Effect of Calcium and Phytic Acid on the Activation of Trypsinogen and the Stability of Trypsin

Robert A. Caldwell

Department of Agricultural Chemistry, University of Sydney, Sydney, NSW 2006, Australia

The kinetics of the activation of trypsinogen to trypsin has been investigated under different combinations of calcium(II) and phytic acid. The complexation of phytic acid and calcium increases (~30-fold) the rate of formation of catalytically inactive protein. The stabilizing effect of calcium on the autocatalytic degradation of trypsin is also substantially reduced (~100-fold). Activation of trypsinogen by enteropeptidase has a positive effect in that it rapidly activates trypsinogen and reduces the degradative impact of inactive protein formation. The data describing the integrated process of active trypsin generation, inert protein generation, and active trypsin stability have been analyzed in terms of two rate equations containing four rate constants. A numerical integration technique and nonlinear regression have been used to estimate the rate constants. The effect of phytic acid on these rate constants at 30 °C and either pH 6.0 or pH 8.1 is given.

INTRODUCTION

Phytic acid is a naturally occurring compound found in the seeds of plants, where it functions during germination as a source of inorganic phosphate and *myo*-inositol. In the seeds of legumes phytic acid accounts for 60–80% of the phosphate content and is structurally integrated with the protein bodies as phytin, a mixed K, Mg, and Ca salt (Lott, 1984). Phytin is soluble at acidic pH but has limited solubility in the more neutral pH region, 5–8. The insolubility of cation–phytate complexes in this pH region is a feature of many multivalent cations [e.g., Ca(II), Mg(II), Fe(III), Zn(II), and Cu(II); Cheryan, 1980; Nolan et al., 1987]. The insolubility and stability of the complexes in the gastrointestinal tract of monogastrics and birds is regarded as the major reason for the reduced bioavailability of some trace elements. In particular, phytate-induced zinc deficiency has been extensively reported [see, for example, reviews by Maga (1982) and Reddy et al. (1982)].

Some nutritional studies have suggested that phytate also affects protein utilization in rats (Atwal et al., 1980) and fish (Richardson et al., 1985; Spinelli et al., 1983). Richardson et al. (1985) suggested that several contributing factors may have been responsible for lowered protein utilization in high-phytate diets. They included the formation of protein–phytate complexes, inhibition of digestive enzyme secretion, and depressed absorption of nutrients in the pyloric caecal region of the intestine.

Although the formation of protein–phytate complexes at acid pH and their effect on acid proteases are well established (Barre, 1956; Knuckles et al., 1985), the existence of similar complexes at basic pH and their resistance to proteolysis by neutral proteases are less certain. Inagawa et al. (1987) have reported that phytic acid did not affect the proteolysis of either casein or soybean protein. Singh and Krikorian (1982) observed moderate inhibition at a very high phytate concentration (90 mM). Furthermore, it was suggested that the activation of trypsinogen could also be affected by the interaction of phytate and calcium ions. This work looks in more detail at the in vitro activation of trypsinogen and the stability of trypsin at 30 °C in the presence of different calcium and phytic acid combinations at pH 6.0 and pH 8.1. These pHs were chosen as being representative of the range of pHs over which trypsin normally functions in vivo.

EXPERIMENTAL PROCEDURES

(1) Materials and Methods. Proteins were commercial products obtained from Sigma (bovine pancreatic trypsin T1005, bovine pancreatic trypsinogen T1143, and porcine intestine enteropeptidase E0885). Solutions of trypsin (EC 3.4.21.4) and trypsinogen were dialyzed overnight against 1 mM HCl, filtered through a 0.45-μm Millipore filter, and used immediately. Solid enteropeptidase (EC 3.4.21.9) was dissolved in sterile, distilled, deionized water and used without further treatment. Protein concentrations were determined using extinction coefficients at 280 nm ($E_{1\%,1cm}$) of 15.0 for trypsin and trypsinogen (Walsh, 1970) and 17.8 for porcine enteropeptidase (Maroux et al., 1971). Dodecasodium phytate and N^{α}-*p*-tosyl-L-arginine methyl ester (TAME) were obtained from Sigma. All other reagents were of analytical reagent grade. The final solute concentrations in reaction mixtures were, for pH 8.1, 100 mM Tris, 100 mM NaCl, $CaCl_2$ and sodium phytate as indicated in Table I, and HCl to pH 8.1 and, for pH 6.0, 50 mM Mes, 100 mM NaCl, $CaCl_2$ and sodium phytate as indicated in Table I, and NaOH to pH 6.0.

The two combinations of calcium chloride and sodium phytate produced precipitates of calcium phytate. Reaction mixtures containing precipitates were stirred using a magnetic stirrer and stirrer bar.

Reaction mixtures were incubated at 30 °C. Sampling commenced immediately after the addition of trypsinogen and either trypsin or enteropeptidase. Enteropeptidase, when added, was to a final concentration between 0.6 and 1.0 μg/mL. Under the assay conditions used (either pH 6.0 or 8.1 and 30 °C) enteropeptidase specific activity was 540 units/mg of enteropeptidase. One unit is equivalent to 1 mg of trypsinogen converted per hour at an initial trypsinogen concentration of 0.9 mg/mL. Final reaction mixture volume was 1.0 mL. Controls consisted of trypsinogen without added trypsin or enteropeptidase. Total tryptic activity of reaction mixtures was determined by assaying 0.5-μL samples in 3.0 mL of standard assay mixture at regular intervals according to the spectrophotometric method of Hummel (1959). This 6000-fold dilution of samples was sufficient to solubilize any precipitate from the reaction mixtures. Standard assay conditions were 40 mM Tris, 10 mM $CaCl_2$, 1.04 mM TAME, and HCl to pH 8.1. Tryptic activity of reaction mixtures was converted to concentration of active trypsin in milligrams per milliliter using a factor of 430 TAME units being equivalent to 1.0 mg of active trypsin. One TAME unit is defined as the quantity of trypsin hydrolyzing 1 μmol of TAME/min under standard assay conditions.

0021-8561/92/1440-0043$03.00/0

Table I. Rate Constants for the Activation of Trypsinogen and Deactivation of Trypsin[a]

	pH 6.0			pH 8.1		
	k_1	k_2	k_4	k_1	k_2	k_4
10 mM Ca^{2+}	14 (1)	6 (2)	0.2 (0.1)	47 (2)	15 (3)	0.8 (0.1)
10 mM Ca^{2+}, 1.5 mM phytate (ppte)	16 (4)	19 (2)	2.5 (0.6)	27 (3)	50 (5)	2.4 (0.4)
10 mM Ca^{2+}, 5 mM phytate (ppte)	17 (3)	70 (28)	4 (2)	33 (2)	410 (200)	49 (16)
1.5 mM phytate	17 (4)	160 (40)	16 (4)	32 (4)	540 (180)	88 (7)

[a] All rate constants have been multiplied by 10^3. Units are mg^{-1} cm^3 min^{-1}. Figures in parentheses are average deviations from the mean.

(2) Data Analysis. Data have been analyzed by assuming the existence of three schemes describing the reactions of trypsin, trypsinogen, and enteropeptidase. These are

$$T + TG \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \begin{matrix} 2T + P \\ T + IP \end{matrix} \quad (1)$$

$$EP + TG \xrightarrow{k_3} EP + T + P \quad (2)$$

$$T + T \xrightarrow{k_4} T + IP' \quad (3)$$

where T, TG, and EP are trypsin, trypsinogen, and enteropeptidase, respectively, and P, IP, and IP′ are enzymically inactive peptides or proteins. The first reaction scheme describes the conversion of trypsinogen to active trypsin or inactive protein as originally described by Kunitz (1939a). The second reaction describes the conversion of trypsinogen by enteropeptidase solely to active trypsin (Kunitz, 1939b; Maroux et al., 1971). The last scheme represents the bimolecular inactivation of trypsin (Kunitz and Northrop, 1934).

The rate equations describing the disappearance of trypsinogen and the appearance and deactivation of trypsin are

$$d[T]/dt = k_1[T][TG] + k_3[EP][TG] - k_4[T][T]$$

$$d[TG]/dt = -k_1[T][TG] - k_2[T][TG] - k_3[EP][TG]$$

An explicit solution for [T] in terms of time only is not possible.

The rate constant k_4 was estimated by analyzing data from the latter part of each activation experiment. Here the concentration of trypsinogen is essentially zero and the disappearance of trypsin is described solely by the bimolecular inactivation term.

Estimation of the rate constants k_1, k_2, and k_3 was carried out according to the Euler method (Bajpai et al., 1975) for computing values of [T] as a function of t for preset values of the rate constants. The values of the rate constants were then adjusted using a least-squares nonlinear regression program based on the Marquardt algorithm (Marquardt, 1963).

The solid lines in Figures 1–3 have been drawn according to best fit values of the rate constants and requirements of the two differential equations listed above. They were generated using a digital plotter, and each point comprising the curve was calculated using the Euler method series

$$A_n = A_{n-1} + (dA/dt)_{n-1}\Delta t$$

where A is either [T] or [TG]. A_0 is the active trypsin or trypsinogen concentration at $t = 0$. The value of $[TG]_0$ and [EP] was determined from the absorbance at 280 nm of the protein stock solutions and the dilution within each reaction mixture, whereas $[T]_0$ was determined from the trypsin activity of the reaction mixture immediately after initiation of the activation reaction. The time interval between each point, Δt, was set arbitrarily small (compared to the duration of the activation experiment) at 1 min.

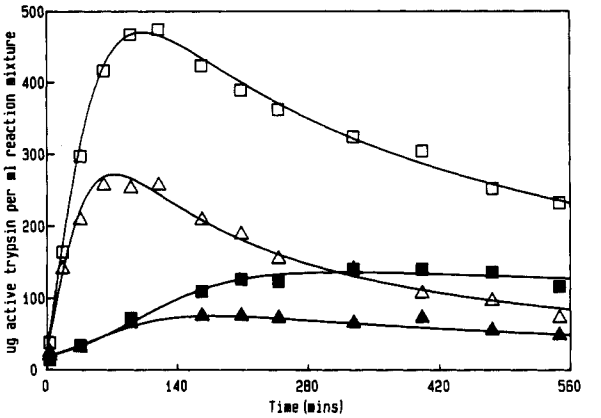

Figure 1. Activation of trypsinogen at pH 6.0 in the presence and absence of enteropeptidase and at different calcium to phytate ratios. (□, ■) 10 mM Ca^{2+}, 5 mM phytate; (△, ▲) 0 mM Ca^{2+}, 1.5 mM phytate. (Open symbols) with enteropeptidase; (solid symbols) without enteropeptidase. $[TG]_0$ = 832 μg/mL.

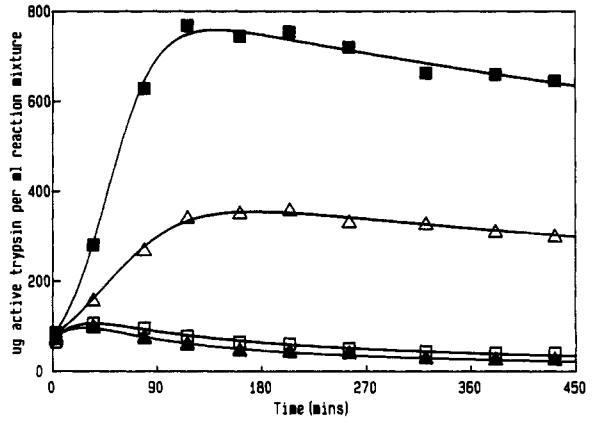

Figure 2. Activation of trypsinogen at pH 8.1 by trypsin alone at different calcium and phytate concentrations. (■) 10 mM Ca^{2+}, 0 mM phytate; (△) 10 mM Ca^{2+}, 1.5 mM phytate; (□) 10 mM Ca^{2+}, 5 mM phytate; (▲) 0 mM Ca^{2+}, 1.5 mM phytate. $[TG]_0$ = 922 μg/mL; $[T]_0$ = 75 μg/mL.

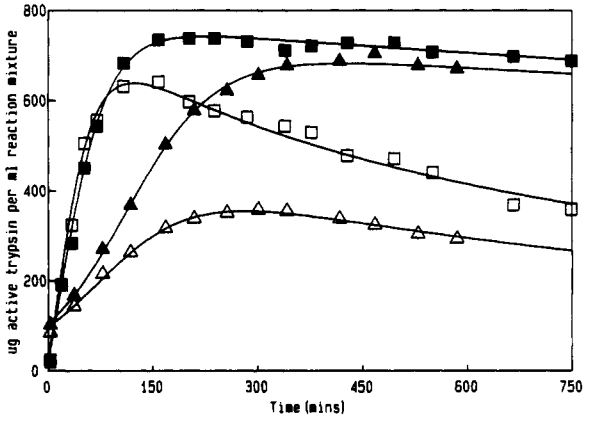

Figure 3. Activation of trypsinogen at pH 6.0 by either trypsin or enteropeptidase. (□, ■) with enteropeptidase, $[TG]_0$ = 820 μg/mL; (△, ▲) with trypsin, $[TG]_0$ = 987 μg/mL, $[T]_0$ = 101 μg/mL. (Solid symbols) 10 mM Ca^{2+}, 0 mM phytate; (open symbols) 10 mM Ca^{2+}, 1.5 mM phytate.

RESULTS AND DISCUSSION

Data obtained from selected activation experiments together with the digitally plotted curves appear in Figures 1–3 to illustrate the extent of the effect of phytate on the in vitro activation of trypsinogen. The mean values for the rate constants obtained from these and other analyses are presented in Table I. The data are adequately fitted

to the rate equations for the three reaction schemes given under Data Analysis. The major effect of phytate is on the rate constants k_2 and k_4, characterizing the inactivating reactions of trypsin. Both rate constants can be related to the function of the Ca^{2+} binding sites of trypsin and trypsinogen. Trypsinogen has two Ca^{2+} binding sites. The first, a tight binding site, occurs in trypsin also and protects both against the formation of inert proteins. The second, a weaker binding site, occurs only in trypsinogen and has been postulated to enhance the formation of active trypsin by increasing the rate of hydrolysis of the Lys(6)–Ile(7) bond in trypsinogen (Delaage and Lazdunski, 1967). However, the data presented in Table I indicate that the rate constant for this hydrolysis, k_1, is relatively unaffected by the calcium status of the reaction mixture, although some increase in k_1 at pH 8.1 is apparent in the reaction mixture containing only calcium.

The rate constant k_2 is greater at pH 8.1 than at pH 6.0. This is consistent with the findings of Kunitz (1939b) that the formation of inert protein from trypsinogen is greatly reduced at pHs less than 6.0. The two combinations of calcium and phytate result in calcium–phytate precipitates. The work of Nolan et al. (1987) suggests that for the 10 mM Ca^{2+}/1.5 mM phytate combination the stoichiometry of the calcium–phytate complex will be predominantly Ca_5 (phytate), indicating an excess of free calcium. That a marked increase in k_2 (and k_4) occurs for this combination, compared to the 10 mM Ca^{2+} solution, suggests that there is some destabilizing interaction on both trypsinogen and trypsin from the formation of the calcium–phytate precipitate. The tri- or dicalcium complex is the more likely for the 10 mM Ca^{2+}/5 mM phytate combination (Nolan et al., 1987), and it is unlikely that there will be a substantial level of free calcium ions in this reaction mixture. That there is a difference in k_2 and k_4 between this reaction mixture and the one containing only phytate suggests that the di- or tricalcium–phytate complex can provide some stabilization against autolysis and inert protein generation for both trypsinogen and trypsin. The reason for such effects may be due either to the formation of ternary complexes or competitive sequestration of Ca^{2+} ions between phytate and protein. Ternary complexes between cations, phytic acid, and protein have been proposed to explain observed interactions between phytate and protein at neutral pH (Omosaiye and Cheryan, 1979; Nosworthy and Caldwell, 1988). The interaction is mediated through a cationic bridge between two anionic components; however, binding is not observed in the absence of divalent cations. The prediction of a cationic bridge between the negatively charged hexapeptide region of trypsinogen and phytate may be a plausible explanation for the increase in the rate constant k_2. Binding of calcium ions also retards autolytic degradation of trypsin, and the existence of a similar interaction between trypsin and the calcium–phytate complex may also explain the increase in the rate constant k_4.

Evidence supporting competitive sequestration of calcium comes from previous work (Chiancone et al., 1985) which has shown that the calcium chelator, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), rapidly reverses the binding of calcium to trypsin and trypsinogen. Other work (Desnuelle and Gabeloteau, 1957) on the role of calcium in trypsinogen activation and trypsin stability showed that a similar chelator, ethylenediaminetetraacetic acid (EDTA), produced results consistent with those given in this work.

The rate constant k_3 was found to be 10 (mg per mL of enteropeptidase)$^{-1}$ min^{-1}. This value was little affected by the calcium or pH status of the reaction mixtures. Maroux et al. (1971) found that the kinetic parameters for porcine enteropeptidase were unaffected by Ca^{2+} ions and the activity of enteropeptidase toward benzoyl-L-arginine ethyl ester only increased by ~8% from pH 6 to pH 8.

Reference to Figure 1 indicates that enteropeptidase has a very positive effect in that it rapidly activates trypsinogen and reduces the degradative impact of inert protein generation in reaction scheme 1. The degradative effect of reaction scheme 3, however, will not be affected by either the presence or absence of enteropeptidase. The essentiality of enteropeptidase in the digestion process has been adequately demonstrated by Tarlow et al. (1970) in children with intestinal enteropeptidase deficiency.

The observed effect of phytate on the in vitro activation of trypsinogen and the stability of trypsin (Figure 2, for example) is considerable and if reproduced in vivo might inhibit growth more so than that shown in nutritional studies (Atwal et al., 1980; Richardson et al., 1985; Spinelli et al., 1983). Some nutritional studies have shown that phytate has no effect on protein utilization (Thompson and Serraino, 1986). The explanation for this is uncertain but may be dependent on the cationic component of the phytate complex in the diets used. Another explanation may be due to the ability of the pancreas to increase secretion of digestive proteases in response to proteolytic dysfunction. Pancreatic enzyme secretion is stimulated by cholecystokinin (CCK), and in many species the release of CCK is under negative feedback control by trypsin. The specificity of this feedback control by trypsin is demonstrated by the ability of trypsin inhibitors in raw soybean flour to increase plasma CCK levels in rats (Liddle et al., 1984) and humans (Calam et al., 1987). Raw soybean meal produces pancreatic hypertrophy in chicks and rats (Garlich and Nesheim, 1966; Booth et al., 1964) and may be related to CCK stimulation of pancreatic growth. It may be coincidental that similar morphological changes have also been observed in fish fed high-phytate diets (Richardson et al., 1985). However, pancreatic hypertrophy does not occur in all species in response to raw soybean meal or soybean trypsin inhibitor (Kakade et al., 1975; Yen et al., 1977), and trypsins from different species have been reported to respond differently to trypsin inhibitors (Struthers and MacDonald, 1983; Temler et al., 1983).

The effect of phytate on in vivo mineral bioavailability is well documented and may be such as to obscure a minor physiological role in protein utilization for some species. In this study the effect of phytate on the activation of bovine pancreatic trypsinogen has been presented. The work should be extended to trypsins from commercially farmed species, especially those for which soybean or other high-phytate meals are used as a major dietary component.

LITERATURE CITED

Atwal, A. S.; Eskin, N. A. M.; McDonald, B. E.; Vaisey-Genser, M. The effects of phytate on the nitrogen utilization and zinc metabolism in young rats. *Nutr. Rep. Int.* **1980**, *21*, 257–267.

Bajpai, A. C.; Calus, I. M.; Fairley, J. A. In *Numerical Methods for Engineers and Scientists*; Taylor and Francis: London, 1975; pp 287–290.

Barre, M. R. *Ann. Pharm. Fr.* **1956**, *14*, 182–193.

Booth, A. N.; Robbins, D. J.; Ribelin, W. E.; DeEds, F.; Smith, A. K.; Rackis, J. J. Prolonged pancreatic hypertrophy and reversibility in rats fed raw soybean meal. *Proc. Soc. Exp. Biol. Med.* **1964**, *116*, 1067–1069.

Calam, J.; Bojarski, J. C.; Springer, C. J. Raw soya-bean flour increases cholecystokinin release in man. *Br. J. Nutr.* **1987**, *58*, 175–179.

Chiancone, E.; Drakenberg, T.; Teleman, O.; Forsen, S. Dynamic and structural properties of the calcium binding site of bovine serine proteases and their zymogens. *J. Mol. Biol.* **1985**, *185*, 201–207.

Delaage, M.; Lazdunski, M. The binding of Ca^{2+} to trypsinogen and its relation to the mechanism of activation. *Biochem. Biophys. Res. Commun.* **1967**, *28*, 390–394.

Desnuelle, P.; Gabeloteau, C. *Arch. Biochem. Biophys.* **1957**, *69*, 475–485.

Garlich, J. D.; Nesheim, M. C. Relationship of fractions of soybeans and a crystalline soybean trypsin inhibitor to the effects of feeding unheated soybean meal to chicks. *J. Nutr.* **1966**, *88*, 101–110.

Hummel, B. C. W. A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. *Can. J. Biochem. Physiol.* **1959**, *37*, 1393–1399.

Inagawa, J.; Kiyosawa, I.; Nagasawa, T. Effects of phytic acid on the digestion of casein and soybean protein with trypsin, pancreatin and pepsin. *Nippon Eiyo Shokuryo Gakkaishi* **1987**, *40*, 367–373.

Kakade, M. L.; Thompson, R. D.; Engelstad, W. E.; Behrens, G. C.; Yoder, R. D.; Crane, F. M. Failure of soybean trypsin inhibitor to exert deleterious effects in calves. *J. Dairy Sci.* **1975**, *59*, 1484–1489.

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.

Kunitz, M. Effect of the formation of inert protein on the kinetics of the autocatalytic formation of trypsin from trypsinogen. *J. Gen. Physiol.* **1939a**, *22*, 293–310.

Kunitz, M. Formation of trypsin from crystalline trypsinogen by means of enterokinase. *J. Gen. Physiol.* **1939b**, *22*, 429–446.

Kunitz, M.; Northrop, J. H. Inactivation of crystalline trypsin. *J. Gen. Physiol.* **1934**, *17*, 591–615.

Liddle, R. A.; Goldfine, I. E.; Williams, J. A. Bioassay of plasma cholecystokinin in rats: Effects of food, trypsin inhibitor and alcohol. *Gastroenterology* **1984**, *87*, 542–549.

Lott, J. N. A. Accumulation of seed reserves of phosphorus and other minerals. In *Seed Physiology*; Academic Press: Sydney, 1984.

Maga, J. A. Phytate: Its chemistry, occurrence, food interactions, nutritional significance and methods of analysis. *J. Agric. Food Chem.* **1982**, *30*, 1–9.

Maroux, S.; Baratti, J.; Desnuelle, P. Purification and specificity of porcine enterokinase. *J. Biol. Chem.* **1971**, *246*, 5031–5039.

Marquardt, D. W. An algorithm for least-squares estimation of non-linear parameters. *J. Soc. Ind. Appl. Math.* **1963**, *11*, 431–441.

McDonald, M. R.; Kunitz, M. The effect of calcium and other ions on the autocatalytic formation of trypsin from trypsinogen. *J. Gen. Physiol.* **1941**, *25*, 53–73.

Nolan, K. B.; Duffin, P. A.; McWeeny, D. J. Effects of phytate on mineral bioavailability. In Vitro studies on Mg^{2+}, Ca^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} (also Cd^{2+}) solubilities in the presence of phytate. *J. Sci. Food Agric.* **1987**, *40*, 79–85.

Nosworthy, N.; Caldwell, R. A. The interaction of zinc(II) and phytic acid with soya bean glycinin. *J. Sci. Food Agric.* **1988**, *44*, 143–150.

Omosaiye, O.; Cheryan, M. Low-phytate, full-fat soy protein product by ultrafiltration of aqueous extracts of whole soybeans. *Cereal Chem.* **1979**, *56*, 58–63.

Reddy, N. R.; Sathe, S. K.; Salunkhe, D. K. Phytates in legumes and cereals. *Adv. Food Res.* **1982**, *28*, 1–92.

Richardson, N. L.; Higgs, D. A.; Beames, R. M.; McBride, J. R. Influence of dietary calcium, phosphorus, zinc and sodium phytate level on cataract incidence, growth and histopathology in juvenile chinook salmon (oncorhynchus tshawytscha). *J. Nutr.* **1985**, *115*, 553–567.

Singh, M.; Krikorian, A. D. Inhibition of trypsin activity in vitro by phytate. *J. Agric. Food Chem.* **1982**, *30*, 799–800.

Spinelli, J.; Houle, C. R.; Wekell, J. C. The effect of phytates on the growth of rainbow trout (salmo gairdneri) fed purified diets containing varying quantities of calcium and magnesium. *Aquaculture* **1983**, *30*, 71–83.

Struthers, B. J.; MacDonald, J. R. Comparative inhibition of trypsins from several species by soybean trypsin inhibitors. *J. Nutr.* **1983**, *113*, 800–804.

Tarlow, M. J.; Hadorn, B.; Arthurton, M. W.; Lloyd, J. K. Intestinal enterokinase deficiency. A newly recognized disorder of protein digestion. *Arch. Dis. Child.* **1970**, *45*, 51–655.

Temler, R. S.; Simon, E.; Amiguet, P. Comparison of the interactions of soya bean protease inhibitors with rat pancreatic enzymes and human trypsin. *Enzyme* **1983**, *30*, 105–114.

Thompson, L. U.; Serraino, M. R. Effect of phytic acid reduction on rapeseed protein digestibility and amino acid absorption. *J. Agric. Food Chem.* **1986**, *34*, 468–469.

Walsh, K. A. Trypsinogens and trypsins of various species. *Methods Enzymol.* **1970**, *19*, 41–63.

Yen, J. T.; Jensen, A. H.; Simon, J. Effect of dietary raw soybean and soybean trypsin inhibitor on trypsin and chymotrypsin activities in the pancreas and in small intestinal juice of growing swine. *J. Nutr.* **1977**, *107*, 156–165.

Received for review May 1, 1991. Revised manuscript received September 9, 1991. Accepted September 16, 1991.

Registry No. Ca, 7440-70-2; trypsinogen, 9002-08-8; trypsin, 9002-07-7; enteropeptidase, 9014-74-8; phytic acid, 83-86-3.