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Myo-inositol Hexasulfate Is a Potent Inhibitor of *Aspergillus ficuum* Phytase

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Myo-inositol hexasulfate (MIHS), a structural analog of the substrate myo-inositol hexaphosphate, is a potent competitive inhibitor of both phyA and phyB enzymes. The K_i of inhibition for the phyA and phyB proteins were estimated to be 4.6 and 0.2 μM, respectively. Thus, the phyB protein is 23-fold more sensitive to MIHS inhibition than the phyA protein. The active-site geometry of phyB protein is presumed to be very different from the phyA protein as deduced by chemical probing of the enzymes by Arg-specific modifiers, i.e., 1,2-cyclohexanedione and phenylglyoxal. Probing the catalytic site of the same proteins by this newly developed specific inhibitor also gives a similar conclusion.

Myo-inositol hexaphosphate, phytic acid, is abundant in food grains. Cotton seeds, soybean meals and seeds of other legumes contain high levels of phytic acid (1). Phytic acid is the predominant storage form of phosphorus in both monocot and dicot plants. Phytic acid is a known antinutrient because it chelates essential minerals (2). Its presence, in excessive amounts, in soybean and cotton seed meal thus poses a nutritional problem in monogastric animals, i.e., chickens, hogs, humans, etc. One enzyme that can degrade phytic acid quite efficiently is phytase (3). We characterized one fungal phosphomonoesterase (EC 3.1.3.8), which is capable of efficiently degrading phytic acid at acidic pH range (4). The enzyme, phytase or phyA, is a member of the subfamily "Histidine Acid Phosphatase." The phyA gene has received a great deal of attention lately from biotechnology companies, especially in Europe. The Dutch company, Gist-brocade, has cloned and overexpressed phyA gene from *Aspergillus ficuum* (5). The reaction *Aspergillus ficuum* phytase catalyzes is shown in Fig. 1. We also identified, purified, and characterized another phytase from the same fungal strain of *Aspergillus ficuum*. The enzyme was referred to as phyB (6). Like phyA the phyB enzyme is also a histidine acid phosphatase (7). Besides the active site similarities, phyA differed from phyB proteins in terms of catalytic properties and pH optima of the enzyme reaction (8). We performed chemical probing of the active site of both phyA and phyB proteins and identified the active site residues in fungal phytases (9, 10). Recently, a Swiss group deduced the three-dimensional structure of A. ficuum phytase (11). The chemical data presented from our laboratory thus far complement very well the X-ray deduced structure of phytase (12). No specific inhibitor of phyA or phyB protein has yet been reported in the literature. In this communication we show that *myo*-inositol hexasulfate is a potent inhibitor of both A. ficuum phyA and phyB enzymes with different K_i for each enzyme.

MATERIALS AND METHODS

Enzymes. PhyA was purified from a commercial preparation obtained from Gist-brocade (Delft, The Netherlands). The PhyA gene from Aspergillus ficuum was cloned and overexpressed in a commercial strain of Aspergillus niger by Gist-brocade researchers. We purified the phyA protein by using low pressure ion-exchange chromatography (4). The phyB proteins were obtained from crude culture filtrate when *Aspergillus ficuum* was grown in a starch-based liquid media under phosphate starvation condition (4). The phyB proteins were also purified to near homogeneity by using soft-gel chromatographies (13).

Substrates and inhibitor. The substrate, myo-inositol hexaphosphate or phytic acid, was obtained as a sodium salt from Sigma–Aldrich (St. Louis, MO). Myo-inositol hexasulfate was also obtained from the same source. A fresh solution of myo-inositol hexasulfate (10 mM) was made in deionized water and diluted to 1.0 mM and 0.1 mM in deionized water prior to dispensing into the enzyme assay media.

Phytase assays. Phytase assays were performed at 58°C using either Sodium Acetate buffer (pH 5.0) or glycine–HCl buffer (pH 2.5). The liberated phosphates were determined spectrophotometrically at 355 nm using the method of Heinonen and Lahti (14). The enzyme unit is defined by moles of substrate hydrolyzed per second (Kat).

RESULTS

Inhibition of phytase activity by myo-inositol hexasulfate. To show the inhibition of phytase by myo-inositol hexasulfate (MIHS), a structural analog of

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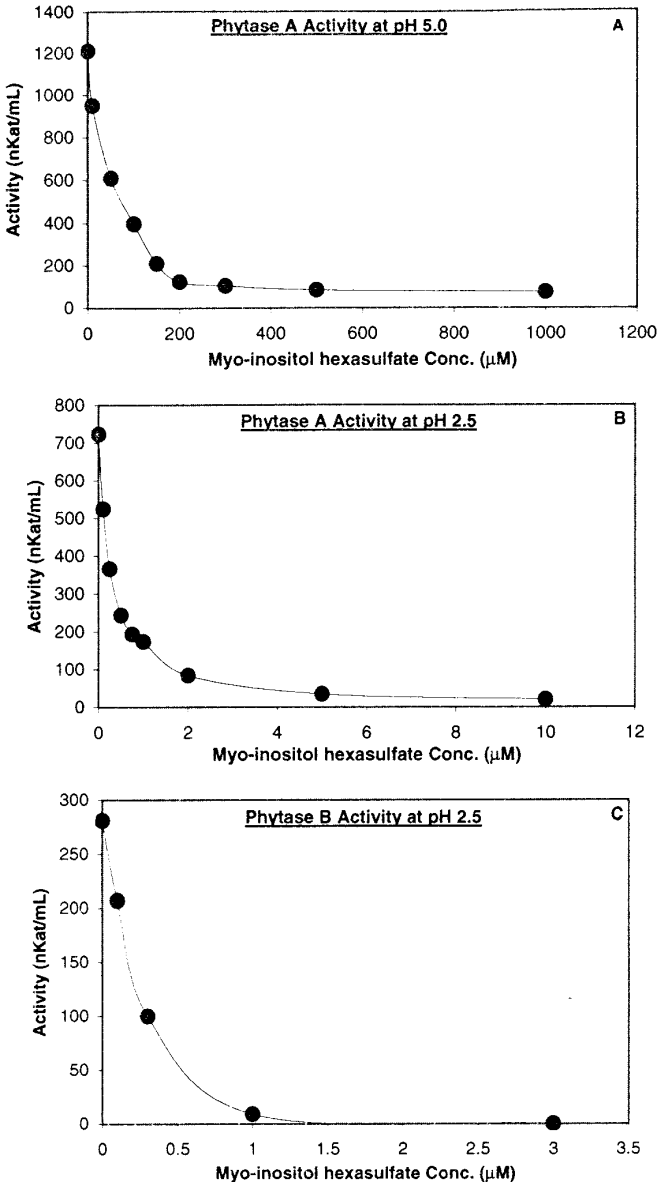

FIG. 1. Inactivation of phytase by myo-inositol hexasulfate. The enzyme and pH of the assay medium are as follows: A, PhyA at pH 5.0; B, PhyA at pH 2.5; C, PhyB at pH 2.5.

phytic acid, we assayed phytase activity in the presence of varying concentration of MIHS. The results are shown in Figs. 1A–1C. PhyA phytase activity was assayed at pH 5.0 in the presence of increasing amounts of MIHS (0 to 1000 μM). The enzyme activity was inhibited linearly with increasing concentrations of myo-inositol hexasulfate. About 92% of the activity was inhibited by 200 μM MIHS. It can be seen that at 50 μM concentration of the substrate analog about 50% of the phytase activity was inhibited. However, at pH 2.5, phytase activity was almost completely inhibited by only 10 μM MIHS (Fig. 1B). At pH 2.5 the substrate analog, MIHS, was so potent that only 0.2 μM concentration inhibited 50% of the phyA activity. The phyB enzyme is inherently more susceptible to inhibitory action of myo-inositol hexasulfate (Fig. 1C). Most phyB activity, however, was inhibited at a concentration of 1.0 μM MIHS at pH 2.5. Moreover, at a inhibitor concentration of only 0.2 μM half the enzyme activity of phyB enzyme was inhibited.

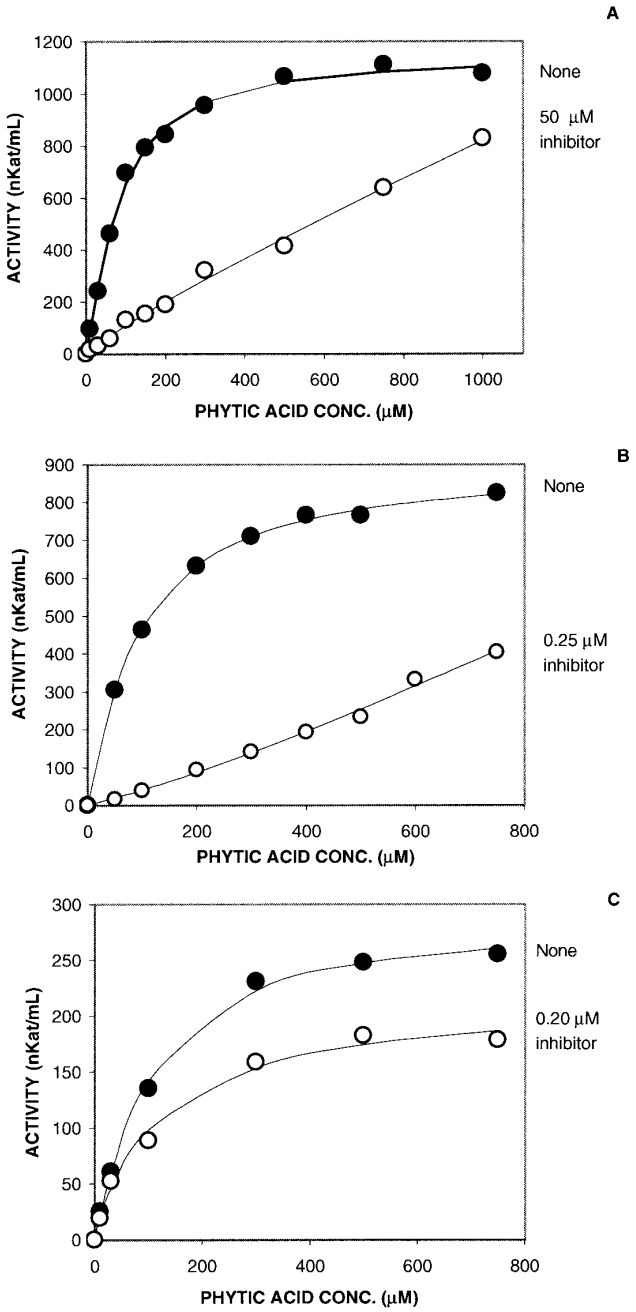

FIG. 2. Phytase activity as function of increasing concentration of phytate in presence and absence of MIHS (inhibitor). A, PhyA at pH 5.0; B, PhyA at pH 2.5; C, PhyB at pH 2.5.

TABLE 1

Changes in Kinetic Parameters in the Presence of Myo-inositol Hexasulfate

Phytase	pH	MIHS[a] (μM)	$V_{max\ (observed)}$ (ηKat/mL)	K_m (μM)	K_i (μM)	Inhibition type
PhyA	5.0	0	1103.08	77	—	—
	5.0	50.00	821.67	910	4.62	Competitive
PhyA	2.5	0	822.63	100	—	—
	2.5	0.25	407.53	1000	0.03	Competitive
PhyB	2.5	0	260.32	114	—	—
	2.5	0.20	187.06	228	0.20	Competitive

[a] Myo-inositol hexasulfate.

Michaelis constant in the presence & absence of myo-inositol hexasulfate. To determine the "Inhibition Constant" (K_i) of myo-inositol hexasulfate for both phyA and phyB phytases, we performed the K_m study by varying substrate concentration in the presence and absence of myo-inositol hexasulfate. The inhibitor concentration for each enzyme was fixed at a value where a 50% inhibition of the activity was obtained (Figs. 1A–1C). These results are shown in Figs. 2A–2C.

The phyA enzyme at pH 5.0 showed an interesting inhibition profile. In the absence of inhibitor, the enzyme showed a typical sigmoidal curve for catalytic activity versus phytate concentration. However, in the presence of inhibitor, the enzyme hydrolyzed phytate linearly at a diminished rate (Fig. 2A). When plotted in a double reciprocal fashion (graph not shown), the data showed that MISH inhibited the reaction competitively. The K_i was 4.6 μM as opposed to the K_m value of 90 μM (Table 1). The same enzyme also showed a similar inhibition profile when the assay was done at pH 2.5 (Fig. 2B). However, the activity was inhibited severely at a much lower concentration of MIHS. This was reflected by a lower K_i (0.03 μM) for phyA protein at pH 2.5 (Table 1). In contrast *Aspergillus ficuum* phyB enzyme activity was also inhibited by MIHS yielding a sigmoidal curve in the presence of the inhibitor (Fig. 2C). By plotting the data in double reciprocal fashion we obtained the K_m value of 114 μM and K_i of 0.2 μM (Table 1). We determined the nature of inhibition by *myo*-inositol hexasulfate in all cases to be a competitive type (plot not shown).

DISCUSSION

Our results show that *myo*-inositol hexasulfate, an analog of *myo*-inositol hexaphosphate, is a potent competitive inhibitor of phytases, phyA and phyB. PhyA and phyB are structurally very different proteins although they share the identical active site residues (11). Thus, the development of this specific inhibitor for all phytases will greatly aid the researchers conducting nutritional experiments in animal models.

We have shown earlier that the active site geometry of phyA and phyB may be different (15). This earlier conclusion was drawn from our observations with the inhibition of both phyA and phyB by specific modifiers of sensitive arginyl residues. The arginyl residues at the active site of phyB were found to be more sensitive to degradation than the similar residues in the phyA enzyme. Similarly, phyB is more sensitive to inhibition by MIHS than phyA. This paralleled the observation that the phyA protein was also more sensitive to MISH at pH 2.5. Perhaps at this low acidic pH phyA and phyB assume a similar structure. It is noteworthy that while phyA can catalyze the reaction at both pH 5.0 and 2.5, phyB can only hydrolyze phytate at pH 2.5. While the 3-D structure for phyA is now known, no such structure is available for the phyB protein.

Considering active site similarities one can assign the phyB protein in the class 'Histidine Acid Phosphatase.' However, phyB phytase is markedly different from phyA phytase showing only about 23.5% homology. Despite the differences in primary structure in these proteins, the inhibitor, MIHS, showed a very similar affinity for the active site as reflected by the lower K_i when the enzyme assays were performed at pH 2.5. It is plausible that at pH 2.5 the active site environment is similar in both phyA and phyB phytases, whereas, at pH 5.0, the phyA phytase assumes a different structure. Computer aided modeling of phyA and phyB active site may reveal these structural differences. In summary, we have developed a specific inhibitor for phosphomonoesterases belonging to the class "Histidine Acid Phosphatases." MISH is a structural analog of the enzyme's primary substrate, myo-inositol hexaphosphate or phytic acid. Interestingly, the K_i (0.03 to 4 μM) for MISH is substantially lower than the K_m (70 to 100 μM) for phytate. At pH 2.5, both phyA and phyB enzyme showed higher affinity for the inhibitor. Despite a low 23.5% sequence homology between phyA and phyB, the active site of these two histidine phosphatases may have structural similarities.

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