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Synergistic effect of the combination of phytases on hydrolysis of phytic acid

5 The invention relates to the synergistic effect of the combination of phytases on phytic acid hydrolysis.

Phytic acid salts (myo-inositol hexakisphosphate) or phytates (myo-inositol hexakis dihydrogen phosphate) are the major storage form of phosphorus in cereals,
10 leguminous plants and oil-yielding plants. They thus constitute the main source of phosphorus in plant-based animal feeds, the main components in the diets of monogastric animals (poultry and pigs). However, the bioavailability of this phosphorus in feeds is limited
15 for these animals. This is because they do not possess the intestinal enzymes that degrade phytates in sufficient amount to allow phytate hydrolysis and thus provide the amounts of inorganic phosphate that they require. In addition, phytic acid is an antinutritional
20 factor, which forms complexes with proteins and ions (Fe^{3+} , Ca^{2+} , Zn^{2+} , Mg^{2+}) and thus decreases the availability of these elements.

The feed intake of poultry and pigs must therefore be
25 supplemented with inorganic phosphate, while the phosphorus of the phytates is excreted and contributes to the eutrophication of surface water in areas where there is intensive rearing of monogastric animals.

30 Phytases (myo-inositol hexakisphosphate 3- and 6-phosphohydrolases EC 3.1.3.8 and 3.1.3.26) are part of the family of histidine acid phosphatases. Phytases are hexakisphosphohydrolases which hydrolyse the phosphoester bonds in phytic acid or phytate. Thus,
35 they catalyse the hydrolysis of myo-inositol hexakisphosphate (phytic acid, InsP_6) to inorganic monophosphates and to myo-inositol phosphates with a



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lower degree of phosphorylation (InsP₅ to InsP₁) and to free myo-inositol in certain cases.

Two classes of phytase exist, differentiated only by the position of the first phosphate hydrolysed. 3-Phytases (EC 3.1.3.8) hydrolyse the phosphate in the 3-position and 6-phytases (EC 3.1.3.26) hydrolyse the phosphate in the 6-position.

These enzymes, used as an additive in animal nutrition, make it possible, firstly, to increase the availability of the phytic phosphorus and, secondly, to improve the digestibility of the feeds. In addition, the release of phytic phosphate considerably decreases the costs due to supplementation with phosphate, and also the pollution caused by an excess of excreted phosphates.

Phytases are produced by a large variety of organisms: plants, animals and especially microorganisms. These phytases have very different biochemical characteristics, in particular their activity as a function of pH and their temperature stability. In addition, these enzymes exhibit differences in terms: (a) of their effectiveness in hydrolysing all the phosphates, (b) of their stereospecificity and (c) of their affinity with respect to inositol phosphates.

Among phytase-producing microorganisms, mention will in particular be made of: fungi of the genera *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus*, bacteria: *Pseudomonas* sp., *Klebsiella* sp., *Escherichia coli*, *Enterobacter* sp., *Bacillus subtilis* and yeast: *Saccharomyces cerevisiae*, *Candida tropicalis*, *Torulopsis candida*, *Debaryomyces castellii*, *Debaryomyces occidentalis* (synonym *Schwanniomyces castellii*), *Kluyveromyces fragilis*.

Numerous microorganism phytases have already been studied and used in various agro-industrial applications. The effectiveness of these enzymes in the feed and in the course of digestion in the animal
5 depend on their ability to maintain their potential, irrespective of the various conditions encountered during the feed preparation steps and in the digestive tract. Preparation of the feeds requires thermoresistant enzymes, whereas the pH values range,
10 for example, between 5.02, 2.75, 6.28, 6.63 and 5.98 during passage through the various crop, stomach, duodenum, jejunum and ileum compartments of poultry.

Most of the phytases described to date only partially
15 hydrolyse phytic acid and some with very slow kinetics. Thus, numerous phytases hydrolyse only 5 phosphate bonds of phytic acid, i.e. 83% of the potential phosphorus.

20 To date, among yeast, only the phytase of the yeast *Schwanniomyces occidentalis* has been described as being capable of hydrolysing all the phosphate bonds of phytate (EP 0 699 762 and Segueilha L., Lambrechts C., Boze H., Moulin G., Galzy P., (1992) Purification and
25 properties of a phytase from *Schwanniomyces castellii*, J. Ferm. Bioeng., 74, 7-11).

Under experimental conditions, complete hydrolysis of phytate or of phytic acid is not, however, obtained.

30

In order to obtain a more effective hydrolysis of phytic acid, it has thus been proposed to combine various phytases.

35 WO 98/30681 describes the combination of phytases having various stereospecificities, and in particular the combination of 3-phytase and of 6-phytase. This document describes in particular the combination of a

Peniophora lycii 6-phytase and an *Aspergillus niger* 3-phytase (phytase NovoTM).

US 6,183,740 also describes the combination of phytases
5 of various specificities and also the combination with other acid phosphatases.

Complete hydrolysis of phytic acid to phosphate and to inositol is not, however, observed.

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The problem that the present invention is intended to solve consists in improving the rate and the effectiveness of hydrolysis of phytic acid in order to obtain complete hydrolysis of phytic acid in various
15 agro-industrial applications.

This problem is solved by the compositions and the methods of the present invention in which at least two specific phytases are combined. Advantageously, the
20 compositions of the present invention allow complete hydrolysis of all the phosphate groups of phytic acid by virtue of the synergistic effect observed. Advantageously, the compositions of the present invention are active in a wide pH range suitable for
25 agro-industrial applications of phytases, and in particular for applications in the animal nutrition field. Another advantage of the present invention is that the phytases used are thermostable.

30 Sequence description

- SEQ ID No. 1: *Schwanniomyces castellii* phytase.
SEQ ID No. 2: *Debaryomyces castellii* phytase.
SEQ ID No. 3: *Aspergillus niger* phytase.
35 SEQ ID No. 4: *Penicillium funiculosum* phytase.
SEQ ID No. 5: *Peniophora lycii* phytase.
SEQ ID No. 6: Sequence encoding wheat phytase.
SEQ ID No. 7: Wheat (*Triticum aestivum*) phytase.

Description of the invention

A subject of the invention is a composition combining
5 at least two phytases for the hydrolysis of phytic acid
(myo-inositol 1,2,3,4,5,6-hexakisphosphate),
comprising:

- a first phytase exhibiting at least 80% identity
with the phytase of SEQ ID No. 1 or exhibiting at
10 least 80% identity with the phytase of SEQ ID
No. 2;
- a second phytase exhibiting at least 80% identity
with the phytase of SEQ ID No. 3.

15 Preferably, the first phytase is a 3-phytase which
catalyses the hydrolysis of the six phosphate bonds of
phytic acid.

Preferably, the first phytase has an optimum
20 temperature of between 55°C and 80°C and an optimum pH
of between pH 3.5 and pH 5.

In a preferred embodiment, the first phytase is a
phytase of the yeast *Schwanniomyces castellii* or a
25 phytase of the yeast *Debaryomyces castellii*.

In another preferred embodiment, the first phytase is
the phytase of SEQ ID No. 1 or the phytase of SEQ ID
No. 2.

30

Preferably, the second phytase is a 3-phytase which
catalyses the hydrolysis of at least five phosphate
bonds of phytic acid.

35 Preferably, the second phytase has an optimum
temperature of between 50°C and 60°C and an optimum pH
of between pH 2 and pH 6.

In a preferred embodiment, the second phytase is an *Aspergillus niger* phytase.

5 In another preferred embodiment, the second phytase is the phytase of SEQ ID No. 3.

Preferably, the compositions according to the invention consist of a nutritional additive for animals or of an animal feed.

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A subject of the invention is also the use of a composition according to the invention, for the manufacture of a nutritional additive for animals or of an animal feed.

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A subject of the invention is also the use of a composition according to the invention, for increasing the availability of phytic phosphorus and improving the digestibility of animal feeds.

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The present invention also relates to a method of hydrolysis of phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) to inorganic monophosphates, to myo-inositols with a lower degree of phosphorylation and to
25 free myo-inositol, comprising the following steps:

- a first phytase exhibiting at least 80% identity with the phytase of SEQ ID No. 1 or exhibiting at least 80% identity with the phytase of SEQ ID No. 2 is provided;
- 30 - a second phytase exhibiting at least 80% identity with the phytase of SEQ ID No. 3 is provided;
- the phytic acid is simultaneously brought into contact with the first phytase and the second phytase.

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Preferably, the first phytase is a 3-phytase which catalyses the hydrolysis of the six phosphate bonds of phytic acid.

Preferably, the first phytase has an optimum temperature of between 55°C and 80°C and an optimum pH of between pH 3.5 and pH 5.

5

In a preferred embodiment, the first phytase is a phytase of the yeast *Schwanniomyces castellii* or a phytase of the yeast *Debaryomyces castellii*.

- 10 In a particularly advantageous embodiment, the first phytase is the phytase of SEQ ID No. 1 or the phytase of SEQ ID No. 2.

Preferably, the second phytase is a 3-phytase which
15 catalyses the hydrolysis of at least five phosphate bonds of phytic acid.

Preferably, the second phytase has an optimum temperature of between 50°C and 60°C and an optimum pH
20 of between pH 2 and pH 6.

In an advantageous embodiment, the second phytase is an *Aspergillus niger* phytase.

- 25 In a particularly advantageous embodiment, the second phytase is the phytase of SEQ ID No. 3.

A subject of the invention is also a kit or assembly for feeding animals, comprising:

- 30 - the *Schwanniomyces castellii* phytase of SEQ ID No. 1 or the *Debaryomyces castellii* phytase of SEQ ID No. 2;
- the *Aspergillus niger* phytase of SEQ ID No. 3.

- 35 The compositions according to the invention combine a first phytase exhibiting at least 80% identity with the phytase of SEQ ID No. 1 or exhibiting at least 80% identity with the phytase of SEQ ID No. 2; and a second

phytase exhibiting at least 80% identity with the phytase of SEQ ID No. 3.

Surprisingly, this specific combination of phytases
5 makes it possible to obtain a synergistic effect on the hydrolysis of phytic acid. The compositions according to the invention thus catalyse a rapid and complete hydrolysis of phytic acid in a wide pH range.

10 The term "phytase" is intended to mean myo-inositol hexakisphosphate phosphohydrolases (EC 3.1.3.8 and 3.1.3.26). These enzymes catalyse the hydrolysis of myo-inositol 1,2,3,4,5,6-hexakisphosphate (phytic acid, InsP_6) to inorganic monophosphates and to myo-inositol
15 phosphate with a lower degree of phosphorylation (InsP_5 to InsP_1) and to free myo-inositol for certain phytases.

The compositions according to the invention comprise a
20 first phytase exhibiting at least 80% identity with the phytase of SEQ ID No. 1 or exhibiting at least 80% identity with the phytase of SEQ ID No. 2.

The phytase of SEQ ID No. 1 is a phytase of the yeast
25 *Schwanniomyces castellii*, while the phytase of SEQ ID No. 2 is a phytase of the yeast *Debaryomyces castellii*.

These phytases have very similar catalytic properties and are therefore interchangeable in the phytase
30 combinations according to the invention.

Preferably, the first phytase exhibits at least 80%, 90%, 95%, 98%, and preferably at least 99%, of amino acids that are identical with the phytase of SEQ ID
35 No. 1 or exhibit at least 80%, 90%, 95%, 98%, and preferably at least 99%, of amino acids identical with the phytase of SEQ ID No. 2.

Preferably, the first phytase has the same properties and in particular the same catalytic properties as the phytases of SEQ ID No. 1 and of SEQ ID No. 2. Preferably, the first phytase is isolated from other
5 strains of *Schwanniomyces castellii* or of *Debaryomyces castellii* or from other yeast. Alternatively, the first phytase can be obtained by site-directed mutagenesis techniques, for example.

10 The expression "amino acids that are identical" is intended to mean amino acids which do not vary between two sequences. The first phytase can exhibit a deletion, an addition or a substitution of at least one amino acid with respect to the phytases of SEQ ID No. 1
15 or of SEQ ID No. 2.

The phytases of SEQ ID No. 1 and of SEQ ID No. 2 have common catalytic properties. According to the invention, the first phytase used in the compositions
20 and the methods according to the invention exhibit a degree of identity with the phytase of SEQ ID No. 1 or of SEQ ID No. 2 and conserve these common catalytic properties.

25 Preferably, the first phytase has 3-phytase activity. Preferably, the first phytase catalyses the hydrolysis of the six phosphate bonds of phytic acid.

The 3-phytases (EC 3.1.3.8) hydrolyse firstly the
30 phosphate in the 3-position.

A phytase capable of hydrolysing all the phosphate bonds of phytic acid (1-, 2-, 3-, 4-, 5- and 6-positions) catalyses the hydrolysis of myo-inositol
35 1,2,3,4,5,6-hexakisphosphate (phytic acid, InsP_6) to inorganic monophosphates and to free myo-inositol.

Many phytases hydrolyse only 5 phosphate bonds of phytic acid, i.e. 83% of the potential phosphorus. These enzymes hydrolyse phytic acid to myo-inositol monophosphate but not to free myo-inositol.

5

The expression "activity of the phytase" is intended to mean the enzymatic activity of the phytase. This activity is expressed in International Units (I.U.) per milligram of protein. One I.U. of enzymatic activity is
10 the catalytic ability to convert one micromole of substrate per unit of time, at a given pH and at a given temperature.

Preferably, the first phytase has an optimum
15 temperature of between 55°C and 80°C and an optimum pH of between pH 3.5 and pH 5.

In a preferred embodiment, the first phytase is a phytase of the yeast *Schwanniomyces castellii* or a
20 phytase of the yeast *Debaryomyces castellii*.

In another particularly advantageous embodiment, the first phytase is the phytase of SEQ ID No. 1 or the phytase of SEQ ID No. 2.
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The compositions according to the invention comprise at least a second phytase exhibiting at least 80% identity with the phytase of SEQ ID No. 3.

30 The phytase of SEQ ID No. 3 is a phytase of the filamentous fungus *Aspergillus niger*.

Preferably, the second phytase exhibits at least 80%, 90%, 95%, 98%, and preferably at least 99%, of amino
35 acids that are identical with the phytase of SEQ ID No. 3.

Preferably, the second phytase conserves the properties and in particular the catalytic properties of the phytase of SEQ ID No. 3. Preferably, this phytase is isolated from other strains of *Aspergillus niger* or
5 from other filamentous fungi. Alternatively, this phytase can be obtained by site-directed mutagenesis techniques, for example.

The expression "amino acids that are identical" is
10 intended to mean amino acids which do not vary between two sequences. The second phytase can exhibit a deletion, an addition or a substitution of at least one amino acid with respect to the phytase of SEQ ID No. 3.

15 According to the invention, the second phytase used in the compositions and the methods of the invention therefore exhibits a degree of identity with the phytase of SEQ ID No. 3 and conserves the catalytic properties of the latter.

20 Preferably, the second phytase is a 3-phytase which catalyses the hydrolysis of at least five phosphate bonds of phytic acid.

25 Preferably, the second phytase has an optimum temperature of between 50°C and 60°C and an optimum pH of between pH 2 and pH 6. Even more preferably, the second phytase has a first optimum pH of between pH 2.5 and 3.5 and a second optimum pH of between pH 4.5 and
30 5.5.

In a preferred embodiment, the second phytase is an *Aspergillus niger* phytase.

35 In an advantageous embodiment, the second phytase is the phytase of SEQ ID No. 3.

The enzymatic activity of a phytase varies in particular as a function of the pH. Phytases thus have pH ranges (or spheres) in which their enzymatic activity is higher or optimal. The term "range" is intended to mean a given pH sphere or interval. Two phytases have enzymatic activities in complementary pH ranges when these phytases have an activity in different pH ranges. Preferably, these ranges overlap or partially superimpose. Typically, the phytases have a higher enzymatic activity at an acidic pH. Two phytases having complementary pH ranges have thus, for example, a maximum enzymatic activity between pH 2 and pH 4 for the first phytase and a maximum enzymatic activity between pH 4 and pH 6 for the second phytase. In the compositions and the methods of the present invention, the first and the second phytase preferably have complementary pH ranges or complementary optimum pHs. The compositions and the methods according to the invention thus allow hydrolysis of phytic acid in a wide pH range.

The methods of measuring and of identifying the degree of identity between polypeptides are known to those skilled in the art. Use may, for example, be made of Vector NTi 9.1.0, alignment program AlignX (Clustal W algorithm) (Invitrogen INFORMAX, <http://www.invitrogen.com>). Preferably, the default parameters are used.

The phytases of the compositions and methods according to the invention are isolated or purified from their natural environment. The phytases can be prepared by means of various methods. These methods are in particular purification from natural sources such as cells naturally expressing these phytases, production of recombinant phytases by appropriate host cells and subsequent purification thereof, production by chemical synthesis or, finally, a combination of these various

approaches. These various methods of production are well known to those skilled in the art. Thus, the phytases used in the compositions and the methods of the present invention can be isolated from
5 *Schwanniomyces castellii*, from *Debaryomyces castellii* or from *Aspergillus niger*. In another embodiment, the phytases of the present invention are isolated from recombinant host organisms.

10 Preferably, the compositions according to the invention consist of a nutritional additive for animals or of animal feed.

A subject of the invention is also the use of a
15 composition according to the invention, for the manufacture of a nutritional additive for animals or of an animal feed.

The present invention therefore also relates to feed
20 additives that provide a phytase activity. The provision of this type of enzymatic activity makes it possible to improve the digestibility of the feed and to improve its nutritional value.

25 The term "nutritional additive" is intended to mean a substance intentionally added to a feed, generally in small amounts, so as to improve its nutritional characteristics or its digestibility. The nutritional additives for animals can, for example, contain
30 vitamins, mineral salts, amino acids and enzymes.

The present invention also relates to the animal feeds. These feeds are usually in the form of meals or of granules into which the additives according to the
35 invention are incorporated. The term "feed" is intended to mean anything that can be used for feeding animals. For the intensive rearing of animals, the animal feeds usually comprise a nutritional base and nutritional

additives. The term "nutritional base" is intended to mean that which constitutes most of the animal's food intake, consisting, by way of example, of a mixture of cereals, of proteins and of fats of animal and/or plant origin.

The nutritional bases for animals are suitable for the diet of these animals and are well known to those skilled in the art. Usually, these nutritional bases comprise, for example, maize, wheat, pea and soybean. These nutritional bases are suited to the needs of the various animal species for which they are intended. These nutritional bases may already contain nutritional additives such as vitamins, mineral salts and amino acids.

In a preferred embodiment, the invention relates to feeds for monogastric animals, and in particular for poultry and pigs. The poultry comprise in particular laying hens, chickens for meat, turkeys and ducks. The pigs comprise in particular growing and finishing pigs and also piglets.

A subject of the invention is also the use of a composition according to the invention, for increasing the availability of phytic phosphorus and improving the digestibility of animal feeds.

The compositions and the methods according to the invention combine a first phytase capable of hydrolysing all the phosphate bonds of phytic acid, chosen from *Schwanniomyces castellii* phytases and *Debaryomyces castellii* phytases, and a second phytase capable of hydrolysing at least five phosphate bonds of phytic acid and having an enzyme activity in a pH range complementary to the pH range of the first phytase.

Figures

Figure 1: Comparison of the kinetics of hydrolysis of phytic acid at various pHs for the 6 phytases alone or combined in pairs. The results are expressed as % of theoretical phosphates released.

Figure 2: Percentage hydrolysis of phytic acid by the various phytases after reaction for 120 minutes. (A) phytases alone, (B) combined phytases. The dotted lines correspond to the hydrolysis of 5 phosphate bonds.

Figure 3: Comparison of the initial rates of hydrolysis of phytic acid ($\mu\text{mol.ml}^{-1}.\text{min}^{-1}$) for 6 phytases at various pHs. (A) phytases alone, (B) combined phytases. The rates are calculated during the first 10 minutes of the reaction.

Examples

Materials and methods

1. Phytase origins

▪ *Aspergillus niger* Natuphos (AN): batch R2503 or batch 057NPHO2

Wim van Hartingsveldt, Cora M. J. van Zeijl, G. Marian Hartevelde, Robin J. Gouka, Marjon E.G. Suykerbuyk, Ruud G. M. Luiten, Peter A. van Paridon, Gerard C. M. Selten, Annemarie E. Veenstra, Robert F. M. van Gorcom and Cees A. M. J. van den Hondel; Cloning, characterization and overexpression of the phytase-encoding gene (*phyA*) of *Aspergillus niger*. Gene, 127, (1) 87-94 (1993)

▪ *Peniophora lycii* (PL): batch 172NHSO2

Lassens, S.F., Breinholt, J., Ostergaard, P.R., Brugger, R., Bischoff, A., Wyss, M. and Fuglsang, C.C.; Expression, gene cloning, and characterization of five novel phytases from four basidiomycete fungi: *Peniophora lycii*, *Agrocybe pediades*, a *Ceriporia* sp.,

and *Trametes pubescens*. Appl. Environ. Microbiol. 67 (10), 4701-4707. (2001)

▪ *Penicillium funiculosum* (PF): Godo A1346 batch 1301

5 WO 03054199

▪ Wheat (B): Sigma reference P1259.

WO 0183763

▪ *Schwanniomyces castellii* (SC): produced in *Candida boidinii* batch 10059 CN 25

10 EP 0931837

▪ *Debaryomyces castellii* strain CBS 2923 (DC).

2. Enzymatic method

The phytase activity is measured by following the release of inorganic phosphate over time.

The activity is measured in the presence of 8 mM of sodium phytate (Sigma) dissolved in a 200 mM sodium acetate buffer, pH 5.5, at 37°C (5 volumes). The reaction is triggered by the addition of the enzymatic extract (1 volume). The reaction is stopped by acidification of the medium with 20% trichloroacetic acid (1 volume of reaction medium + 1 volume of acid). The amount of phosphate released is determined after various incubation times.

One enzymatic unit (U) is defined as the amount of enzyme which releases one μ mole of inorganic phosphate in one minute.

30

3. Conditions for hydrolysis of phytates at various pHs

The hydrolysis is carried out at 40°C in 10 ml of 250 mM acetate buffer for pHs 4 to 6.5 or of 200 mM glycine buffer at pH 3, containing: 0.340 mM of sodium phytate, 0.04 or 0.08 U/ml of phytase. Samples are taken at various times for 120 minutes. The reaction is stopped by the addition of trichloroacetic acid.

The phosphates are assayed directly without dilution.

4. Phosphate assay

The amount of phosphate released is visualized by colorimetry. The visualizing solution, prepared extemporaneously, contains iron sulphate (380 mM, 1 volume) and ammonium heptamolybdate (12 mM, 4 volumes). The absorbance is measured at 700 nm after visualization for 30 minutes at ambient temperature (1 volume of reaction medium + 1 volume of visualizing solution), using a UV/visible spectrophotometer (Beckman DU 530).

A calibration line is established beforehand with potassium dihydrogen phosphate.

Results

1. Phytases tested

The phytases tested differ from one another according to various criteria: (1) biochemical characteristics (optimum pH, stereospecificity: 3, 4, or 6 phytases), (2) number of phosphate bonds hydrolysed.

All these phytases have an optimum hydrolysis pH close to 5; however, the AN and PL phytases have a second optimum pH at 2.5. Three completely hydrolyse the phosphates (SC, DC, wheat). The position hydrolysed first is either the 3-position (AN, PL, DC) or the 6-position (PL), or the 4-position (wheat) (Table 1).

Table 1: Comparison of the biochemical characteristics of the phytases

Origin	Km (μ M)	Optimum pH	Optimum temperature (°C)	Number of phosphate bonds hydrolysed	Stereospecificity (order of hydrolysis of phosphate bonds)
<i>Aspergillus niger</i> ⁽¹⁾	40/27	2.5-5.5	55/58	5	3/4/5/6/1

<i>Peniophora lycii</i> ⁽¹⁾	33	2.5-5.5	58	5	6/3 (pH5.5) 3.6 (pH3 and 5.5)
<i>Penicillium funiculosum</i> ⁽²⁾	550	4-5	50	5 or 6	3/4
Wheat ⁽³⁾	22	5-7	55	6	4/3/5/6/1/2
<i>Schwanniomyces castellii</i> ⁽⁴⁾	38	4.4	77	6	?
<i>Debaryomyces castellii</i>	236	4-4.5	60	6	3/4/5/6/1-2

(1) Ullah, A.H.J. and Sethumadhavan, K., (2003) PhyA gene product of *Aspergillus ficuum* and *Peniophora lycii* produces dissimilar phytases. Biochemical and Biophysical Research Communications 303: 463-468

5 (2) WO 03054199 filed June 13, 2003 Bohlmann, R., Moussu, F., Nore, O., Pierrard, J., Saunier, D., Testeniere, O., New polynucleotide encoding fungal phytase, useful as feed additive to improve phosphate assimilation, also new strain of *Penicillium*.

10 (3) Nakano, T., Joh, T., Narita, K., Hayakawa, T. (2000) The pathway of dephosphorylation of myo-Inositol Hexakisphosphate by Phytases from Wheat Bran of *Tricicum aestivum* L. cv Nourin#61. Biosci. Biotechnol. Biochem., 64:995-1003

15 (4) Segueilha, L., Lambrechts, C., Boze, H., Moulin, G. and Galzy, P. (1992) Purification and Properties of the Phytase from *Schwanniomyces castellii*. J. Ferment. Bioeng. 74(1):7-11.

20 2. Comparison of the effectiveness of phytic acid hydrolysis by 6 phytases at various pHs

The study is carried out in the presence of 0.340 mM of phytate. The amount of enzyme introduced corresponds to
 25 0.08 U/ml of phytase, measured under standard conditions at pH 5.5. The pH values tested are 3, 4, 5.5, 6, 6.5. The reaction is followed for 120 minutes. Samples are taken over time, the reaction is stopped by

acidification, and the phosphates are assayed by colorimetry.

5 The comparison of the various phytases is carried out as a function of the hydrolysis efficiency, i.e. the percentage of phosphate released relative to the theoretical percentage. The results are expressed in Figure 1A.

10 According to the literature, 4 phytases (PF, SC, DC, B) are capable of completely hydrolysing phytic acid to inositol and phosphate; 2 phytases (AN and PL) hydrolyse only 5 bonds, i.e. 83% of the potential phosphorus.

15 Under the conditions tested at pH 3 and pH 4, 4 phytases (SC, PF, DC and PL) hydrolyse at least 80% of the phytic acid. The wheat phytase is not very active at pH 3 and pH 4. For pH values above pH 5.5, no
20 phytase hydrolyses more than 70% of the phytic acid. The PF and DC phytases are not very active at pH 6.5. The AN phytase is the least sensitive to variations in pH; however, it releases only 60 to 70% of the phosphates, i.e. values 10 to 20% lower than those
25 expected (Fig. 2A).

The comparison of the initial rates of phosphate release show a great deal of heterogeneity between the phytases (Fig. 3A). The most efficient phytase at
30 acidic pH is that of *Schwanniomyces castellii*, whereas that of *A. niger* is the most efficient at pH values above 5.5. The wheat phytase is the least efficient irrespective of the conditions tested.

35 3. Influence of pH on the rate of phosphate release in the presence of combined phytases

In order to improve the efficiency of phytic acid hydrolysis, several combinations of two phytases are tested. Each phytase is introduced at 0.04 U/ml, i.e. a total value of 0.08 U/ml in the reaction tube. The DC
5 phytase, which hydrolyses 6 phosphate bonds, is systematically tested in combination with the other phytases, which hydrolyse 5 or 6 phosphate bonds. Two other combinations are also used to compare, firstly, another combination of phytases able to release 6 and 5
10 phosphates (SC/AN) and, secondly, two phytases, PL/AN, having very similar biochemical characteristics (Fig. 1B).

An overall analysis makes it possible to show that the
15 combination of 2 phytases mostly promotes the efficiency of phytic acid hydrolysis. Although, under the same conditions, no phytase completely hydrolyses phytic acid, for 2 mixtures (DC/AN and SC/AN), 100% to 85% of the phosphate bonds are hydrolysed whatever the
20 pH. For the DC/PL combination, a gain of approximately 20% at acidic pHs is also observed. The PL/AN, DC/PF and DC/SC combinations provide little improvement (Fig. 2B).

25 The comparison of the initial hydrolysis rates shows that a strong synergistic effect exists for the SC/AN combination and for the DC/AN combination compared with the phytases alone and compared with the other phytase combinations. The pH-related differences in hydrolysis
30 rate are minimized when the combination is used. The other combinations are less efficient, the synergistic effect is visible only for certain pHs, for example for DC/PL at pH 3 and pH 4 (Fig. 3).

35 The synergistic effect appears to be due, to a large extent, to the ability of the phytases to hydrolyse all the phosphates. In addition, the best combinations concern phytases having complementary optimum pHs for

activity (2.5-4.5). It should, however, be noted that there is little synergistic effect for the phytases that are the least effective in terms of hydrolysis efficiency, such as that of wheat and of *Penicillium*
5 *funiculosum*.

4. Influence of the concentrations and of the proportion of phytases on the efficiency of phytic acid hydrolysis

10

We compared the influence of the amount of phytase (dose effects) and of the proportion of the mixture of phytases on phytic acid hydrolysis. Three combinations were used, DC/AN, DC/PL and AN/PL, and 2 phytase
15 concentrations (0.04 and 0.08 U/ml) were tested (Tables 2 and 3).

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Table 2: Comparison of the percentage phytic acid hydrolysis for the various combinations, tested at two final concentrations, 0.04 and 0.08 U/ml. The first three columns give the values for the three phytases alone at the concentration 0.08 U/ml.

	AN (0.08U)	DC (0.08U)	PL (0.08U)	AN/DC (0.08U)	AN/DC (0.04U)
pH 3	66	82	80	92	67 (73%)
pH 4	69	85	83	95	96 (101%)
pH 5.5	55	63	67	92	81 (87%)
pH 6	72	42	64	94	78 (83%)
pH 6.5	63	6	34	75	42 (56%)

	PL/DC (0.08U)	PL/DC (0.04U)	AN/PL (0.08U)	AN/PL (0.04U)
pH 3	94	70 (74%)	79	56 (72%)
pH 4	100	93 (94%)	86	76 (89%)
pH 5.5	85	71 (84%)	70	66 (94%)
pH 6	85	47 (56%)	74	67 (90%)
pH 6.5	35	10 (30%)	61	52 (86%)

The values between parentheses represent the ratio of the values obtained with 0.04 U/ml to those in the presence of 0.08 U/ml.

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The percentages of hydrolysis during the study with the mixture of enzymes (overall concentration 0.08 U/ml) are higher than those determined with the enzymes alone (0.08 U/ml) and close to 100% for the AN/DC and PL/DC mixtures, in a pH range varying from 3 to 6. The AN/PL mixture does not significantly increase the percentage hydrolysis observed with each phytase taken separately. The 2-fold reduction of the overall amount provided (0.04 U/ml) does not induce a 2-fold decrease in released phosphates. In certain cases, AN/DC and PL/DC at pH 4 and pH 5.5, and AN/PL at pHs 4 to 6, the percentages of hydrolysis are equivalent to those observed in the presence of 0.08 U/ml (Table 3).

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Table 3: Comparison of the initial rates of hydrolysis ($\mu\text{mol/ml/min}$) of phytic acid for the various combinations, tested at two final concentrations 0.04 and 0.08 U/ml.

- 5 The first three columns give the values for the three phytases alone at the concentration 0.08 U/ml.

	AN (0.08U)	DC (0.08U)	PL (0.08U)	AN/DC (0.08U)	AN/DC (0.04U)
pH 3	2.04	1.78	2.52	4.00	1.70 (42%)
pH 4	4.98	3.96	5.36	6.49	2.85 (44%)
pH 5.5	7.76	1.56	4.00	7.26	1.86 (26%)
pH 6	7.91	0.39	2.87	7.53	1.04 (14%)
pH 6.5	5.09	0.00	0.85	3.71	0.26 (7%)

	PL/DC (0.08U)	PL/DC (0.04U)	AN/PL (0.08U)	AN/PL (0.04U)
pH 3	3.88	1.45 (37%)	2.18	1.60 (73%)
pH 4	8.61	3.33 (39%)	4.29	2.73 (63%)
pH 5.5	3.79	0.85 (22%)	4.56	2.82 (62%)
pH 6	2.10	0.04 (2%)	3.96	2.60 (66%)
pH 6.5	0.22	0.00 (0%)	1.89	0.74 (39%)

- 10 The values between parentheses represent the ratio of the rates obtained with 0.04 U/ml to those in the presence of 0.08 U/ml.

15 The combination of phytase therefore makes it possible to decrease the amount of phytase in the reaction medium by a factor two, while maintaining an identical hydrolysis efficiency, within a broad pH range. There is a clear dose effect on the release of phosphate.

20 On the other hand, the initial rates of phytic acid hydrolysis are at least two times lower in the presence

of 0.04 U/ml of phytase than in the presence of 0.08 U/ml (Table 3). These results support the hypothesis that the synergy between the phytases essentially allows complete hydrolysis of the phosphates present.

The proportion of each phytase in the reaction medium does not appear to have a great deal of influence on the hydrolysis efficiency (Table 4). In all the cases, an increase in the amount of phosphate released is observed, with a slight advantage for a combination containing 50% of each phytase, mainly for pHs above 5.5.

Table 4: Comparison of the percentage phytic acid hydrolysis as a function of the proportion of each phytase in the mixture. In all cases, the overall amount remains 0.08 U/ml.

Proportion of phytases	AN	DC	AN/DC (1/1)	AN/DC (1/2)	AN/DC (2/1)
pH 3	66	82	92	90	88
pH 4	69	85	95	93	93
pH 5.5	55	63	92	79	79
pH 6	72	42	94	87	86
pH 6.5	63	6	75	52	64

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Under our experimental conditions, no phytase completely hydrolyses phytic acid to inositol and phosphates, although 4 of them are capable of releasing all the phosphates according to the literature. Four phytases SC, PF, DC and PL, hydrolyse 80% of the phosphate bonds at pH values of 3 and 4. Two mixtures, DC/AN and SC/AN, hydrolyse from 80% to 100% of the phosphate bonds at all the pHs tested.

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The combination of phytase of 3-phytase (AN, DC, PF), 4-phytase (B) or 6-phytase (PL) type, with one another, does not provide any notable improvement.

- 5 The complementation and the synergy between phytases appears to depend on 2 criteria specific to each phytase: (1) on their profile of activity as a function of pH, (2) on their ability to hydrolyse, or not, all the phosphates.

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- The use of such combinations in animal nutrition could make it possible to decrease the amounts of phytases used by at least 20% and therefore to reduce costs. The content of phosphate of phytic origin in the feed would
15 also be increased, improving the food value and thus making it possible to decrease the intake of inorganic phosphate. The pollution caused by unavailable phosphate waste would thus be decreased.

CLAIMS

1. Composition combining at least two phytases for the hydrolysis of phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate), characterized in that it comprises:
 - a) a first phytase exhibiting at least 80% identity with the phytase of SEQ ID No. 1 or exhibiting at least 80% identity with the phytase of SEQ ID No. 2;
 - b) a second phytase exhibiting at least 80% identity with the phytase of SEQ ID No. 3.
2. Composition according to Claim 1, characterized in that the first phytase is a 3-phytase which catalyses the hydrolysis of the six phosphate bonds of phytic acid.
3. Composition according to one of the preceding claims, characterized in that the first phytase has an optimum temperature of between 55°C and 80°C and an optimum pH of between pH 3.5 and pH 5.
4. Composition according to one of the preceding claims, characterized in that the first phytase is a phytase of the yeast *Schwanniomyces castellii* or a phytase of the yeast *Debaryomyces castellii*.
5. Composition according to one of the preceding claims, characterized in that the first phytase is the phytase of SEQ ID No. 1 or the phytase of SEQ ID No. 2.
6. Composition according to one of the preceding claims, characterized in that the second phytase is a 3-phytase which catalyses the hydrolysis of at least five phosphate bonds of phytic acid.

7. Composition according to one of the preceding claims, characterized in that the second phytase has an optimum temperature of between 50°C and 60°C and an optimum pH of between pH 2 and pH 6.
- 5 8. Composition according to one of the preceding claims, characterized in that the second phytase is an *Aspergillus niger* phytase.
- 10 9. Composition according to one of the preceding claims, characterized in that the second phytase is the phytase of SEQ ID No. 3.
- 15 10. Composition according to one of the preceding claims, characterized in that it consists of a nutritional additive for animals or of an animal feed.
- 20 11. Use of a composition according to one of Claims 1 to 9, for the manufacture of a nutritional additive for animals or of an animal feed.
- 25 12. Use of a composition according to one of Claims 1 to 10, for increasing the availability of phytic phosphorus and improving the digestibility of animal feeds.
- 30 13. Method of hydrolysis of phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) to inorganic monophosphates, to myo-inositols with a lower degree of phosphorylation and to free myo-inositol, characterized in that it comprises the following steps:
- 35 a) a first phytase exhibiting at least 80% identity with the phytase of SEQ ID No. 1 or exhibiting at least 80% identity with the phytase of SEQ ID No. 2 is provided;

- b) a second phytase exhibiting a least 80% identity with the phytase of SEQ ID No. 3 is provided;
- c) the phytic acid is simultaneously brought into contact with the first phytase and the second phytase.
14. Method according to Claim 13, characterized in that the first phytase is a 3-phytase which catalyses the hydrolysis of the six phosphate bonds of phytic acid.
15. Method according to either of Claims 13 and 14, characterized in that the first phytase has an optimum temperature of between 55°C and 80°C and an optimum pH of between pH 3.5 and pH 5.
16. Method according to one of Claims 13 to 15, characterized in that the first phytase is a phytase of the yeast *Schwanniomyces castellii* or a phytase of the yeast *Debaryomyces castellii*.
17. Method according to one of Claims 13 to 16, characterized in that the first phytase is the phytase of SEQ ID No. 1 or the phytase of SEQ ID No. 2.
18. Method according to one of Claims 13 to 17, characterized in that the second phytase is a 3-phytase which catalyses the hydrolysis of at least five phosphate bonds of phytic acids.
19. Method according to one of Claims 13 to 18, characterized in that the second phytase has an optimum temperature of between 50°C and 60°C and an optimum pH of between pH 2 and pH 6.

20. Method according to one of Claims 13 to 19, characterized in that the second phytase is an *Aspergillus niger* phytase.
- 5 21. Method according to one of Claims 13 to 20, characterized in that the second phytase is the phytase of SEQ ID No. 3.
- 10 22. Kit or assembly for feeding animals, characterized in that it comprises:
- a) the *Schwanniomyces castellii* phytase of SEQ ID No. 1 or the *Debaryomyces castellii* phytase of SEQ ID No. 2;
 - b) the *Aspergillus niger* phytase of SEQ ID No. 3.