Inhibition of Trypsin Activity in Vitro by Phytate

In vitro activity of the proteolytic enzyme trypsin using casein as the substrate was substantially inhibited by low levels of phytic acid (*myo*-inositol hexaphosphate). The possible significance of this finding for protein availability in nutrition is discussed.

Phytates or salts of phytic acid, *myo*-inositol hexaphosphate or more properly *myo*-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate), have long been recognized as components of certain seeds and plant tissues—especially cereal grains and oilseeds (Cosgrove, 1966). It has also been appreciated for some time that if phytates comprise a substantial part of the diet (greater than 1% or so), they can interfere with the bioavailability of elements such as zinc, calcium, magnesium, etc. (Erdman, 1979). Because of the nutritional implications of the binding of phytic acid with these and related ions, there has been a renewed and substantial interest in it [see Oberleas (1973) and Cheryan (1980 and references cited therein)]. Although less attention has been placed on phytate–protein interactions (de Rham and Jost, 1979), it has been reported that phytic acid can inhibit pepsin activity (Camus and Laporte, 1976) and α-amylase activity in vitro (Cawley and Mitchell, 1968; Sharma et al., 1978). In this short communication we report that phytic acid can also inhibit the proteolytic enzyme trypsin.

EXPERIMENTAL SECTION

Materials. Trypsin from bovine pancreas, 2× crystallized, dialyzed, and essentially salt free (Lot No. 79C-8015), casein, essentially vitamin free (Lot No. 69C-0416), and phytic acid—ex Corn, Type V, sodium salt (Lot No. 118C-0067)—were all obtained from Sigma Chemical Co., St. Louis, Mo. Other chemicals were reagent grade.

General Procedures. The assay system for trypsin activity consisted of 2.0 mL of Tris-HCl buffer, 0.2 M, pH 7.5, 1.0 mL of 2.5% casein solution, pH 7.5, and 0.2 mL of enzyme (100 μg of enzyme), 0.2 M sodium phytate, pH 7.5, was added to suply the various concentrations, and the volume was made up to 5.0 mL with water. The mixture was heated for 5 min at 37 °C, and this was followed by the addition of enzyme to start the reaction. The incubation was carried out for 20 min with stirring. The reaction was stopped by the addition of 0.5 mL of 50% w/v cold trichloroacetic acid. Controls were run including the appropriate amount of phytate wherever necessary and enzyme was added after the addition of Cl_3AcOH. After the mixture was chilled for about 1 h at 0–4 °C, clear supernatants were separated on a refrigerated centrifuge at 3000g for 20 min.

The clear supernatants were suitably diluted (generally 4×), and the optical d. was read at 280 nm. In some experiments, the enzyme was preincubated with sodium phytate, pH 7.5, at 4 °C and also at 37 °C for 30 min and then used for assay. In another set of similar experiments, Tris-HCl buffer containing 0.025 M $CaCl_2$ was also used. The experiments were repeated several times.

RESULTS AND DISCUSSION

The inhibition of trypsin activity at pH 7.5 in the presence of phytate under different conditions is given in Table I. The data indicate that the inhibition of trypsin activity by phytate varies with the phytate concentration. It was immaterial whether the buffer used for the assay contained any calcium ion or not. Preincubation of the enzyme with phytate at a higher temperature (37 °C) produced a higher level of inhibition than that at the lower temperature (4 °C), and the degree of inhibition was not significantly changed with more added phytate. Similarly, the preincubation of the enzyme with phytate (0.1 M, pH 7.5) at 37 °C for 30 min diminished the enzyme activity by about 46%. The inclusion of $CaCl_2$ in the assay buffer did not significantly alter the inhibition pattern whether the enzyme was preincubated or not.

Trypsinogen, the inactive precursor of trypsin, is known to bind calcium ions at two sites, one being located in the body of the molecule (Delaage and Lazdunski, 1967; Abita et al., 1969; Radhakrishnan et al., 1969), whereas trypsin has only one binding site for calcium (Delaage and Lazdunski, 1967). Calcium ion retards trypsin autolysis and promotes activation of trypsinogen to form trypsin (Gomez et al., 1974). Thus, the inhibitory effect of phytic acid on trypsin might be due to binding of trypsin calcium, and it is suggested that under some circumstances in vivo it could well affect the conversion of inactive trypsinogen into trypsin by virtue of its binding affinity for calcium. The possibility of the direct interaction of protein, either enzymic or nonenzymic (i.e., substrate), with phytic acid

Table I. Percent Inhibition of Trypsin Activity in Vitro by Phytate

experimental conditions	phytate concn, mM	% inhibition of enzyme act.
Tris-HCl buffer without $CaCl_2$	10	2.7 ± 0.21
no preincubation of enzyme	20	5.6 ± 0.40
	40	9.9 ± 1.60
	60	12.4 ± 1.73
	80	15.3 ± 1.67
	90	19.6 ± 2.10
enzyme preincubated with 0.1 M phytate at 37 °C for 30 min; 0.2 mL of enzyme-phytate solution used for assay	no additional phytate	42.5 ± 2.34
	phytate added[a] to make 40 mM	45.9 ± 2.21
Tris-HCl buffer containing 0.025 M $CaCl_2$, no preincubation of enzyme	with 40 mM phytate added	13.2 ± 1.32
enzyme preincubated with 0.1 M phytate at 4 °C for 30 min	no additional phytate	7.9 ± 1.00
enzyme preincubated with 0.1 M phytate at 37 °C for 30 min	phytate added[a] to make 40 mM	45.8 ± 2.45

[a] Phytate present in the preincubated enzyme plus added phytate gave a 40 mM final strength with respect to phytate.

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could also, to some extent, be responsible for the phytate-induced inhibition of trypsin.

We appreciate that the measurement of proteolytic activity in vitro by the use of casein as a substrate has its limitations and entails uncertainties. One of these is the measurement of enzyme activity by the increase in ultraviolet absorbency. Since the proteolytic action is observable only when UV-absorbing amino acids are released, the observed proteolysis would be somewhat less than the real activity (Reimerdes and Klostermeyer, 1976). We submit, therefore, that the inhibition of trypsin activity by phytate is likely to be even greater than that observed by the method used here. The question naturally arises whether the phytate-based inhibition reported here has any significance in animal nutrition in terms of possible effects on protein *availability*.

Phytate is known to affect the bioavailability of zinc and calcium, possibly by forming very stable complexes with these cations (Erdman, 1979). Vohra et al. (1965), give stability constants as $Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$ at pH 7.4. Both diminished intestinal retention of calcium due to the presence of phytate and diminished absorption have been demonstrated (Cosgrove, 1966). Phytate is also involved in iron deficiency (Sharpe et al., 1950) and zinc deficiency (Maddaiah et al., 1964). Some of the digestive enzymes contain calcium (α-amylase) or zinc (phosphatase, carboxypeptidases, leucine aminopeptidase). Moreover calcium activates some enzymes such as phospholipases and also the conversion of trypsinogen to trypsin. Yet another important role of calcium and zinc is in the secretion of insulin. Calcium deficiency completely blocks insulin secretion; a requirement for zinc in the release of stored insulin has been suggested (Grodsky and Forsham, 1966). The conversion of proinsulin into insulin is also carried out by trypsin. Thus, phytate inhibits not only trypsin activity but also its formation from trypsinogen. The formation of insoluble complexes between proteins and phytic acid are well-known (Cosgrove, 1966). To our knowledge there is no report regarding the suitability of phytic acid–protein complexes as substrates for various proteases. In short, trypsin inhibition by phytate could be one of a number of potential effects of phytate in human nutrition.

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