight)

Dose levels:

0, 100, 500 and 1000 mg/kg bw were administered.

Controls received equal volumes of the vehicle (distilled water).

Nature of major observations:

Mortality:

Viability was checked twice daily.

Body weight and food utilization:

Body weight was recorded weekly.

Food consumption was recorded once each week.

Ophthalmology

Ophthalmoscopy was carried out on 10 animals before commencement of treatment and on each 10 animals of the control group and the highest dose group at week 13 of the study.

Blood sampling:

Samples were taken from 10 rats of each group during the weeks 5,9 and 13 of dosing from the orbital sinus after 18h deprivation of food.

Hematological parameters examined:

Hemoglobin, red blood cell count, hematocrit, prothrombin time, mean corpuscular volume, mean corp. hemoglobin and mean corp. hemoglobin concentration, reticulocyte count, reticulocyte fluorescence ratios, nucleated erythrocytes, red cell morphology, mean corpuscular volume, whole and differential white blood cell count, coagulation parameters (PT, APPT).

Clinical chemistry parameters examined:

Urea, glucose, total protein, protein/electrophoresis (=> albumin, AG ratio), total and direct bilirubin, creatinine, cholesterol, phospholipids, triglycerides, calcium, phosphate, sodium, potassium, chloride, aspartate aminotransferase , alanine aminotransferase , lactate dehydrogenase, alkaline phosphatase, γ-glutamyl transferase, creatinine kinase.

Study termination:

Surviving animals were killed by i.p. injection of pentobarbitone followed by exsanguination.

Statistical analysis:

Hematology data, clinical chemistry, organ weight, body weight and food consumption data were statistically analyzed. If group variances appeared to follow a normal distribution, the Dunnett-test (many to one t-test) was applied. If the variances were heterogeneous, the Steel-test (many one rank test) was applied.
Ophthalmoscopy and modified irwin screen data were evaluated by the exact Fisher-test.

Organ examination

Organ	Examined macroscopically Weight determination	Examined histologically
Liver	x	x
Heart		x
Kidneys	x	x
Lung		x
Spleen	x	x
Adrenals	x	x
Thymus	x	x
Testes	x	x
Prostate		x
Epididymis		x
Seminal vesicles		x
Uterus		x
Vagina		x
Urinary bladder		x
Brain	x	x
Spinal cord		x
Exorbital lachrymal glands		x
Thigh muscle		x
Pancreas		x
Submaxillary salivary gland		x
Submandibular lymph node		x
Pituitary		x
Skin and mammary gland		x
Eyes		x
Optic nerves		x
Auricles		x
Nasal turbinates		x
Harderian gland		x
Tongue		x
Aortic arch		x
Mesenteric lymph nodes		x
Thyroids	x	x
Trachea		x
Larynx		x
Oesophagus		x

Organ	Examined macroscopically Weight determination	Examined histologically
Stomach		x
Duodenum		x
Jejunum		x
Ileum		x
Caecum		x
Colon		x
Rectum		x
Sciatic nerve		x
Sternum		x
Femur		x

Results:

Clinical condition:

No treatment related clinical signs were observed.

Ophthalmology:

No ophthalmoscopic findings as a result of the treatment with the pectin lyase enzyme preparation in either sex at either point of time were reported.

Mortality:

The animals male no. 66 (group 4) and female no.140 (group 3) were found dead on day 2 of treatment.
Both animals showed evidence of misdosing (fluid in thoratic cavities).

Body weight changes and changes in food consumption or utilization

No notable intergroup differences in either sex were observed with respect to body weight, food and water consumption.

In conclusion, there were no treatment-related effects on body weight and food consumption.

Hematology

There are a few statistically significant differences from controls which are considered to be in the range of normal biological variation.
Thus, no treatment related changes in hematology were found.

Clinical chemistry

The differences from controls noted were considered to be of metabolic nature and without toxicological relevance. Moreover, the findings were within the range of normal background data.

Organ weights:

In male rats at the 1000 mg/kg group, slight increases in relative organ weight of liver, spleen and kidneys was observed. These increases are considered to be incidental and to reflect normal biological variation.

Necropsy- and histological findings:

At necropsy, there were no findings in either sex that were considered to be related to the treatment with the pectin lyase enzyme preparation.

In both animals which died on treatment day 2 (cf. 'Mortality'), minor grades of inflammatory processes in the lungs and/or esophagus were observed.

All other findings were considered to be typical for rats of this age and strain.

Conclusions:

The pectin lyase enzyme preparation administered by gavage for 13 weeks produced no treatment related effects at 100, 500 or 1000 mg/kg bw/day.

Therefore, a NOAEL ≥ 1000 mg/kg bw/day can be derived from this study.

References:

Sponsor: Röhm GmbH
Investigator: H. Schmid
Laboratory: RCC Research & Consulting Co. Ltd., CH-4452 Itingen / Switzerland
RCC Project No. 610918
Date: July 28, 1998

SUBMISSION END

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[redacted]

Reference List for Industry Submission, GRN 000032

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000222 - 000231	NA	Regulatory aspects of microbial food enzymes	NA	The Association of Microbial Food Enzyme Producers	pgs 2-11
000234 - 000242	NA	Safety aspects of Enzymes Produced by Genetically Modified Micro-Organisms	1996	The Association of Manufacturers of Fermentation Enzyme Products	pgs 1-7

NA- Not applicable

AM

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Dr. Rudaina H. Alrefai
Office of Premarket Approval (HFS-206)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, SW
Washington, DC 20204

1999 OCT 22 P 3:14

Re: Röhm Enzyme GmbH GRAS Notification for Pectin Lyase Enzyme Preparation Produced with *Trichoderma reesei* Containing a Gene from *Aspergillus niger* – Data Availability

Dear Dr. Alrefair:

As attorneys for Röhm Enzyme GmbH ("Röhm Enzyme"), we wish to clarify that while the cover letter to this GRAS Notification did not state that Röhm Enzyme would make the records supporting the GRAS Notification available to the Food and Drug Administration ("FDA") for copying, Röhm Enzyme intends to make available for FDA review and copying the data and information that are the basis for Röhm Enzyme's GRAS determination. Pursuant to proposed 21 C.F.R. § 170.36(c)(1)(v), this information will be made available to FDA at reasonable times at Röhm Enzyme's address in Germany and/or at our address listed above, or this information will be sent to FDA upon request.

Please feel free to contact me at (202) 496-7645 if you have any questions.

Very truly yours

Gary L. Yingling

GLY/so