Effect of Phytate on the in Vitro Activity of Digestive Proteinases

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Phytate inhibits the action of pepsin on the proteins but does not affect the pepsin hydrolysis of a low molecular weight substrate, acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine. The inhibition is maximal at pH 2–3 and drops to zero when the pH is increased to 4.0–4.5. Trypsin hydrolysis of both a low molecular weight substrate (benzoyl-D,L-arginine p-nitroanilide) and proteins is insensitive to the presence of phytate. The proteins tested include RNase and lysozyme charged positively at the pH of the trypsin hydrolysis. Thus, the inhibitory action of phytate is manifested only when it is bound with the protein substrate. The overall positive charge of protein is not a sufficient condition of phytate binding. Evidently, the protonation of protein carboxylate groups is also required.

INTRODUCTION

The effect of phytate on the bioavailability of zinc and some other cations is well established (Maga, 1982). Its inhibiting action on the activity of digestive proteinases, pepsin (Barré, 1956; Camus and Laporte, 1976; Knuckles et al., 1985) and trypsin (Singh and Krikorian, 1982), was also reported. According to different authors the inhibition of pepsin at comparable phytate concentrations varies from about 10% (Knuckles et al., 1985) to about 80% (Camus and Laporte, 1976). Considerable inhibition of trypsin action was observed only at high phytate concentrations (Singh and Krikorian, 1982). On the contrary, in a recent work (Reddy et al., 1988) no inhibiting effect of phytate on trypsin hydrolysis was detected, and the authors conclude that phytate does not act on the activity of digestive proteases. In this work an attempt is made to look into these contradictions and to determine the conditions of the inhibitory action of phytate on enzymatic proteolysis.

MATERIALS AND METHODS

Reagents. Proteinases used were porcine pepsin (Olajne) and bovine trypsin (Spofa). Protein substrates used were bovine serum albumin (BSA) from Serva, casein (Reachim), soybean 11 S protein (phytate content 0.4%) isolated according to the procedure of Thanh and Shibasaki (1976), hemoglobin, RNase, and lysozyme (Reanal). The last two proteins were reduced by 2-mercaptoethanol and dialyzed against 0.2 M Tris-HCl buffer after blocking the resulting sulfhydryl groups with N-ethylmaleimide. Synthetic substrates used were benzoyl-D,L-arginine p-nitroanilide (BAPA) from Merck and acetyl-L-phenylalanyl-3,5-diiodotyrosine (APIT) synthesized according to the method of Baker (1951).

Sodium phytate was prepared from calcium phytate by ion exchange on Dowex 50 in H^+ form with subsequent neutralization. The sodium phytate formed was precipitated by ethanol and dried in vacuo over H_2SO_4.

Enzymatic Hydrolysis. Pepsin hydrolysis was performed at pH values from 2.0 to 4.5 (1.0–4.5 for casein) and that by trypsin at pH 7.5. The assay mixture consisted of 1.5 mL of 2.5% protein dispersion in an appropriate buffer (0.1 M phosphate–citrate for pepsin and 0.2 M Tris-HCl for trypsin hydrolysis), of enough phytate stock solution in the same buffer to obtain the desired concentration of phytate and enough buffer solution to bring the volume of the mixture up to 3 mL. Phytate was omitted in control experiments. The mixture was preincubated at 37 °C for 30 min, and then 1.5 mL of enzyme solution was added. The enzyme/protein ratio was 1:400 for trypsin and 1:250 for pepsin. When the hydrolysis was performed at pH lower than 2.0, the protein and phytate were dissolved in 0.05 M HCl and the desired pH was adjusted by adding 2.0 M HCl. The concentrations of phytate stock solutions were 11.25 and 160 mg/mL (0.2 M). The latter was the same as in the work of Singh and Kirkorian (1982) and was used while the effect of high phytate concentrations on trypsin hydrolysis was studied. By adjusting the pH of the concentrated stock solution its diluted samples were used for all measurements to avoid the distortion of the pH meter readings due to the effect of the high ionic strength on the glass electrode. At pH 7.5 the charge of the phytate ion exceeds 7.0 (Costello et al., 1976) and the ionic strength of the concentrated stock solution was higher than 5.6.

The assay mixture was incubated for 2 h at 37 °C with continuous stirring. Samples (0.3 mL) were taken for analysis every 0.5 h, and the reaction was stopped by the addition of 0.1 mL of 15% TCA. The degree of hydrolysis was characterized by the content of NH_2 groups in the TCA-soluble fraction which was determined by the reaction with 2,4,6-trinitrobenzenesulfonic acid (Satake et al., 1960). In several experiments the absorbance of the TCA-soluble fraction at 280 nm was measured directly. Pepsin hydrolysis of casein at pH 2.0 was also carried out with simultaneous dialysis against the buffer solution. In this case the action of the enzyme was stopped by raising the pH. The residual nonhydrolyzed protein was determined by its reaction with Coomassie brilliant blue (Bradford, 1976).

APIT was hydrolyzed by pepsin at pH 2.0. The concentration of the substrate was 4×10^{-4} M and that of enzyme 0.05 mg/mL. The reaction was stopped by adjusting the pH of the incubation mixture to 9, and the NH_2 groups formed were determined by the trinitrobenzenesulfonic method. Trypsin hydrolysis of BAPA was measured as described by Erlanger et al. (1961).

Each determination was performed in triplicate. The results were expressed in absorbance units. The standard deviation was equal to 0.02 absorbance unit. The degree of inhibition was calculated as the relative decrease of the hydrolysis in the presence of phytate.

RESULTS

Pepsin Hydrolysis. The effect of increasing phytate concentration on the course of hemoglobin hydrolysis is shown in Figure 1. Similar results where obtained when the hydrolysis of several other proteins (casein, BSA, and soybean 11 S protein) was studied. The calculated values of inhibition decline with the increase in digestion time (Figure 2). The values obtained after 0.5 h of digestion are given below. The continuous removal of digestion products by dialysis did not alter significantly either the proteolysis course or its inhibition by phytate.

The degree of inhibition rises rapidly with the increase in phytate concentration, but after attaining a definite value characteristic of each of the investigated proteins,

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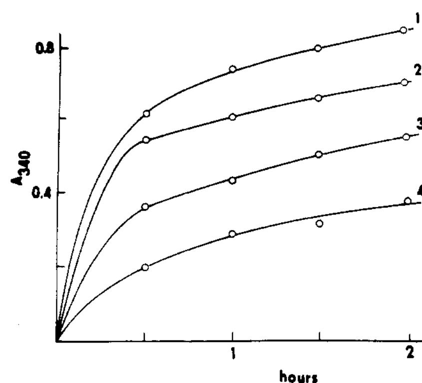

Figure 1. Effect of phytate on the pepsin hydrolysis of hemoglobin at pH 2.0. Phytate concentrations: 0 (1), 0.83 (2), 1.67 (3), and 2.5 (4) mg/mL.

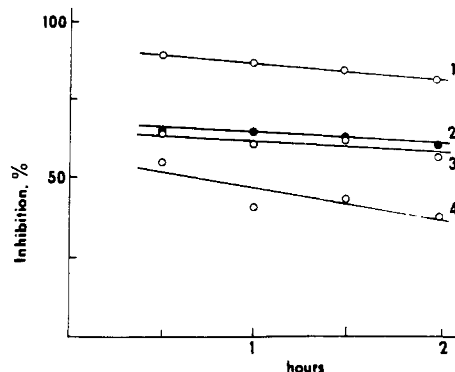

Figure 2. Effect of the hydrolysis time on the calculated values of the inhibition of pepsin hydrolysis by phytate. Conditions: pH 2.0; phytate concentration, 2.5 mg/mL. Substrates: BSA (1); hemoglobin (2); casein (3); soybean 11 S protein (4).

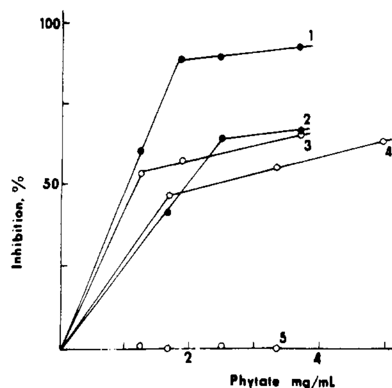

Figure 3. Effect of phytate concentration on the inhibition of pepsin hydrolysis. Conditions: pH 2.0; hydrolysis time, 0.5 h. Substrates: BSA (1); hemoglobin (2); casein (3); soybean 11 S protein (4); APIT (5).

the increase of the inhibition slows significantly (Figure 3). The inhibition percent at the inflection point also depends on the substrate used and varies from 60% (11 S soybean protein) to 92% (BSA).

The hydrolysis of APIT is not affected by the addition of phytate (Figure 3).

The inhibition of protein substrates by phytate is strongly affected by pH. Maximal inhibition is observed at pH 2–3 (Figure 4). It decreases rapidly at higher pH until it reaches zero at pH 4.0–4.5. Decreasing of pH below 2.0 also diminishes the degree of inhibition.

The high inhibition of protein hydrolysis by phytate is in agreement with the results of Camus and Laporte (1975) and with the early work by Barré (1956). A considerably lower inhibition was observed by Knuckles et al. (1985),

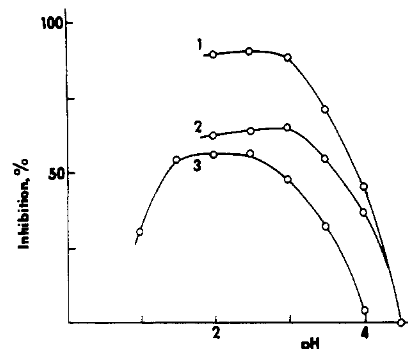

Figure 4. Effect of pH on the phytate inhibition of pepsin hydrolysis. Conditions: phytate concentration, 2.5 mg/mL; hydrolysis time 0.5 h. Substrates: BSA (1); hemoglobin (2); casein (3).

Table I. Effect of Phytate on Trypsin Activity

substrate	phytate concn, mg/mL	absorbance	
		−phytate	+phytate
casein	2.5	0.737	0.747
casein	9.3	0.560[a]	0.546[a]
casein	13.5	0.775	0.755
casein	20.6	0.727	0.754
casein	23.0	0.740	0.736
casein	47.5	0.742	0.730
casein	71.3	0.715	0.708
RNase	2.5	0.210	0.205
lysozyme	2.5	0.093	0.097
BAPA	3.6	0.655[b]	0.654[b]

[a] A_{280} of the TCA-soluble fraction. [b] A_{410}.

who performed the hydrolysis at pH 1.0 with simultaneous dialysis. Our results show that low inhibition is not due to the removal of phytate-containing hydrolysis products and may be at least partially explained by a lower inhibition effect of phytate at pH 1.0.

Trypsin Hydrolysis. The data given in Table I indicate that the hydrolysis of none of the investigated substrates is inhibited by phytate even at concentrations as high as those used by Singh and Kirkorian (1982). All differences observed are within experimental error. The hydrolysis of BAPA also is not inhibited.

Singh and Kirkorian (1982) reported an inhibition of trypsin action from 2.7 to 20% at very high phytate concentrations (from 7.8 to 71.3 mg/mL). The inhibition increased considerably if the enzyme was preincubated with phytate at 37 °C for 30 min. However, if the distorting effect of high ionic strength is not taken into consideration during the adjustment of the pH of the phytate stock solution, its real pH is essentially higher and results in a higher pH of the incubation mixture (e.g., 8.9 instead of 7.5 at a final phytate concentration of 31 mg/mL). The decrease in trypsin activity at higher pH may simulate the inhibition by phytate. The higher inhibition observed when the preincubation of trypsin with phytate was performed may also be apparent and may be due to the trypsin autolysis. According to our data, preincubation of the trypsin alone at 37 °C for 30 min diminishes its activity by 34%. It is possible that these error sources had not been taken into consideration by Singh and Kirkorian (1982).

DISCUSSION

The presence of phytate inhibits only the digestion of proteins and does not affect the hydrolysis of low molecular weight substrates by pepsin. These data confirm that the inhibition is due to the phytate interaction with protein substrate (Barré, 1956; Camus and Laporte, 1976).

It was suggested that the binding of phytate with proteins occurs via the positively charged groups of the latter and proceeds at pH lower than the isoelectric point of the proteins (Camus and Laporte, 1976; Okubo et al., 1976, and the works cited therein).

If the inhibition by phytate is accepted as proof of its binding with proteins, it may be suggested that the pH range of phytate–protein binding is considerably narrower. Indeed, the action of trypsin on RNase and lysozyme is not inhibited by phytate, although these proteins are at pH lower than their isoelectric point under the hydrolysis conditions. The inhibition of pepsin hydrolysis stops at the same pH (4.00–4.5) for the casein, BSA, and 11 S soybean proteins (p*I* 4.7–5.0) and for hemoglobin (p*I* 7.0). Apparently the phytate–protein binding is conditioned not only by the overall positive charge of the latter but also by the suppression of ionization of its carboxyl groups. The inhibition begins at pH corresponding approximately to the p*K* of carboxyl groups and reaches its maximum when their dissociation is completely suppressed. A significant decrease of inhibition at lower pH is evidently due to the decrease of phytate binding caused by suppression of the ionization of phosphate groups of phytate, their p*K* being equal to 1.5 (Costello et al., 1976). A direct study of the effect of pH on the phytate–protein interactions is still needed to check the assumptions expressed.

ABBREVIATIONS USED

APIT, acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine; BAPA, benzoyl-D,L-arginine *p*-nitroanilide; BSA, bovine serum albumin.

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Registry No. Sodium phytate, 14306-25-3; pepsin, 9001-75-6; trypsin, 9002-07-7; phytic acid, 83-86-3.