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CYCLOHEXANEDIONE MODIFICATION OF ARGININE AT THE ACTIVE SITE OF ASPERGILLUS FICUUM PHYTASE

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Reaction of *Aspergillus ficuum* phytase with the arginine specific modifier 1,2-cyclohexanedione causes a rapid loss of activity. The inactivation can be partially reversed by 0.2 M hydroxylamine and exhibits pseudo-first order kinetics. The reaction order and second order rate constant of inactivation were 0.87 and 6.72 M^{-1} Min^{-1}, respectively. Amino acid analysis of modified phytase indicates that about 7 arginine of the total 19 were modified. While the chymotryptic maps of treated and untreated phytase were virtually identical, the tryptic maps had 4 peaks of altered mobility. An Arg containing tripeptide was identified in the phytase which is also present in other phosphohydrolases and may represent one of the labile Arg involved in the formation of the active site.

Phytase (Myo-inositol-hexaphosphate 3-phosphohydrolase, E.C.3.1.3.8) catalyses the hydrolysis of inorganic orthophosphate from phytate (myo-inositol hexaphosphate). An extracellular and highly active phytase has been purified in this laboratory from *Aspergillus ficuum* NRRL3135 (1). Biochemical characterization and N-terminal sequence have been reported (2) and immobilization studies have been performed (3,4). This enzyme sequentially hydrolyzes phosphate groups starting at the 3 position, with the final product being myo-inositol monophosphate (5). The enzyme has broad substrate specificity, as evidenced by its capacity to hydrolyze phosphate groups from a variety of phospho-monoesters (2) under acidic conditions (pH 5.0). Thus, it can be considered a special form of acid phosphatase (E.C. 3.1.3.2). We are currently elucidating the covalent structure of this protein and characterizing the residues involved in active site formation. Fungal phytase can be totally inactivated by phenylglyoxal, indicating involvement of Arg at the active site. However, sulfosuccinimidyl acetate and acetyl imidazole, which modify Lys and Tyr, respectively, do not affect activity (A. H. J. Ullah, unpublished results). In both alkaline and acid phosphatases, Arg has been implicated as an important residue for catalytic activity (6,7). In this communication we report inactivation of phytase by 1,2-

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cyclohexanedione, a modifier specific for Arg and identify an arginyl residue which may be essential to catalytic activity.

MATERIALS AND METHODS

Chemicals: 1,2-cyclohexanedione (98%) and hydroxylamine hydrochloride (99%) were purchased from Aldrich Chemical Co. Phytate, phenylphosphate, 4-nitrophenylphosphate bis, Na_2H_2 pyrophosphate, fructose 1,6 diphosphate, ATP and glucose-6-phosphate were obtained from Sigma Chemical Co. Trypsin and chymotrypsin were purchased from Boehringer Mannheim.

Source of phytase and enzyme assay: Purification and assay procedures for phytase were as previously described (1,2). A specific activity of 1800-2000 nKat/mg was obtained for the purified enzyme, using sodium phytase as the substrate. Various phosphomonoesters were used for substrate specificity studies (Table 1).

Modification of arginyl residues in phytase: Derivatization of native phytase was performed under conditions similar to those of Patthy and Smith (8). The enzyme was dialyzed aginst 20 mM sodium borate, pH 9.5 and then concentrated to raise the buffer concentration to 50 mM. Upon addition of 1,2-cyclohexanedione (CHD) solutiom (8 mM), the buffer concentration was 37.5 mM, and the CHD concentration was 2 mM. The mixture was incubated at 37°C for 2 hours and placed on ice. Purification of the derivatized enzyme was on a C8 HPLC column (SynChrom, Inc. Lafayette, IN).

HPLC system: The HPLC system consisted of an ISCO V^4 variable absorbance detector, model 2350 pumps. Data acquisition and analysis, as well as system control, were achieved using ISCO's ChemResearch™ software and an IBM system 2 model 30 PC.

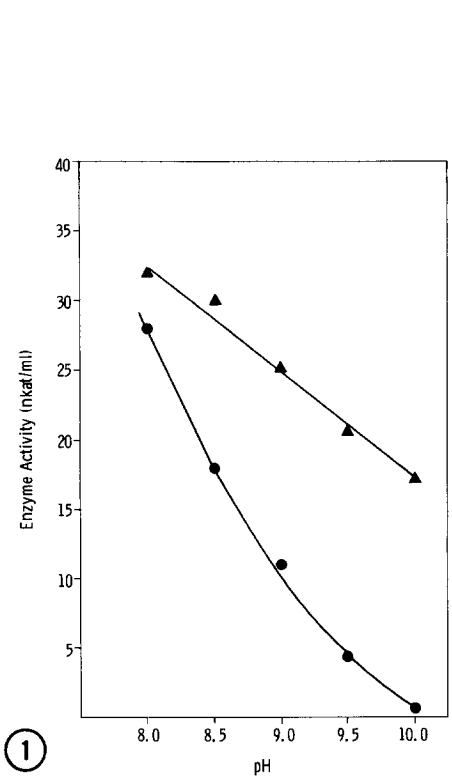
Amino acid analyses: Aliquots of CHD-derivatized phytase were dialyzed sequentially against 50%, 10%, and 5% acetic at 0°C. These were lyophilized, hydrolyzed, and derivatized according to the method of Tarr (9), except that phenol was replaced by 0.5% mercaptoacetic acid in the hydrolysis mix in to prevent regeneration of arginyl residues. Analysis was on a 0.39 x 30.0 cm Novapak C18 column (Waters, Milford, MA).

Peptide mapping: Aliquots of modified and native phytase containing approximately 0.6 mg were dissolved in 200 μl of 50 mM sodium borate, pH 9.0 buffer. The protein-to-protease ratio was adjusted to 100 : 1 and incubated at 37°C for 120 min. The reaction mixture was injected directly into a C-18 HPLC column (218TP54, Vydac). The solvent systems for eluting peptides were 0.1% TFA in water (solvent A) and 0.1% TFA (trifluoroacetic acid) in 99.9% acetonitrile (solvent B). The acetonitrile gradient is shown in Figure 5.

Protein sequencing: Primary structures of the trypsin and chymotrypsin-derived peptides were deduced using a Porton model 2090 on-line gas phase microsequencer using procedure 40 as the sequencing program. The typical initial and repetitive yields were 50 and 90%, respectively.

RESULTS

Inactivation of phytase by 1,2-cyclohexanedione: The reaction of 1,2-cyclohexanedione with *A. ficuum* phytase cause loss of phosphohydrolytic activity. Inactivation conditions were optimized for pH (in borate buffer), time, and CHD concentration. The effect of pH



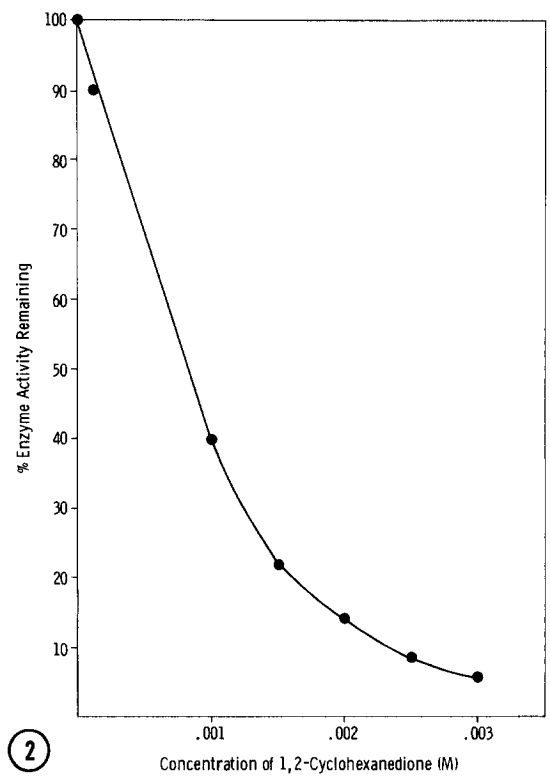


Figure 1. The effect of pH on the inactivation of phytase by 1,2-cyclohexanedione (– ●–). The effect of pH on native phytase is shown in the upper curve (– ▲–).

Figure 2. Phytase activity remaining after 2.0 h at 37°C incubation with varying concentrations of 1,2-cyclohexanedione at pH 9.0.

on inactivation of the enzyme by 1 mM modifieris shown in Figure 1. The native fungal phytase is unstable at alkaline pH; an increase of two pH units (from 8.0 to 10.0) decreased activity 53%. Under identical buffer conditions, in the presence of modifier, enzyme activity decreased 97.6%. The modifier-induced inactivation of phytase was 14%, 49%, 56% and 94% at pH 8.0, 8.5, 9.0, and 10.0, respectively. A pH of 9.0 was chosen for subsequent inactivation studies. The concentration dependence of phytase inactivation by 1,2-cyclohexanedione at pH 9.0 over a 2.0 h period is shown in Figure 2. The enzyme was inactivated 60%, 85% and 94% at modifier concentrations of 1mM, 2mM and 3mM, respectively. Inactivation of phytase by different concentrations of 1,2-cyclohexanedione as a function of incubation time in borate buffer (pH 9.0) at 37°C is depicted in Figure 3. The inactivation follows pseudo-first order kinetics over the first 50% of the reaction. The initial pseudo-first order rate constant is independent of enzyme concentration (0.7 - 7.0 μM), but is dependent on CHD concentration, yielding rate constants of 0.017, 0.0356, 0.0485, 0.066 and 0.102 min^{-1} for inactivation with 2, 5, 7.5,

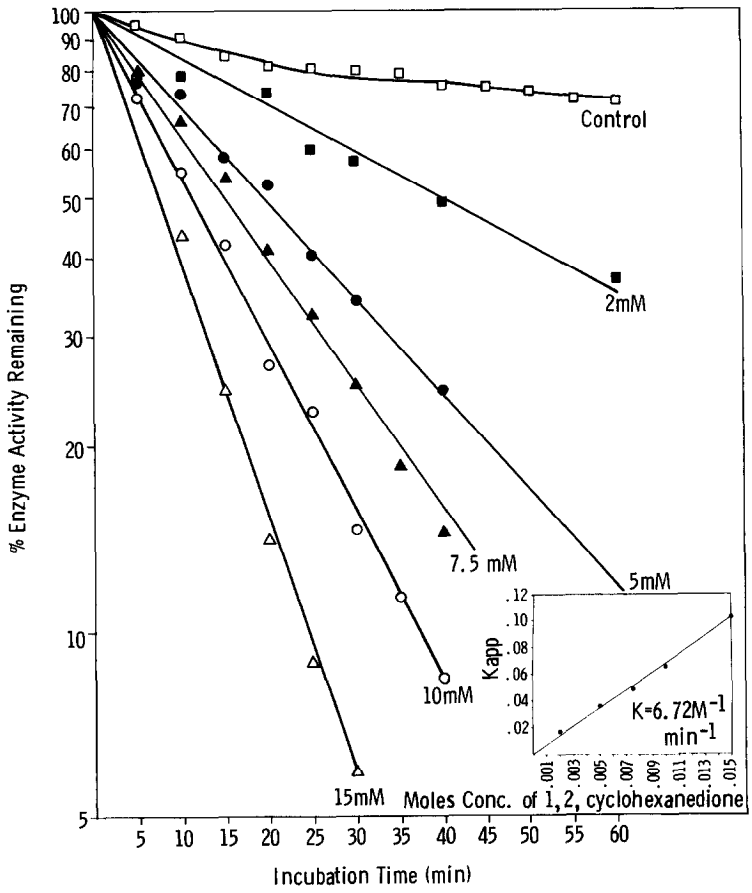


Figure 3. Inactivation of phytase by varying concentrations of 1,2-cyclohexanedione as a function of time. The inset shows the first order rate constant derived from these curves.

10 and 15 mM modifier, respectively. The reaction order for the inactivation was 0.87. The second order rate constant for CHD inactivation of phytase was also computed from these data ($K = 6.72\ M^{-1}\ min^{-1}$) and is in the inset (Figure 3). Modification of essential Arg under these conditions lead to loss of activity of phytase for a variety of substrates (Table 1).

After derivatization, native arginyl residues can be regenerated by treatment with neutral hydroxylamine (12). The partial recovery of phytase activity by the derivatized enzyme (15 mM CHD used) upon the addition of neutral 0.2 M hydroxylamine is shown in Figure 4. Ten min after addition of modifier, the enzyme lost approximately 66% of the activity. At this time hydroxylamine was added, and after 20 min of incubation the treated enzyme regained about 50% of the lost activity. The inability of hydroxylamine-treated enzyme to regain total activity was due to the alkaline conditions (borate buffer, pH 9.0) as shown in Figure 1.

Table 1. Substrate selectivity of native and 1,2-cyclohexanedione inactivated phytase

Substrate	Enzyme activity (nKat/ml)	
	Native	Modified
Phytate	32.0	0
Phenyl phosphate	3.6	0
4-nitrophenyl phosphate bis	8.0	0
Pyrophosphate (Na_2H_2)	4.6	0
Fructose 1,6 diphosphate	19.3	0
ATP	13.0	0
Glucose-6-phosphate	3.6	0

The specificity of cyclohexanedione for Arg modification in fungal phytase is in agreement with the results of amino acid analysis. *A. ficuum* phytase has a characteristic picomole ratio for Leu to Ile, Asx to Glx and Arg to Asx (2). The untreated phytase paralleled the picomole yield and ratio as observed earlier (2); however, cyclohexanedione-treated phytase showed a decrease in number of Arg residues. For

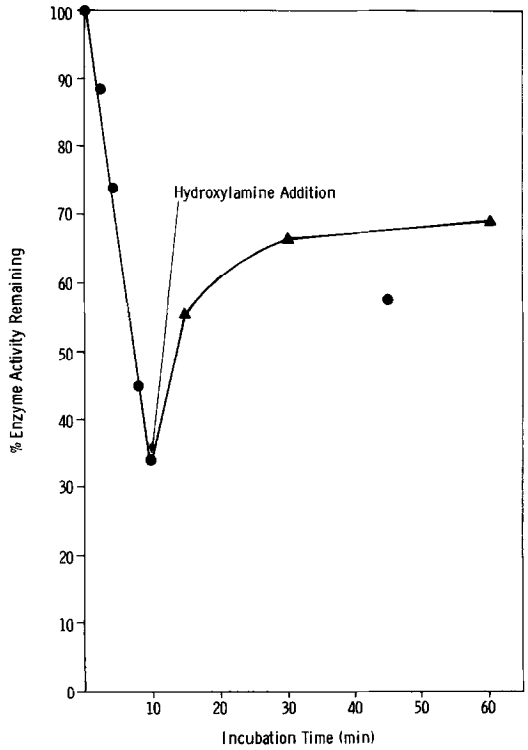


Figure 4. Reversibility of 1,2-cyclohexanedione-induced inactivation of phytase by the addition of 0.2 M hydroxylamine.

example, while the characteristic ratios of Leu to Ile (2.43) and Asx to Glx (1.32) were within experimental error (2.63 and 1.33 for modified protein), the ratio for Arg to Asx was reduced by 38% for modified protein (0.34 for native protein and 0.21 for modified protein). From these data, we concluded that of 19 Arg residues only 7 were modified by cyclohexanedione.

Tryptic maps of native and modified phytase: Reverse phase (RPC18) tryptic maps of both native and modified phytase were obtained (Figure 5). On comparison, it was observed that while the overall profiles were similar for both sets of peptides, eight new peaks emerged in the case of derivatized protein. A total of 36 peaks were seen for native protein, whereas for modified phytase an extra 8 peaks showed up in the chromatogram. Six of these (A through F) were eluted from 20 to 23 minutes and 2 (G and H) appeared between 39 and 40 min. At the same time peak 29, present in the native protein, disappears from the map of the derivatized protein. Chymotryptic maps developed under the same conditions were identical for the native and derivatized phytase (data not shown).

Protein sequencing: In order to locate the modified Arg residues, we separated the tryptic fragments of both modified and native phytase by RPC18 chromatography and sequenced these peptides. Although a majority of the peaks contain one peptide, some peaks contain as many as three distinct peptides (*e.g.*, peak D, Figure 5B). The presence of unmodified Arg residues in these peptides was determined from sequence information. We accounted for at least 14 unmodified Arg residues out of total 19 residues. We have been unable to detect any modified Arg in the tryptic fragments. Upon sequencing chymotryptic fragments of the modified phytase, however, we detected one peptide where a known Arg residue gave a blank cycle in the PTH-AA analysis, indicating modification of Arg (data not shown). Another peptide containing the sequence **KDPR** was homologous to an internal region of *Aspergillus niger* acid phosphatase (10). Finally, a chymotryptic fragment of the phytase containing the sequence **SRHGARYPTD** showed homology for **RHG** with *A. niger* acid phosphatase (10) and a variety of enzymes with phosphohydrolase activity (Figure 6).

DISCUSSION

Previous studies have implicated both arginyl (7) and histidyl (11) residues in the mechanism of action of human prostatic acid phosphatase. *A. ficuum* phytase is readily inactivated by either 1,2-cyclohexanedione or phenylglyoxal, using established methods of arginine modification (8, 12). In this communication, we have detailed the inactivation by 1,2-cyclohexanedione. Amino acid analysis of CHD-treated phytase shows that the

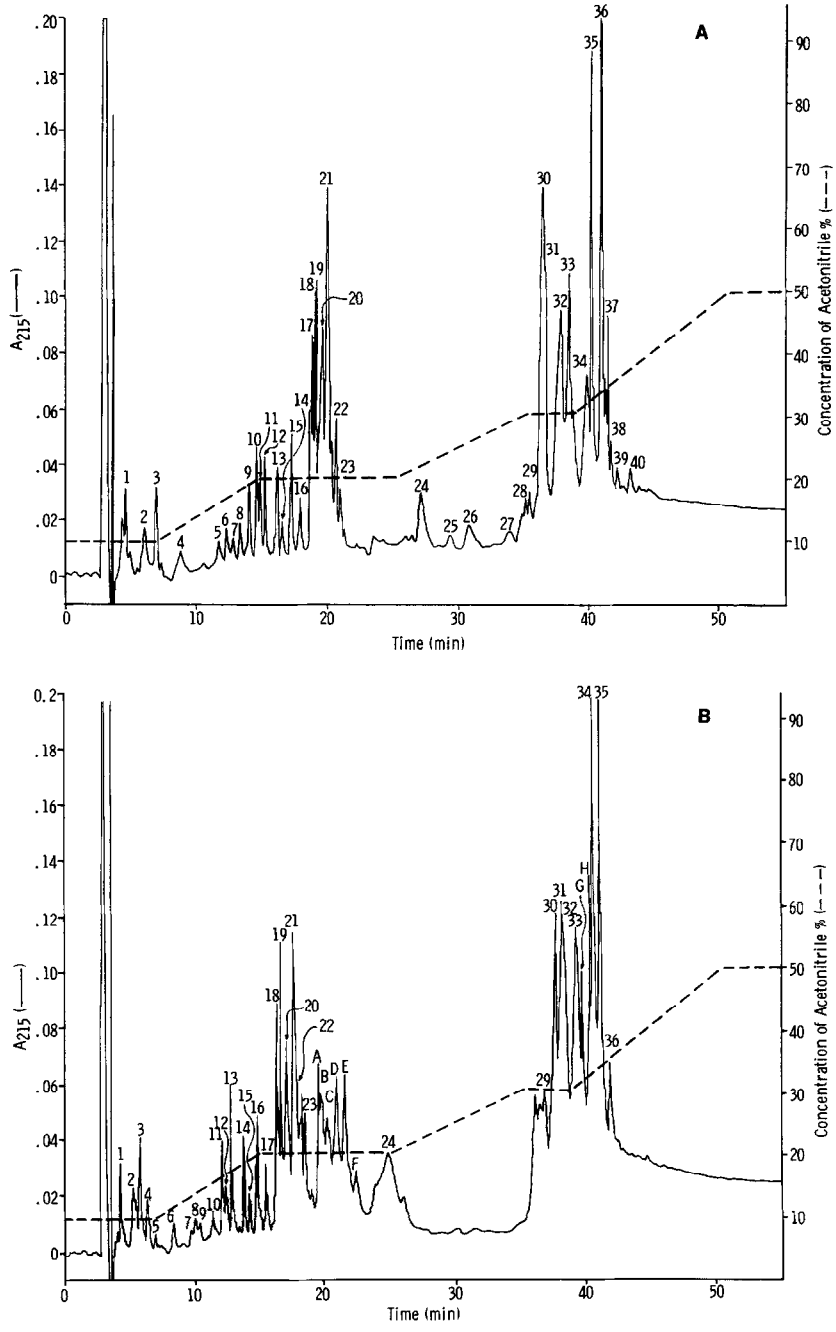


Figure 5. Reverse-phase C-18 tryptic map of native phytase (A) and 1,2-cyclohexanedione treated phytase (B).

loss of enzyme activity was correlated with the loss of arginyl residues. Furthermore, the recovery of the phytase activity upon treatment with hydroxylamine is in accord with the chemical details of arginine modification by cyclohexanedione (8, 12). The loss of phoshpohydrolase activity for phosphomonoesters in addition to phytate implies that the

AfPhytase	(52)	C R V T F A Q V L S **R H G** A R Y P T D S K G K
EcACP	(6)	L K L E S V V I V S **R H G** V R A P T K A T Q L
HuPACP	(1)	K E L K F V T L V F **R H G** D R S P I D T F P T
HuLACP	(1)	R S L R F V T L L Y **R H G** D R S P V K T Y P T
YspACP1	(29)	C K I K Q V H T L Q **R H G** S R N P T G G N A A
YscACP3	(46)	C E M K Q L Q M L A **R H G** E R Y P T Y S K G A
YscACP5	(46)	C E M K Q L Q M V G **R H G** E R Y P T V S L A K
AnACP	(129)	P G I V Q R R R A G **R H G** L H Q R S R N T Q A
RtF2,6BP	(250)	. . . P R S I Y L C **R H G** E S E L N L R G R I
YePGM	(1)	. . . . P K L V L V **R H G** Q S E W N E K N L F
HuPGM-M	(1)	. M A T H R L V M V **R H G** E T T W N Q E N R F
HuBPGM	(1)	. . S K Y K L I M L **R H G** E G A W N K E N R F

Figure 6. Alignment of *A. ficuum* phytase sequence with published acid phosphatase, fructose-2,6-biphosphatase, and phosphoglycerate mutase sequences. EcACP = *E. coli* acid phosphatse (16), HuPACP = human prostatic acid phosphatase (17), HuLACP = human lysosomal acid phosphatse (18), YspACP1 = *Sacchromyces pombe* acid phosphatase (19), YscACP3 = *S. cerevisiae* acid phosphatse (20), YscACP5 = *S. cerevisiae* acid phosphatase (21), AnACP = *A. niger* acid phosphatse (10), RtF2,6 BP = rat fructose-2,6-bisphosphatase (13), YePGM = yeast phosphoglycerate mutase (22), HuPGM-M = human muscle phosphoglycerate mutase (23), and HuBPGM = human brain phosphoglycerate mutase (24, 25).

essential arginyl residues are involved in the catalytic site, rather than the substrate binding site. This active site might therefore be structurally similar to those in other acid optimum phosphomonoesterases.

We have compared sequence data from arginine containing fragments of phytase with published sequences of acid phosphatases from diverse sources. Two regions of homology were identified; the sequences are: **SRHGARYP** (residues 61-68) and **KDPRA** (residues 162-166). The tripeptide **RHG** from the first region is found in diverse acid phosphatases, mutases and biphosphatases (13), but is absent in low molecular weight constitutive acid phosphatases (14, 15); see Figure 6.

The function of an essential Arg residue in fungal phytase and acid optimum phosphomonoesterase (7) is not fully understood; however, similar inactivation has been demonstrated for an alkaline phosphatase (6). A His residue has been implicated in the hydrolysis of phosphomonoesters in human prostatic acid phosphatase (11). Therefore, it is plausible that electrostatic interaction between the positively charged Arg at the active center and the negatively charged phosphate group of the substrate is crucial in the formation of the 'ES' complex with proper geometric alignment for catalysis in this class of enzyme. It was demonstrated for *E. coli* alkaline phosphatase by site directed mutagenesis of an essential arginine (Arg 166) that the residue is not essential for the catalysis but appears to be important for binding substrates (26, 27). A similar role for the essential arginine in phytase is proposed in this communication.

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