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Research Article

Also in Crayfish: How Phytase Inclusion Avoids Phytic Acid Effects on Hepatopancreas Enzymes of Redclaw *Cherax quadricarinatus*

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This work aimed to evaluate the effects of dietary phytic acid on the enzymatic activities of the hepatopancreas of the redclaw crayfish, *Cherax quadricarinatus*. For this purpose, a completely randomized *in vitro* trial was conducted with three phytic acid levels (0.56, 1.68, and 2.80%) and three phytase doses (0, 250, and 500 PU/kg DM). Solubilized protein, reducing sugars, and soluble phosphorus showed significant responses to the interaction between phytic acid and phytase ($p < 0.001$). Only the main effects were detected on the released amino acids, in keeping with the main effects of alkaline protease activity, which are negatively affected by phytic acid ($p < 0.001$) and improved by phytase inclusion ($p < 0.001$). Differences in released reducing sugars were attributed to a reduction in amylase activity by increased levels of phytic acid and not to cellulase activity, where only a negative trend of phytic acid was found ($p = 0.068$). Phytic acid depresses calcium availability, which would explain the decrease in amylase activity. A 500 PU/kg DM dose improved amino acid, reduced sugars, and phosphorus release. These *in vitro* results might have *in vivo* implications for the digestibility of proteins, minerals, and energy. Further investigations are required to determine the chelated calcium effect on redclaw amylase activity, molting, and survival.

1. Introduction

The redclaw crayfish (*Cherax quadricarinatus*) is a freshwater species native to northern Queensland (Australia) and southeast Papua New Guinea. Redclaw presents a rapid growth rate and high productivity, easy general management, tolerance to wide variations in water quality, adaptability to intensification, and an invasive potential risk [1, 2]. Its commercial culture has increased by 40,000 metric tons/year since the end of the 1990s [3], being the second most cultured and caught crayfish species globally [4].

Feed can constitute up to 70% of the operational cost in aquaculture [5, 6], making feed efficiency a critical factor for aquaculture worldwide. The decrease in supply and the rising cost of fishmeal, coupled with problems related to its sometimes controversial origin and ecological sustainability, have promoted research on finding cheaper and more sustainable alternatives for redclaw crayfish nutrition.

Several studies revealed various protein sources from plants and terrestrial animals as alternatives for the formulation of redclaw feed [7–9]. Several of them highlighted the performance of proteins of plant origin, as well as their good palatability and growth efficiency [10–13], but others, not from any plant source [14].

The presence of antinutritional factors (ANF) in protein sources from plant origin, represents a current challenge in the aquafeeds industry. The ANF group includes phytic acid (PA), protease inhibitors, nondigestible carbohydrates, phytoestrogens, quinolizidine alkaloids, lectins, and saponins [15,16]. Special attention should be paid to the presence of PA due to its antinutritional properties resulting from strong negative charges under gastrointestinal conditions [17]. The considerable number of phosphate ions gives PA the ability to chelate numerous divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} and shapes strong phytate complexes [18]. Hence, this interaction reduces the bioavailability of dietary minerals [19–21], resulting in poor retention in crustaceans and fish [22, 23]. Furthermore, the binding of proteins with PA in insoluble binary and ternary structures makes proteins unavailable for digestion [24, 25]. In addition, the ability of PA to bind proteins involves the inhibition of commercial bovine trypsin [26] and fish gastric enzymes, decreasing protease activity at the stomach level by up to 60% according to in vitro [27] and in vivo studies [28]. *In vitro* evidence also indicated that PA is a potent noncompetitive inhibitor of α-amylase activity, probably due to phytate complexing with the enzyme or by blocking its active sites [29–31]. This leads to incomplete digestion of starch, thereby reducing its digestibility [32]. Khan and Ghosh [33] reported *in vitro* assays with enzyme extracts from three species of carp (*Cyprinidae*) where PA inhibited proteases and α-amylase. The use of exogenous phytases in fish diets has proven to be beneficial for fish growth, but it is not commonly used in crustaceans [34]. Research is still lacking in this regard. On the other hand, Divakaran and Ostrowski [35] reported an additional problem: the inhibitory effect of phytase on amylase present in shrimp hepatopancreas extracts from *Litopenaeus vannamei* through hydrolysis products of phytase action.

In view of this, we decided to work with the redclaw as a decapod crustacean model through *in vitro* assays. Previous research on this species involved the incubation of an ingredient with enzymes [36], a pH drop [37], and bioreactors with a semipermeable membrane [38], checking its performance for nutritional studies. This work carried out a completely randomized *in vitro* assessment of the effects of PA and phytase on the enzymatic activities of the hepatopancreas of *C. quadricarinatus*, and the hydrolysis products released. For this purpose, we simulated the digestive conditions of the hepatopancreas with the following major variables: pH, temperature, and redclaw enzymatic extract.

2. Materials and Methods

2.1. Source of Hepatopancreas Enzyme Extract and Exogenous Phytase. For this in vitro assay, the reproductive stock was provided by the Centro Nacional de Desarrollo Acuícola (CENADAC), Corrientes, Argentina. In the laboratory, animals were acclimated and once ovigerous females were obtained, they were individually kept under optimal rearing conditions based on previous studies [39]. From hatching until the start of the experiment, juveniles were grown in glass aquaria ($60 \times 40 \times 30$ cm) containing dechlorinated water (pH 7-8, hardness 70–100 mg·L^{-1} as $CaCO_3$ equivalents), under continuous aeration to maintain a dissolved oxygen concentration of >5 mg·L^{-1}. Water temperature was held constant at 27 ± 1°C and the photoperiod was 14L : 10D according to Jones [40]. Animals were daily fed at 1.5% of body weight (BW) with a commercial complete feed for tropical fish tetracolor, TETRA® with 95% dry matter (DM), 48% crude protein (CP), and 0.56% PA. This feed guarantees good growth and reproduction performance in this species under laboratory conditions [39, 41]. Upon the size of 15.1 ± 0.8 g, ten redclaw were fasted (48 h), anesthetized with cold water, weighed, sacrificed, and hepatopancreas were dissected, and pooled. The enzymatic extract was prepared according to Yasumaru and Lemos [42]. The pooled hepatopancreas (HP) were homogenized (1 : 10 w/v) in cold distilled water (4°C) by pulses with Ultra-Turrax T25 (IKA-Works Inc., Wilmington, NC 28405, USA), and then centrifuged at $17000 \times g$ for 15 minutes at 4°C. After extraction of the superficial lipid layer, the supernatant was stored overnight at −20°C to be utilized for enzyme activity analysis. The concentration of soluble protein was determined by the Coomassie blue dye method according to Bradford [43]; using 1 mg/ml bovine serum albumin (BSA) as a standard at 595 nm (Lambda 20 UV/VIS Spectrophotometer, Perkin Elmer, Norwalk, CT, USA).

A commercial 6-phytase from *Escherichia coli* (FITA-MAX HTR 10000, Magiar Feed®) was extracted at 0.1% w/v in chilled distilled water (4°C) at 500 rpm in a magnetic stirrer for four hours [44]. The solution was centrifuged at $17000 \times g$ and 4°C for 15 minutes. The supernatant was pooled, aliquoted, and stored overnight at −20°C for enzymology.

2.2. Enzyme Activities. The total proteinase activity was assayed using 10 μl of enzyme extract and 1 ml of a solution of 0.5% casein in 50 mM Tris-HCl buffer pH 7.5 [45], as modified by [46, 47]. The mixtures were incubated for 30 minutes at 27°C, and the reaction was stopped by adding 0.5 ml of 20% trichloroacetic acid (TCA). Similarly, blank tubes were prepared, but the enzyme extract was added after the TCA solution. The absorbance of the TCA-soluble peptides was measured at 280 nm. One unit of enzyme activity was defined as 1 μg of tyrosine released per minute, using the molar extinction coefficient for tyrosine = 1.490/M/cm.

Total α-amylase activity present in each crude extract was determined according to an adaptation of the method proposed by Bernfeld [48]. The assay mixture consisted of 50 μl of enzyme extract and 950 μl of a 1% soluble starch solution in 50 mM phosphate buffer (pH 7.5). The mixture was incubated for 30 minutes at 27°C. Phosphate buffer was used instead of Tris-HCl buffer due to the inhibitory effect of

the latter on α-amylase [49, 50] and other enzymes from the glycoside hydrolase family [51, 52]. The incubation was stopped by adding 500 μl of DNS reagent and boiling it for 10 minutes. Blank tubes were prepared as mentioned above, but the extract was added after the DNS solution. Samples were centrifuged and measured by spectrophotometry at 540 nm. The amylase activity was determined by measuring the production of reducing sugars using maltose as the standard. One unit of activity was defined as 1 mg of released maltose from starch per minute at the pH and temperature previously indicated.

Cellulase activity was estimated adapting the assay of König et al. [53], by determining the capacity of endoglucanase (CMCase) using glucose as the standard. The assay mixture consisted of 50 μl of enzyme extract and 950 μl of carboxymethylcellulose 1% w/v (pH 7.5) incubated for 30 minutes at 27°C. The incubation was stopped by adding 500 μl of DNS reagent and boiling it for 10 minutes. Blank tubes were prepared as mentioned above, but the extract was added after the DNS solution. Samples were centrifuged and measured by spectrophotometry at 530 nm. The unit of cellulase activity will correspond to the amount of enzyme that releases one μmol of glucose per minute under the detailed pH, temperature, and substrate conditions.

Phytase activity was determined for both HP extracts and phytase based on the work of Engelen et al. [54] and expressed in phytase units (PUs). PUs were the amount of enzyme that releases one μmol of inorganic orthophosphate per minute from a 5.1 mM sodium phytate substrate in Tris-HCl buffer at pH 7.5 and 27°C (in vitro simulated hepatopancreas conditions). Sodium phosphate was used as the standard and ammonium molybdovanadate as the reagent. The assay mixture consisted of 300 μl of enzyme extract and 600 μl of the substrate. The incubation was stopped by adding 600 μl of the reagent. Samples were centrifuged and measured by spectrophotometry at 415 nm. All measurements were carried out in triplicates.

2.3. In Vitro Digestion. The commercial diet tetracolor, TETRA® was employed as a reference diet. The PA content of the reference diet was determined according to Latta and Eskin [55]. The triplicated nine treatments consisted of the combination of three levels of PA in diet: 0.56% (basal level in reference diet), 1.68% (3x basal level), and 2.80% (5x basal level) by phytate sodium inclusion; and three levels of phytase dose: 0, 250, and 500 PU/kg of DM. The ionic strength level from diets was matched with NaCl. *In vitro* digestion was performed at 27°C and pH 7.5 in 27 glass tubes in a thermal water bath (Precisdig 6001236, JP Selecta, Abrera, Barcelona, Spain) for four hours, where they were also regularly vortexed. The amount of HP enzyme extract added was calculated based on the enzyme-substrate ratio (E : S) formula (equation (1)). The digestion mixture consisted of 5 mg of CP (11 mg for the reference diet) with 550 μl of HP extract according to the E : S ratio (equation (1)). Phytase dose was added in the liquid form. The volumes were matched with distilled water in the following manner:

$$\text{E: S} = \frac{\text{Alkaline protease activity } i\,[\text{U/g}]\text{x HP weight } i\,[\text{g}]}{\text{Daily protein feed intake } i\,[\text{g DM/day}]/\text{number of rations}\,[\text{r/day}]}. \quad (1)$$

After the incubation, the final soluble phosphorus released was measured by a molybdovanadate reagent at 415 nm [54]. The total concentration of amino acids (AA) released by hydrolysis was measured as m·eq. of tyrosine (Tyr) at 280 nm using 1490/M/cm as the molar extinction coefficient. The reducing sugars (RS) were determined with glucose as standard and DNS reagent at 540 nm [56]. The concentration of soluble proteins was determined by the Coomassie blue dye method according to Bradford [43] using 1 mg/ml bovine serum albumin (BSA) as the standard at 595 nm.

The remaining enzymatic activity from alkaline proteases, α-amylases, and cellulases after four hours of incubation was determined according to the techniques previously mentioned. To obtain these final enzyme activity results, corrections were made for the aliquot of the enzyme used, according to the dilution factor of the incubation mixture.

2.4. Experimental Design and Statistical Analysis. The experiment consisted of a completely randomized design of two factors (PA and phytase) with three levels each one resulting in nine triplicated treatments. After verification of the assumptions of normality and homoscedasticity by Kolmogorov–Smirnov and Bartlett's tests, data were analyzed by ANOVA. Tukey's multiple comparison tests were used to compare means between treatments. Differences between means are significant at $p < 0.05$. In the absence of interaction, regressions were fitted to a linear and/or quadratic function for the main effect of the factor. All the analyses were performed using Minitab® 18 software (Minitab Inc.). Mean values are reported ± standard deviation of the mean (SD).

3. Results

The main enzyme activities from *C. quadricarinatus* HP before in vitro digestion are presented in Table 1. The phytase activity capacity of this species was below the sensitivity threshold of the technique, and therefore, negligible. The exogenous *E. coli* phytase had an activity of 17.1 ± 0.4 PU/g product under the simulated HP conditions (pH 7.5; 27°C). However, the activity was 8446.7 ± 378.2 PU/g product under classical conditions (pH 5.5; 37°C). The E : S ratio was 4.40 U of alkaline proteinase per mg of CP intake in DM.

Table 1: Enzyme activities from *Cherax quadricarinatus* hepatopancreas extract performed at 27°C. Values are expressed as means ± SD.

	U/ml of HP extract	U/g of fresh tissue	U/mg of soluble protein
Alkaline protease	40.09 ± 0.87	400.91 ± 8.66	7.21 ± 0.16
α-Amylase	8.35 ± 0.28	83.53 ± 2.77	1.50 ± 0.05
Cellulase (CMCase)	10.20 ± 0.10	102.02 ± 1.01	1.84 ± 0.02
Phytase	nd	nd	nd

HP, hepatopancreas; nd, not detected; U, units.

The solubilized proteins detected by the Bradford method are shown in Figure 1. The PA × phytase interaction was statistically significant ($p = 0.0007$). Low PA treatments with added phytase (250 or 500 PU/kg DM) were statistically higher. Medium (1.68%) and high (2.80%) PA contents negatively affected protein solubility. However, a phytase dose of soluble protein 500 PU/kg DM on a medium PA content achieved statistically the highest levels, but not on high PA content.

The released AA are shown in Figure 2. No interaction between PA and phytase was detected ($p = 0.7571$). The main effects of PA ($p = 0.0006$) and phytase ($p < 0.0001$) were significant on released AA. Low PA contents (0.56%) determined superior AA release than medium and high PA contents. The released AA responded quadratically to the phytase dose ($p < 0.0001$; $R^2 = 0.6596$), but just a linear trend with low adjustment was registered in PA ($p = 0.0579$; $R^2 = 0.1365$)

The released RS are shown in Figure 3. Interaction with PA × phytase was significant on released RS ($p = 0.0014$). The highest values resulted from the combination of higher phytase doses and both low and medium PA contents. The released RS was negatively affected by incremental PA contents. These reductions were partially compensated by the phytase doses assayed.

The released soluble P is shown in Figure 4. Interaction with PA × phytase was significant on released soluble P ($p = = 0.0007$). In this case, the highest value was present in the combination of medium PA content and 500 PU/kg DM as well as in a high PA content with either 250 or 500 PU/kg DM.

The remaining enzymatic activities are presented in Table 2. No interaction effects were detected for alkaline protease activity ($p < 0.2049$), but the main effects of PA and phytase were significant on it ($p < 0.0001$). PA fitted to a quadratic regression ($p = 0.0002$; $R^2 = 0.5212$). Phytase fitted to a quadratic regression ($p = 0.0016$; $R^2 = 0.4159$). There was a decrease in the remaining activity of around 20%, which could not necessarily be attributed to the treatments tested. The inclusion of phytases helped to soften the negative effects of a high PA content against the proteases, although they were not fully eliminated. In the case of α-amylase activity, the interaction between PA and phytase over the response was significant ($p = 0.0402$). The high dose of phytase tested (500 PU/kg DM) was largely able to counteract the effects of a high dietary phytate content. However, for all dietary PA levels, a marked decrease in activity (35 to 50%) was observed compared to the initial one. Nevertheless not all the effects can be attributed to dietary phytate. For cellulase activity, as for proteases, there were no significant effects due to the interaction between PA and phytase. Furthermore, no main effects on cellulase activity were detected, although a trend ($p = 0.0683$) was detected by PA level.

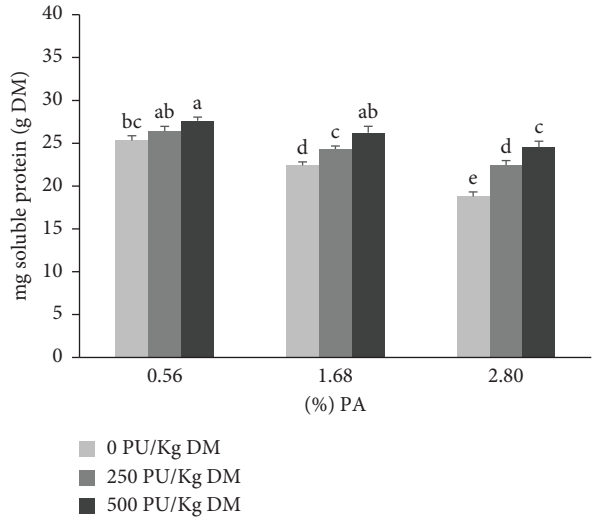

Figure 1: Solubilized proteins (as mg/g substrate in DM) after four hours *in vitro* digestive incubation of *Cherax quadricarinatus* ($n = 3$). Means with different letters indicate significant differences ($p < 0.05$). ANOVA p values: $p < 0.0001$ (PA); $p < 0.0001$ (phytase); and $p = 0.0007$ (interaction PA × Phytase). DM, dry matter; PA, phytic acid; PU, phytase units. Values are expressed as means ± SD.

4. Discussion

4.1. General Comments. Regarding redclaw enzymatic HP extract activities, initial alkaline protease activity was slightly lower and the initial α-amylase activity was just above the activities reported by Casaretto et al. [38]. The present experiment was conducted with larger animals and protease activity decreases, and carbohydrases activity increases as individuals grow [57]. The absence of phytase activity in the HP enzymatic extract was consistent with other crustaceans studied and the lack of PA in aquatic plants [58]. The medium and top phytic acid levels assayed (1.68 and 2.80%) could be considered exceptionally high, but they were in accordance with high vegetable protein inclusion diets. Particularly diets that include byproducts of nuts reaching PA values of up to 4–9% [59], such as *Acrocomia totai* kernel meal, a novel raw material recently tested [44].

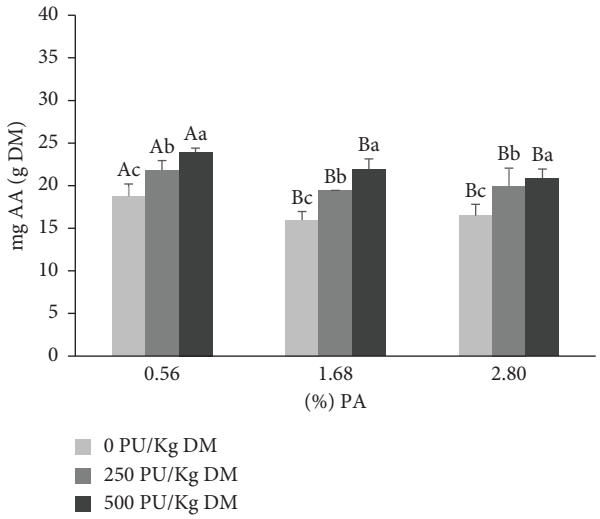

Figure 2: Released amino acids (as mg eq. Tyr/g substrate in DM) after four hours *in vitro* digestive incubation of *Cherax quadricarinatus* ($n = 3$). Means without a common uppercase differ ($p < 0.05$) for % of phytic acid (PA). Means without a common lowercase differ ($p < 0.05$) for phytase doses (in PU/kg DM). ANOVA p values: $p = 0.0006$ (PA); $p < 0.0001$ (phytase); $p = 0.7586$ (interaction PA × phytase). PA fitted to a linear regression ($p = 0.0579$; $R^2 = 0.1365$). Phytase fitted to a quadratic regression ($p < 0.0001$; $R^2 = 0.6596$). AA, amino acids; DM, dry matter; PU, phytase units. Values are expressed as means ± SD.

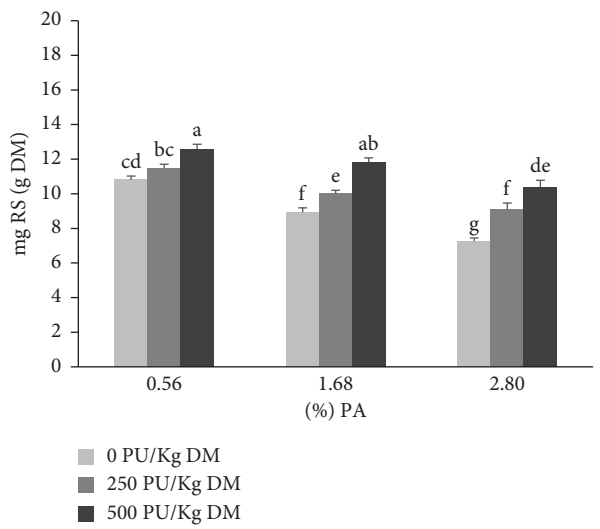

Figure 3: Released reducing sugars (as mg glucose/g substrate in DM) after four hours in vitro digestive incubation of *Cherax quadricarinatus* ($n = 3$). Means with different letters indicate significant differences ($p < 0.05$). ANOVA p values: $p < 0.0001$ (PA); $p < 0.0001$ (phytase); $p = 0.0014$ (interaction PA × phytase). DM, dry matter; PA, phytic acid; PU, phytase units; RS, reducing sugars. Values are expressed as means ± SD.

4.2. Effects on Nitrogenous Compounds. Phytic acid is claimed to affect protein, energy, and mineral digestibility. Amino acids responded significantly to PA and phytase as main effects, but no interaction was detected. However, the soluble protein was statistically sensitive to the interaction. This concurs with the results reported in birds and pigs [60] and fishes [24, 25, 27, 61]), where PA caused a decrease in protein and amino acids digestibility by binding to them. The isoelectric point of myofibrillar proteins is in a pH range of 4.8–6.2, whilst globulins are close to pH 4.8 [62, 63]. As the pH was higher than these isoelectric points, PA carried a negative charge through a cationic bridge (likes Ca^{2+}) and linked itself to proteins forming ternary complexes [60], thereby affecting protein digestibility. The remaining alkaline protease activity of the redclaw showed PA and phytase's main effects. It agreed with Yang et al. [64], who found higher protease activities in red swamp crayfish (*Procambarus clarkii)* hepatopancreas with increased phytase doses. Divakaran and Ostrowski [35] indicated that phytase may exert an indirect but positive effect on soybean meal proteolysis, measured as tryptic activity in in vitro assays with white shrimp (*L. vannamei*) HP extract. In their research, protein increased linearly with increasing phytase units, and it was not significantly affected from pH 5.5 to pH 8.5. Furthermore, in the same species but in an *in vivo* trial, a positive dose-response up to 750 FTU/kg with protein digestibility improvement of 17%, was found by Suprayudi et al. [65]. Future *in vivo* trials are warranted to validate the existence of beneficial effects in protein digestibility with redclaw as well.

4.3. Effects on Carbohydrates Compounds. The values of released reducing sugars did not distinguish per se the sources of polysaccharides that originated them. The remaining α-amylase and cellulase activities clarified this. In phytase absence, increasing PA contents from 0.56 to 1.68 and 2.80%, inhibited α-amylase activity in *C. quadricarinatus* HP enzyme extract 17% and 22%, respectively, but did not inhibit cellulase activity. Several previous in vitro studies have already indicated that phytate is a potent noncompetitive inhibitor of α-amylase activity [31, 66] confirming a marked reduction in the degradation path of carbohydrates [67] by the formation of phytate-carbohydrate complexes negatively affecting digestibility and absorption of glucose [68]. The formation of these complexes includes direct and indirect mechanisms. Among the first, Blennow et al. [69] inferred that phytic phosphates could bind covalently to glucose. These resulting starch polymer complexes could depress energy utilization. Indirectly, starch granules are so closely associated with a protein matrix in the endosperm of cereals [70], allowing for starch-protein interactions and the formation of ternary complexes together with PA, thus affecting starch digestibility [71]. PA may also have inhibited amylase activity both directly and indirectly, through the chelation of calcium, which is the required cofactor of amylase [72]. This supports the existence of energetic benefits due to the inclusion of phytase, as recently found by Yang et al. [64] for red swamp crayfish (*P. clarkii*) on amylase activity and it was again confirmed here. Suprayudi et al. [65] worked with juveniles of the whiteleg shrimp *L. vannamei* and a 500 FTU/kg dose from an *Aspergillus niger* phytase, finding a 10% improvement in energy digestibility. In contrast, Divakaran

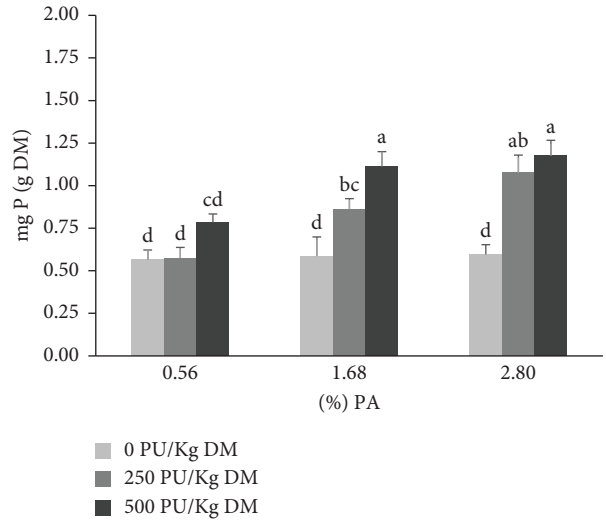

Figure 4: Released soluble phosphorus (as mg P/g substrate in DM) after four hours of in vitro digestive incubation of *Cherax quadricarinatus* ($n = 3$). Means with different letters indicate significant differences ($p < 0.05$). ANOVA p values: $p < 0.0001$ (PA); $p < 0.0001$ (phytase); $p = 0.0007$ (interaction PA × phytase). DM, dry matter; P, soluble phosphorus; PA, phytic acid; PU, phytase units. Values are expressed as means ± SD.

Table 2: Remaining enzymatic activities after four hours of in vitro digestive incubation of *Cherax quadricarinatus* ($n = 3$). Values are expressed as means ± SD.

Remaining Activities (U/ml)	Phytase (PU/kg DM)	PA level (%)			SEM	p-value		
		0.56	1.68	2.80		PA level	Phy level	PA × Phy
Alkaline protease	0	28.92 ± 0.98^{Ac}	26.32 ± 0.74^{Bc}	23.23 ± 0.93^{Cc}	0.23	<0.0001	<0.0001	0.2049
	250	30.83 ± 0.27^{Ab}	29.33 ± 0.49^{Bb}	26.40 ± 0.60^{Cb}				
	500	31.89 ± 0.91^{Aa}	30.77 ± 0.57^{Ba}	28.13 ± 0.34^{Ca}				
α-Amylase	0	5.42 ± 0.10^{ab}	4.52 ± 0.08^{def}	4.22 ± 0.12^{f}	0.10	<0.0001	0.0001	0.0402
	250	5.49 ± 0.08^{a}	4.85 ± 0.39^{cde}	4.44 ± 0.24^{df}				
	500	5.53 ± 0.10^{a}	5.11 ± 0.03^{abc}	4.97 ± 0.13^{bcd}				
Cellulase	0	8.13 ± 0.40	8.09 ± 0.37	7.36 ± 0.12	0.24	0.0683	0.7603	0.6837
	250	8.06 ± 0.34	8.03 ± 0.57	7.68 ± 0.58				
	500	7.93 ± 0.44	7.72 ± 0.27	7.68 ± 0.47				

$^{a-c}$Different lowercased letters indicate a significant effect ($p < 0.05$) of phytase dose. $^{A-C}$Different uppercased letters indicate a significant effect ($p < 0.05$) of phytic acid level. U, units; PU, phytase units; DM, dry matter; PA, phytic acid; Phy, phytase; SEM, standard error of the mean.

and Ostrowski [35] registered an inhibitory effect on amylase extracted from the hepatopancreas of *L. vannamei* by phytase. The same authors suggested that the byproducts of soybean meal PA, obtained because of phytase activity, inhibited amylase activity. Their argument was based on previous demonstrations by Knuckels and Betschart [29], who found that myo-inositol phosphate esters resulting from PA hydrolysis inhibited pancreatic and salivary α-amylase. From our study, we could conclude that phytate continues to be more negative than the products resulting from its hydrolysis.

Cellulase activity is present in the redclaw [73, 74], like in other crustaceans [75–77]. Consistent with the results of Björck and Nyman [78]; there were no statistically significant variations in cellulase activity with increasing inclusion of PA. This was advantageous considering that redclaw can obtain energy (glucose) from carboxymethyl cellulose [79]. However, Pavasovic et al. [80] did not detect nutritional benefits in the inclusion of insoluble cellulose (α-cellulose) in a redclaw feed. For all these reasons, Saoud et al. [81] assumed that there was still no conclusive evidence about the nutritional value of this polysaccharide.

4.4. Effects on Mineral Compounds. The present work presented a clear effect on the release of soluble phosphorus because of the interaction between the current PA content and the action of phytase in the *in vitro* simulated digestion of redclaw. The optimal pH reported for phytases of bacterial origin is between pH 4 and 6 [82]. It is generally accepted that most crustaceans lack an acid stomach digestion phase [75, 83, 84]. This represented a limitation to a large part of the phytases inclusion in diets for shrimps [24]. This may explain why no positive effects on growth performance and nutrient digestibility have been recorded due to phytase supplementation in the whiteleg shrimp [85], the black tiger shrimp [86], and the kuruma shrimp [87]. In contrast, Divakaran and Ostrowski [35] reported an additive effect between

trypsin and possibly other proteolytic enzymes in HP extract and phytase on the release of phosphorus from soybean meal phytate for whiteleg shrimp in *in vitro* tests. Furthermore, an *in vivo* response of +4% P digestibility was found by Suprayudi et al. [65] for whiteleg shrimp juveniles with 500 FTU/kg on a diet with 31% soybean meal and 23% wheat pollard (around 1% PA diet content, according to our own calculations). Other authors opted for the use of neutral phytases such as Bacillus or Pedobacter in juvenile whiteleg shrimp. One of such examples is Cheng et al. [88], who obtained increases in weight gain and feed efficiency by adding a recombinant phytase from Bacillus subtilis. For our study, the phytase employed was selected after scouting among eight commercial phytases because of the great activity around pH 7 (personal communication). As a result, a 15% soluble P increase was achieved with a dose of 500 PU/kg DM for a 0.56% PA content in the diet, compared to a 0 PU/kg DM dose, and values were even higher in diets with 1.68 and 2.80% PA levels. However, *in vivo* tests should be carried out to evaluate its performance, especially considering that P is an essential macromineral for the hardening of the exoskeleton and, as seen previously, the availability of calcium can be diminished by the presence of PA. Consequently, dietary PA could inhibit molting activity, weaken antibacterial ability, and survival as shown by Liu et al. [89] with diets without calcium in the Chinese mitten crab. The phytases in shrimp deserve to be further explored, considering that the addition of sodium phytate has already been shown to be detrimental to the growth rate in decapod crustaceans such as *L. vannamei* [90].

5. Conclusion

Our study provided evidence about the dynamics of the digestive enzymes of *Cherax quadricarinatus* hepatopancreas through different levels of phytic acid and phytase in the diet. The use of exogenous phytase in a proper dose when formulating redclaw diets should be considered. The implications of the results shown here are encouraging for more efficient use of nitrogen from vegetable protein sources and maximizing digestibility of phosphorus and energy with a 500 PU/kg DM dose. Future *in vivo* tests are necessary to move forward in the study of the detrimental effects of phytic acid on this species and the key role of phytases.

Data Availability

The authors confirm that all relevant data are available within the article.

Disclosure

This study is part of the MEC postgraduate scholarship in Consejo Nacional de Investigaciones Científicas y Técnicas.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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