Reference 5-3

Confirmation of safety related to "heat-resistant α-amylase-producing maize line 3272" (draft)

I Introduction

About heat resistant α-amylase-producing maize line 3272, “Recombinant DNA technology applied feed”
And procedures for confirming the safety of feed additives ”(Announcement No. 1780, Ministry of Agriculture, Forestry and Fisheries, November 26, 2002
No.) was deliberated.

II Summary of feed to be confirmed

Feed name: Thermostable α-amylase-producing maize 3272 line
Properties: Thermostable α-amylase productivity
Applicant: Syngenta Seed Co., Ltd.
Developer: Syngenta Seeds, Inc. on behalf of Syngenta Crop Protection AG and its affiliates

Thermostable α-amylase-producing maize line 3272 (hereinafter referred to as “3272 maize”)
Yeah.) Is a chimera derived from the three α-amylase genes of the archaeon Thermococcales thermophile
Modified α-amylase gene (hereinafter referred to as “ amy797E gene”) and E. coli mannose residue
An acid isomerase gene (hereinafter referred to as “ pmi gene”) is introduced. Be produced
The thermostable α-amylase exhibits activity even under high temperature conditions in the starch liquefaction process for industrial use.
In general, corn is mainly used as feed for livestock etc.
The residue after the process is also used as feed.

III Contents of deliberation

1. Matters concerning the equivalence of existing products

(1) Matters concerning genetic material

The plant used as the host was a corn of the genus Zea (Zea mays)
L.) and belongs to the Dent species. 3272 The amy797E gene introduced into maize
Derived from the three α-amylase genes of thermophilic bacteria (Reference 1),
The pmi gene is derived from the mannose phosphate isomerase gene of Escherichia coli

To do.

(2) Matter about safe breeding experience such as domestic animals

Maize, the host, has been used for a long time as feed in various countries around the world.

(3) Matters concerning feed components

Major components in corn kernels and foliage

(Protein, lipid, dietary fiber, ash, carbohydrate), secondary metabolites (ferulic acid, p -bear)

Luric acid, furfural, inositol, phytic acid, raffinose, trypsin inhibitor

The amount of-) has been made clear.

(4) Matters concerning differences in usage between existing and new varieties

3272 Maize is mainly used for ethanol production, and the ethanol distillation process

The latter residue (DDGS) is used as feed for livestock and the like. Existing corn is also steamed with ethanol

Residues after the distillation process are used as livestock feed. Harvest time and storage method, Feeding of livestock, etc.

Edible part, intake of livestock, etc. preparation and processing methods in combination with existing corn

There is no difference.

From the above (1) to (4), in order to evaluate the safety of 3272 corn as feed,

It was judged that the method using existing corn as a comparison target can be applied.

2. Matters concerning the purpose and method of use of recombinants

3272 Corn is mainly used for ethanol production from grain, but the ethanol distillation process

The latter residue (DDGS) is rich in protein, fiber and fat, so it is used as feed for livestock.

3. Matters concerning the host

(1) Matters related to taxonomic position such as scientific name, variety, strain name, etc.

Host, Gramineae corn (Zea) genus of corn (Zea Mays is dent type a L.)

Belonging to.

(2) Matters concerning genetic ancestry

In general, Mexico or Guatemala in 5000 BC is considered the origin, and the breeding process
The theory that it is derived from butter sorghum, a wild relative, is considered to be prominent in Japan (reference text)
2).

(3) Matters concerning the production of harmful physiologically active substances

Maize is not known to produce harmful bioactive substances that are considered nutritionally harmful.
(Reference 3).

(4) Matters concerning parasitic property and fixing property

There is no report that corn is infested or settled in livestock.

(5) Matters concerning not being contaminated with pathogenic foreign factors such as viruses

Pathogens that infect corn are known (Reference 4), but pathogens for livestock, etc.
Sex has not been reported (Reference 5).

(6) Matters concerning survival and proliferation ability under experimental conditions that reflect the natural environment

Maize is a cultivated crop and has not been reported to survive or propagate in the natural environment.

(7) Matters concerning sexual reproductive cycle and crossability

The cultivation period of corn varies depending on the variety, region and cultivation form, but it is mainly sown in spring.
Harvested in autumn (Reference 6).
The related species of corn are pork sorghum and trypsacum,
Only pig sorghum can be crossed (Reference 4). Theosinte grows naturally in our country
Therefore, it is not possible to cross with 3272 corn.

(8) Matter about history used for feed

The origin of corn is considered to be Mexico or Guatemala in about 5000 BC.
Currently, it is cultivated worldwide from 58 degrees north latitude to 40 degrees south latitude (references).
7). Throughout this cultivation history, corn has been used as feed worldwide.

(9) Matter about safe use of feed

As described in (8) above, corn is safely used as feed.

(10) Matters concerning conditions that limit survival and proliferation ability

Corn is a crop that has been artificially improved to be suitable as a cultivated crop.
Without intervention, they cannot survive or proliferate (Refs. 4, 6, 8, 9).

(11) Matters concerning production of harmful physiologically active substances of related species

The related species that can be crossed with corn are butterfly sorghum and trypsum.
Production of harmful physiologically active substances has not been reported (References 8 and 10).

4 Vector matters

(1) Matter about name and origin

3272 The vector pNOV7013 used to produce corn was obtained from Danisco Biotechnology
Derived from the binary vector pVictor.

(2) Matters concerning properties

The total number of bases of pNOV7013 is 11,439 bp, and the base sequence has been clarified. Known
Is not included (Reference 11).

(3) Matters concerning drug resistance

pNOV7013 contains the aadA gene from E. coli Tn7 as an antibiotic resistance marker.
However, it has been confirmed that 3272 maize does not contain the aadA gene.

(4) Matters concerning transmission

PmNOV7013 includes VS1ori derived from Pseudomonas involved in transduction (reference document 12), E.
ColE1ori derived from Escherichia coli (Ref. 13) and Rhizobium radiobacter (Agrobacterium
virG derived from tumefaciens) (reference document 14).

(5) Items related to host dependence

host range capable of transmitting the pNOV7013 is, Pseudomonas spp, R . radiobacter (A .
tumefaciens), R. leguminosarum and their related bacterial species and mono- and dicotyledonous species, homes
Livestock is not considered to be a host.

one two Three (6) Matters concerning the method of creating expression vectors

Based on the binary vector pVictor of Danisco Biotechnology, pmi gene expression cassette ([ZmUbiInt promoter]-[pmi gene]-[NOS terminator])
In addition, amy797E gene expression cassette fragment ([GZein promoter]-[amy797E gene]-[PEPC intron # 9]-[35S terminator]) was introduced to create pNOV7013 (references) 15).

(7) Matters concerning the method and position of inserting the expression vector into the host
The Agrobacterium method was used to introduce pNOV7013 into the host.

5. Matters concerning inserted genes
(1) Matter about donor
Matters concerning name, origin and classification
The amy797E gene is inherited from three α-amylase genes of the thermophilic bacterium Archaea Thermococcales
It is a chimeric modified gene (Reference 1) derived from a child (BD5031, BD5063 and BD5064).
Of the three genes, BD5031 and BD5064 are shallow ocean hydrothermal systems at 95 ° C and pH 7.0.
DNA library of Thermococcus species collected at 85 ° C and pH 6.0
Isolated from. BD5063 was also isolated from the deep sea Pacific Ocean at 90 ° C and pH 6.5.
It was isolated from an unidentified thermophile DNA library.
Thermococcales Consider either Pyrococcus spp. Or Thermococcus spp.
(Reference 1).
The pmi gene is a mannose phosphate isomerase cloned from E. coli K-12.
It is the manA gene that encodes (Reference 16).

Safety matters
Archaea Thermococcales, donor of amy797E gene
The eating experience is not known. However, α-amylase can be used in many plants (Ref. 17) and animals.
It is widely present in eukaryotes and prokaryotes in the natural world.
Ingests various α-amylases and their genes from many microorganisms and animals and plants.
E. coli , the donor of the pmi gene, is widely present in the natural and animal digestive organs
So far, livestock etc. have been ingested indirectly through feed. Also pmi remains
Toxicity to animals is denied for the E. coli K-12 strain, which is the donor of the gene (reference)
References 18, 19, 20, 21).

(2) Matters concerning gene insertion methods

pNOV7013 is a binary vector pVictor, pmi gene expression cassette fragment and
It was created by introducing the amy797E gene expression cassette fragment. The introduction method to the host
A bacterial method was used, and after the introduction, transformants were selected on a medium supplemented with mannose.

(3) Structure matters

The promoter of the amy797E gene expression cassette is the maize 27 kDa storage tamper.

It is the GZein promoter derived from the protein gene (Reference 22). pmi gene expression cassette
The promoter extends to the first intron region from the maize polyubiquitin gene.
ZmUbiInt promoter (Reference 23).

The terminator of the amy797E gene expression cassette is the cauliflower mosaic virus
A 35S terminator containing a polyadenylation signal from 35S RNA (reference 24),
pmi terminator gene expression cassette, R . radiobacter (A. tumefaciens) of
NOpa terminator containing a polyadenylation signal derived from the nopaline synthase gene (see
Reference 25).

The properties of all genes in pNOV7013 have been elucidated and known harmful bases
Does not contain sequences.

(4) Matters concerning properties

The results are summarized in Table 1.

table 1 Origin and function of each component DNA of expression vector pNOV7013

Composition DNA	Origin and function
amy797E gene expression cassette	
GZein promoter	Endosperm-specific promoter from the maize 27 kDa storage protein (zein) gene Column (reference 22).
amy797E	Archaea Thermococcales Chimera derived from 3 α-amylase genes of thermophilic bacterium Genetically modified gene encoding thermostable AMY797Eα-amylase (references) 1). In order to transport and accumulate the expressed protein in the endoplasmic reticulum, Γ-zein signal sequence (GZein ss) consisting of 19 amino acids and 6 amino acids The endoplasmic reticulum residual signal sequence (ER rs) is added to its N-terminal and C-terminal respectively. (References 26 and 48). Introns from maize phosphoenolpyruvate carboxylase gene.

PEPC intron # 9	# 9 sequence (Ref. 27), used to increase the expression of target genes in seeds (grains) The
35S Terminator	Polyadenyl directs transcription termination from cauliflower mosaic virus 35S RNA A sequence containing an activation signal (Reference 24).
pmi gene expression cassette	
ZmUbiInt promoter	Contains up to the first intron region (1,010 bp) from maize polyubiquitin gene Promoter sequence for monocotyledons (Reference 23).
pmi	The manA gene from E. coli K-12 (Reference 16), a selectable marker for transformants Mannose phosphate isomerase (Reference 28). pmi gene was introduced In cells that produce PMI protein, fructose Mannose can be added to the tissue culture medium because it can be converted to acid and grown. This makes it possible to select transformants.
NOS Terminator	Directs termination of transcription from R. radiobacter (A. tumefaciens) nopaline synthase gene A sequence containing a polyadenylation signal (Reference 25).

(5) Purity matters

pNOV7013 is spec (aadA) as a bacterial selection marker gene in the exoskeleton region of its T-DNA. Gene) and purified through selection and propagation of vectors in bacteria.

(6) Matters concerning stability

3272 Using four generations of maize, the expected amount of inserted gene in each backcross generation As a result of comparing the separation ratio and the measured value, the inserted gene is inherited based on Mendel's separation law (Reference 29).

In addition, in order to confirm the stability of the inserted gene, the result of Southern blot analysis showed that the inserted gene was It was shown to be inherited stably in the progeny (Reference 30).

(7) Items related to the number of copies

Southern blot analysis shows that the 3272 corn genome contains an expression vector. One copy of the complete amy797E gene expression cassette and pmi gene expression cassette from pNOV7013 Inserted into the expression vector pNOV7013 outside the T-DNA region. It was shown that there is no exoskeleton region (Reference 31).

Moreover, from the results of PCR analysis, both sequences of the inserted gene are derived from the maize genome.
(Reference 32).

(8) Matter about expression site, expression time and expression level

As a result of ELISA analysis, AMY797E α-amylase grains from the ripening period to the harvest period
The range of the average analysis value of the expression level is 838 to 1,627 μg / g fresh weight (1,004 to 3,365 μg / g dry weight)
However, almost no expression was observed in leaves, roots and pollen. Therefore,
It was confirmed that the expression of AMY797E α-amylase is grain specific (Reference 33).

In addition, the range of the average analysis value of the expression level of PMI protein is determined by the leaves from the growing season to the harvesting season.
Less than volume limit (LOQ)-5.0 μg / g fresh weight (<LOQ-17.1 μg / g dry weight), from growing season to harvesting season
<0.1-0.8 μg / g fresh weight at root (<0.6-5.3 μg / g dry weight), 8.0-8.5 μg / g fresh weight at flowering pollen
(17.0-18.2 μg / g dry weight), <0.4-0.8 μg / g fresh weight (<0.5-1.8
μg / g dry weight) (Reference 33).

(9) Matters concerning the safety of antibiotic resistance marker genes

3272 The absence of antibiotic resistance marker genes in maize
Confirmed by analysis (Ref. 31).

(10) Existence of exogenous open reading frames and the possibility of transcription and expression
Matter

From the results of analysis using InforMax's VNTi (Ver9.0)
A total of 7 open reading frames were detected (Reference 34). This openly
Homology with known toxins and known allergens in the translated amino acid sequence of the coding frame
A search was performed to confirm that there was no significant homology.

6. Matters concerning recombinants

(1) Matters concerning properties newly acquired by recombinant DNA manipulation

In 3272 maize, the inserted amy797E and pmi genes

AMY797E α-amylase and PMI protein are expressed.

(2) Matter related to toxicity of gene products

AMY797Eα-amylase

To confirm the structural homology between AMY797E α-amylase and known toxic proteins,
National Center for Biotechnology Information (NCBI) Entrez Protein Database (Reference
Reference 35) and blastp search program (version 2.2.6) (Reference 36)
(Reference 37). As a result, a known toxin having significant structural homology with AMY797E α-amylase
It was not shown.

In addition, a single-dose toxicity study using mice has also been conducted.
No toxic effects were observed due to Z (net dose: 1,511 mg / kg) (Reference 38).

PMI protein

Regarding the structural homology between PMI protein and known toxic protein, AMY797Eα-Ami
Evaluation was performed using a method similar to that for ase (Reference 39). As a result, significant with PMI protein
It was shown that there are no known toxins with similar structural homology.

In addition, single-dose toxicity studies using mice have been conducted, but PMI protein (net administration)
No toxic effects were observed due to the amount (3,030 mg / kg) (Reference 40).

(3) Matters concerning susceptibility of gene products to physicochemical treatment

3272 High production of AMY797Eα-amylase in corn kernels, extraction from cereal grains
AMY797Eα-amylase was extracted directly from corn kernels
did.

On the other hand, the production of PMI protein in 3272 corn is extremely small, and the following physical
It is necessary to extract from 3272 corn the amount necessary for the sensitivity evaluation test for chemical treatment.
Because it was extremely difficult, the one produced and extracted with the E. coli overexpression system was used.

The PMI protein from the E. coli overexpression system is a PMI tamper expressed in 3272 maize.
It had the same enzyme activity and immunological reaction as those of the cuticle (Reference Document 41). Therefore, the following physical
PMI derived from E. coli overexpression system for susceptibility testing and single-dose toxicity testing for chemical treatment
It was determined that the use of protein did not affect the results.

Sensitivity to artificial gastric juice

(I) AMY797Eα-amylase

SDS-PAGE analysis of digestibility of AMY797E α-amylase preparation in artificial gastric juice (SGF)
And Western blot analysis (Reference 42). As a result, 1 minute after the start of the reaction
The complete AMY797Eα-amylase band is no longer detected and a new AMY797Eα
-Bands of two peptide fragments that are degradation products of amylase were detected. further,
5 minutes after the start of the reaction, no peptide fragment bands were detected and Western blotting was performed.
In the analysis, a band showing immunoreactivity with AMY797Eα-amylase was detected 5 minutes after the start of the reaction.
It was not issued.

(Ii) PMI protein

The digestibility of PMI protein in artificial gastric juice (SGF) was evaluated by SDS-PAGE analysis (Reference 43). As a result, PMI protein is easily degraded immediately after the start of the reaction. Even in SGF with a pepsin concentration 1/1000 times lower than the standard, it breaks down into small fragments in a 2 minute reaction It was done. In addition, in a digestion experiment over time using SGF with a pepsin concentration of 1 / 10000-fold dilution. However, it breaks down into small fragments 10 minutes after the start of the reaction and those 60 minutes after the start of the reaction. No fragment was detected.

Alkaline treatment with artificial intestinal fluid and enzyme (pancreatin) treatment

(I) AMY797Eα-amylase

SDS-PAGE analysis of digestibility of AMY797E α-amylase preparation in artificial intestinal fluid (SIF) Evaluated by Western blot analysis (Ref. 44). As a result, AMY797Eα-Amirror Ze was not degraded 60 minutes after the start of the reaction.

(Ii) PMI protein

The digestibility of PMI protein in artificial intestinal fluid (SIF) was analyzed by SDS-PAGE analysis and Western blot. Evaluation by network analysis (References 43 and 45). As a result, the PMI protein is in standard SIF. It was shown to decompose easily in a 2 minute reaction. 1/10 times more SIF reaction start In 30 minutes, both SDS-PAGE analysis and Western blot analysis Bands derived from PMI protein are no longer detected, and the SIF reaction starts 1/100 times. It was shown to decompose over time (reaction start 0-30 minutes).

Heat treatment

(I) AMY797Eα-amylase

3272 AMY797E α-amylase produced in corn is a thermostable α-amylase It has been confirmed that it exhibits maximum activity under high temperature conditions (Reference 46). Did not.

(Ii) PMI protein

As a result of evaluation of heat treatment sensitivity by enzyme activity assay and ELISA analysis, PMI protein

Quality was confirmed to completely lose enzyme activity and immune response activity after standing at 95 ° C for 30 minutes.
(Reference 47).

(4) Matters concerning the influence of gene products on metabolic pathways

AMY797Eα-amylase

3272 AMY797Eα-amylase in maize

It accumulates in the endoplasmic reticulum (References 26 and 48). On the other hand, starch in seeds is not
It is synthesized and stored in amyloplasts, which are different intracellular organs, as water-soluble granules.
49, 50, 51).

Based on the above, the expression of AMY797Eα-amylase in 3272 maize
The possibility of affecting the metabolic system of the main plant is considered to be extremely low.

PMI protein

PMI protein expressed by pmi gene is mannose 6-phosphate and fructose
It is a catalytic enzyme protein that reversibly interconverts 6-phosphate, and its reaction is mannose-6-
Specific for phosphate and fructose-6-phosphate, other natural groups for PMI proteins
The quality is not known (reference 52).

Based on the above, PMI protein expression in 3272 maize
The possibility of affecting the metabolic system is considered extremely low.

(5) Matters concerning differences from the host

Ingredients for non-recombinant corn in 3272 and control in 2003 and 2004
An analysis was performed (Ref. 53). The 2003 cultivation test included 3272 corn hybrids.
Two lines (hereinafter referred to as “hybrid A1” and “hybrid B1”) and a non-recombinant tow
Two sorghum hybrids were used. In the 2004 cultivation test, 3272 corn
Hybrid (hereinafter referred to as “hybrid B3”) and non-recombinant maize hybrid
Used.

Based on the report on the amount of DDGS components using 3272 corn (Reference 54).
I considered it.

Analysis results of main components and mineral components in grains and foliage

Main components of grains and foliage (moisture, protein, lipid, ash, carbohydrates, acidity) Gent fiber, neutral detergent fiber, total dietary fiber, starch) and mineral components Lucium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc, selenium Nium) was analyzed.

As a result, the major constituents of the grain are ash in the hybrid A1 and the hybrid B1 For proteins, carbohydrates, total dietary fiber (TDF) and starch, for hybrid B3, acid With natural detergent fiber (ADF), neutral detergent fiber (NDF) and total dietary fiber (TDF) A statistically significant difference (p <5.0%) was observed with non-recombinant maize. Within the range of literature values reported for corn varieties, and between test materials or year of cultivation There was no consistent consistency between the degrees.

The main components of the foliage are the protein and ADF in the hybrid B1, the hybrid B3 is a carbohydrate and has a statistically significant difference (p <5.0%) from non-recombinant maize. However, it is within the range of literature values reported for general commercial corn varieties, and There was no consistent consistency between the foods or the cultivation years.

In terms of the mineral content of the grain, the hybrid B1 manganese and non-recombinant maize A statistically significant difference (p <5.0%) was reported in general commercial corn varieties. And consistent consistency between the test materials and the year of cultivation is not observed. It wasn't.

The mineral content of the foliage is statistically different from that of non-recombinant maize with hybrid B1 iron. A significant difference (p <5.0%) was observed, but consistent consistency between test materials or cultivation years

Was not seen.

Analysis results of vitamins, amino acid composition and fatty acid composition in grain

Grain vitamins (β-carotene, cryptoxanthin, folic acid, vitamin B1 (thiamine), Vitamin B2 (riboflavin), niacin, vitamin B6 (pyridoxine), vitamin C, vitamin Tamine E (tocopherol)), amino acid composition and fatty acid composition were analyzed.

For grain vitamins, hybrid A1 is vitamin B6 and hybrid B1 is Vitamin B1, Vitamin B6 for hybrid B3, and non-recombinant maize A statistically significant difference (p <5.0%) was observed but has not been reported for common commercial corn varieties.

And consistent consistency is seen between the test materials and the year of cultivation.

There was no.

In terms of the amino acid composition of the grain, hybrid B1 excludes methionine and tyrosine.

All 16 amino acids, tryptophan in hybrid B3, non-recombinant corn

A statistically significant difference (p <5.0%) was observed with Rokoshi, but general commercial corn varieties

Within the range of literature values reported in, and consistent between the test materials or the cultivation year

There was no consistency.

The fatty acid composition of the grain was statistically different between 3272 corn and non-recombinant corn.

There was no significant difference.

(3) Analysis results of secondary metabolites and anti-nutrients in grain

Secondary metabolites and anti-nutrients of grains (inositol, phytic acid, raffinose, chicken

Analysis of pussin inhibitor, ferulic acid, p-coumaric acid, furfural)

Statistics of hybrid B3 inositol and ferulic acid with non-recombinant maize

Significant difference (p <5.0%) was observed. Hybrid A1 and B1 are both inositol and

There was no statistically significant difference in ferulic acid, and there was no consistent consistency among the test materials

The In addition, with regard to ferulic acid, the literature values reported for general commercial corn varieties

It was within the range.

DDGS component amount

Mixing 3272 corn kernels with conventional corn kernels at a rate of 3%

Report of analysis of the components, crude protein, crude lipid, crude fiber and ash (references

According to 54), all component analysis values were within the range of DDGS on the market. In addition,

Residual amino acids in DDGS in both the 3272 corn mixed sample and the control

No enzyme activity was detected.

(6) Matters concerning survival and proliferation ability in the outside world

Through field trials conducted in the United States, 3272 corn survival and growth ability (seed dormancy,

Low-temperature tolerance in early growth, wintering ability of adults and seed production and shedding ability)

It was comparable.

(7) Matters concerning limitations on survival and proliferation ability

3272 Reproductive and proliferative ability of corn is considered to be the same as that of conventional non-recombinant corn Be

(8) Matters concerning the inactivation method

3272 Maize, like conventional non-recombinant maize, is physically controlled (tillage) and chemically It is inactivated by conventional methods of killing corn such as control (use of sensitive herbicides).

(9) Matters concerning approvals in foreign countries

In August 2007, the US Food and Drug Administration (FDA) confirmed the safety of food and feed.

The Canadian Food Inspection Agency (CFIA) confirmed its safety as feed in March 2008.

In March 2008, the Australian and New Zealand Food Standards Organization (FSANZ) The safety was confirmed. To the European Union in February 2006, food and feed As an application for imports.

(10) Matter about production, breeding and cultivation method

There is no difference from conventional corn in terms of cultivation method.

(11) Matters related to seed production and management

3272 Seed production and management methods for corn There is no difference.

7 If knowledge about feed safety is not obtained from the materials listed in 2 to 6, Items related to required test results Not applicable.

IV Deliberation result

About heat resistant α-amylase-producing maize line 3272 As a result of deliberation based on `` Procedure for Confirmation of Safety of Feed Additives '', It was decided that it would be safe to do so.

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