In Vitro Studies on the Interaction of Phytase with Trypsin and Amylase Extracted from Shrimp (*Penaeus vannamei*) Hepatopancreas

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Phytases are added to livestock feeds to enhance bioavailability of phytate phosphorus. The possibility of adding phytase to shrimp feeds was investigated by studying the in vitro interaction of phytase on trypsin and amylase extracted from the hepatopancreas (HP extract) of *Penaeus vannamei*. Proteolysis of soybean meal (SBM) protein measured as tryptic activity increased linearly with increasing phytase units, indicating that phytase may exert an indirect but positive effect on SBM protein hydrolysis. Increase in tryptic hydrolysis was not significantly affected by changes in pH from 5.5 to 8.5. An additive effect was observed between trypsin and possibly other proteolytic enzymes in the HP extract and phytase on the release of phosphorus from phytate in SBM. An inhibitory effect on HP extract amylase activity by phytase, reported here for the first time, may have important implications on direct addition of phytase to shrimp feeds.

Keywords: *Phytase; shrimp; trypsin; amylase; interaction*

INTRODUCTION

Phytases have been added to feeds as a cost-effective alternative to inorganic phosphorus in intensive livestock production (Campbell and Bedford, 1992). Addition of phytase to swine and poultry diets containing corn and soybean meal (SBM) increased bioavailability of phosphorus and lowered phosphorus and nitrogen excretion (Ravindran et al., 1995). Phytase added to diets containing SBM as one of the ingredients fed to rainbow trout (*Oncorhynchus mykiss*) increased bioavailability of phosphorus and reduced phosphorus in the effluent (Cain and Garling, 1995). Garling (1995) obtained comparable biological performance of trout fed a diet containing phytase-pretreated SBM supplemented with 0.4% inorganic phosphorus to those fed a diet supplemented with 0.8% inorganic phosphorus. The benefits achieved by the addition of phytase enzymes in these studies are a result of digestion of phytate, a component present in all plant-based ingredients used in feeds. As a means of delineating the harmful effect of phytates, Civera-Ceracedo and Guillame (1987) found that adding sodium phytate to diets of Pacific white shrimp (*Penaeus vannamei*) decreased growth rate and increased feed consumption. Phytates induce many undesirable effects such as inhibition of protein digestibility (Knuckles et al., 1985) and amylase digestibility of starch (Knuckles and Betschart, 1987; Li et al., 1993). Although addition of phytase has been documented to ameliorate many of the negative effects of phytates, it is not clear if the enzyme phytase added to diets could in itself have any undesirable physiological effects in monogastric animals. Beers and Jongbloed (1992) found no pathological changes in internal organs and no residual phytase activity in the ileal digesta of pigs fed diets containing phytase. The objective of this study was to determine the interaction of phytase with trypsin and amylase extracted from hepatopancreas of Pacific white shrimp (*P. vannamei*). The intent was to investigate the possibility of direct addition of phytase to shrimp diets as a simple means of reducing inorganic phosphorus supplementation.

MATERIALS AND METHODS

Shrimp (*P. vannamei*) were maintained in 600 L raceways and fed a mixture of commercial diets. Hepatopancreas was collected from five batches of 10 10−12 g shrimp that were killed by immersion in ice-cold water. Each batch of hepatopancreas was washed in 0.2 M cold saline and homogenized with 50 mL of 0.2 M NaCl containing 20% glycerol (Maugle et al., 1982). The homogenate was centrifuged at 4000*g* for 30 min in a Sorvall refrigerated centrifuge. The clear supernatant (HP extract) was saved, distributed in 5.0 mL amounts, stored at −20 °C, and used as the source of trypsin and amylase. Protein content of HP extract was determined by the method of Lowry et al., (1951).

Presence of trypsin in HP extract was confirmed by comparison with commercial (Sigma Chemical Co., St. Louis, MO) trypsin (EC 3.4.21.4, 13 700 BAEE units/mg of protein) using the specific substrate *N*α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA). Commercial trypsin solution (75 μg/mL) was prepared in 0.01 N HCl. BAPNA solution (1 mg/mL) was prepared by first dissolving BAPNA in 1 mL of dimethyl sulfoxide and then diluting with distilled water. Buffer, pH 8.5, was prepared by adjusting the pH of 0.2 N triethanolamine hydrochloride containing 0.01 N $CaCl_2$ with 2.0 N NaOH (TEA buffer). TEA buffer (0.8 mL), BAPNA solution (1 mL), and 0.2 mL of trypsin or HP extract (HP extract diluted × 10 in TEA buffer) were prewarmed to 37 °C and taken in a 4 mL cuvette and mixed using a Pasteur pipet. Enzyme−substrate reaction was followed for 10 min at 1 min intervals by measurement of absorbance (A_{405}) resulting from *p*-nitroanilide released by the action of enzyme on BAPNA. The measurements were made using a spectrophotometer (Beckman DU 70) with a heated cell holder maintained at 37 °C. Specific activity of trypsin in HP extract was calculated based on the molar absorbtivity of *p*-nitroanilide (Liu and Markakis, 1989).

Presence of amylase in the HP extract was confirmed by comparing with commercial α-amylase (EC 3.2.1.1, 23 units/mg of solid, pH 6.9, Sigma) using amylase azure (Sigma) as

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the substrate (Rinderknecht et al., 1967). Amylase azure is an amylase covalently linked with Remazol Brilliant Blue R (RBB). All amylase−substrate reactions were carried out in TEA buffer (pH 6.9) containing 0.01 N $CaCl_2$. A solution of α-amylase (0.1 units/mL) was prepared in TEA buffer. A 2% suspension of amylase azure in TEA buffer was prepared fresh every time before use. Amylase azure suspension (1 mL) and 1 mL of α-amylase or HP extract (HP extract diluted × 100 in TEA buffer) both prewarmed to 37 °C were taken in a series of test tubes and placed in a Dubnoff shaker water bath at 37 °C. Enzyme−substrate reaction was stopped by adding 2 mL of 5% TCA to one of the test tubes every 5 min and reaction was followed until 30 min. Blanks were prepared by adding 2 mL of 5% TCA before addition of enzyme. The contents of the test tubes were then centrifuged at 10000*g* for 15 min, and the clear supernatant solution containing the RBB dye released by hydrolysis of amylase azure by amylase was saved for analysis. Absorbance of the solution was measured at 595 nm with a spectrophotometer (Beckman DU 70). Specific activity of amylase in the HP extract was calculated by comparison with α-amylase of known specific activity.

Phytase (EC 3.1.3.26, 0.04 units/mg of solid; Sigma; 0.1 unit/0.4 mL of stock solution) was prepared in TEA buffer of required pH by homogenizing 125 mg of phytase in 20 mL of buffer. The stock solution was then serially diluted with buffer of the same pH to obtain the phytase units tested.

Interaction between trypsin and phytase in the presence of casein and SBM was used to measure proteolysis of casein and the protein present in the SBM (45% crude protein, 0.71% P) and simultaneous hydrolysis of phytate P present in SBM by phytase. The enzyme−substrate interactions were performed at pH 8.5, 7.5, 6.5, and 5.5 in TEA buffer at 37 °C for 30 min using a Dubnoff shaker water bath. The reaction mixture at each pH tested consisted of 0.2 mL of HP extract (Trypsin specific activity 525 units/mg of protein), a suspension of 100 μg of SBM in 0.4 mL of buffer, 0.1% casein in 1.0 mL of buffer, and phytase at 0.1, 0.08, 0.04, 0.02, and 0.01 units in 0.4 mL buffer. The reaction was stopped at 30 min by adding 1 mL of 5% aqueous trichloroacetic acid (TCA). The resulting solution was then centrifuged at 10000*g* for 30 min in a Sorvall refrigerated centrifuge, and the supernatant was saved for analysis. The absorbance measured as tyrosine residues with a spectrophotometer at A_{280} and subtracted from absorbance at A_{320} was used as a measure of protein hydrolysis (Maugle et al., 1982, Stevens, 1992). Phosphorus released by hydrolysis of phytate present in SBM by phytase could not be measured in this series of experiments due to interference from traces of P present in the enzyme−substrate mixture. Therefore, a separate series of assays was conducted using 25 mg of SBM instead of 100 μg of SBM to compensate for background interference. Rapid agitation was used to keep the SBM in suspension during the reaction. Phosphorus was measured by phosphovanadomolybdate method (AOAC, 1990, method 965.17) and expressed as percent P hydrolyzed from total P in SBM. Blanks for the above assays contained the substrates in buffer without the addition of enzymes.

Additional enzyme−substrate reactions were conducted to determine the extent to which protein hydrolysis from SBM and casein was affected by the interaction between trypsin (HP extract) and phytase. An orthogonal array of tests was conducted at 4 pH levels and under conditions similar to those outlined above: the test consisted of HP extract + SBM; HP extract + casein; HP extract + SBM + Casein; HP extract + casein + phytase 0.1 unit; HP extract + casein + phytase 0.01 unit; and HP extract + Casein + SBM + phytase 0.1 unit. Protein hydrolysis was measured as described earlier. Blanks in all the reactions analyzed contained the substrates in buffer with no enzyme.

Trypsin inhibitor (TI) content of SBM was measured by the method of Liu and Markakis, (1989) using BAPNA as the substrate for trypsin. To check if phytase had any action on TI, content of TI was measured in enzyme−substrate reactions between HP extract + SBM in the presence and absence of phytase in TEA buffer at pH 8.5, 7.5, 6.5, and 5.5 at 55 °C for 30 min. The reaction was stopped by rapid cooling and filtered using a syringe filter (Acrodisc, 0.45 μm, Gelman Sciences), and TI content was measured in the filtrate. The TI measured was quantified as the difference between active trypsin left after the reaction and trypsin in fresh HP extract.

Table 1. Effect of Interaction between Trypsin (HP Extract) and Phytase on Protein Hydrolysis of a Mixture of Soybean Meal and Casein[a]

phytase units	protein hydrolysis measured as tyrosine residues ($A_{280} - A_{320}$)			
	pH 8.5	pH 7.5	pH 6.5	pH 5.5
0.01	0.54 ± 0.01^{b}	0.54 ± 0.01^{b}	0.55 ± 0.01^{a}	0.54 ± 0.01^{b}
0.02	0.78 ± 0.01^{ab}	0.78 ± 0.01^{ab}	0.76 ± 0.01^{bc}	0.74 ± 0.03^{c}
0.04	1.13 ± 0.01^{b}	1.13 ± 0.01^{b}	1.11 ± 0.01^{b}	1.16 ± 0.021^{a}
0.08	1.65 ± 0.11^{bc}	1.64 ± 0.02^{bc}	1.70 ± 0.02^{ab}	1.74 ± 0.00^{a}
0.10	1.91 ± 0.03^{d}	1.95 ± 0.02^{c}	1.99 ± 0.01^{b}	2.01 ± 0.02^{a}

[a] Protein hydrolysis measured as tyrosine residues ($A_{280} - A_{320}$) with increasing units of phytase (1 unit of phytase will release 1 mol of P/min at 37 °C, pH 5.5, from sodium phytate) at four pH levels. Values represent the mean ± standard deviation of five replicate samples. Values across rows not sharing the same superscript letter are significantly ($P < 0.05$) different.

Interaction between amylase and phytase was measured in TEA buffer at pH 6.9, at which amylase from HP extract manifested maximum activity. Maugle et al. (1982) also found only one type of amylase with optimum activity at pH 6.8 in *Penaeus japonicus*. The HP extract (amylase specific activity 0.76 units/mg of protein) diluted to contain 0.1 unit/mL was combined with 0.5 mL serial dilutions of phytase containing 0.01, 0.02, 0.04, 0.08, and 0.1 units phytase, SBM 100 μg in 0.5 mL and 1 mL of 2% RBB starch. The interaction between amylase, SBM, and RBB starch in the presence of serial dilutions of phytase was allowed to proceed at 37 °C for 30 min in a Dubnoff shaker water bath. The reaction was stopped by adding 1 mL of 5% aqueous TCA. Amylase control (0 phytase) contained 0.5 mL of buffer with no enzyme, and the blank consisted of SBM, RBB starch, and 1.5 mL of buffer and no enzymes. Amylase−substrate (RBB starch) interaction under these specified conditions was measured by following the same procedure used for the evaluation of amylase in HP extract. The influence of varying quantities of phytase on HP extract (amylase) was quantified as change in the unit activity of amylase based on the linear relationship between amylase units and hydrolysis of RBB starch.

STATISTICAL ANALYSIS

Statistical analyses were conducted using the StatView software package (Abacus Concepts, Inc., Berkeley, CA). One-way and two-way analyses of variance were conducted when appropriate to determine the effects of pH and/or phytase units on hydrolysis. All data were subject to tests for assumptions for the analysis of variance. Remedial actions (e.g., transformations) were implemented when necessary. Percentage data was subjected to the arcsine transformation and transformed back to percentages for presentation. Fisher's protected least significant difference test was used to compare differences between treatment means. Simple linear regression was used to predict responses of the dependent variable where appropriate.

RESULTS AND DISCUSSION

Protein content of HP extract was 13.20 ± 0.98 mg/mL. Specific activity of trypsin in the HP extract was 525 units of trypsin/mg of protein. Specific activity of amylase in HP extract using α-amylase as standard was 0.76 unit of amylase/mg of protein. The SBM with 48% crude protein used in the studies had 0.71% P and contained 500 mg TI/kg SBM.

Table 2. Effect of Protein Hydrolysis of Soybean Meal and Casein Due to Interaction between Trypsin (HP Extract) and Phytase Using an Orthogonal Array of Tests Conducted at Four pH Levels[a]

enzyme−substrate mixture	protein hydrolysis measured as tyrosine residues ($A_{280} - A_{320}$)			
	pH 8.5	pH 7.5	pH 6.5	pH 5.5
HP extract + SBM	0.05 ± 0.01^{b6}	0.09 ± 0.01^{a6}	0.08 ± 0.01^{a5}	0.05 ± 0.01^{b6}
HP extract + casein	0.20 ± 0.00^{b4}	0.23 ± 0.01^{a4}	0.23 ± 0.01^{a3}	0.20 ± 0.01^{b4}
HP extract + SBM + casein	0.11 ± 0.01^{c5}	0.15 ± 0.00^{b5}	0.16 ± 0.01^{a4}	0.14 ± 0.00^{b5}
HP ext + casein + Phy 0.1 unit	0.35 ± 0.01^{a2}	0.34 ± 0.01^{b2}	0.33 ± 0.00^{b2}	0.33 ± 0.00^{b2}
HP ext + casein + Phy 0.01 unit	0.24 ± 0.01^{a3}	0.24 ± 0.01^{a3}	0.24 ± 0.01^{a3}	0.24 ± 0.00^{a3}
HP + casein + SBM + Phy 0.1 unit	0.91 ± 0.02^{a1}	0.70 ± 0.02^{c1}	0.71 ± 0.10^{c1}	0.83 ± 0.02^{b1}

[a] Protein hydrolysis measured as tyrosine residues ($A_{280} - A_{320}$) with increasing units of phytase enzyme at four pH levels. Values represent the mean ± standard deviation of five replicate samples. Values across rows not sharing the same superscript letter are significantly ($P < 0.05$) different. Values within columns not sharing the same superscript number are significantly ($P < 0.05$) different.

Table 3. Phosphorus Released by Phytase at Four pH Levels by Hydrolysis of Phytate Present in Soybean Meal in the Presence and Absence of Casein and HP Extract[a]

enzyme substrate mixture	% phosphorus released from total P in soybean meal			
	pH 8.5	pH 7.5	pH 6.5	pH 5.5
SBM + phytase 0.1 unit	11.75 ± 0.30^{b2}	11.75 ± 0.46^{b2}	18.63 ± 1.27^{a2}	0.04 ± 0.87^{a4}
SBM + phytase 0.01 unit	3.49 ± 1.04^{b4}	2.41 ± 0.86^{b4}	6.73 ± 0.29^{a3}	7.32 ± 0.64^{a2}
SBM + casein + phytase 0.1 unit	8.09 ± 0.31^{c3}	9.33 ± 0.53^{c3}	17.41 ± 1.29^{b2}	19.54 ± 1.73^{a1}
SBM + casein + phytase 0.1 unit + HP	17.46 ± 0.49^{c1}	17.08 ± 0.90^{c1}	27.07 ± 2.88^{b1}	4.32 ± 2.20^{a3}

[a] The results are expressed as a percentage of total P released from soybean meal. Values represent the mean ± standard deviation of three replicate samples for each substrate mixture without casein and five replicate samples for each substrate mixture with casein. Values across rows not sharing the same superscript letter are significantly ($P < 0.05$) different. Values within columns not sharing the same superscript number are significantly ($P < 0.05$) different. [b] The low % P values for SBM + Phytase 0.1 U, pH 5.5 and SBM + Cas. + Phytase 0.1 U + HP pH 5.5 were caused by accidental dilution of samples by leak from water bath.

Interaction between trypsin and phytase in the presence of SBM and casein using 100 μg of SBM showed that protein hydrolysis measured as absorbance at A_{280} increased linearly with increasing phytase units from 0.01 to 0.1 ($y = 0.442 + 15x$; $r^2 = 0.989$, $P < 0.0001$, where y = tyrosine residues ($A_{280} - A_{320}$) and x = phytase units. Protein hydrolysis in the presence of phytase under these test conditions was not greatly affected by shift in pH from pH 8.5 to 5.5, although optimal tryptic activity occurs at about pH 8.0 (Gibson and Barker, 1979). However, several significant ($P < 0.05$) interactions between pH and phytase units did occur (Table 1). Differences in protein hydrolysis with change in pH was most pronounced at 0.10 phytase units.

Additional enzyme−substrate reactions conducted to determine the extent to which protein hydrolysis from SBM and casein was affected by the interaction between trypsin (HP extract) and phytase by using an orthogonal array of tests at 4 pH levels indicated that protein hydrolysis was enhanced ($P < 0.05$) in the presence of phytase (Table 2). Low level of protein hydrolysis in the reaction between SBM and trypsin in the HP extract was caused by TI present in SBM. This was confirmed by comparing the level of hydrolysis between HP extract and casein where no T1 was present, with HP extract, SBM, and casein. Protein hydrolysis was reduced ($P < 0.05$) by approximately one-third in the latter at all pH ranges due to contribution from TI present in SBM. The increase recorded in protein hydrolysis may have occurred in the interaction between HP extract, casein, SBM, and phytase by assuming that a combined catalysis of phytase and trypsin or other proteolytic enzymes in the HP extract could have somehow increased protein hydrolysis. The possibility of inactivation of TI by phytase was not established in this study. The reason for a 37% increase ($P < 0.05$) in hydrolytic activity of HP extract on casein (Table 3) in the enzyme substrate mixture (HP extract + casein + SBM + phytase 0.1 unit) when phytase units were increased from 0.01 to 0.1 is unclear. Phosphorus release by phytase hydrolysis of phytate present in SBM expressed as % P released from total P present in SBM, was dependent on pH (Table 3) which was expected. Maximum ($P < 0.05$) phosphorus release occurred at pH 6.5. There was nearly a 3-fold increase ($P < 0.05$) in hydrolytic activity as phytase units increased from 0.01 to 0.1 in the enzyme−substrate mixture containing SBM + phytase. Phosphorus release from SBM in the presence of phytase was not ($P > 0.20$) affected by the presence of casein at pH 5.5, at which pH phytase activity is optimal. Maximum release of phosphorus ($P < 0.05$) was obtained with addition of HP extract at this pH (pH 5.5), indicating an additive effect between trypsin or possibly other proteolytic enzymes from HP extract and phytase on release of P from SBM.

Table 4. Inhibition of Amylase Hydrolysis by Increasing Units of Phytase[a]

phytase units tested	hydrolysis measured in amylase units
0.00	0.19 ± 0.01^{a}
0.01	0.20 ± 0.02^{a}
0.02	0.15 ± 0.03^{b}
0.04	0.06 ± 0.02^{c}
0.08	0.01 ± 0.01^{d}
0.10	0.00 ± 0.00^{d}

[a] Results are expressed as amylase units (1 unit of amylase will hydrolyze 1 mg of starch to maltose in 3 min at 20 °C at pH 6.9) measured by hydrolysis of amylase azure by amylase present in HP extract at pH 6.9. Values represent the mean ± standard deviation of five replicate samples. Values within column not sharing the same superscript letter are significantly ($P < 0.05$) different.

Amylase from HP extract was clearly inhibited by phytase at pH 6.9, although optimal phytase activity occurs at pH 5.5 and declines considerably near neutral pH. (Table 4). Amylase activity was more than halved ($P < 0.05$) at 0.04 phytase unit and was not detected at all at 0.10 phytase unit. The change in amylase hydrolysis with increasing phytase units was described by the regression, $y = 0.192 - 0.22x$ ($r^2 = 0.88$, $p < 0.0001$),

where y = amylase hydrolysis and x = phytase units. This type of inhibition of amylase activity in vitro has not been reported earlier. Knuckels and Betschart (1987) demonstrated that myo-inositol phosphate esters resulting from hydrolysis of phytate inhibit pancreatic and salivary α-amylase. In this study, the in vitro inhibition of amylase extracted from shrimp HP could have resulted from the byproducts of hydrolysis of SBM phytate formed by phytase activity.

These in vitro studies indicated that proteolysis of SBM by shrimp HP extract increases linearly with increasing phytase units from 0.01 to 0.1 and is not greatly affected by changes in pH between 5.5 and 8.5 or the presence of another substrate, casein. Such increases may be due to a combined catalysis by trypsin, other proteolytic enzymes in HP extract, and phytase. Possible inactivation of TI present in SBM by phytase was not established in this study to explain the observed increase. However, phytase may exert other indirect but positive effects on protein hydrolysis.

Phytase hydrolysis of phosphorus from SBM also increases with increasing phytase units from 0.01 to 0.1, but is maximum at pH 5.5 and 6.5, respectively, in the interaction between SBM and phytase. There appears to be an additive effect between trypsin or other proteolytic enzymes in the HP extract and phytase at pH 6.5 on the release of phosphorus from SBM.

The inhibitory effect of phytase on amylase present in shrimp HP extract is being reported for the first time to our knowledge. This may have important implications in the use of phytase enzymes in shrimp feeds. Pretreatment of SBM with phytase to enhance phytate P availability in shrimp diets may be appropriate.

LITERATURE CITED

AOAC. AOAC Method 965.17. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 15th ed.; AOAC Inc., Arlington, VA, 1990; p 88.

Beers, S.; Jongbloed, A. W. Effect of supplementary *Aspergillus niger* phytase in diets for piglets on their performance and apparent digestibility of phosphorus. *Anim. Prod.* **1992**, *55*, 425−430.

Cain, K. D.; Garling D. L. Pretreatment of soybean meal with phytase for salmonid diets to reduce phosphorus concentrations in hatchery effluents. *Prog. Fish Cult.* **1995**, *57*, 114−119.

Campbell, G. L.; Bedford, M. R. Enzyme applications in monogastric feeds: A Review. *Con. J. Animal Sci.* **1992**, *7* (3), 449−466.

Civera-Ceracedo, R.; Guillame, J. Effect of sodium phytate on the Japanese shrimp *Penaeus japonicus*. *International Council for the exploration of the sea*. Copenhagen, Denmark, 1987; p 13.

Garling, D. L. (Unpublished). Tropical aquaculture feeds and culture technology development annual report, Submitted to the U.S. Department of Agriculture by The Oceanic Institute, Makapuu Point, Waimanalo, Hawaii, April 1995.

Gibson, R.; Baker, P. L. The decapod hepatopancreas. *Oceanogr. Mar. Biol. Annu. Rev.* **1979**, *17*, 285−346.

Knuckles, B. E.; Betschart A. A. Effect of phytate and other myo-inositol phosphate esters on α-amylase digestion of starch. *J. Food Sci.* **1987**, *52*, 719−721.

Knuckles, B. E.; Kuzmicky D. D.; Betschart A. A. Effect of phytate and partially hydrolyzed phytate on *in vitro* protein digestibility. *J. Food Sci.* **1985**, *50*, 1080−1082.

Li, K.; Alli, I.; Kermasha, S. *In−Vitro* α-amylase inhibitor activity-Phytate relationships in proteins from *Phaseolus* beans. *Food Res. Int.* **1993**, *26*, 195−200.

Liu, K.; Markakis, P. An improved colorimetric method for determining antitryptic activity in soybean products. *Cereal Chem.* **1989**, *66* (5), 415−422.

Lowry, O. H.; Rosenberg, N. J.; Farr, A. L.; Randal, R. J. Protein determination with folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Maugle, P. D.; Deshimaru, O.; Katayama, T.; Simpson, K. L. Characteristics of amylase and protease of the shrimp *Penaeus japonicus*. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48* (12), 1753−1757.

Ravindran, V.; Kornegay, E. T.; Denbow, D. M.; Yi, Z.; Hulet, R. M. Response of turkey poults to tiered levels of Natuphos phytase added soybean meal-based semi-purified diets containing three levels of nonphytate phosphorus. *Poult. Sci.* **1995**, *47*, 1372−1374.

Rinderknecht, P.; Wilding, P.; Haverback, B. J. A new method for the determination of α-amylase. *Experienta* **1967**, *23*, 805.

Stevens, L. Buffers and the determination of protein concentration. In *Enzyme assays, A practical approach*. Eisenthal, R.; Dnason, M. J., Eds.; Oxford University Press: NY, 1992; Chapter 11, pp 317−335.

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