European Food Research and Technology (2023) 249:2377–2386
https://doi.org/10.1007/s00217-023-04299-6

ORIGINAL PAPER

Cross-linked phytase aggregates for improved phytate degradation at low pH in animal feed

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Received: 10 April 2023 / Revised: 22 May 2023 / Accepted: 28 May 2023 / Published online: 16 June 2023

Abstract

Phytases are widely used food and feed enzymes to improve phosphate availability and reduce anti-nutritional factors. Despite the benefits, enzyme usage is restricted by the harsh conditions in a gastrointestinal tract (pH 2–6) and feed pelleting conditions at high temperatures (60–90 °C). The commercially available phytase Quantum® Blue has been immobilized as CLEAs using glutardialdehyde and soy protein resulting in a residual activity of 33%. The influence of the precipitating agent, precipitant concentration, cross-linker concentration and cross-linking time, sodium borohydride as well as the proteic feeders gluten, soy protein and bovine serum albumin (BSA) has been optimized. The best conditions were 90% (*v/v*) ethyl lactate as precipitating reagent, 100 mM glutardialdehyde and a soy protein concentration of 227 mg/L with a cross-linking time of 1 h. The intrinsically stable phytase remained its high thermal stability and temperature optimum. The phytase-CLEA achieved a 425% increase of residual activity under harsh acidic conditions between pH 2.2 and 3.5 compared to the free enzyme. The free and immobilized phytase were deployed in an in vitro assay simulating the acidic conditions in the gizzard of poultry at pH 2. The hydrolysis of phytate was monitored using a novel high-performance thin-layer chromatography (HPTLC) analysis and DAD scanner to study the $InsP_x$ fingerprint. All lower inositol phosphate pools ($InsP_1$–$InsP_6$) and free phosphate were separated and analyzed. The phytase-CLEA efficiently released 80% of the total phosphate within 180 min, whereas the free enzyme only released 6% in the same time under the same conditions.

Keywords Phytase · Cross-linked enzyme aggregates (CLEA) · Phytase immobilization · Phytate degradation · Animal feed

Introduction

Phytases (EC: 3.1.3.26) are a class of enzymes that catalyze the stepwise dephosphorylation of phytate (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate or $InsP_6$), the natural storage component of phosphate in plants. Phytases are mainly applied as a feed additive for non-ruminant animals such as swine, fish or poultry, which lack the enzymes in their gastrointestinal tract impairing the release of bound phosphate [1, 2]. To meet the phosphate demand in animal feed, either phosphate salts are added as an external source or alternatively phytases are used to facilitate the release of naturally contained phosphate [3]. To increase the efficiency and stability of phytases as a feed additive, different approaches are pursued, such as directed evolution or semi-rational/rational design to engineer the protein structure or, alternatively, coating of the enzymes is applied as an external protection [4]. Furthermore, enzyme immobilization can be a promising strategy. In general, immobilization can offer several advantages, including increased activity and shift of operational parameters such as pH optimum or thermal stability, simplified reactor operation as well as product separation and reusability [5, 6].

Currently, either carrier-bound or carrier-free methods are used for immobilization. Suitable carriers include agarose, polysaccharides, cellulose or ceramics as well as inorganic products such as zeolite or mesoporous silica [5]. However, carriers are often expensive and selection of suitable materials requires high level of labor due to the trial and error process [5, 7]. Carrier-free immobilization methods

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are applied by directly cross-linking enzyme preparations. Depending on the preparation technique, the immobilization results in cross-linked enzyme crystals (CLECs), cross-linked spray dried enzymes (CLSDs) or cross-linked enzyme aggregates (CLEAs) [7]. CLEAs are characterized by their robustness and simplicity of preparation as well as the low purity requirement of the used enzyme solution [6]. The preparation procedure for CLEAs consists of two main steps: precipitation of enzyme and cross-linking. The free enzyme is first precipitated in a suitable precipitating agent such as ammonium sulfate, an organic solvent or ionic/nonionic polymers. Subsequently, cross-linking is started by the addition of a cross-linking agent [6]. Glutardialdehyde is commonly used as the cross-linker as it results in a high degree of cross-linking [8]. Other possible cross-linkers are for example dextran poly-aldehyde, dialdehyde starch or genipin [6]. Cross-linking occurs via the reaction of free amino groups of lysine residues on the surface of enzyme molecules [8]. Free carboxyl groups from glutamic acid and aspartic acid can also be utilized for cross-linking using, e.g., ethylene glycol-bis [succinic acid N-hydroxy succinimide] [9] or polyethyleneimine [10] as a cross-linking agent. The process of CLEA production can be optimized through varying the concentration of precipitating and cross-linking agents as well as cross-linking time. The residual activity may further be enhanced through the addition of, e.g., protein stabilizers or amino-group feeders [6]. CLEAs have been proven to be more stable than soluble enzymes for extreme conditions such as temperature and organic solvents [6]. These advantages of CLEAs serve the special requirement profile for phytases as feed additives. These include thermal stability between 60 and 90 °C to withstand the pelleting process during feed formulation as well as a broad pH profile (pH 2–6 in poultry's gastrointestinal tract) to maintain activity under the harsh conditions of a gastrointestinal tract [11, 12].

The aim of this study was to immobilize Quantum® Blue, a commercially available enzyme preparation, using the CLEA technique to increase enzyme activity over a broad acidic pH range while maintaining the intrinsic thermal stability. The changes in the biochemical properties of phytase-CLEA compared to free phytase will be investigated. In addition, the dephosphorylation process of the free and immobilized phytase at pH 2 will be studied by HPTLC analysis.

Materials and methods

Chemicals and reagents

All chemicals and reagents were of analytical grade and were obtained from either Carl Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany), unless stated otherwise. Glutardialdehyde 25% was purchased from Carl Roth (Karlsruhe, Germany), soy protein (isolated) from MP Biomedicals (Solon, OH, USA) and ethyl L-lactate 99% from Alfa Aesar (Kandel, Germany). The commercial phytase product used in this study was Quantum® Blue (AB Vista, Woodstock, UK) in solid formulation and a liquid formulation of Quantum® Blue 5G (AB Vista, Woodstock, UK) was obtained from AB Vista. The phosphate standard for IC (TraceCERT®, 1000 mg/L ± 4 mg/L) was ordered from Merck (Darmstadt, Germany). For a system suitability test (SST) the inositol phosphate standards Ins(3)P_1 (sodium salt), Ins(2,4)P_2 (sodium salt), Ins(1,4,5)P_3 (sodium salt), Ins(2,3,5,6)P_4 (sodium salt), and Ins(1,3,4,5,6)P_5 (sodium salt) were sourced from Cayman Chemical (Ann Arbor, Michigan, USA). Ins(1,2,3,4,5,6)P_6 (sodium salt) was purchased from Merck (Darmstadt, Germany).

Chemicals and instrumentation

A ThermoMixer® C manufactured by Eppendorf (Hamburg, Germany) was used to incubate all reaction tubes. Spectrophotometric measurements in microtiter plates were performed in an Epoch 2 manufactured by Biotek (Winooski, Vermont, USA). An ICP-OES spectrometer iCAP 7000 series from Thermo Fisher Scientific (Dreireich, Germany) was utilized. Centrifuge 5424 R and Centrifuge 5810 R (Eppendorf, Hamburg, Germany) were used. The ultrasonic processor UP200St from Hielscher Ultrasonic GmbH was utilized (Teltow, Germany). High-performance thin-layer chromatography (HPTLC) was performed on HPTLC Cellulose F 20 × 10 Glass plates (1.15036.0001, Lot: HX69191936) from Merck (Darmstadt, Germany) in a 20 × 10 Twin Trough Chamber with glass lid from CAMAG (Muttenz, Switzerland). Samples were applied by the Automatic TLC Sampler 4 by CAMAG (Muttenz, Switzerland), derivatized in the CAMAG® Derivatizer (CAMAG, Muttenz, Switzerland) and visualized in a CabUVIS from DESAGA (Wiesloch, Germany) equipped with a Canon EOS 600D camera. For direct DAD-spectrophotometry of UV spectra from HPTLC plates, a TIDAS TLC S700 system from J&M (Aalen, Germany) was used.

Standard phytase activity assay

Phytase activity was determined in triplicates according to a slightly modified method of Greiner et al. [13]. 200 µL of a phytate solution (50 mM NaOAc and 2.5 g/L sodium phytate buffer, set to pH 5.5) was pre-incubated for 5 min at 37 °C. Hydrolysis was started by adding 10 µL of an appropriately diluted enzyme solution and stopped after 5 min with 100 µL of an acidic molybdate-vanadate reagent according to DIN EN ISO 30024 [14]. Any precipitate formed was removed by centrifugation (17,000xg for 2 min) and the supernatant was

measured photometrically at 405 nm. The stop reagent consists of one-part solution A (10% (w/w) ammonium molybdate in 0.25% (*v/v*) ammonia solution), one-part solution B (aqueous 0.24% (w/w) ammonium vanadate solution) and two parts of 21.7% of nitric acid. Stock solution A and B can be stored refrigerated and dark for up to 2 months, the stop reagent must be prepared fresh daily [14].

Production of cross-linked enzyme aggregates (CLEA)

The standard procedure for the CLEA preparation was conducted according to a modified method of Stressler et al. [15]. The Quantum® Blue solution (25 µL, 227 mg/L soy protein) was added to ice-cold ethyl lactate (225 µL, 80% (v/v)). After a cooling period of 15 min, the solution was centrifuged (17,000xg, 4 °C, 10 min) and glutardialdehyde (100 mM final concentration) was added to induce the cross-linking. After gentle mixing, samples were stored on ice for 1 h. Subsequently, the samples were washed twice with 1 mL NaOAc buffer (50 mM, pH 5.5). The samples were centrifuged after each washing step and the supernatant was discarded. Phytase activity of the immobilized Quantum® Blue and the free enzyme were determined using the standard phytase activity assay. The residual activity was expressed as enzyme activity of the CLEA compared to the free enzyme. To study the pH dependency of the CLEA, the washing step during CLEA preparation was carried out with citrate buffer (50 mM, pH 2.2–6) at the respective pH. For the $InsP_6$ degradation assay, CLEA was prepared with 20 times the amount of the standard preparation. Subsequently, the CLEA was broken down into smaller aggregates by ultrasound using three short pulses (1 s each at 50 W).

Optimization of the CLEA preparation

Several parameters that effect the CLEA-preparation process were investigated to optimize residual activity with a one-factor-at-a-time strategy. Multiple precipitating agents (acetone, acetonitrile, ethanol, methanol, ethyl lactate, tert-butyl alcohol, 1-propanol or 2-propanol) were applied as well as different precipitating agent concentrations (60–100% (v/v)). Soy protein, gluten and bovine serum albumin (BSA) were tested as proteic feeders and soy protein content was varied in concentration (0–0.46 g/L). Furthermore, the concentration of glutardialdehyde (10–400 mM final concentration) as the cross-linking agent was investigated as well as cross-linking time (0.25–24 h).

Biochemical characterization

The temperature optimum of the phytases was determined using the standard phytase activity assay with varying incubation temperature from 30 to 90 °C. To determine the thermal stability, the residual enzyme activity was measured after incubation of the enzyme solution at 65–83 °C for 30 min and subsequently cooling it on ice for 10 min. To study the pH dependency of phytase activity, a citric acid buffer pH 2.2–6 was used. To determine kinetic constants, the incubation mixture consisted of 50 mM NaOAc buffer pH 5.5 containing increasing contents of phytate (0.05–2 mM). Kinetic constants (K_M, K_i) were determined from Michaelis–Menten plots of the data according to Chmiel et al. [16].

$InsP_6$ degradation assay

The degradation assay was conducted using a slightly modified version as described by Henninger et al. [17]. In a 50 mL falcon tube, 9 mL of phytate solution (1.66 g/L sodium phytate, set to pH 2 with 5 M HCl) was pre-incubated at 37 °C for 10 min on an Eppendorf ThermoMixer® C at 800 RPM. Hydrolysis was started by adding 1 mL of enzyme solution to a resulting final activity of 10 U/L. Samples of 500 µL were taken periodically (after 5, 15, 30, 60, 120, 180, 240, 300 min and 24 h) and stopped by heat, diluting the sample twofold in 95 °C H_2O_{dd} and inactivating it for 1 h. For the blank, an appropriately diluted enzyme solution was inactivated for 1 h at 95 °C before adding it to the phytate solution and leaving it at 95 °C for 1 h.

HPTLC analysis

HPTLC analysis of inositol phosphates as well as free phosphate was performed as described in a previous work [17]. In brief, with the Automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland), 6 mm wide bands were sprayed onto cellulose F glass plates (Merck, Darmstadt, Germany). The plate was developed in a twin trough chamber (200 × 100 mm with a glass lid) which was previously saturated with mobile phase consisting of an aq. salt solution (20 mM NaOAc, 10 mM NH_4Cl)/2-propanol/1,4-dioxane/acetic acid 5/5.2/2/0.06 (*v/v/v/v*) for 30 min at ambient temperature (~ 20 °C). Consequently, the plate was dried at 105 °C in a drying oven over night to remove all solvent residues. To visualize inositol phosphates, the plate was treated with a phytase solution (250-fold diluted Quantum® $Blue_{liquid}$ 5G in 50 mM NaOAc, pH 4.5). Subsequently, the plate was dried in the drying oven at 105 °C for 30 min. The hydrolyzed free phosphate was then stained with a molybdate reagent (5 mL of a 10 g/L ammonium molybdate heptahydrate aq. solution mixed with 200 µL of concentrated sulfuric acid) in combination with UV light (254 nm) exposure and scanned afterwards using a DAD scanner (J&M, Aalen, Germany) in the wavelength range 200–900 nm. The resulting blue zones were evaluated at the absorption

maximum of 774 nm using the Kubelka–Munk equation. For a more detailed description of the analysis method, please refer to the previous work.

Statistical analysis

Standard deviation was used for data evaluation and calculated with Excel (Microsoft, Redmond, USA). All experiments were conducted at least in duplicate.

Results and discussion

Animal feed enzymes such as phytases, xylanases or amylases have to withstand the harsh pasteurization conditions during the feed pelleting process [11, 12]. In poultry in particular, phytases must exhibit rapid phytate degradation due to the short residence time (ca. 3 h) [18] in the gastrointestinal tract (GIT). Furthermore, in the GIT, there is a large pH variance from pH 2–6 depending on the animal species and composition of the feed [19].

In the bio-industrial industry, protein engineering is usually applied to optimize the operational pH range, thermal stability and specific activity. Increased production costs and strict approval procedures in the feed industry may be reasons that the immobilization by cross-linking of the enzymes for the animal feed industry is not yet commercially applied.

Production of phytase-cross-linked enzyme aggregates (p-CLEA)

The immobilization of Quantum® Blue as phytase-cross-linked enzyme aggregates (p-CLEAs) has been optimized varying different parameters: precipitating agents and concentration, concentration of cross-linking agent (glutardialdehyde), cross-linking time, proteic feeders and their concentrations.

Initially, six organic solvents (ethyl lactate, methanol, ethanol, acetonitrile, 1-propanol and 2-propanol) have been screened as precipitating agents for the initial step of p-CLEA production (Online Resource 1). The highest residual activity (RA) has been obtained using ethyl lactate with 33% RA, followed by the solvents methanol, acetonitrile and 1-propanol with a RA of approximately 20%. The lowest RAs were achieved using ethanol and 2-propanol as precipitating agents (8%). Precipitation with organic solvents may inactivate enzymes due to hydrophobic interactions between the non-polar groups of the enzyme and the solvent [6]. The inactivation effect is highly dependent on the amount of non-polar groups on the proteins surface and active site and thus may be different from protein to protein.

Following, the concentration of the precipitating agent ethyl lactate was investigated. The optimum was determined to be at 80% (v/v) ethyl lactate as seen in Fig. 1a. The optimal concentration range of cross-linker was determined to be in the range of 25 and 100 mM glutaraldehyde (Fig. 1b) using a cross-linking time between 1 and 4 h (Fig. 1c). As proteic feeders, three proteins have been evaluated, soy protein, gluten and bovine serum albumin (BSA). The coaggregation with proteins serves as an amino-group feeder and can compensate the low cross-linking efficiency of some enzymes. Furthermore, the addition of proteins can stabilize the structure of the immobilized enzyme significantly and improve, e.g., the pH tolerance [6]. However, the amount and type of protein stabilizer is determined by the highest RA measured. Even at a low concentrations, BSA showed a negative effect on the RA (Fig. 1d). Increasing concentrations of gluten showed a slight decrease in RA (43% at 5 mg/L), whereas soy protein showed a tendency of a positive effect on the RA (117%), the optimum was obtained at 227 mg/L of soy protein. Sodium borohydride showed an overall negative effect on the RA of p-CLEAs (data not shown). Generally, sodium borohydride stabilizes the glutardialdehyde bond and, therefore, the stability of the CLEA and not the enzyme activity [15]. After all optimization steps were performed, a RA of 33% was obtained.

Previously, immobilization using the cross-linked enzymes aggregates (CLEA) technique has been reported for a 3-phytase [20]. An increase of activity from initial 4428 U for the free enzyme used for immobilization to 8000 U for the phytase-CLEA was achieved. The RA resulting after immobilization by cross-linking is highly dependent on each individual enzyme and its method of cleaving phosphate groups from phytate. Since the cross-linking occurs on the surface of the enzyme, the active site might be negatively affected in terms of activity loss [21]. Moreover, the conformational flexibility of the enzyme decreases, which may affect the catalytic activity [22].

Apart from direct cross-linking, different immobilization techniques for phytases have been employed, such as entrapment in, e.g., alginate gel, adsorption to mesoporous silica or zeolite as well as via covalent bonds for example to chitosan microspheres or carbon nanotubes and Pragya et al. have written a comprehensive review about the subject [5]. An overview of the phytases discussed here is given in Table 1.

It has to be mentioned that the given RAs are not directly comparable to the results in the present work as for the RA the immobilization yield was considered [23–25]. Lopes et al. [23] immobilized a phytase that was produced from a genetically modified strain from *A. niger* via adsorption onto zeolites that were modified with metallic ions. The zeolite modified with nickel ions showed an improved catalytic activity with a RA of 150%. The high RA was attributed to the presence of metallic ions on the support. A similar observation was made by Coutinho et al. [24] who immobilized the commercial phytase Natuphos by adsorption onto

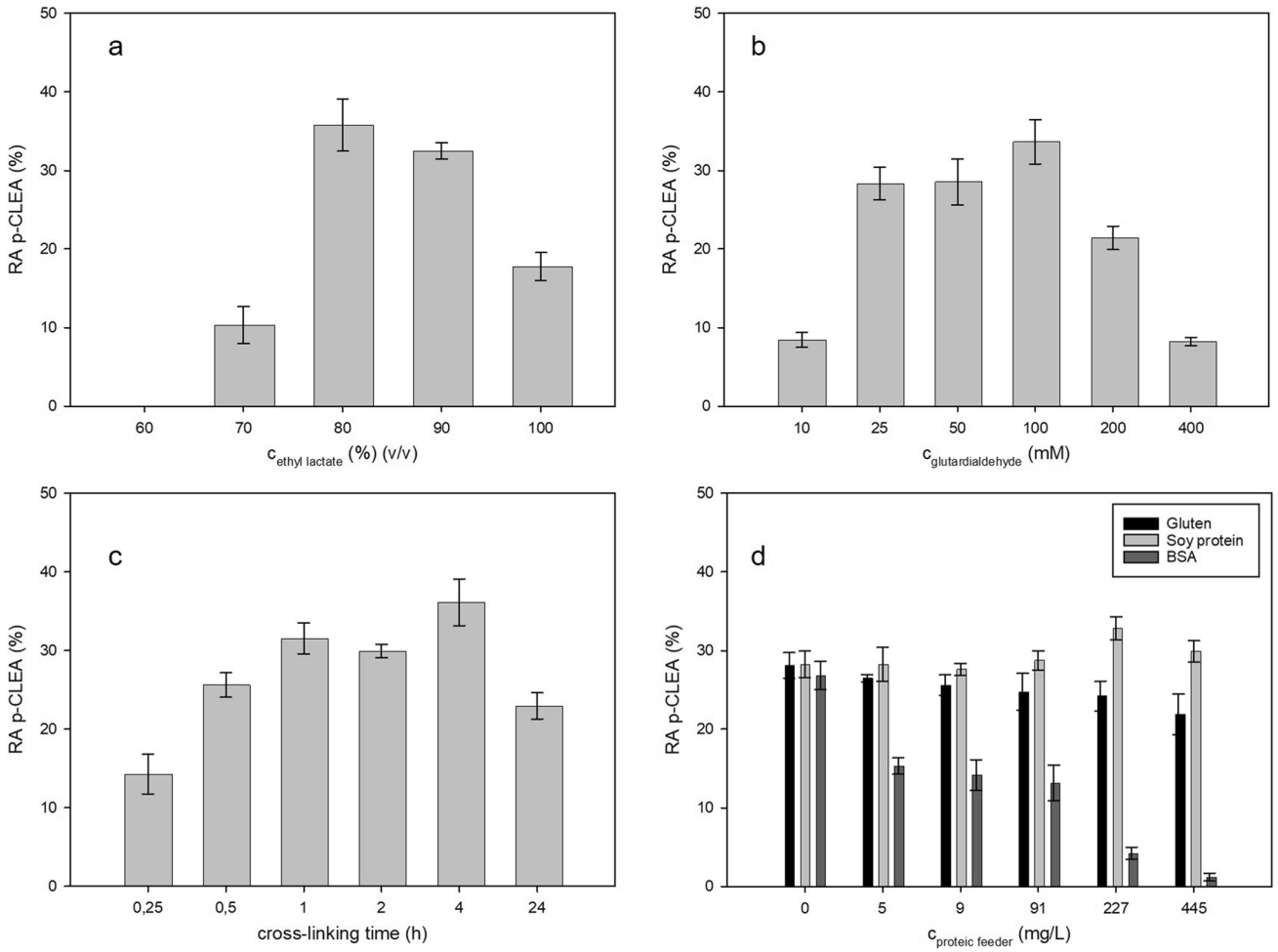

Fig. 1 Optimization of p-CLEA production process: concentration of precipitating agent ethyl lactate (**a**), glutardialdehyde concentration as cross-linking agent (**b**), cross-linking time (**c**) as well as concentrations of the proteic feeders gluten, soy protein and BSA (**d**)

Table 1 Overview of literature studies dealing with phytase immobilization including source of enzyme, modes of immobilization, support material and application

Phytase source	Support material	Mode of immobilization	Application	References
Quantum® Blue (commercial phytase)	Free of support	CLEA	In vitro phytate degradation	Present work
Phytase (E.C. 3.1.3.8)	Free of support	CLEA	Biochemical characterization	Tirunagari et al. [20]
Natuphos (commercial phytase)	Zeolite–Fe	Adsorption	Investigation of immobilization with biochemical characterization	Lopes et al. [23]
Natuphos (commercial phytase)	Hydroxyapatite nanoparticles	Adsorption	Hydrolysis of soybean milk	Coutinho et al. [24]
Aspergillus niger and *Escherichia coli* phytase	Synthetic iron-coated allophanes and natural montmorillonite	Adsorption	Analysis of phytase immobilization patterns	Menezes-Blackburn et al. [26]
Oakbug milkcap mushrooms phytase	Modified chitosan with nano-Fe_3O_4	Covalent bonding	Usability in food industry, hydrolysis of phytate in cereal	Onem et al. [27]

hydroxyapatite nanoparticles, complemented by ionic bonds. The RA after immobilization was higher than 100% and was explained by the presence of metal ions (Na^+, Ca^{2+}, Mg^{2+}, and Mn^{2+}) in the immobilization process which can have a positive effect on enzyme activity. Overall, the immobilized phytase showed a broader profile in terms of pH and temperature. However, desorption of the enzyme from the support material occurred at very acidic conditions (pH 2–3).

Biochemical characterization

Regarding the temperature optimum of the p-CLEA, no differences were observed between free and immobilized Quantum® Blue and was determined to be 70 °C. A similar thermal stability of the p-CLEA and free enzyme was found. After 30 min of incubation at a temperature range (65–83 °C), a residual activity of 50% remained at 77 °C and 79 °C, respectively. Enzymes immobilized with the CLEA technique often show an increase in thermal stability [6]. However, Quantum® Blue, the phytase utilized in the present work, is an intrinsically stable enzyme and the thermal stability was maintained after the immobilization. Tirunaragi et al. [20] could observe an increased temperature stability for the prepared p-CLEA; however, no background information about the type of phytase they used was undisclosed. After incubation for 6 h at temperatures ranging from 30 to 70 °C, the free enzyme lost 50% activity at 50 °C, whereas the immobilized enzyme retained 70% of its activity at 70 °C. Furthermore, Tirunaragi et al. found also a shift in pH range, as the immobilized phytase maintained a higher level of activity especially under very acidic conditions.

In the present work, the pH optimum of Quantum® Blue p-CLEA was determined to be 4.5, which is the same as the pH optimum of the free enzyme (Fig. 2). However, the pH range of the p-CLEA was extended to the acidic range. At slightly acidic conditions (pH 5–6), the p-CLEA shows a slightly lower activity than the free enzyme. In moderate to very acidic conditions (pH 2.2–3.5), the relative enzyme activity of the p-CLEA is approximately 200–425% higher compared to the free enzyme at pH 2.2–3.5. This higher pH tolerance was only achieved by the addition of proteic feeder (soy protein). The cross-linking of molecules on the enzyme surface results in a decrease of conformational flexibility but in an increase of structure rigidity. The stability gained reduces the loss of activity in harsh environments such as low pH and high temperatures [22]. This stabilizing effect can also be observed with other immobilization techniques. For example, Menezes-Blackburn et al. [26] immobilized an *A. niger* phytase and an *E. coli* phytase onto differently modified nanoclay supports. Whereas the *A. niger* phytase was inhibited by the immobilization, the immobilized *E. coli* phytase showed an increased activity at pH 2–3

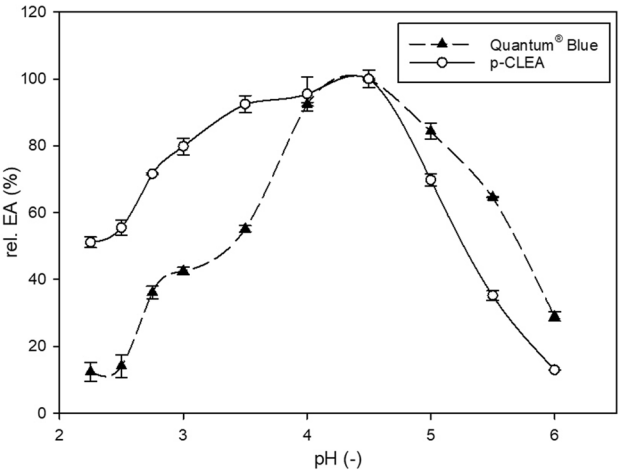

Fig. 2 pH range of the free enzyme (Quantum® Blue) and the p-CLEA determined in 50 mM citrate buffer pH 2.2–6

compared to the free enzyme. Onem et al. [27] described a stabilization for the pH range 2–9 for a phytase purified from oakbug milkcap (*Lactarius quietus*) immobilized on the surface of modified chitosan with nano-Fe_3O_4 nanoparticles. The pH optimum remained at pH 6 as with the free enzyme.

The stabilization of the phytase due to immobilization can be advantageous in the application in animal feed to withstand the harsh conditions in a gastrointestinal tract while remaining a high level of catalytic activity.

The kinetic constants (K_m, K_i) were determined for the free Quantum® Blue and p-CLEA under standard conditions (50 mM NaOAc pH 5.5, 37 °C). To compare the immobilized and free enzyme, an amount of enzyme was used that gives the same volumetric activity at a phytate concentration of 0.25 mM. The results were plotted and analyzed in a Michaelis–Menten diagram [16], as shown in Fig. 3. For both, the p-CLEA and free Quantum® Blue, a substrate inhibition was observed with a similar K_i value of 1.7 mM and 1.9 mM, respectively. The K_M value of the p-CLEA increased (0.09 mM) compared to the free enzyme (0.05 mM), indicating a reduced affinity for the substrate $InsP_6$ of the p-CLEA. Rix et al. [28] also observed substrate inhibition when studying a soil phytase and reported a K_M value of 0.65 mM and a K_i value of 2.23 mM. Greiner et al. mentions substrate inhibition for a phytase from an earthworm cast bacterium to be 5 mM [29]. For a different bacterial phytase, an inhibition effect for a phytate concentrations > 7 mM is reported [30].

Unfortunately, there is very little data on phytate levels within a gastrointestinal tract nor the phytate-release rate especially since the phytate concentration highly depends on the type of animal feed [31]. Furthermore, phytic acid is often present as insoluble calcium-phytate or phytate-protein

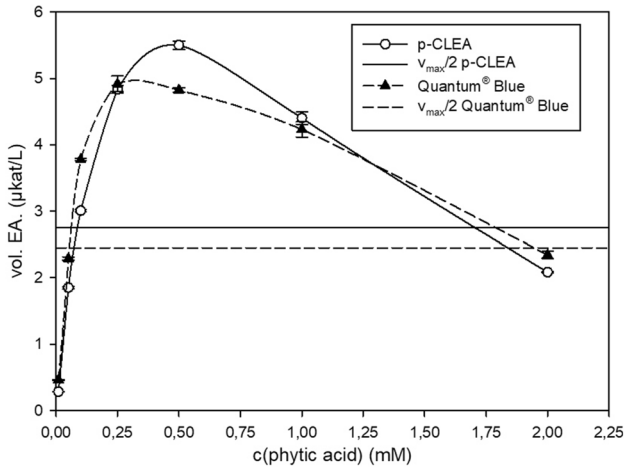

Fig. 3 Determination of the kinetic parameters of Quantum® Blue and p-CLEA for the substrate phytate according to Michaelis–Menten analysis

complexes [18], with no data on how this effects the substrate inhibition or the P-bioavailability. However, in animal feed trials, Quantum Blue showed the ability to reduce phytate contents in laying hens [32] and pigs [33].

$InsP_x$ fingerprint

Phytate is mainly hydrolyzed in the gizzard under acidic conditions that range from pH 2 to 5 [11] depending on the animal species. As previously shown in the biochemical characterization (Fig. 2), the p-CLEA maintained a significantly higher RA than the free enzyme (Δ425%) under very acidic conditions. These results were transferred to a simplified in vitro GIT-assay which simulates the phytate dephosphorylation under conditions similar to acidity levels in the gizzard of poultry (pH 2–3.6) [11]. The assessment of the differences of the $InsP_x$ fingerprint of the free enzyme compared to the immobilized phytase (p-CLEA) was performed using the previously established $InsP_6$ degradation assay [17].

The phytate degradation was performed at pH 2 using 10 U/L initial activity at 37 °C for up to 24 h (Fig. 3). For the free enzyme (Fig. 4a), $InsP_{5+6}$ was degraded at a slow rate and approximately 40% of the initially applied phytate was still detectable after 300 min of hydrolysis. Accordingly,

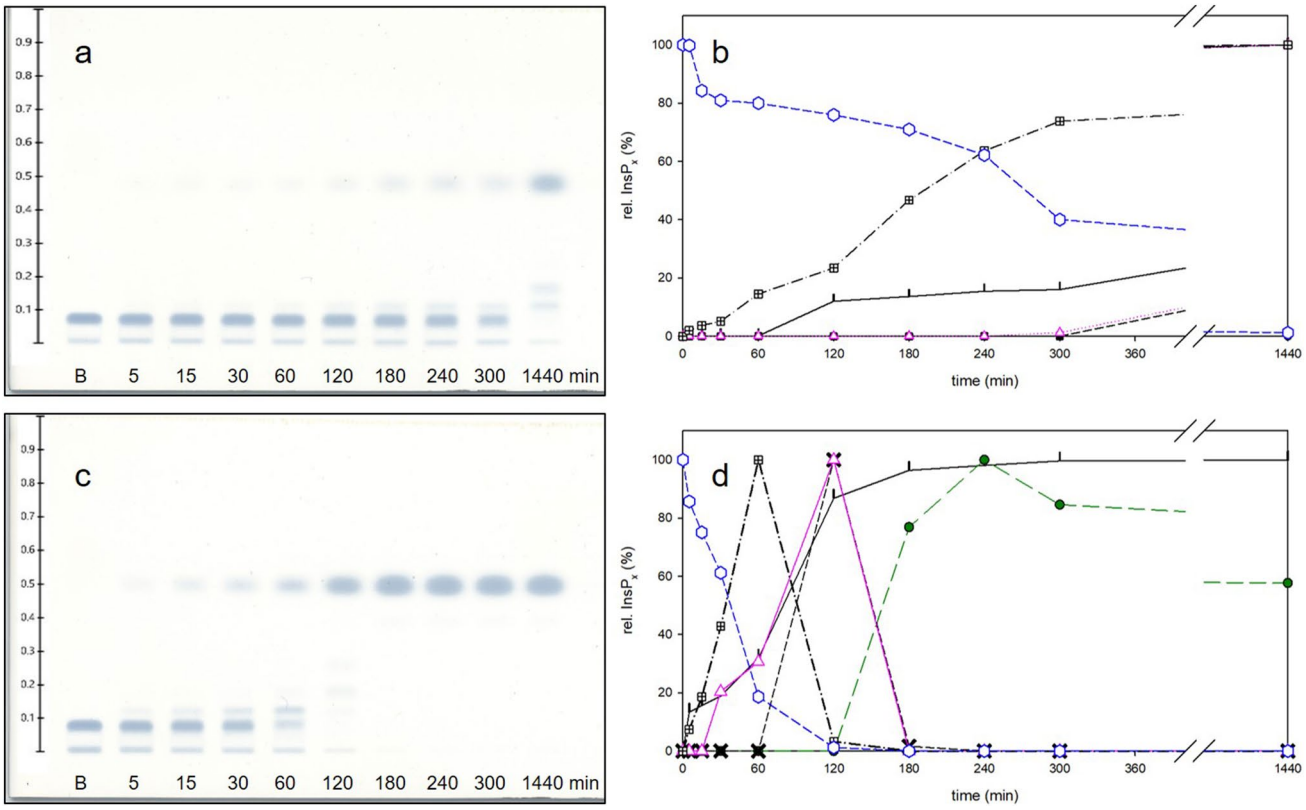

Fig. 4 Monitoring phytate hydrolysis at pH 2 using Quantum® Blue **a** and p-CLEA **c** (10 U/L, 37 °C) at the time points 0 (blank), 5, 15, 30, 60, 120, 180, 240, 300 min and 24 h in ascending order. Relative amounts of $InsP_x$ and P_i during enzymatic hydrolysis according to the $InsP_x$ fingerprint for **b** Quantum® Blue and **d** p-CLEA. Figure legend (b + d): ' = P_i, o = $InsP_1$, x = $InsP_2$, Δ = $InsP_3$, □ = $InsP_4$, ⬡ = $InsP_{5+6}$

$InsP_4$ and free phosphate were generated slowly as shown in Fig. 4b. The lower inositol phosphates $InsP_3$ and $InsP_2$ could only be detected after 1440 min.

The p-CLEA (Fig. 4d) showed a depletion of $InsP_{5+6}$ within 120 min. Subsequently, $InsP_4$ was produced, reached its maximum at 60 min of hydrolysis time and was almost completely degraded within the following 60 min. $InsP_3$ was built up between 30 and 120 min, followed by its degradation after another 60 min. The generation and depletion of $InsP_2$ occurred within 60 min. $InsP_2$ was detected at 120 min for the first time and was almost completely degraded (98.4%) after 180 min. The generation of $InsP_1$ started after 120 min and reached its maximum after 240 min followed by a slow and incomplete hydrolysis until the assay was terminated (1440 min). Degradation of the inositol phosphates $InsP_6$ to $InsP_2$ by the p-CLEA occurred at a much higher velocity compared to the free enzyme. The liberated phosphate P_i reached its plateau after 180 min.

As previously indicated by the determination of the pH optimum (Fig. 2), the immobilization of Quantum® Blue by cross-linking with glutardialdehyde stabilizes the phytase in particular under very acidic conditions (pH 2) compared to the free enzyme. With the help of the HPTLC [17] method, differences in inositol phosphate degradation at different pH values as well as phosphate release of the free and immobilized enzymes can be observed. The $InsP_x$ fingerprint (Fig. 4b) shows the inhibition of the free Quantum® Blue at pH 2 as the degradation of inositol phosphates occurs at a very low rate and $InsP_6$ degradation is still incomplete over the hydrolysis time of 24 h. In contrast, the p-CLEA is able to hydrolyze phytate completely to free phosphate and $InsP_1$ at pH 2. The p-CLEA partially hydrolyzed $InsP_1$ further at a slow rate as it has been observed in a previous work for free Quantum® Blue at pH 3.6 [17]. Furthermore, the degradation kinetic for the hydrolysis of $InsP_{5+6}$ by p-CLEA changed at higher pH levels compared to the free enzyme. Whereas the free Quantum® Blue (at pH 3.6 and 5.5) degraded $InsP_{5+6}$ within 60 min, the p-CLEA required twice the time. However, as soon as $InsP_{5+6}$ was completely degraded the dephosphorylation rate increased significantly and the degradation process was almost completed within the following 60 min of hydrolysis time. The slower degradation rate for higher inositol phosphates correlates with the increased Michaelis Menten constant of p-CLEA compared to the free enzyme as the kinetic parameter is determined for $InsP_6$ as a substrate.

There are possible reasons for the change in substrate affinity of the immobilized phytase. Glutardialdehyde is a very short cross-linking molecule resulting in dense aggregates and, therefore, substrate mass transfer into the aggregate may be limited [34, 35]. Furthermore, blocked amino groups on the enzyme surface result in charge differences within the phytase [6, 36] that may influence the affinity towards the highly charged substrates ($InsP_6$-$InsP_1$) or even increase the substrate inhibition towards phytate compared to the free enzyme (Fig. 3).

Phosphate release

To evaluate the efficiency of the free and immobilized Quantum® Blue at pH 2, the released phosphate was quantified and referenced to the maximum amount of phosphate released after acidic hydrolysis in 0.5 M HCl at 100 °C for 5 days (Fig. 5). The free enzyme released approximately 6% of the total phosphate over 300 min of hydrolysis time. After 24 h, a total of only 42% phosphate was liberated (free Quantum® Blue). The phosphate liberation for the p-CLEA was slower at the beginning of the hydrolysis (0–60 min) which correlates with the lower substrate affinity towards phytate compared to free Quantum® Blue at higher pH levels [17]. After 60 min, the phosphate release rate increases, reaching 50% free phosphate after 90 min. At 180 min of hydrolysis time, the phosphate release stagnates at 80% (570 mg/L P_i) and after 24 h a maximum of 84% P_i was reached. Lopes et al. [23] applied an enzymatic loading of 30 IU/g phytase and was able to observe a 20% increase in phosphate release when comparing the immobilized phytase (adsorption onto zeolite) to the free enzyme, however, no maximum phosphate content was given.

A stagnation at around 80% total phosphate released could also be observed in a previous work for several phytases [17] which indicates either a low affinity towards $InsP_1$ which is degraded at a very low rate or a possible product inhibition caused by the release of phosphate. Product inhibition of phytases is commonly recognized [29, 37–39], however, only very little data is available and it represents

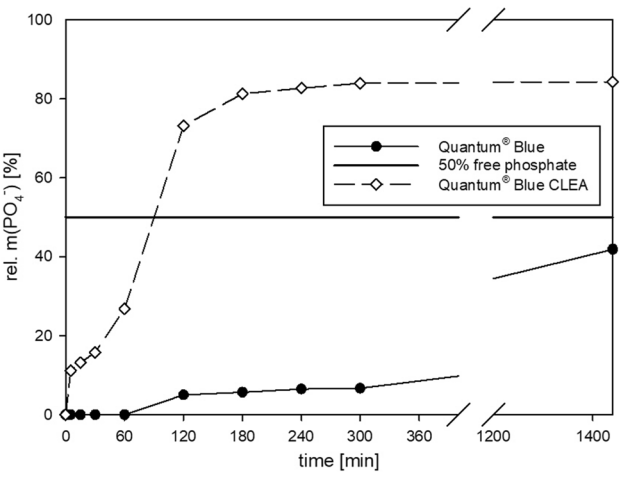

Fig. 5 Phosphate release during phytase (EA of 10 U/L) hydrolysis of phytate (1.66 g/L) at pH 2 over 24 h relative to the total phosphate content for Quantum® Blue and p-CLEA

one more challenge to overcome when designing novel phytases.

The $InsP_6$ degradation assay was applied to investigate the degradation pattern of a phytase on a decelerated time scale and detect possible accumulations of lower inositol phosphates. The adjusted dose of 10 U/L is not comparable to the doses used in animal feed, where, e.g., a dose of 500 FTU/kg feed [40, 41] is generally recommended for chickens. The higher phytase concentration should compensate for the decreased affinity for $InsP_{5+6}$. In addition, studies will be necessary to evaluate the behavior of the immobilized enzyme in animal feed and its performance under gastrointestinal conditions such as high dry matter, high viscosity and high levels of calcium ions.

Conclusion

In the present study, the commercially available phytase Quantum Blue was immobilized for the first time by the CLEA technique using soy protein as a stabilizer. The p-CLEAs were biochemically characterized and showed a significantly broader pH range and improved residual activity at very acidic conditions compared to the free enzyme. The improved pH profile of the p-CLEAs meets the requirements of the animal feed industry. The increase in activity in the acidic range may allow a more efficient phytate degradation in the gastrointestinal tract during the short residence time. The applied in vitro assay coupled to HPTLC $InsP_x$ analysis has proven to be a powerful tool to evaluate the potential of the p-CLEA over the free phytase. For an industrial application of p-CLEAs, the achieved residual activity needs to be improved to become a viable economic option. Further optimizations of the immobilization process and the use of other phytases to increase residual activity could make the CLEA technique an interesting addition to the stabilization process of phytases for use in animal feed.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-023-04299-6.

Funding Open Access funding enabled and organized by Projekt DEAL. This work has not been funded by a third party.

Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest There is no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human participants or animals performed by any of the authors.

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