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Novel Long-Term Phytase from *Serratia odorifera*: Cloning, Expression, and Characterization

Ignasi Salaet,[⊥] Ramon Marques,[⊥] Tula Yance-Chávez, Judit Macías-Vidal, David Gimémez-Zaragoza, and Rosa Aligue*

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ABSTRACT: The *appA-So* gene, encoding a phytase from *Serratia odorifera*, was cloned and heterologously expressed in *Komagataella phaffii*. The open reading frame of *appA-So* comprised 1281 bp that encoded a 426-amino acid protein, including a 27-amino acid signal peptide. The encoded phytase, AppA-So, showed 52% homology with other histidine acid phosphatases. The purified recombinant phytase showed optimal activity at 55 °C and pH 4.5, exhibiting enzymatic activity between pH 3.7 and 5.8, with a specific activity of 1123 U/mg at pH 4.5 and 37 °C. The AppA-So protein retained more than 85% of its initial activity after incubation in different pH conditions (pH 2.5−6.5) at 37 °C for 3 h. AppA-So activity was maintained over time and displayed a low Michaelis−Menten constant (Km) of 0.093 g/L. To the best of our knowledge, this is the first report of the cloning and characterization of the phytase from *S. odorifera*. Comparison of AppA-So with other well-known phytases suggests that the *S. odorifera* phytase has the lowest Km and highest stable activity over time, making it very suitable for use in the animal feed industry.

KEYWORDS: *phytase enzyme, histidine acid phosphatase, Serratia odorifera, Komagataella phaffii*

INTRODUCTION

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphophate) is the primary source of phosphorus in cereals and legumes (80% of the total phosphorus).[1] However, monogastric animals have very low or no phytase activity in their digestive tracts and are unable to digest phosphorus in the form of phytate. This results in phosphorus pollution in areas of intensive animal production because almost all of the dietary phytate phosphorus ingested by the animals is excreted into the environment.[1−3] Moreover, phytate decreases the nutritional value of animal feed because it can form insoluble complexes with nutritionally important metals, thereby preventing their uptake.[2,4−7] To overcome these problems, phytases (myo-inositol hexakisphosphate phosphohydrolases, E.C.3.1.3.8) have been widely used in animal feed.[8−10] Phytases belong to a special group of phosphatases that are capable of hydrolyzing phytate, thereby releasing free phosphorus. The addition of phytases to feed for monogastric animals increases phosphorus availability (thereby increasing its nutritive value), decreases the need for inorganic phosphorus supplementation, and prevents phosphorus pollution.[11−13] Therefore, most of the recent studies on phytases have focused on identifying novel phytases, increasing phytase production to decrease costs, and improving the desired enzymatic characteristics.[14−16] Although several phytase genes have been isolated from various sources,[15,17,18] only a few phytases have been widely used in industry because of their relative instability and their high costs.[10,15,19] The industrial demand for phytases with high specific activity and stability under the high temperature conditions used for feed pellet production and under the acidic conditions found in the stomach of monogastric animals continues to drive the search for new enzyme sources. Phytases that are stable under acidic and high temperature conditions can remain active in the digestive tracts of monogastric animals, therefore reducing the quantity needed and lowering the cost of animal production.

In this paper, the *appA-So* gene encoding a phytase from *Serratia odorifera* was cloned and heterologously expressed in *Komagataella phaffii*. We performed a detailed biochemical characterization of the recombinant phytase and found that it was highly stable over time with a low Michaelis−Menten constant (Km), showing significant advantages over other conventional phytases.

MATERIALS AND METHODS

Strains and Chemicals. The strains used were *S. odorifera 867T* (strain ATCC 33077/CECT 867) and *Komagataella phaffii 11047* (strain ATCC 76273/CBS 7435/CECT 11047), originally from the genus *Pichia* and recently reassigned to *Komagataella*.[20] Phytic acid, the sodium salt, was purchased from Sigma (68388). All other chemicals were of analytical grade.

Cloning of the *appA-So* and *appAs-OP* Genes. The coding region of the *S. odorifera* phytase gene (GenBank accession no. EFE96977.1), without the part encoding the first 26 amino acids containing the signal peptide, was inserted into a plasmid containing: the alcohol oxidase (*AOX1*) gene promoter region, the α-factor from *Saccharomyces cerevisiae*, and the *Eco*RI and *Xba*I restriction sites as well as the *AOX1* terminator. The phytase gene was inserted between

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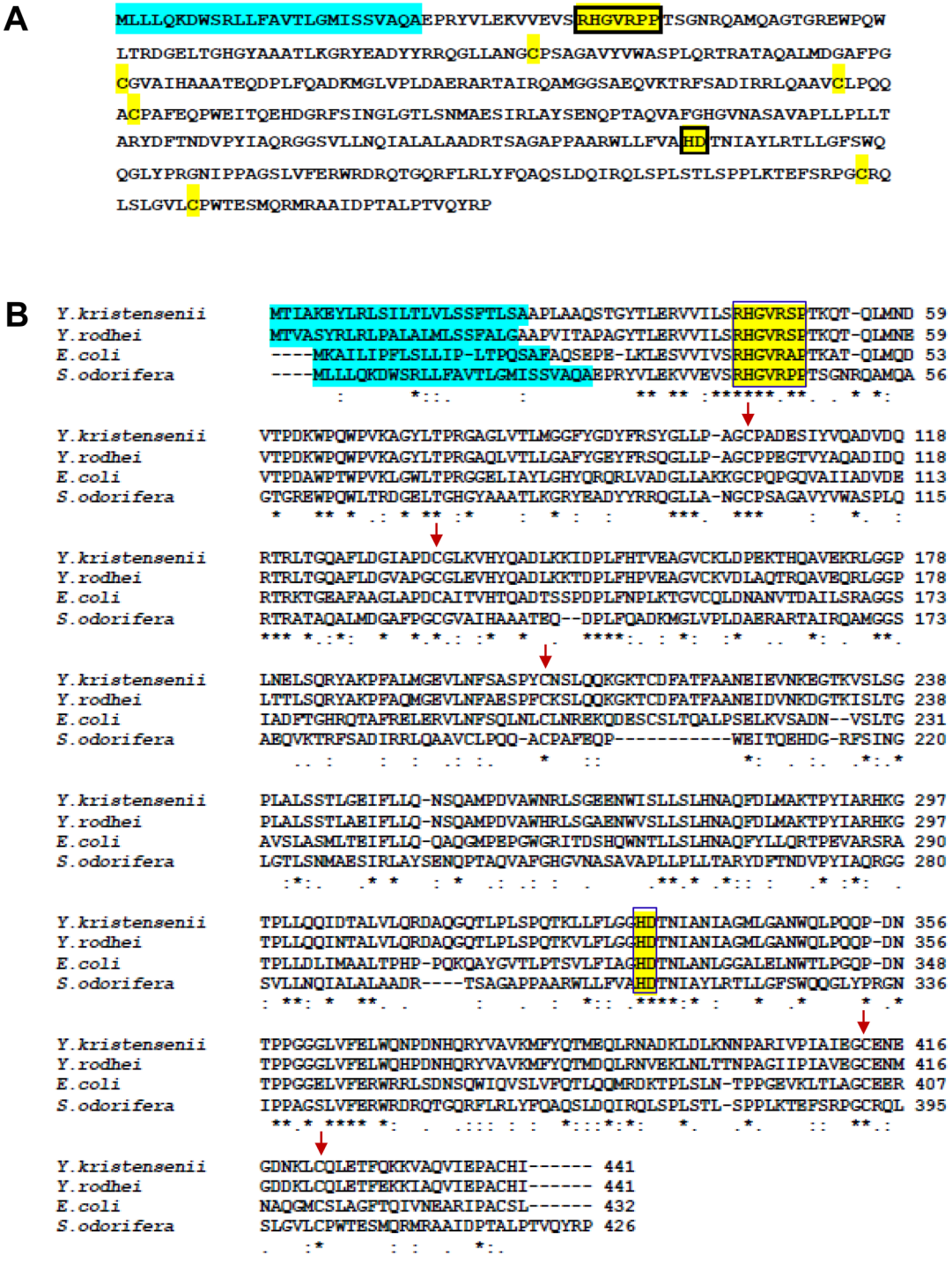

Figure 1. AppA-So phytase from *Serratia odorifera*. (A) Amino acid sequence of the AppA-So phytase from *S. odorifera*. The conserved active site motifs of the HAP family are shown in the yellow boxes. The signal peptide conserved among bacterial phytases is shown in the blue box. (B) Multiple sequence alignment of AppA-So with the other HAP phytases, using the Clustal Omega program. Conserved active site motifs are boxed in blue and yellow, while the cysteine residues are indicated by the arrows. The GenBank accession numbers of the proteins are *S. odorifera* EFE96977.1; *E. coli* AMH85921.1; *Y. kristensenii* ABX75421.1; and *Y. rohdei* EEQ02229.1.

*Eco*RI and *Xba*I sites to create the *appA-So* plasmid, which was generated by Genescript. The *appAs-OP* plasmid contained the optimized sequence of *appA-So* inserted between the *Eco*RI and *Xba*I sites.

Expression of the Recombinant Phytases AppA-So and AppAs-OP in *Komagataella phaffii*. The *appA-So* and *appAs-OP* genes were integrated by homologous recombination into the *K. phaffii* chromosome after linearization of the vector using the *Pme*I restriction enzyme. The integrated colonies were selected by zeocin resistance. The clone with the highest phytase activity was selected and incubated in a shaker flask containing 300 mL of BMGY medium (1% yeast extract, 2% peptone, 1.34% YNB [yeast nitrogen base with ammonium sulfate and without amino acids], 0.00004% biotin, and 1% glycerol) at 30 °C and 220 rpm for 24 h. The cells were pelleted and resuspended in 100 mL of BMMY medium (1% yeast extract, 2% peptone, 1.34% YNB, 0.000 04% biotin, and 0.5% methanol) to induce the expression of the phytase. The enzyme activity of the culture supernatant was examined every 24 h after the addition of

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ACS Food Sci. Technol. 2021, 1, 689−697

0.5% methanol to the BMMY medium. When it reached the maximum value, the culture supernatant was collected by centrifugation (7000*g* for 5 min), and the cell precipitate was discarded. The supernatant was concentrated by centrifugation with VIVASPIN 20 (50 000 MWC Sartorius Studium). The concentrated solution was then dialyzed in sodium acetate buffer (0.25 M, pH 5.5). Finally, the phytase was purified by gel filtration (Sephacryl S-200) using the same buffer used in the dialysis. This process was used to concentrate the supernatant 10 times.

Protein samples were analyzed by 10% SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) stained by Comassie blue.

To determine the specific activity of a known concentration of the purified enzyme, it was used the Bradford method,[21] with bovine serum albumin as the reference.

Enzyme Activity of AppA-So/AppAs-OP. Phytase activity was determined using the vanadate-molybdate method in acid medium (European ISO 30024; https://www.iso.org/standard/45787.html). Briefly, 40 μL of enzyme solution were incubated with 1160 μL of substrate solution (5 mM sodium phytate in 0.25-M sodium acetate buffer, pH 5.5) at 37 °C for 30 min. The reaction was stopped by adding 800 μL of the STOP solution (14% nitric acid, 3.3% ammonium heptamolybdate tetrahydrate, and 0.078% vanadate). As a control, the STOP solution was added to the enzyme solution before incubation with the substrate solution. After 10 min in the STOP solution, the released inorganic phosphate was analyzed by colorimetric quantification at 415 nm. One unit (U) of phytase activity was defined as the amount of enzyme required to liberate 1 μmol of phosphate per minute at 37 °C.

Enzyme Properties of AppAs-OP. The effect of pH on phytase activity was evaluated by incubating the purified enzyme with sodium phytate at 37 °C for 30 min in the following buffers (100 mM): glycine−HCl, pH 1.0−3.5; sodium acetate−acetic acid, pH 3.5−6.0; Tris−HCl, pH 6.0−8.5; and glycine−NaOH, pH 8.5−12.0. The buffers used to dilute the phytase contained 0.05% bovine serum albumin and 0.05% (w/v) Tween. To assess the stability of the phytase, the purified enzymes were preincubated in the aforementioned buffer systems in the absence of the substrate for 3 h before their phytase activity was measured at pH 4.5 and 37 °C (European ISO 30024).

The effect of temperature on phytase activity was measured by performing the phytase activity assay over a temperature range of 35−85 °C at 5 °C intervals in sodium acetate (pH 4.5). The thermostability of phytase was investigated by assessing enzyme activity after incubating the purified enzyme at each temperature for 10 min in sodium acetate (pH 4.5).

To determine the effects of proteases on AppAs-OP activity, phytase activity was also assayed, following the European ISO 30024 method, by incubating the phytase for different amounts of time with 5 mg/mL of pepsin or 50 mg/mL of trypsin at 37 °C.

The Km value of AppAs-OP and phytases from different commercial companies (whose names have been changed to capital letters in this work) were determined by incubating the purified enzyme (0.1 U at optimum pH) with phytic acid (5 mM) at 37 °C in 0.25 M sodium acetate (pH 4.5 to pH 5.5). The different phytases were supplied by BASF, Novozymes, ABvista, and Dupont. The solid phytase preparations were extracted with sodium acetate buffer (0.1 M) to give a final activity of 50 U/mL, the same activity that for the liquid phytases.

RESULTS

Sequence Analysis and Molecular Cloning of *appA-So*. After analyzing genes showing homology with those encoding phytases from the HAP (histidine acid phosphatase) family, we focused on a gene from *Serratia odorifera DSM 4582* that corresponded by homology to a phytase gene we called *appA-So*. *appA-So* has an open reading frame of 1281 bp that encodes 426 amino acid residues. The amino acid sequence encoded by *appA-So* contains the highly conserved active sites RHGXRXP and HD of the HAP family[22] and the putative signal peptide cleavage site located between residues 26 and 27 (Figure 1A), as predicted by the SignalP 4.0 software.

The AppA-So protein was compared with several bacterial phytases, exhibiting 34%, 32%, and 31% identity with the phytases from *Yersinia rohdei*, *Yersinia kristensenii*, and *Escherichia coli*, respectively (Figure 1B). The AppA-So protein also contains six cysteine residues that are involved in the formation of disulfide bonds, five of which are conserved among enteric bacterial phytases.

The full-length phytase gene of *S. odorifera* (GenBank accession no. EFE96977.1), lacking the part encoding the first 26 amino acids containing the signal peptide, was cloned into an *AOX1* promoter-derived vector (see Materials and Methods).

Expression of the AppA-So Phytase in *K. phaffii*. The recombinant plasmid (p-*appA-So*) was expressed in *K. phaffii*. One of the transformants was chosen for the induction of phytase expression by methanol. Culture samples were collected at different times. The supernatant was obtained it was concentrated (see Materials and Methods). The molecular weight of the novel phytase determined by SDS-PAGE was about 45 kDa, similar to the value predicted based on the amino acid sequence (Figure 2A).

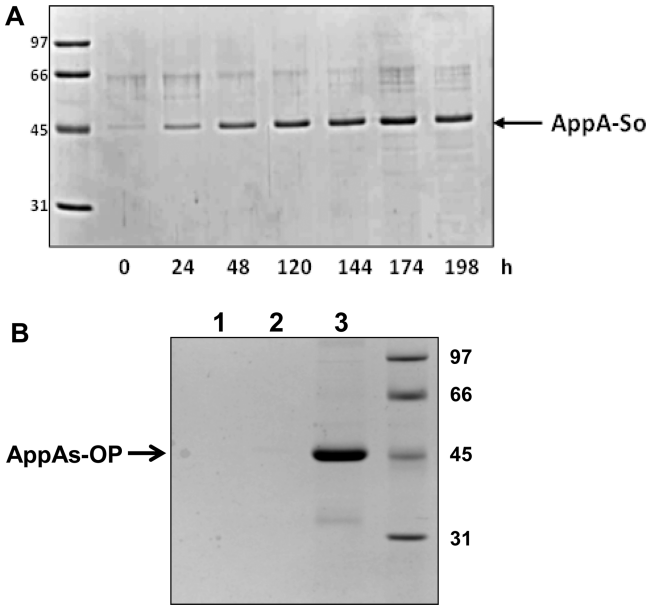

Figure 2. Expression of AppA-So and AppAs-OP codon optimized in *K. phaffii*. (A) Supernatant samples of expressed AppA-So were collected at different time points and analyzed by 10% SDS-PAGE stained with Coomassie blue. (B) AppA-So and AppAs-OP proteins were expressed in *K. phaffii*. A 60 μL of the supernatant was loaded onto a 10% SDS-PAGE gel from a control culture (not induced) (lane 1) or from cultures induced for 5 days with 0.5% of methanol to express the original phytase AppA-So (lane 2) or the optimized phytase AppAs-OP (lane 3). The SDS-PAGE gel was stained with Coomassie blue.

As shown in Figure 2A, the expression of the protein did not increase significantly after 120 h (5 days) of induction.

The phytase activity of the same samples (starting at 48 h) was measured following the ISO 30024 method. The activity obtained was about 155.5 U/mL at 48 h (Table 1), increasing by 2 to 3 units at the later time points (Table 1).

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ACS Food Sci. Technol. 2021, 1, 689−697

Table 1. Quantification of AppA-So Phytase Activity[a]

time (h)	48	120	144	174	198
time (days)	2	5	6	7.25	8.25
activity (FTU)	155.5 ± 10.60	152.8 ± 10.32	156 ± 8.69	162.1 ± 9.33	156.9 ± 8.62

[a]The activity, expressed in FTU (U/mL), was measured following the ISO 30024 method at the indicated hours/days. The values are from two independent experiments.

Table 2. Phytase Activity Profiles of the Original AppA-So and the Optimized AppAs-OP Proteins[a]

(A) Phytase AppA-So								
induction time (h)	0	19	31	43	55	67	79	91
units/mL	0	0	0	0	0	0	0	0
units/L	1 ± 0	1 ± 0	1 ± 0	1 ± 0	18.5 ± 2.5	52.5 ± 4.5	64 ± 4	140.5 ± 2.5

(B) Phytase AppAs-OP								
induction time (h)	0	19	31	43	55	67	79	91
units/mL	0	2.5 ± 9.5	5.3 ± 14.8	13 ± 17.43	15.5 ± 22.8	19.7 ± 27.3	24.5 ± 31.6	28.5 ± 36.1
units/L	1	2593 ± 450	5505 ± 925	13152 ± 1762	15619 ± 560	19658 ± 138	24572 ± 3274	28656 ± 1316

[a]Activity was measured with the ISO 30024 method, collecting equivalent supernatant samples of induced cultures expressing (A) AppA-So and (B) AppAs-OP at the indicated induction times. The values are from three independent experiments.

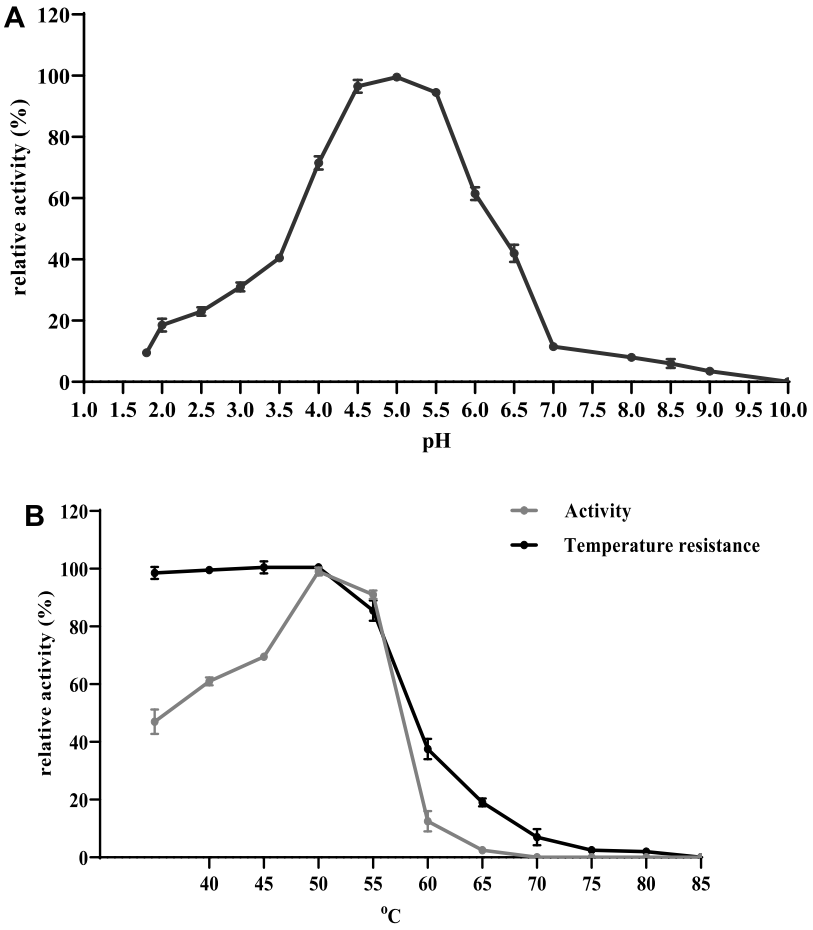

Figure 3. Effect of pH and temperature on the enzymatic activity of AppAs-OP. (A) Relative activity (% maximal activity) determined in the following buffers: glycine−HCl (0.25 M; pH 1.5−3.5), sodium acetate (0.25 M; pH 3.5−6.0), Tris−HCl (0.25 M; pH 6.0−8.0), and glycine−NaOH (0.25 M; pH 8.5−10.0). All buffers contained 0.05% IGPAL. (B) Relative activity determined using the ISO 30024 method at the indicated temperatures (gray line). Temperature resistance was analyzed by incubating the phytase protein for 10 min at the indicated temperatures before quantifying its activity with the ISO 30024 method at 37 °C (black line). Values are the means of three replicates ± standard deviation (S.D.).

Modification and Optimization of the *S. odorifera* Phytase Gene for Its Expression in *K. phaffii*. Previous analyses have revealed a strong relationship between tRNA multiplicity and codon selection, suggesting that the use of codons might be one of the factors that leads to inefficiency of the translation process and limited protein production.[23−25] We carried out codon optimization based on the codon usage database created by Kazusa (http://www.kazusa.or.jp/codon/) and the work published by Bai et al.,[26] in which they took into

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ACS Food Sci. Technol. 2021, 1, 689−697

account the use of gene codons that are highly expressed in *K. phaffii*.

The codons were analyzed one by one and exchanged for the preferred codons for each amino acid. A total of 166 out of the 400 codons of the *appA-So* sequence without the N-terminal signal peptide were modified, corresponding to 41.5% of the codons. This optimized sequence was called *appAs-OP* (Figure S1A). The *appAs-OP* gene sequence was compared with the original *appA-So* sequence to visualize the changes made (Figure S1B).

We analyzed the protein encoded by the modified sequence (Figure S2A) and compared it to that encoded by the original sequence (Figure S2B). As expected, the encoded proteins were the same.

The modified *appAs-OP* gene was then cloned and expressed in *K. phaffii*. Some colonies were randomly selected and inoculated in liquid medium. After 72 h of growth, phytase expression was induced. During the expression process, phytase activity was measured to evaluate the level of the phytase protein. All clones expressed similar levels of protein (Figure S3A); however, clone 6 displayed slightly higher activity (Figure S3B). Therefore, we continued the analysis of the AppAs-OP phytase using clone 6.

To corroborate the optimization of AppAs-OP versus the original AppA-So, the levels of protein synthesis and activity were compared. The fact that we were able to detect the AppAs-OP protein directly in the supernatant (Figure S3A) indicated that the levels of expression were increased with codon optimization for *K. phaffii*. To verify this, we inoculated liquid medium with the *K. phaffii* expressing AppA-So and the optimized AppAs-OP. After 72 h of growth, the expression of phytase was induced with 0.5% methanol. After 5 days of induction, samples of the supernatant from each culture were collected and analyzed on SDS-PAGE gels. As shown in Figure 2B, the phytase protein was only detected in the supernatant from the cultures expressing AppAs-OP, which had a molecular weight of 46 kDa, corresponding to the estimated molecular weight of the polypeptide sequence derived from the cloned DNA sequence.

The activities of the two phytases at different expression times were also compared (Table 2). Alongside protein levels, the activity of the AppAs-OP phytase was detected after 19 h of induction at U/mL levels (Table 2B). In contrast, the activity of the original phytase AppA-So was only detected at U/L levels after 55 h of induction (Table 2A).

Therefore, we verified that the optimization of the sequence had significantly increased the expression level.

Effect of pH and Temperature on the Enzymatic Activity of AppAs-OP. The effect of pH on phytase activity was evaluated at 37 °C and adjusted to different pH levels (Figure 3A).

Phytase activity varied depending on the pH, reaching maximum activity at pH 4.5. At pH 4.0, 70% of the initial activity was retained, while at pH 5.5, more than 80% of the initial activity was retained (Figure 3A).

To define the optimal temperature, the activity of the AppAs-OP phytase was measured at temperatures between 35 and 85 °C. Maximum activity was observed at 55 °C (Figure 3B). To determine the temperature resistance of the phytase, the phytase was subjected to different temperatures (between 35 and 85 °C) for 10 min before its activity was analyzed at 37 °C and pH 5.5. As shown in Figure 3B, the phytase retained 100% of its activity at temperatures up to 55 °C. Above this temperature, its activity began to fall. It should be noted that, at 60 °C, it still retained 40% of its initial activity (Figure 3B).

Effect of Proteases on the Enzymatic Activity of AppAs-OP. To determine its resistance to proteases, purified AppAs-OP was incubated with pepsin (5 mg/mL) or trypsin (50 mg/mL) at 37 °C. After incubation for different amounts of time, the remaining phytase activity was quantified in the sample, following the ISO 30024 method. As shown in Figure 4, the phytase retained more than 70% of its initial activity 1 h

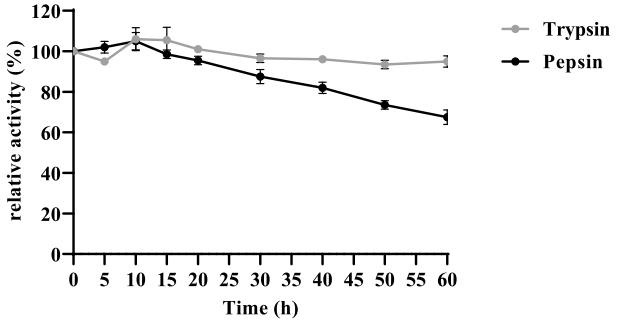

Figure 4. Protease resistance of the AppAs-OP protein. The effect of the proteases pepsin (black line) and trypsin (gray line) was determined by quantifying the relative activity of AppAs-OP at the indicated times, following the ISO 30024 method at 37 °C. Values are the means of three replicates ± standard deviation (S.D.).

after incubation with pepsin. Moreover, it retained 90% of its initial activity 1 h after incubation with trypsin. Thus, the phytase was resistant to protease activity.

Determination of the Specific Activity and Preservation of Activity over Time. The specific activity of a known concentration of AppAs-OP was determined. The resulting specific activity was 1123 ± 0.112 units/mg (U/mg).

To determine the activity of the AppAs-OP phytase over time, the specific activity was determined at different time points after the addition of the substrate. As seen in Figure 5,

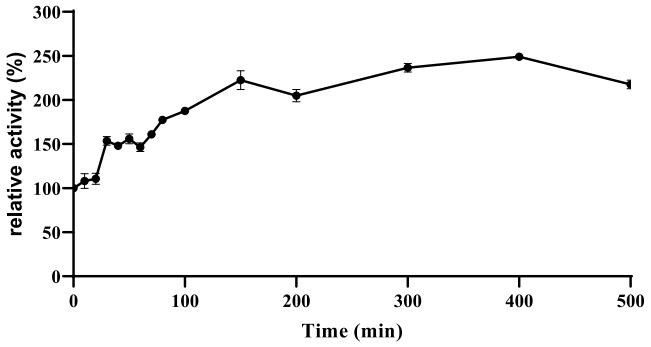

Figure 5. AppAs-OP activity over time. The relative activity of the AppAs-OP phytase was measured after the addition of the substrate at the indicated time points, following the ISO 30024 method. Values are the means of three replicates ± standard deviation (S.D.).

AppAs-OP was still active at least 8 h after substrate addition. It can also be observed that the initial specific activity doubled after 4.5 h. This increased and sustained activity over time is a novel characteristic among the phytases described to date.

Michaelis–Menten Constant (Km) of AppAs-OP. The Km of AppAs-OP, which corresponds to the substrate concentration at which the reaction rate is half of the Vmax (the maximum rate achieved by the system), was determined

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ACS Food Sci. Technol. 2021, 1, 689–697

Table 3. Kinetic Parameter Km[a]

phytase	Z	Y	X	AppAs-Op	R	N
Km (mM)	1.14 ± 0.0066	0.5886 ± 0.0213	1.3709 ± 0.0025	0.0391 ± 0.0032	0.3168 ± 0.0041	0.1478 ± 0.005

[a]Km values of different commercial phytases (see Materials and Methods) compared to that of AppAs-OP at pH 4.5 and 37 °C. The values are from three experiments.

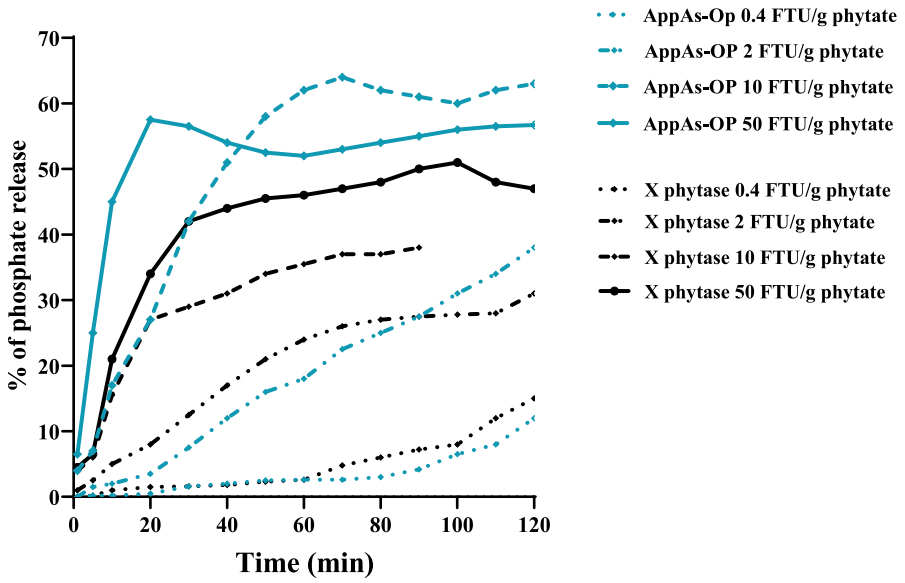

Figure 6. Comparison of the Km values of AppAs-OP and phytase X. Different FTU values (amount of enzyme that liberates 1 μmol of inorganic phosphate per minute) per gram of phytate at pH 5.5 and 37 °C of AppAs-OP and the commercial phytase X.

at pH 4.5 and 37 °C. The Km values of several different commercial phytases were also analyzed (Table 3). The Km of AppAs-OP was the lowest among all the phytases studied.

To corroborate the result, we examined phosphorus release kinetics using different concentrations of the phytase. We compared the AppAs-OP phytase with the X phytase, whose Km was the most different to that of AppAs-OP. At low enzyme concentrations, there was no difference between the two phytases since the phytate concentration was high and the two phytases acted at the same speed (Figure 6). At high concentrations of the enzyme, the percentage of phosphorus released was lower for the X phytase when around 30 and 40% of the phytate had been consumed, whereas AppAs-OP increased the release rate.

DISCUSSION

In the present study, we cloned, expressed and characterized the phytase gene *appA* from *Serratia odorifera* (*appA-So*). This gene has an open reading frame of 1281 bp that encodes a 426-amino acid protein containing an N-terminal 26-amino acid signal peptide. The AppA protein from *S. odorifera* contains the conserved N-terminal RHGXRXP and the C-terminal HD active site motifs and five cysteine residues, which are characteristic of phytases belonging to the HAP family.

Previously, phytases were reported in three species of *Serratia* (*S. plymuthica*, *Serratia sp. TN49*, and *Serratia sp. PSB-15*),[45−47] but they were not isolated. Thus, AppAs-OP is the first phytase purified from the genus *Serratia*.

The AppA-So phytase was expressed in *K. phaffii* and presented 163 U/mL of activity at 48 h of methanol induction after concentrating the sample 10 times. To improve the expression and activity of AppA-So, we synthesized AppAs-OP based on the codon preference of *K. phaffii*. Codon optimization not only improves protein expression in the host of choice, but it also reduces sequence complexity, such as secondary structures, and facilitates protein folding. Previous studies have shown the effect of codon optimization on the expression of recombinant enzymes expressed in *P. pastoris*.[27,28] AppAs-OP activity was notably higher than that of AppA-So. However, although AppAs-OP activity was higher than that reported for the neutral phytases encoded by the *phyC* gene of *Bacillus subtilis* that were expressed in *K. phaffii* (12.5 U/mL),[29] the yield was lower than that reported for AppA phytases from *Citrobacter braakii* (197 U/mL) and *E. coli* (237.2 U/mL).[30−32]

Phytase expression is usually evaluated by measuring enzyme activity. Although this may seem simple, it can be misleading and involves some vagueness. The measurement of phytase activity is usually based on the analysis of phosphate release using colorimetric methods.[33,34] In general, this analysis is sufficient, since the amount of phosphate released over time is usually the most relevant parameter in food supplies. We used the ISO 30024 method, approved by the European Food Safety Authority (EFSA), to determine the amount of phosphate release in feed materials. However, the measurement of inorganic P released over time is a black box and may not indicate only the enzymatic activity on the specific phytate substrate (IP6).

Several studies have assessed differences in phytase activities using different methods, discriminating between real phytase activity (defined as the hydrolysis of IP6) and total inositol phosphate activity (defined as the sum of all IPn hydrolysis).[33,34] Based on these comparative studies, the Bae method[35] appears to produce significantly higher values of phytase activity (U/mL) than the ISO 30024 method, for instance. In fact, the Bae method was used to determine the

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ACS Food Sci. Technol. 2021, 1, 689−697

AppA2 phytase activity of *E. coli* mentioned earlier.[32] Therefore, the activity of our AppAs-OP phytase might not actually be so low, considering the quantification method used. It would be useful to measure the expression of different phytases and compare their activities using the same method of quantification.

The purified AppAs-OP exhibited several characteristics that were similar to those of other bacterial phytases. AppAs-OP exhibited optimal activity at pH 4.5 and was stable from pH 3.7 to pH 5.8, indicating that it would be active in the stomach (pH 2.0−4.5) when used as an animal feed additive. Enzyme activity was highest at 55 °C, displaying 90% and 40% of its initial activity after incubation at 45 and 60 °C, respectively, for 10 min. The specific activity of AppAs-OP was 1.123 U/mg, which is higher than that of phytases from *Y. enterolitica*,[36] *Shigella sp.*,[37] and *Bacillus*,[38] but lower than that of phytases from *E. coli*,[32] *Y. kristesenii*,[39] and *Y. rohdei*.[40]

The most remarkable features of the purified AppAs-OP were its Km and its sustained activity over time. The Km of AppAs-OP, corresponding to the substrate concentration at which the reaction rate is half of the Vmax, was 0.039 mM. The Km of AppAs-Op was the lowest among all the commercial phytases analyzed in this study. It was also lower than the values reported for bacterial phytases, such as those from *Y. intermedia* (0.125 mM), *Y. rohdei* (0.206 mM), *Y. enterolitica* (0.19 mM), *Shigella sp.* (0.22 mM), *O. proteus* (0.34 mM), *E. coli* (0.55 mM), and *K. pneumonia* (0.28 mM).[32,37,39,41,42] In addition, AppAs-OP was still active 8 h after substrate addition, which is a good property for solubilized phytase.

In summary, the low Km observed in this study indicates that AppAs-OP maintains a high speed even with low concentrations of the substrate. Among all the commercial phytases assessed in this study, AppAs-OP had the lowest Km.

Given these features, the AppAs-OP phytase is a good candidate for improving the utilization of phytate phosphorus in animal feed. AppAs-OP has already been used in several studies. For example, it has been used to demonstrate that the age of the pigs being fed barley- and wheat-based diets affect the efficacy of AppAs-OP in phosphorus and calcium digestibility, with improvements in growth, feed conversion, and bone development observed until the pigs are 154 days in age.[43]

Furthermore,[44] evaluated the effects of phytase and different levels of calcium in a phosphorus-limited diet on the performance, bone mineralization, and calcium and phosphorus retention of broilers. They observed that the group receiving the highest dose of AppAs-OP showed significantly higher average daily gains and greater tibia weight.[44]

Thus, the AppAs-OP phytase will be a useful additive for improving the utilization of phytate phosphorus in animal feed, thereby reducing the need for inorganic phosphorus supplementation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.0c00074.

Codon optimization of *appAs* gene (Figure S1), protein sequence of the optimized phytase AppAs-OP (Figure S2), and protein level and phytase activity of AppAs-Op colonies (Figure S3) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Rosa Aligue − *Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University of Barcelona, Institute of Biomedical Researh, Barcelona 08036, Spain;* orcid.org/0000-0002-2085-325X; Email: aliguerosa@ub.edu

Authors

Ignasi Salaet − *Fertinagro Biotech, Teruel 44002, Spain*

Ramon Marques − *Fertinagro Biotech, Teruel 44002, Spain*

Tula Yance-Chávez − *Fertinagro Biotech, Teruel 44002, Spain*

Judit Macías-Vidal − *Global Feed S.L.U., Huelva 21001, Spain*

David Gimémez-Zaragoza − *Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University of Barcelona, Institute of Biomedical Researh, Barcelona 08036, Spain*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsfoodscitech.0c00074

Author Contributions

⊥I.S. and R.M. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ATCC, American Type Culture Collection; CBS, Netherlands Central Bureau of Fungal Cultures; CECT, Spanish Type Culture Collection; HAP, histidine acid phosphatase; Km, Michaelis−Menten constant; FTU, phytase unit

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