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Stable compositions comprising transgenic plant material

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(54) Title: STABLE COMPOSITIONS COMPRISING TRANSGENIC PLANT MATERIAL

(57) Abstract

The present invention discloses thermomechanically processed compositions comprising as active ingredient a polypeptide. These compositions are obtainable by subjecting raw materials containing transgenic plant material in which the polypeptide has been biosynthesized due to the expression of a transgene therein, to a thermomechanical process, such as e.g. pelleting, extrusion or expansion. The compositions of the present invention are preferably used as feed for animals or as a premix therefor.

STABLE COMPOSITIONS COMPRISING TRANSGENIC PLANT MATERIAL

Field of the invention

The present invention relates to the application of enzymes in industrial processes such as the manufacture of food or feed compositions.

Background of the invention

The use of enzymes in animal feed for livestock has become almost common practice. These enzymes are produced by micro-organisms grown in large scale fermenters operated by industrial enzyme producers. At the end of the fermentation period, broths of micro-organisms are commonly subjected to a series of filtration steps to separate biomass from the enzyme. The enzyme solution is either sold as a liquid after addition of various stabilizers or processed to a dry formulation.

Enzyme liquids and dry formulations are used on commercial scale by the feed industry. Liquids may be applied to the feed after pelleting to prevent heat inactivation of the enzymes by the pelleting process. The problem is that the amounts of enzyme in the final feed preparations are very small which makes it difficult to realize a homogenous distribution of the enzyme molecules over the feed. Liquids are notoriously more difficult to mix than dry ingredients. One needs specific equipment to add liquids to the feed after pelleting which is not currently available at most feed mills.

Dry formulations of enzymes, on the other hand, have the disadvantage of heat inactivation of the enzymes during pelleting. During pelleting, feed is pressed through a matrix and the formed stripes are cut into suitable pellets of variable length. The moisture of the mass when arriving into the pelleting equipment is generally between about 18% and about 19%. Directly after pelleting, temperatures may rise to 60-95°C. The combined effect of high moisture content and high temperatures is detrimental to most enzymes.

These disadvantages equally apply to other types of thermomechanical treatments of feed, such as extrusion and expansion.

In order to overcome these problems a method for producing a heat stable enzyme premix containing one or more enzymes which retain an effective level of activity during commercial feed pelleting processes has been published (Haarakilta, A (1988) European Patent Application #0257 996 B1). The method comprises the steps of:

> mixing a grain carrier which is physiologically acceptable and capable of absorbing an aqueous enzyme solution, with an aqueous enzyme solution for a period of time sufficient to absorb the enzyme or enzymes onto the carrier to form a carrier/enzyme complex; pelleting the carrier/enzyme complex; and drying the pelleted carrier/enzyme complex to a moisture content of between 7% and 15% preferably less than about 10% by weight.

This method is laborious and in our hands does not result in an improved pelleting resistance.

Various enzyme manufacturers have developed different formulation methods to improve the stability of dry enzyme products during feed pelleting and storage. It has been suggested, for example, that a formulation characterized in that the product particles are coated granulates would improve the resistance to pelleting conditions (WO 92/12645). These efforts emphasize the importance of the problem and the need in the industry to solve this application problem.

Summary of the invention

We have now surprisingly found that enzymes are much more resistant to thermomechanical processing, such as pelleting, when they are added to the feed in the form of transgenic plant material in which these enzymes have been produced by means of expression of a transgene. Thus, the present invention discloses a process for improving stability of a polypeptide as an active ingredient in a thermomechanically processed composition comprising a polypeptide as an active ingredient, said process including the steps of

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(i) providing transgenic plant material in which the polypeptide as an active ingredient is biosynthesized due to the expression of a transgene therein, and

(ii) subjecting the transgenic plant material in its entirety with said polypeptide to a thermomechanical process.

The preferred thermomechanical processes are pelleting, extrusion and expansion.

The invention discloses the use of these compositions as a feed for animals or as a premix therefor. Specifically, the compositions of the invention are used in processes for promoting the growth of animals in which the animals are fed diets which comprises these compositions.

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Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

Description of the invention

The thermomechanical processes to be applied in the present invention are frequently used in commercial feed processing, where they occur in a number of ways such as e.g. expansion, extrusion, and pelletization, or combinations thereof. All these processes are characterized by an input of thermal energy, usually in the form of steam, and mechanical energy by e.g. the drive of the screw to the feed.

Pelleting is the classical processing method in feed production which may also be applied as thermomechanical treatment as such to any raw material.

The raw materials are mixed and after a low pressure steam addition of several bars are conveyed into a rotating die. The die contains holes, usually several hundred, through which the product passes. It is pushed by rotating rolls inside the die.

Extrusion is an alternative way for feed production which may also be applied as thermomechanical treatment as such to any raw material.

The raw materials are put onto an endless screw consisting generally of several pressure rings of decreasing shape. This screw drives the product and leads it to the die. The temperature rise is caused by product friction between the screw and the jacket and by an increase in pressure. The pressure difference between the interior of the extruder and the exterior leads to a

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partial vaporisation of the water at the exit point and thus to an expansion of the product.

Expansion is a process which is quite similar to extrusion but does not shape the material in a defined matter since it is generally not driven through a die.

An alternative way of producing enzyme for application in animal feed has been invented by expression of genes encoding feed enzymes, such as e.g. microbial phytase, in plants and plant seeds (Pen et al. EP-A-0 449 375). Use of plants or plant seeds expressing these enzymes, either direct or after processing, is compatible with the application in animal feed.

The transgenic plant materials of the invention preferably contain one or more desired polypeptides which are the expression product of transgenes contained in the plant from which the plant material is derived. This is achieved via the introduction into the plant of expression constructs comprising a DNA sequence encoding the desired polypeptides having a relevant activity such as for example the enzyme phytase (of which detailed descriptions may be found in Pen et al. EP-A-0 449 375). Transgenic plants and transgenic plant material, are herein defined to include plants (as well as parts and cells of said plants) and their progeny, which have been genetically modified using recombinant DNA techniques to cause, enhance or alter the production of a desired polypeptide in the plant or plant organ. In this context a transgene is understood to refer to the specific genetic modification, i.e. nucleotide sequences, in the transgenic plant. In the compositions of the invention polypeptides are applied as active ingredients, whereby an active ingredient is defined as being encoded by a transgene.

The polypeptides to be used in the invention may be produced constitutively in the transgenic plants in all tissues, during all stages of development. In some instances, e.g. in the case of plant cell wall degrading enzymes, it may be required to limit the expression of the transgene to specific parts of the plant in which the expressed desired polypeptide do not interfere with the

development of the host plant. The genes encoding the desired polypeptides can therefore also be expressed in a stage- and/or tissue-specific manner, or, alternatively, these genes can be put under control of an inducible promoter. In a preferred embodiment seeds are used, either intact or after milling or grinding, in which the desired polypeptide has been specifically expressed using a seed-specific promoter. Nevertheless, it will be evident to those skilled in the art that expression of polypeptides in other plant organs is equally feasible and may lead to similar advantages during application as disclosed in the present invention.

The choice of the plant species is primarily determined by the intended use of the plants or parts thereof and the amenability to transformation. Within the context of the present invention, plants to be selected include, but are not limited to crops producing edible flowers such as cauliflower (*Brassica oleracea*), artichoke (*Cynara scolymus*), fruits such as apple (*Malus*, e.g. *domesticus*), banana (*Musa*, e.g. *acuminata*), berries (such as the currant, *Ribes*, e.g. *rubrum*), cherries (such as the sweet cherry, *Prunus*, e.g. *avium*), cucumber (*Cucumis*, e.g. *sativus*), grape (*Vitis*, e.g. *vinifera*), lemon (*Citrus limon*), melon (*Cucumis melo*), nuts (such as the walnut, *Juglans*, e.g. *regia*; peanut, *Arachis hypogeae*), orange (*Citrus*, e.g. *maxima*), peach (*Prunus*, e.g. *persica*), pear (*Pyra*, e.g. *communis*), plum (*Prunus*, e.g. *domestica*), strawberry (*Fragaria*, e.g. *moschata*), tomato (*Lycopersicon*, e.g. *esculentum*), leafs, such as alfalfa (*Medicago*, e.g. *sativa*), cabbages (e.g. *Brassica oleracea*), endive (*Cichoreum*, e.g. *endivia*), leek (*Allium*, e.g. *porrum*), lettuce (*Lactuca*, e.g. *sativa*), spinach (*Spinacia* e.g. *oleraceae*), tobacco (*Nicotiana*, e.g. *tabacum*), roots, such as arrowroot (*Maranta*, e.g. *arundinacea*), beet (*Beta*, e.g. *vulgaris*), carrot (*Daucus*, e.g. *carota*), cassava (*Manihot*, e.g. *esculenta*), turnip (*Brassica*, e.g. *rapa*), radish (*Raphanus*, e.g. *sativus*), yam (*Dioscorea*, e.g. *esculenta*), sweet potato (*Ipomoea batatas*) and seeds, such as bean (*Phaseolus*, e.g. *vulgaris*), pea (*Pisum*, e.g. *sativum*), soybean (*Glycin*, e.g. *max*), wheat (*Triticum*, e.g. *aestivum*), barley (*Hordeum*, e.g. *vulgare*), corn (*Zea*, e.g. *mays*), rice (*Oryza*, e.g. *sativa*), rapeseed (*Brassica napus*),

millet (*Panicum* L.), sunflower (*Helianthus annus*), oats (*Avena sativa*), tubers, such as kohlrabi (*Brassica*, e.g. *oleraceae*), potato (*Solanum*, e.g. *tuberosum*) and the like.

The advantages of the present invention are that polypeptides when added to a composition as active ingredient and in the form of a transgenic plant material are more resistant to inactivation during the thermomechanical process steps such as pelleting, as compared to conventional forms in which polypeptides were added to such compositions. It will be apparent that these advantages are not necessarily limited to the specific embodiments of the specific examples of the present application, i.e. enzymes such as phytase or xylanase which are added to compositions to be pelleted and used as feed for animals. In contrast, the advantages of the present invention should apply to any composition which is to be subjected to a thermomechanical treatment and which contains one or more polypeptides as active ingredients. The advantages of the invention will be most pronounced in the case of polypeptides with a sensitive biological activity, e.g. sensitive to thermal inactivation. Thermosensitive polypeptides are herein defined as polypeptides of which a substantial amount of biological activity is lost upon being subjected to the thermomechanical processes applied in the invention.

In a preferred embodiment of the invention the polypeptides and/or the DNA sequences encoding them are heterologous with respect to the transgenic host plant in which the DNA sequences are expressed. Heterologous is herein understood to mean that the DNA sequences and/or polypeptides are not native to the host organism, i.e. which are derived from other plant species, mammals, or microorganisms, such as fungi or bacteria. The present invention is particularly advantageous when glycosylated proteins are expressed in the transgenic plants, whereby their glycosylation may differ from the naturally occurring counterparts of these proteins.

Preferred enzymes to be included in the present invention are enzymes to be applied in animal feed. Animal feed is herein understood to also include pet food. The function of these enzymes is to improve the feed conversion rate, e.g. by reducing the viscosity or by reducing the antinutritional effect of certain feed compounds. Alternatively, feed enzymes (such as e.g. phytase) can be applied to reduce in the manure, the amount compounds which are harmful to the environment. Preferred enzymes for these purposes are: phosphatases, of which preferably phytases and/or acid phosphatases; carbohydrases, such as amylolytic enzymes and plant cell wall degrading enzymes of which preferably cellulases, hemicellulases, or galactanases; proteases of which preferably proteases with a neutral and/or acidic pH optimum; and lipases of which preferably phospholipases such as the mammalian pancreatic phospholipases A2. In addition, the polypeptides to be included in the invention could also comprise non-enzymatic biological activities such as antigenic determinants to be used as vaccines and/or polypeptides engineered to have an increased content of essential amino acids.

The skilled person will understand that the invention equally applies to combinations of desired polypeptides, wherein such combinations comprise combinations of different transgenic plants expressing different desired polypeptides as well as combinations of different desired polypeptides which are co-expressed in one transgenic plant.

In one specific embodiment of the present invention, the pelleting-stability of *Aspergillus niger* phytase has been tested when the enzyme is contained in milled seeds of oilseed rape (*Brassica napus*) in which the *A.niger* phytase gene has been expressed. Cloning of the *A.niger* phytase gene has been described elsewhere (van Gorcom et al. EP-A-0 420 358). In another specific embodiment of the present invention, a pelleting-stable formulation of the *Aspergillus* endoxylanase is provided by expression of the fungal gene encoding this enzyme in tobacco seeds. The cloning of the *Aspergillus*

endoxylanase gene has been described in detail by de Graaff et al. EP-A-0 463 706.

Example 1

Stable seed specific expression of the *Aspergillus niger* phytase gene under the control of a cruciferin promoter in oilseed rape

Details of methods applied for the seed-specific expression of the fungal phytase in oilseed rape have been described by Pen et al. (1991) in EP-A-0 449 375, which is incorporated herein by reference. In essence, the binary vector pMOG23 (deposited, in *E.coli* K12 DH5α, at the Centraal Bureau voor Schimmel Cultures on January 29, 1990, under accession number CBS 102.90) was used as starting point for the construction of the seed-specific expression vector for phytase. A seed-specific phytase expression-cassette was assembled in the polylinker of pMOG23, comprising the following essential elements. The seed-specific transcription of the phytase cDNA and termination thereof are controlled by the 5'- and 3'-regulatory sequences derived from gene encoding the *Brassica napus* 12S storage protein cruciferin (the cruA gene; Ryan et al., 1989, Nucl. Acids Res. 17: 3584), respectively. The Alfalfa Mosaic Virus RNA4 leader sequence is used to stabilize the mRNA (Bredero et al., 1980, Nucl. Acids Res. 8:2213) and the Tobacco PR-S signal peptide (Cornelissen et al., 1986, Nature 321: 531) is coupled in frame to the portion of the phytase cDNA encoding the mature protein in order to achieve extracellular expression of phytase. This binary vector containing the chimeric phytase gene was mobilized, in a triparental mating with the *E. coli* K-12 strain RK2013 (containing plasmid pRK2013) (Ditta et al., 1980, Proc. Natl. Acad. Sci. USA 77: 7347), into *Agrobacterium* strain LBA4404 (Hoekema et al., 1983, Nature 303: 179) that contains a plasmid with the virulence genes necessary for T-DNA transfer to the plant. This strain was used to transform rapeseed (*Brassica napus* cv. Westar). To this aim, surface-sterilized stem segments taken from 5 to 6 week-old plants, just before flowering, are preconditioned for 24 h on MS medium (Fry et al. (1987) Plant Cell Reports 6, 321) and then co-cultivated for 48 h with *Agrobacterium* on fresh plates with the same medium. Transgenic plantlets were regenerated from shoots that grow on selection medium (500 mg/l carbenicilline, 40 mg/l paromomycin) and further analyzed as described in by Pen et al. in EP-A-0 449 375.

Using the above methods, expression levels of phytase in oilseed rape reached levels up to 10% of soluble protein corresponding to about 500 FTU/g. One unit of phytase activity (FTU) is defined as that amount of enzyme which liberates inorganic phosphorus from 1.5 mM sodium phytate at the rate of 1 μmol/min at 37°C and at pH 5.5.

Example 2

Stability of phytase in oilseed rape during pelleting

Pelleting conditions were such that the pellets obtained a temperature of 55°C after conditioning and 75°C after pelleting. Two independent trials have been conducted, one on labscale and one on industrial scale.

Surprisingly, we found that the *Aspergillus niger* phytase produced in oilseed rape is substantially more resistant to pelleting conditions than the current commercial *Aspergillus niger* phytase formulations. Since milled seeds were used, the stability of the enzyme is apparently not dependent on the integrity of the seeds.

Natuphos® is a commercial product containing the *Aspergillus niger* phytase, which may be obtained from BASF, Ludwigshafen, Germany.

Table 1

Effects of pelleting on the stability of different enzyme formulations.

	Percentage of remaining activity	**Percentage of remaining activity**
Product	Labscale	Industrial scale
Commercial Natuphos® powder	43	38
Milled seeds of oilseed rape expressing 100 FTU/g	62	95

Example 3

Stability of phytase in oilseed rape during milling of the seeds

Commercial development of phytase in oilseed rape implies the milling of seeds. As a consequence, different milling conditions were compared to assess the stability of phytase activity as expressed in oilseed rape during milling. The milling methods used are well known to persons skilled in the art. Results show that the way of milling (Table 2) does not drastically affect the stability of the enzyme.

Table 2

Effects of milling conditions on the stability of phytase expressed in oilseed rape.

Milling method	**Percentage of remaining activity**
Flaking	79
Flaking/reflaking	90
Flaking/hammer-milling	91%
Flaking/pinmilling	90
Flaking/frozen pinmilling	89
Hammermilling	75
Pinmilling	87

Example 4

Stable expression of endoxylanase in tobacco seeds under the control of the CaMV 35S promoter

The *Aspergillus* endoxylanase gene has been cloned as described in detail by de Graaff et al. EP-A-0 463 706. The *E.coli* plasmid pIM100 containing the *Aspergillus* endoxylanase gene has been deposited at the Centraal Bureau voor Schimmelcultures on July 19, 1990 and was assigned the designation CBS 322.90. Using a fusion PCR with pIM100 as template, the single intron in the xylanase gene was removed. This artificial cDNA was expressed in tobacco seeds following procedures similar to those described in detail for the expression of phytase in tobacco (Pen et al., 1993, Bio/technology 11: 811). In essence, the cDNA fragment encoding the mature enzyme was fused in-frame to the sequence encoding the signal peptide of the tobacco PR-S protein (Cornelissen et al., 1986, Nature 321: 531) in the binary expression vector pMOG29 (Pen et al., 1992, Bio/technology 10: 292). The expression cassette in pMOG29 consists of the following elements: the constitutive cauliflower mosaic virus (CaMV) 35S promoter with double enhancer (Guilley et al., 1982, Cell 30: 763); the synthetic Alfalfa Mosaic Virus RNA4 leader sequence to stabilize the mRNA (Bredero et al., 1980, Nucl. Acids Res. 8:2213); and the *Agrobacterium tumefaciens* nopaline synthase (NOS) terminator (Bevan, 1984, Nucl. Acids Res. 12: 8711). This binary vector containing the chimeric endoxylanase gene was mobilized, in a triparental mating with the *E. coli* K-12 strain RK2013 (containing plasmid pRK2013) (Ditta et al., 1980, Proc. Natl. Acad. Sci. USA 77: 7347), into *Agrobacterium* strain LBA4404 (Hoekema et al., 1983, Nature 303: 179) that contains a plasmid with the virulence genes necessary for T-DNA tranfer to the plant. This strain was used to transform Tobacco (*Nicotiana tabacum* cv Petit Havana SR1) leaf discs according to the procedure of Horsch et al. (1985, Science 227: 1229). Transgenic plants were selected on media containing 100 mg/l kanamycin.

Constitutive expression of this endoxylanase gene in tobacco resulted in maximum expression levels of 1.5% of soluble protein in seeds corresponding

with about 1625 EXU per gram of seed. One unit of endoxylanase activity (EXU) is defined as the amount of enzyme which liberates 3.346 micromoles of reducing sugars measured as xylose equivalents from 1% xylan from oat spelt at 40°C and pH 3.5.

Example 5

Stability of endoxylanase in tobacco seeds during pelleting

Pelleting conditions were such that the feed obtained a temperature of 65°C during conditioning and 75°C after pelleting. Experiments have been conducted on lab scale.

Milled seeds and the microbial enzyme product sold under the brandname Lyxasan Forte® (obtainable from BASF, Ludwigshafen, Germany) were added to the meal to a final concentration of 6500 EXU/kg of feed

It is concluded that endoxylanase produced in seeds is substantially more resistant to pelleting than the current formulation containing the microbial enzyme.

Table 3

Effects of pelleting on the stability of different formulations of endoxylanase

	Percentage of remaining activity
Commercial Lyxasan Forte® powder	15
Milled tobacco seeds expressing endoxylanase	50

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for improving stability of a polypeptide as an active ingredient in a thermomechanically processed composition comprising a polypeptide as an active ingredient, said process including the steps of

(i) providing transgenic plant material in which the polypeptide as an active ingredient is biosynthesized due to the expression of a transgene therein, and

(ii) subjecting the transgenic plant material in its entirety with said polypeptide to a thermomechanical process.

2. A process according to claim 1 wherein the thermomechanical process is selected from the group including pelleting, extrusion or expansion.

3. A process according to claim 1 or claim 2, wherein the transgenic plant material is seed.

4. A process according to any one of claims 1 to 3 wherein the polypeptide is heterologous to the transgenic plant.

5. A process according to any one of claims 1 to 4 wherein the polypeptide is an enzyme.

6. A process according to any one of claims 1 to 5, wherein the polypeptide is an enzyme selected from the group consisting of a phosphatase, a carbohydrase, a protease, and a lipase.

7. A process according to any one of claims 1 to 6, wherein the enzyme is a fungal phytase and/or a fungal xylanase.

8. A composition produced by the process according to any one of claims 1 to 7.

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9. Use of a composition according to claim 8, as a feed for animals or as a premix therefore.

10. A process for promoting the growth of animals wherein the animal is fed a diet which comprises a composition according to claim 8.

11. A process according to claim 1 substantially as hereinbefore described with reference to any one of the Examples.

DATED: 29 September, 2000
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