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Unfolding and Refolding of *Aspergillus niger* PhyB Phytase: Role of Disulfide Bridges

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The role of disulfide bridges in the folding of *Aspergillus niger* phytase pH 2.5-optimum (PhyB) was investigated using dynamic light scattering (DLS). Guanidinium chloride (GuCl) at 1.0 M unfolded phytase; however, its removal by dialysis refolded the protein. The thiol reagent tris(2-carboxyethyl)phosphine (TCEP) reduces the refolding activity by 68%. The hydrodynamic radius (R_H) of PhyB phytase decreased from 5.5 to 4.14 nm when the protein was subjected to 1.0 M GuCl concentration. The active homodimer, 183 kDa, was reduced to a 92 kDa monomer. The DLS data taken together with activity measurements could indicate whether refolding took place or not in PhyB phytase. The correlation between molecular mass and the state of unfolding and refolding is a very strong one in fungal phytase belonging to histidine acid phosphatase (HAP). Unlike PhyA phytase, for which sodium chloride treatment boosted the activity at 0.5 M salt concentration, PhyB phytase activity was severely inhibited under identical condition. Thus, PhyA and PhyB phytases are structurally very different, and their chemical environment in the active site and substrate-binding domain may be different to elicit such an opposite reaction to monovalent cations.

KEYWORDS: Phytase; HAP; unfolding; refolding; guanidinium chloride

INTRODUCTION

Some members of the enzymes grouped under phosphohydrolase such as fungal phytase that are produced industrially are under scrutiny for their stability; therefore, these enzymes are perfect candidates for folding pathway studies (*1*). *Aspergillus niger* phytase's folding, unfolding, and refolding mechanisms will provide insight into how its structure dictates stability as it is one of the better heat-tolerant enzymes, exhibiting optimum temperature for activity at 58 °C (*2*). The fungal phytase degrades when exposed to 80 °C for a brief period; thus, it may not be pelletized with soybean meal, which is exposed to this high temperature for less than a minute in production facilities (*3*). Therefore, the task before researchers is to render higher temperature stability in phytase through knowledge-based protein engineering.

The phytase *myo*-inositol hexakisphosphate phosphohydrolase produced by *A. niger* belongs to the histidine acid phosphatase (HAP) family of enzymes (*4*). Some other phytases produced by Gram-positive bacteria and plants belong to other classes of proteins with a very different mechanistic enzymology as compared to HAP (*5*). So far, two members of HAP, one from an ascomycete and the other from a basidiomycete, were developed by animal feed industries as feed supplements. Both of these phytases belong to HAP and subclass PhyA (*6*). In 1987, an acid phosphatase cleaving *p*-nitrophenol phosphate (*p*-NPP) with a pH optimum of 2.5 was reported from *A. niger* (*7*). Later, it was shown that the same enzyme was capable of hydrolyzing phytate quite efficiently at pH 2.5; however, unlike PhyA, this enzyme has very little activity at pH 5.0 (*8*). This protein, because of its unique sequence and narrow pH optima as compared to PhyA protein, was placed in a separate subclass under HAP, PhyB (*6, 9*).

PhyB phytase also had received attention from enzymologists and protein chemists because of its high catalytic activity and enhanced thermal stability. Knowledge-based protein engineering could alter the narrow pH optimum, which is a negative attribute for the enzyme; the pH optima of PhyA phytase were altered by this technique (*10*). As a prelude to altering the thermostability of PhyB phytase, unfolding and refolding studies were undertaken. Furthermore, a dynamic light scattering instrument was used to measure the hydrodynamic radius (R_H) of PhyB protein under denaturing (unfolding) and refolding states. The light scattering data in conjunction with activity measurements under various states of chemically induced denaturation indicate that the molecular mass of denatured protein increases considerably but that when the protein is refolded by the removal of denaturant, the activity of PhyB reappears and the molecular mass returns to the predictable level. Therefore, for hydrolytic enzymes such as phytase, a positive correlation exists between molecular mass and the state of unfolding and refolding. The use of a dynamic light scattering

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instrument had augmented the monitoring of these overall conformational changes.

MATERIALS AND METHODS

Source of Phytase. *A. niger* NRRL 3135 was grown in starch media supplemented with glucose, sodium nitrate, and other salts (*2*). The culture medium containing PhyB activity was harvested by centrifugation at 12000 rpm in a Sorval GSA rotor in ice-cold conditions. PhyB phytase was purified using the previously established protocols involving sequential ion-exchange chromatographies (*7*). The elution buffers contained 5% glycerol to provide stability and prevent activity loss during purification. The purified phytase was a homogeneous preparation with >95% purity as judged by SDS−PAGE (data not shown) and had a specific activity of 3570 nkat/mg of protein.

Sample Preparation. The purified phytase preparations were dialyzed against 25 mM glycine/HCl, pH 2.5, buffer to remove glycerol to aid in precise measurements of the hydrodynamic radius (R_H) using dynamic light scattering. The glycerol-free sample was also used for monitoring enzyme activity.

Phytase Assay. Phytase assays were carried out in 1.0 mL volume at 58 °C in 25 mM Gly/HCl, pH 2.5 (assay buffer), similar to the *A. niger* phytase assay (*2*). The assay was initiated by the addition of 75 μL of 10 mM sodium phytate (Sigma-Aldrich). The liberated inorganic *o*-phosphates were quantified spectrophotometrically using a freshly prepared acetone−molybdate−acid (AMA) reagent consisting of acetone, 10 mM ammonium molybdate, and 2.5 M sulfuric acid (2:1:1, v/v). Adding 2.0 mL of AMA solution per assay tube terminated the phytase assay. After 30 s, 0.1 mL of 1.0 M citric acid was added to each tube to fix the color generated by the AMA reagent. Absorbance was read at 355 nm after the spectrophotometer had been blanked with an appropriate control. Enzyme activity was expressed as nanokatals per milliliter (katal = moles of substrate converted per second). One International Unit (IU) is equivalent to 16.67 nkat.

Acid Phosphatase Assay. The phytase enzyme samples in the above assay buffer were incubated with 1.25 mmol of *p*-NPP in a final volume of 1.0 mL at 58 °C for 1 min. The reaction was terminated by using 0.1 mL of 1.0 M NaOH, and the liberated *p*-nitrophenol was measured spectrophotometrically at 400 nm. Enzyme activity was expressed as nanokatals per milliliter.

Dynamic Light Scattering (DLS) Measurements. The PhyB samples under unfolding and refolding conditions were subjected to DLS using a PDDLS/Batch Instrument (Precision Detectors, Bellingham, MA). The software PrecisionDeconvolve (ver 2.1) was run to collect, process, and store light scattering data. The measurements were made at the instrument's ambient temperature (29 °C) using run time 7 (milliseconds) and smoothness 8. Using Precision DeconView, R_H values were obtained from the size distribution plot. The values represent a minimum of three to five measurements of the same sample. The observed hydrodynamic radius, R_H, was converted to molecular mass (kilodaltons) by assuming the empirical globular structure of proteins using a manufacturer-supplied Excel spreadsheet. The formula to calculate molecular mass from observed R_H was $1.549907 \times R_H^{2.430134}$.

Unfolding of the PhyB Phytase. An aliquot of the PhyB enzyme was mixed with guanidinium chloride (GuCl; Pierce, Rockford, IL) to achieve a concentration of 0−2.0 M of GuCl in a total volume of 0.925 mL of assay buffer. After 20 min of incubation at room temperature, the phytase assay was performed at 22, 37, and 58 °C. Similar experiments were performed in the presence of sodium chloride.

Refolding of PhyB Phytase. A 45 μg portion of the PhyB was mixed in 1.0 M GuCl in 250 μL of assay buffer and incubated for 20 min at room temperature. To facilitate refolding, the GuCl was removed by buffer exchange using a PM10 Centricon concentrator. Similar experiments were conducted in the presence of 2.0 mM TCEP (Sigma-Aldrich), which is a reducing agent for the disulfide bridge (*11*).

Time Course of PhyB Phytase Unfolding. A 15 μL aliquot of the GuCl-treated phytase was mixed with 910 μL of assay buffer and kept at 22, 37, and 58 °C for 3−45 min. Phytase assay was measured at 58 °C for 30 s after the addition of 75 μL of 10 mM phytate.

Effect of Temperature on PhyB Phytase Unfolding. A 15 μL aliquot of phyB was mixed in 100 μL of 1.0 M GuCl and kept at room temperature for 20 min. A 9 μL aliquot was then withdrawn from the mix and added to assay tubes containing 916 μL of assay buffer kept at various temperatures (22−60 °C). After 5 min, phytase activity was measured at 58 °C as described earlier (*2*). Parallel control experiments were done in the absence of GuCl under identical conditions.

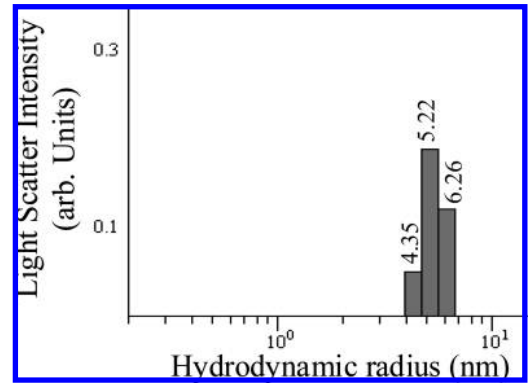

Figure 1. DLS-deduced determination of the hydrodynamic radius of PhyB phytase.

RESULTS

Molecular Mass of PhyB Phytase Deduced by DLS. The native *A. niger* PhyB phytase was first reported to be a homodimer (*7*). However, the schematic diagram of X-ray crystallography-deduced structure showed a tetrameric structure (*12*). Therefore, to figure out whether the PhyB phytase functions as a dimer or tetramer, we decided to analyze the molecular mass distribution of the purified and catalytically active enzyme using dynamic light scattering. The results are shown in **Figure 1**. Three distinct species of nanoparticles could be seen with R_H ranging from 4.35 to 6.26 nm. The average size was computed to be 5.21 nm. This would indicate the molecular mass to be 160 kDa, signifying that the catalytically active native PhyB is a homodimer. Also, gel filtration chromatography shows that the purified active form of the enzyme corresponds to a dimer (*7*).

Unfolding of PhyB Phytase by GuCl. GuCl is a well-known protein denaturant, which had been used to study the unfolding and refolding of phytase (*13*). To elucidate the concentration at which PhyB will be totally inactivated, experiments were performed at 22, 37, and 58 °C while the concentration of the denaturant in the assay medium was increased. The results are shown in **Figure 2**. In all three temperatures, the protein was completely unfolded at 1.0 M denaturant concentration. However, at 58 °C, the protein showed more resistance to denaturation. The concentration at which a 50% inactivation of the enzyme activity took place was dependent on the incubation temperature. For example, the protein was more sensitive to denaturation at 22 °C, which was followed by 37 °C. At 22 °C, a mere 0.03 M GuCl had inhibited the phytase activity, whereas at 37 °C the GuCl concentration had to be raised to 0.125 M to achieve the same level of inhibition. Finally, at 58 °C, a 50% inhibition by the denaturant was achieved at 0.187 M (**Figure 2**). The R_H value (in nanometers) of PhyB at various concentrations of GuCl (at 58 °C) is also shown. The hydrodynamic radius (R_H) of PhyB was found to be 5.5 nm in the absence of GuCl, which corresponds to a molecular mass of 183 kDa; this, however, fell as the concentration of the denaturant was raised. This trend continued up until 1.0 M GuCl, when the R_H value reached 4.14 nm, giving a molecular mass of 92 kDa.

Effect of Incubation Time at Various Temperatures on Refolding of PhyB Phytase. For this set of experiments, PhyB protein was subjected to 1.0 M GuCl at room temperature for

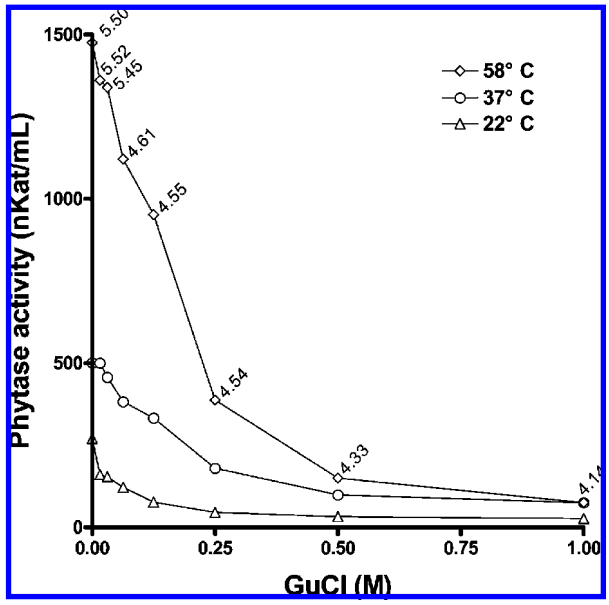

Figure 2. Unfolding of phytase as a function of GuCl concentration at various temperatures.

30 min. Then refolding was allowed to take place for 0−50 min at 22, 37, and 58 °C by simple dilution of GuCl-treated phytase (15 μL) in the assay medium (910 μL). Refolded protein was then assayed at 58 °C. As can be seen, when GuCl-treated phytase was diluted into assay medium containing no denaturant, the protein refolded as a function of time (**Figure 3A**). Most renaturation took place at 58 °C. At this temperature, the treated phytase completely refolded within 4 min. However, renaturation was somewhat slow at both 37 and 22 °C.

Effect of Temperature on Refolding of PhyB Phytase. To assess if temperature plays any role in the refolding process, phytase was first unfolded in 1.0 M GuCl for 30 min at room temperature. Then the unfolded phytase (15 μL) was added to 910 μL of assay medium and allowed to refold at different temperatures starting from 22 °C and ending at 60 °C for 5 min. Then the refolded protein was assayed for phytase activity at 58 °C after preincubation of the sample for an additional 2 min at the same temperature. The results are shown in **Figure 3B**. The unfolded protein refolded in 5 min at temperature from 25 to 60 °C. The extent of refolding did not vary much as a function of temperature except at 22 °C, at which about 88% refolding took place (**Figure 3B**). Therefore, temperatures ranging from 22 to 60 °C are ideal for protein renaturation in this class of hydrolytic enzyme. The control experiment showed that both unfolded GuCl-treated enzyme and untreated enzyme refold in a similar fashion. We have not tested any temperature higher than 60 °C lest the refolded protein be subjected to irreversible heat denaturation.

Hydrodynamic Radius of Phytase in the Presence and Absence of Thiol Reagent. To assess the role of disulfide bridges in refolding, we have unfolded phytase by subjecting the protein to 1.0 M GuCl. To refold the unfolded protein, we removed the denaturant by Centricon PM10 membrane. The unfolded phytase was refolded by this method in the presence and absence of thiol reagent, TCEP. Hydrodynamic radii (R_H) of GuCl-induced unfolded and refolded phytase along with the native phytase are shown in **Table 1**. Activity measurement of phytase at various stages of unfolding and refolding process was done to ascertain whether the refolding process took place or not. First, phytase was unfolded completely in the presence of 1.0 M GuCl. The addition of TCEP (2 mM) during unfolding also made the protein completely unfolded. Second, when GuCl was removed by buffer exchange through Centricon, 83% of the treated phytase refolded. Third, inclusion of TCEP during refolding prevented the refolding of phytase. Fourth, addition of GuCl increased the R_H by about 7% (**Table 1**). Other than that, the R_H dropped about 6% when phytase was treated with GuCl and TCEP, which indicates that as phytase refolded, the protein became somewhat smaller. Fifth, when refolding of phytase was done in the presence of 2 mM TCEP, the R_H dropped about 12%. Therefore, it follows that disulfide bridge formation is an obligatory step in phytase refolding.

Effect of Salt on PhyB Phytase Activity and Hydrodynamic Radius. Sodium chloride at a concentration of 1.0 M was found to be inhibitory when assays were done at pH 2.5 (unpublished observation). To further explore the inhibition of phytase by monovalent cations, we subjected phytase to NaCl, KCl, and $NaNO_3$ in the assay medium. The salt-treated phytase was then assayed using both phytate and a synthetic substrate (*p*-nitrophenyl phosphate). Results are shown in **Table 2**. About 88% of the total activity was lost when PhyB was subjected to a 1.0 M solution of KCl or NaCl and then assayed for activity using phytate. Sodium nitrate was even more inhibitory at the same concentration. The treated phytase did, however, catalyze the hydrolysis of *p*-nitrophenyl phosphate slightly better under identical conditions.

Figure 4 shows the concentration dependence of the inhibition of phytase by NaCl. The concentration of NaCl at which 50% inhibition of activity took place was computed to be 0.125 M. Therefore, PhyB protein is sensitive to monovalent cations at about pH 2.5. We also compared the R_H value of native phytase, which was 5.25 nm, with that of salt-treated phytase (4.95 nm). Thus, the salt-treated phytase shrunk about 4.8% as compared to the native phytase. This compaction in the overall size of phytase was enough of a perturbation to cause enzymatic demise. Unquestionably, either the substrate binding domain or the catalytic site or both were affected by compaction due to salt treatment at low pH.

DISCUSSION

The DLS data indicated that catalytically active native phytase was a homodimer (**Figure 1**). This finding matched with the molecular mass deduced by gel filtration column (*7*). Kostrewa and co-workers, while deducing the crystal structure of *A. niger*, pH 2.5, acid phosphatase (PhyB), indicated that the protein forms a tetramer, which is composed of two homodimers (*12*). They gave an explanation on dimer formation by the PhyB protein by stating that in the N terminus, residues 14−24 stretch out and lie on the surface of the neighboring molecule. The interaction between two monomers is thought to be electrostatic.

The role of disulfide bridges in the unfolding and refolding of *A. niger*, pH 2.5, optimum phytase (PhyB) has been probed by GuCl. Whereas the native phytase was unfolded by chemical denaturant and refolded to become catalytically active upon dialysis via a Centricon, subsequent addition of the thiol active reagent TCEP profoundly affected the refolding process. Activity measurements during various states of unfolding and refolding in the presence and absence of TCEP indicated that disulfide bridge formation is an integral part of folding in fungal phytase. A similar conclusion was drawn when unfolding refolding studies were performed in PhyA phytase (*1*).

Wang and co-workers also probed the role of disulfide bonds in the conformational stability and catalytic activity of phytase in the presence of urea (*13*). They studied the kinetics of unfolding of the protein with or without thiol reagent, DTT,

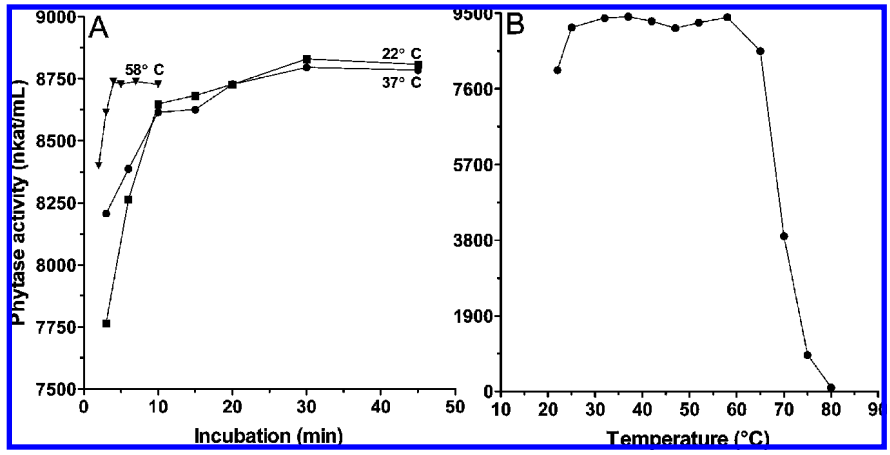

Figure 3. Effect of incubation time (**A**) and incubation temperature (**B**) on refolding of PhyB phytase.

Table 1. Summary of the Unfolding and Refolding of PhyB Phytase in Respect to Activity, Hydrodynamic Radius (R_H), and Molecular Mass

			phytase activity	
unfolding and refolding state	$R_H \pm$ SD	mol mass ± SD	nkat/mL	%
no GuCl	5.23 ± 0.06	162 ± 4.5	499	100
GuCl (1.0 M)	5.61 ± 0.1	192 ± 8.5	16	3
GuCl (1.0) + 2 mM TCEP	4.9 ± 0.07	138 ± 4.8	14	3
GuCl removed by Centricon	4.9 ± 0.09	138 ± 6.1	416	83
GuCl removed by Centricon in the presence of 2 mM TCEP	4.6 ± 0.1	119 ± 5.9	160	32

Table 2. Effect of Monovalent Cations on PhyB Phytase Activity

	phytase activity		acid phosphatase activity[a]	
salt exposure	nkat/mL	%	nkat/mL	%
control, no salt	8332	100	1626	100
NaCl, 1.0 M	1009	12	229	14
KCl, 1.0 M	1020	12	388	24
$NaNO_3$, 1.0 M	575	7	240	15

[a] Determined by using *p*-NPP as the substrate.

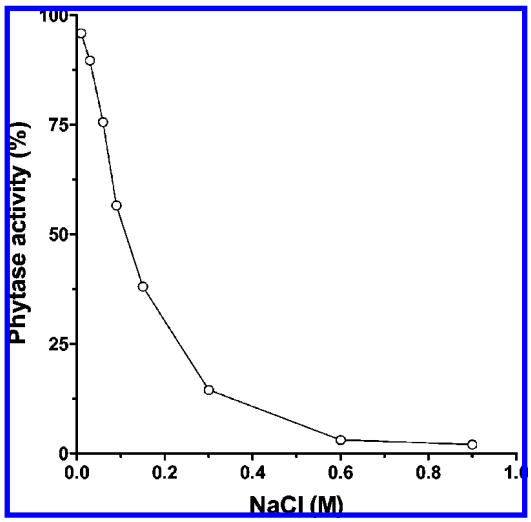

Figure 4. Effect of salt on inhibition of PhyB phytase activity.

using fluorescence spectrometry, far-UV circular dichroism (CD), and enzyme activity measurement. They observed that in the presence of 2 mM DTT both inactivation and unfolding were greatly enhanced at the same concentration of the denaturant. The kinetics of unfolding was a biphasic process consisting of two first-order reactions in the absence of DTT and a monophasic process in the presence of thiol reagent. The kinetic demise, according to authors, was most likely due to a conformational change in the active site; disulfide bridges may have played a crucial role in the optimization of the three-dimensional structure of the active center of fungal phytase (*13*).

Fungal phytase has the ability to cleave phosphomonoesters at elevated temperature, 58−60 °C. However, at 70 °C, catalytic demise sets in. Wang and co-workers probed the stability of phytase's active site and its overall structure as a function of heat treatment. The authors studied the kinetics of inactivation and unfolding during thermal denaturation when phytase is hydrolyzing phytate and synthetic substrate, p-NPP. The loss of phytase activity during thermal denaturation was observed to be a reversible process, whereas two phases of thermal inactivation were noted for acid phosphatase activity. Moreover, thermal denaturation did irreversible damage to acid phosphatase activity; between 45 and 50 °C the heat-induced inactivation was found to be an irreversible process. Inactivation at temperature above 55 °C led to a complete catalytic demise for acid phosphatase activity. Fluorescence studies have indicated that at temperatures below 60 °C for 60 min, the conformation of the enzyme did not change. However, at temperatures above 60 °C, some fluorescence red shift could be observed with a concomitant decrease in emission intensity. When all of these observations are considered together, inactivation of phytase occurs before significant conformational changes of the protein. Therefore, it follows that the active site of fungal phytase is more fragile than the overall conformation of the protein (*14*).

Besides the members of HAP, there are other proteins in which disulfide bridges play an active role in protein folding. The role of each disulfide bridge in the transition state in the folding−unfolding reaction of four species of the three-disulfide variant of hen lysozyme was probed (*15*). There, too, disulfide bridges are implicated as having a role in protein folding. The effects of redox conditions on the folding of phospholipase D (PLD) of *Streptomyces antibioticus* were investigated (*16*). Although the enzyme was very stable even in the presence of 1.0 M GuCl, the coexistence of thiol reagent, DTT, and GuCl inactivated the enzyme completely. The inactivated enzyme, however, recovered its activity by dialysis in which DTT was removed prior to GuCl, whereas its activity was not recovered when GuCl was removed prior to DTT.

Finally, researchers found that disulfide-bonding patterns can be used to discriminate structural similarities among proteins. On the basis of the hierarchical clustering scheme, Chuang and co-workers were able to detect the structural similarities of proteins of low sequence identities (<25%). Their results show

the surprisingly close relationship between disulfide-bonding patterns and protein structures; moreover, their findings should be useful in protein structure modeling (*17*).

The PhyB phytase's reactivity to various monovalent cations markedly contrasted with that of PhyA phytase. In the case of PhyB phytase the salts severely inhibited the activity (**Table 2**); however, sodium chloride significantly boosted the activity of PhyA phytase (*18*). In the case of PhyB phytase, the salt treatment had reduced the hydrodynamic radius (R_H) of the protein, indicating that the overall size of the molecule was reduced. Therefore, it appears that these two groups of phytases are structurally different. The members of PhyA are active, being a monomer (PhyA), but the other is active only as a homodimer (*7*) or homotetramer (*12*). It is worth mentioning here that PhyB phytase is endowed with a higher turnover number (K_{cat}), but due to its restrictive and narrow pH optima the biocatalyst never drew attention from animal feed industries or enzyme producers. That may, however, change when knowledge-based protein engineering will change PhyB phytase's pH optima as was done in PhyA phytase (*10*).

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