In Silico Comparison of Disulfide-Bearing and Disulfide-Free Phytases among Microorganisms

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Abstract: Phytases are degrading enzymes that hydrolyze phytate (myo inositol hexa kis phosphate) to release a series of lower phosphate esters of myoinositol and orthophosphate. Phytase successfully used as an animal feed additive to increase the bioavailability of phosphate from phytic acid in the grain-based diets of poultry and swine. In order to investigate structural relationships between disulfide-bearing phytases and disulfide-free phytases, 9 phytases with resolved three-dimensional (3D) structure were retrieved as pdb and FASFA format from Protein Data Bank server and were analyzed using various tools and software. The results showed that 6 out of 9 phytases carry three or more disulfid bonds while the others lack any disulfide bonds. Our results also demonstrated that there is a remarkable correlation between the presence of disulfide bond and the number of amino acid in each phytase which means the larger enzymes contain three or more disulfide bonds whereas the enzymes containing less than 400 amino acids lack any disulfide bond. Additionally, in order to dig out the structure of phytases, some chemical and physical characteristics of phytases such as aliphatic index (AI), isoelectric pH (PI), amino acids percentage, molecular weights (MW) and 3D structure of phytases were analyzed. Results showed that phytases containing disulfide bonds have some identical characteristic including glycine percentage, AI, and 3D structure rather than disulfide-free phytases do. Moreover, evolutionary surveys by means of alignment studies and evaluations were conducted. Evolutionary analysis represented that phytases with disulfide bond most probably exhibited the same evolutionary course.

Keywords: *In silico* analysis, phytase, disulfide bond, protein stability.

INTRODUCTION

Phytases are enzymes that release phosphorus from phytate or myo inositol hexakis phosphate (IP6), thereby generating less-phosphorylated myoinositol derivatives [1]. Basically, phytate is the main source of inositol and a principal storage form of phosphorus in many plant tissue especially bran and seed, thus essential in seed-based animal feed [2].

Due to the chelating ability of phytate, it strongly binds to a vast variety of metal ions such as calcium, zinc, magnesium, and iron under neutral and alkaline conditions and proteins and starch under acidic conditions, and thereby forms insoluble complexes [3]. Moreover, phytate decreases the bioavailability of inorganic phosphorus and inhibits the activity of enzymes and thus regarded as an antinutrient factor [4]. Since human and monogastric animal like pigs and poultry are incapable of digesting phytate phosphorus due to lack or low level of phytase activity in their intestine, the resulting is lower level absorption of important metal ions, proteins and starch [5]. Further undigested phytates in fecal phosphorus excretion cause significant environmental pollution in areas of intensive livestock production [6, 7], Therefore phytase is an important solution for this problem [8]. Using of phytase as an animal feed additive in corn-soybean meal diets for pigs and poultry effectively improves phytate phosphorus absorption by these animals and reduces their fecal phosphorus excretion by up to 50% [9, 10].

Phytases belong to phosphatase family enzymes and are widely distributed among bacteria, fungi, plants and some animal tissue [1]. Since phytases represent a various mechanism of digestion, structure, and property, classification of this enzyme is a hard job [11]. According to three-dimensional (3D) structure and presence of a specific motif they classified into: histidine acid phosphatases (HAPS), B-propeller phytases, purple acid phosphatases, and most recently, protein tyrosine phosphatase-like phytases (PTP-like phytases) [12]. Among these classes histidine acid phosphatase have the important role in practical application because of their optimal property for tolerance acidic pH in animal intestine [7]. In another classification based on the carbon ring position where removal of phosphate groups from phytate is initiated, phytases classified into three different groups:

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3-phytase or 1-phytase (EC 3.1.3.8), 4-phytase or 6-phytase (EC 3.1.3.26), and 5-phytase (EC 3.1.3.72) [4].

Stability of proteins in condition that they are used is an important point that should be considered [13, 14]. There are several factors for stability of protein such as aliphatic index (AI), amino acid sequence, secondary structure, ionic interaction, hydrogen bond, disulfide bond and etc. Disulfide bond is of most important factors, particularly in extracellular and secretory proteins [15]. Consideration of these factors in designing or engineering of a protein gives one more stable form of a protein.

In this study, phytases were thoroughly analyzed by several tools and software to shed light on relationships between phytases that contains disulfide bonds and those lack disulfide bond. Phytases with disulfides bond have more than 400 amino acid and others that lack any disulfide bond have less than 400 amino acid. Phylogenic analysis revealed that phytases bearing disulfide bond had identical parameters and same evolutionary course.

MATERIAL AND METHOD

In order to analyze phytases various tools and softwares were employed. Both pdb and fasta files were obtained from Protein Data Bank server [16].

Sequence and PDB Retrieval of Phytases

The protein sequences of 9 phytases from various microorganisms were retrieved in fasta format for analyzing of them by softwares CLC protein workbench, Geneious and ProtParam protein tool. In order to study 3-D structure and disulfide bridge, PyMol and Swiss PDB Viewer (SPDBV) softwares used.

Visualization of 3D Structure and Disulfide Bond and In Silico Modeling

SPDBV (version 4.0.4) and PyMol (version 1.1.1) softwares were used for visualization of 3D structure and disulfide bonds, respectively. PyMol is an open source program that created by Warren Lyford Delano and enables to produce high-quality 3D image of small molecules and biological molecules such as protein [17]. PyMol is extensible by Python programming language [17, 18]. SPDBV is an application that can analyze several proteins at the same time and superimpose proteins to compare active site of proteins and deduce structural alignments[19]. SPDBV is tightly linked to Swiss-Model, an automated homology modeling server [20].

Statistical Information and Characteristics of Phytases

Using CLC protein workbench (version 5.2), sequence statistics such as length, isoelectric point (IP), weight and aliphatic index (AI) was obtained. This software enabling users to make a large number of advanced protein sequence analyses, project and data management, analyzing on multiple sequence in one work-step [21]. Some chemical and physical characteristics such as type of amino acid and their percentage was obtained using ProtParam protein tool of expasy proteomic server that is a tool which compute various chemical and physical parameter for sequences that user entered [22].

In Silico Protein Modeling and Refinement

Homology or comparative modeling of proteins refers to modeling a protein 3D structure using a known experimentally determined structure of a homology protein as a template [23]. In silico comparative modeling of phytases that their 3D structures were not exist in Protein Data Bank server was done using SWIIS-MODEL tool that is a fully automated protein structure homology modeling server, accessible via the expacy web server or from software SPDBV [24].

Refinement of initial modeled proteins carried out using $3D^{refine}$ web server, a computationally service for consistent and efficient protein structure refinement based on optimization of hydrogen bonding and atomic level energy minimization. The goal of this server is improvement in structural quality of the initial models to bring it closer to the native state [25].

Superimposition

The term structural superposition refers to rotations and translations performed on one molecular structure to make it match another structure or structures in order to comparison 3D structure of giving structures. It is possible to define superposition in terms such as root mean square deviation (RMSD) [26]. Superposition for phytases with unresolved 3D structures carried out using software SPDBV.

Alignment and Phylogenic Tree

Multiple sequence alignment of proteins is a way of arranging the sequence of them to find identical regions that may be a consequence of structural, functional or evolutionary relationships between the sequences [27].

Phylogenic tree is a diagram that shows evolutionary relationships among a set of organisms or proteins and DNAs [28]. Geneious software and CLC protein workbench are helpful tools for representing phylogenic tree and multiple alignments of protein sequences. It was used in this study for constructing multiple alignment of 9 phytase from different microorganisms and drawing phylogenic tree for them [29].

RESULT

3D Structure and Disulfide Bond in Phytases

9 phytase from various microorganisms in pdb format downloaded from Protein Data Bank server, their pdb IDs are as follows: 1SK9, 1DKP, 2WNH, 2GFI, 3AMR, 4ARU, 303L, 3F41, 3K4P. Visualization of these proteins by SPDBV and PyMol revealed that 6 out of these phytase (1SK9, 2WNH, 2GFI, 4ARU, 3K4P, 1DKP) contain three or more disulfide bonds and others (3AMR, 303L, 3F41) lack any disulfide bond. Information about these proteins that was obtained from SPDBV and Pymol depicted in Table **1**. Considering the Table **1** carefully, it is noticeable that the number of amino acids in phytases containing disulfide bond is more than 400. Moreover, analyzing 3D structure of them by means of above-mentioned softwares revealed that 3D structure of phytases with disulfide bond is identical and are more extensive than that of phytases with no disulfide bond (Figures **2** and **3**).

Homology Modeling and Refinement

Homology modeling carried out by Swiss modeling tool for phytases from hamiltonella defensa subsp, shingella flexnery serotype, Shewanella oneidensis that their 3D structures did not exist in Protein Data Bank server. For refinement of these modeled protein 3D [refine] web server was used. Interestingly, the results of modeling and refinement indicated that in phytase with more than 400 amino acids the formation of disulfide bond is most likely and in that of have less than 400 amino acids disulfide bridge formation do not occur. Moreover, extensive protein structure in phytase contain more than 400 amino acids were observed.

Chemical and Physical Characteristic of Phytases

Using Protparam protein tool from expacy server and CLC protein workbench software several parameters for phytases have been obtained and represented in Table **2**, as it can be seen in Table **2** aliphatic index (AI) in phytase that carry disulfide bond is almost more than those that lack disulfide bond.

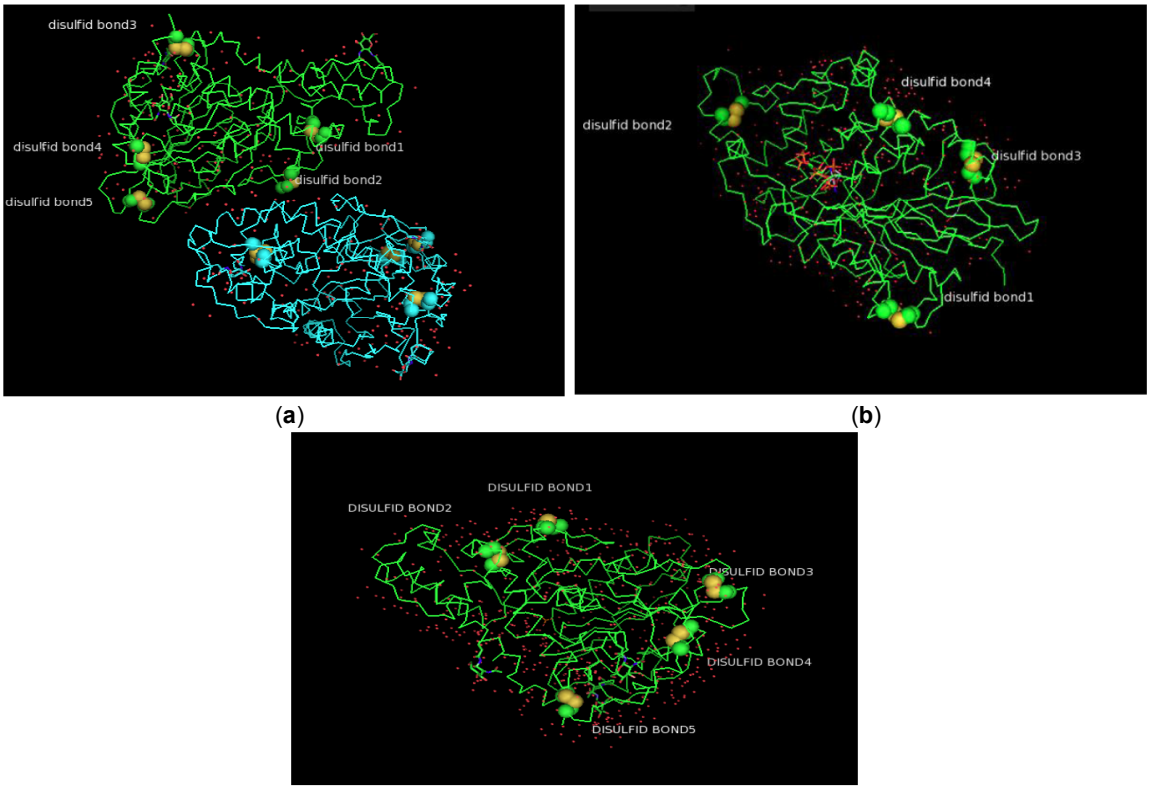
In order to survey on primary structure of phytases, the percentage of amino acids evaluated using Protparam protein tool and tabulated in Table **3**. As shown in Table **3** the percentage of amino acid glycine in phytase that have disulfide bond is approximately equal with a little variations because of structural properties, while the percentage of other amino acids was variable in both groups of phytases.

Superposition of Phytases with Disulfide Bond

Superposition of 3D structure of proteins is a convenient way for unearthing similarity in their folding. For comparison and examination of folding and 3D structure of phytases with disulfide bond they are superposed against each other using software SPDBV. Result showed that most of phytases with disulfide bond are identical in 3D structure and their folding is

Table 1: Information about Phytases Obtained from SPDBV and Pymol Softwares

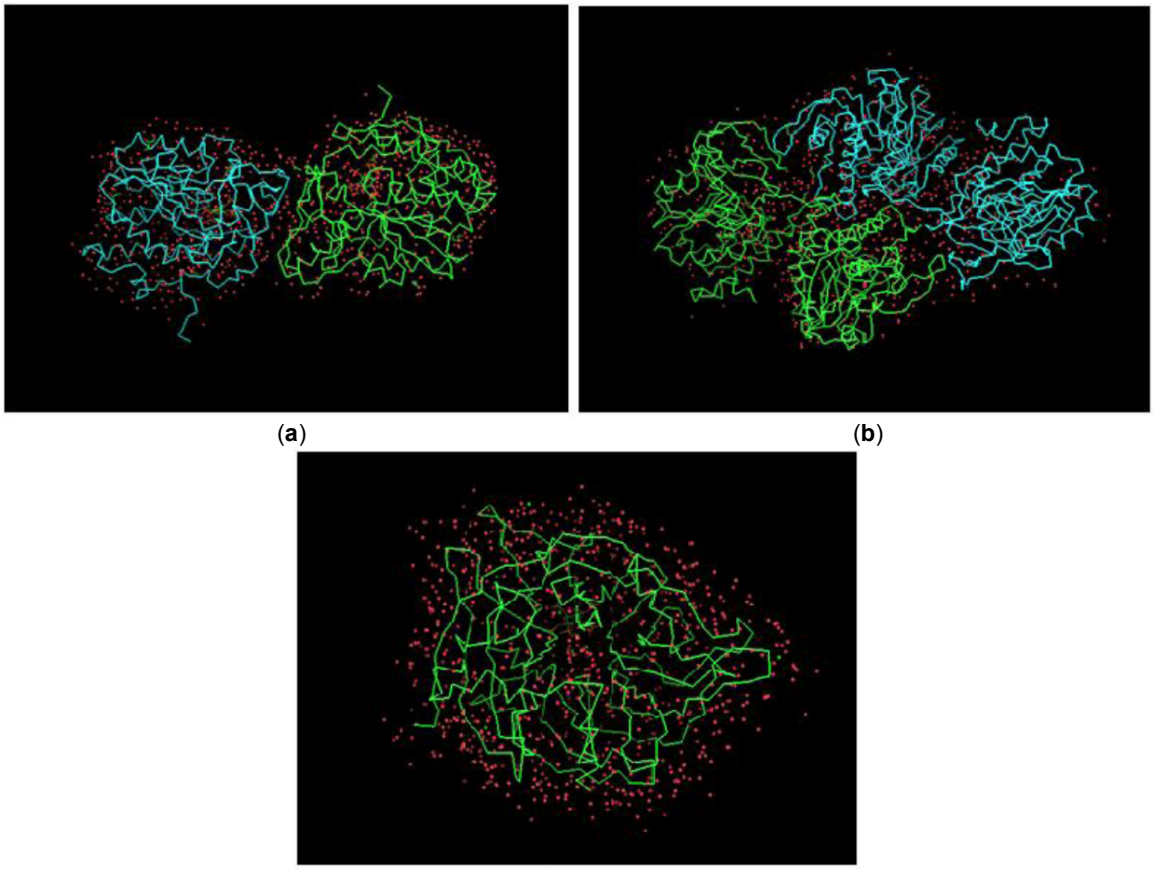
PDB ID	Number of chains	Number of amino acid in each chain	Number of disulfide bonds in each chain	source
4ARU	1	413	4	*Hafnia alvei*
303L	2	346	0	*Selenomonase ruminantum*
3K4P	2	444	5	*Aspergilus nigre*
3F41	2	636	0	*Mitsuokella multacida*
3AMR	1	353	0	*Bacillus sabtilis*
2GF1	2	461	4	*Debaryomyce scastellil*
1DKP	1	410	4	*E. coli*
1SK9	1	443	5	*Aspergiluse fumigatuse*
2WNH	2	405	3	*Klebsiella pneumonia*



(**a**) (**b**)

(**c**)

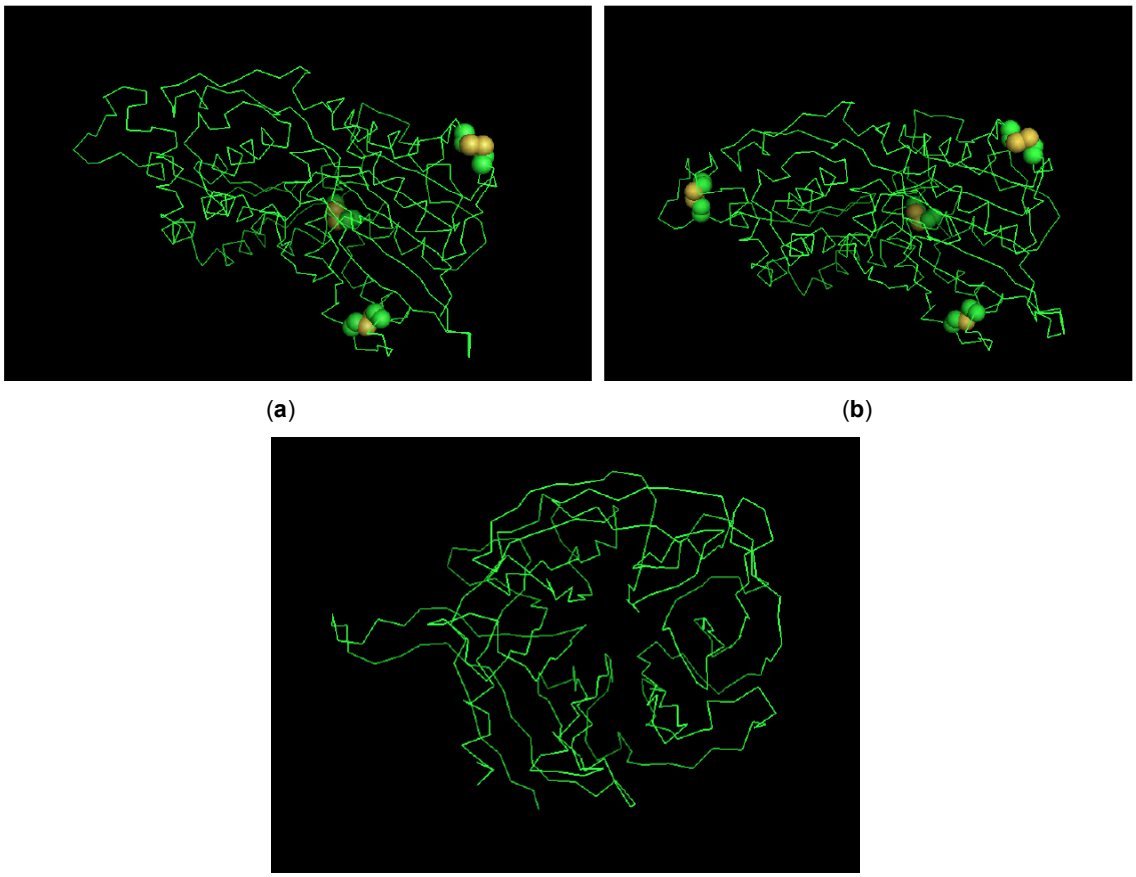
Figure 1: 3D structure of 3K4P with 5 disulfide bond in each chain (**a**), 1DKP with 4 disulfide bond (**b**), 1SK9 with 5 disulfide bond (**c**). Picture of other disulfide-bearing phytases not shown.



(**a**) (**b**)

(**c**)

Figure 2: 3D structure of 303L (**a**), 3F41 (**b**), and 3AMR (**c**) which all lack any disulfide bond.



(**a**) (**b**)

(**c**)

Figure 3: 3D structure of modeled protein, phytases from Hamiltonella defensa subsp that modeled using 1DKP as template with 96% similarity in sequence (**a**), 3D structure of phytase from Shingella flexnery serotype that modeled using 1DKP as template with 58% similarity in sequence (**b**), 3D structure of phytase from Shewanella oneidensis using 3AMR as target with 38% similarity in sequence (**c**).

Table 2: Chemical and Physical Characteristics that were Obtained from ProtParam Tool and CLC Protein Workbench

PDB ID	Molecular weight	Total charge	pI	AI
4ARU	45.31kDa	-4	6.74	82.47
1SK9	48.27KDa	0	7.57	74.23
3K4P	48.84KDa	-38	5.22	72.25
2GFI	51.02KDa	-59	4.8	71.98
2WNH	46.58KDa	+6	8.79	84.59
1DKP	44.65KDa	-4	6.26	89.73
303L	38.90KDa	0	8.87	66.05
3F41	72.13KDa	+13	9.01	71.16
3AMR	39.085	-15	5.22	67.12

Table 3: Percentage of Glycine in Phytases. Phytases with Disulfide Bond has Equal Percentage of Glycine

PDB ID	1DKP	1SK9	2GFI	2WNH	4ARU	3K4P	303L	3F41	3AMR
Disulfide bond	+	+	+	+	+	+	-	-	-
Glycine percentage	7.1	7.1	7.2	7.3	7	6.6	6.6	5.9	10.1

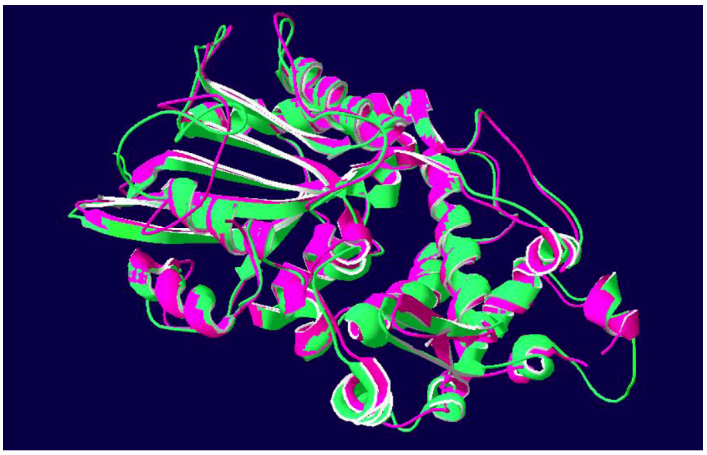


Figure 4: Superimposed structure of 1DKP and 1SK9. 1DKP colored in green and 1SK9 colored in pink.

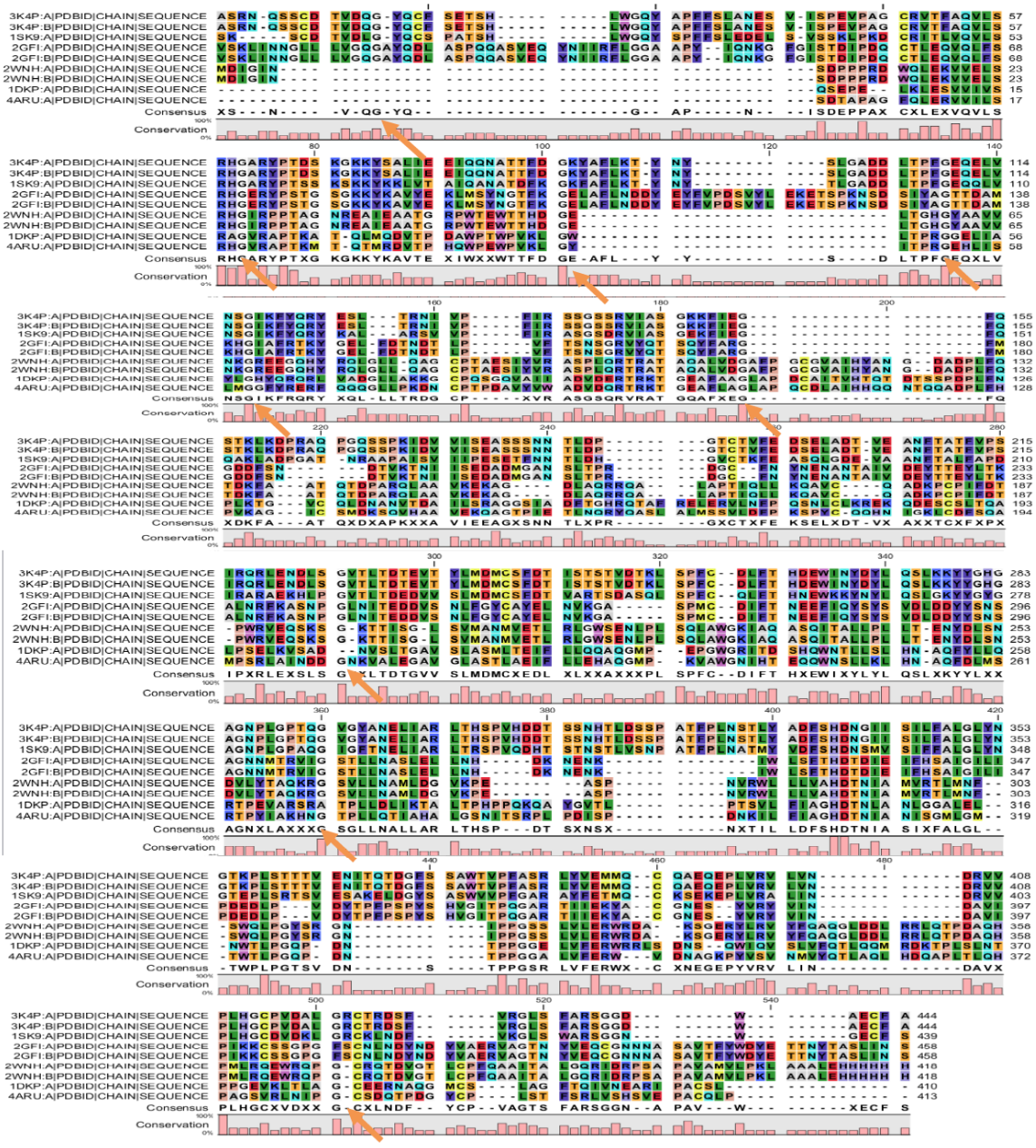


Figure 5: Multiple alignments of phytases with disulfide bond. Most of the glycines are conserved in these phytases.

similar. As an example one of the superposed structure represented in Figure **4** and other data not shown.

Evolutionary Analysis

Sequences of phytases in fasta format that was obtained from UniProt server were aligned in CLC protein workbench. Multiple alignments of sequences constructed in global alignment with free end gaps. As indicated by black arrows in Figure **5** glycine is the most conserved amino acids among phytases. In addition, leucin , arginin, proline and etc are conserved in phytases, as well but the conservation of glycine is more significant. In Figure **5** consensus sequence and conservation percentage histogram is represented at the bottom of the alignment. Following alignment, phylogenic tree has been drawn. A phylogenic tree is consisting of nodes, branches and tips or leaves. Nodes or internal nodes of a tree represent the inferred common ancestor of the sequence that are grouped under them, branches length is a measure of divergence between two nodes in a tree, tips or leaves of a tree represent the sequences that used for constructing a tree [30, 31]. Phylogenic tree that constructed from sequences of phytases broken down into three nodes, each node exhibit a cluster of phytase, first node or cluster consists of two tips or sequences (303L and 3F41 that both of them free from disulfide bonds), second node consists of two tips (2GFI carries disulfide bond and 3AMS lacks disulfide bond), third node consists of tow internal nodes and four tips (4ARU, 2WNH, 1SK9 and 1DKP which all of them possess disulfide bond). Phytases with disulfide bond except 2GFI places in one cluster (third node) that its ancestor is prior to ancestor of other clusters or other word branch of third node have larger length in comparison to other nodes that exhibit more divergence (Figure **6**).

DISCUSSION

Evaluation of 3D Structure and Disulfide Bond

Surveying PDB server revealed that only 3D structures of phytases from 9 various microorganisms was available. To analyze these phytases, all of them were downloaded in pdb format. Protein structures that obtained from Protein Data Bank Server encoded by atomic coordinate PDB files, a molecular graphics visualization tool is required to view and to be able to manipulate the images to view the molecule from various perspectives [32]. SPDBV and PyMol are open sources softwares were selected for visualization 3D structure and disulfide bonds of phytases [17, 20].

Evaluation of 3D structure and disulfide bond Using SPDBV and Pymol softwares revealed that only phytases that has more than 400 amino acids in each chain have disulfide bond and others that have less

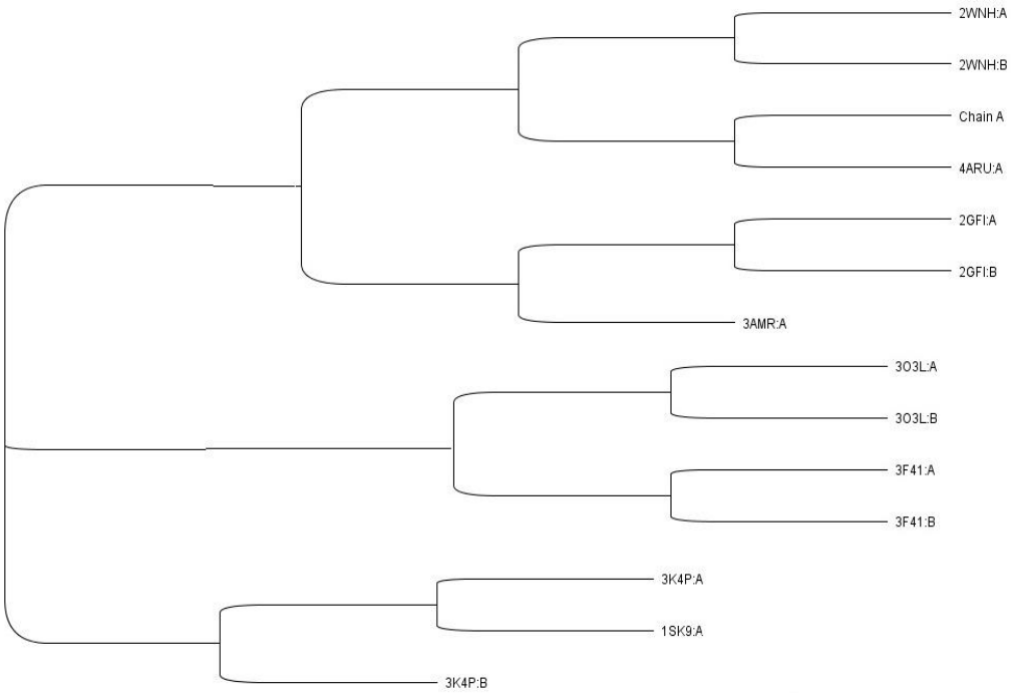


Figure 6: Phylogenic tree constructed from sequence of phytases. This tree consists of three node, each node represents a cluster with same ancestor. Ancestor phytase bearing disulfide cluster has larger branch thus is older than ancestor of another cluster.

than 400 amino acids lack any disulfide bond. Considering Table **1**, at first glance it seems that 3F41 with more than 400 amino acids should probably contain one or more disulfide bond but structural analysis by softwares indicate it is the only phytase that refuses the relationship between number of amino acid and presence of disulfide bond. However, with paying more attention, it could be seen that this phytase have two chains with each containing two domains that are identical in sequence and secondary and tertiary structures. Hence, it could be considered as a protein with 4 chains with less than 400 amino acid and therefore not only it did not refuse this reasonable relationship but also confirmed it.

To verify the accuracy of this relationship more analysis were conducted on phytase which their 3D structures have not yet been resolved. In order to fulfill this, their amino acid sequence were retrieved in fasta format from Uniprot server and their 3D structures were constructed using SWISS modeling tool from expasy server. Interestingly, the modeled structure exhibited that phytases with more than 400 amino acids are comparable with 3D-resolved phytases, structurally. On the other hand, within their modeled structure the formation of disulfide bond occurred, as well. It has been reported that disulfide bond in phytase play an important role in maintaining native structure of the enzyme [33]. Moreover, 4 of 410 amino acids in *E. coli* were glycine, being the highest level content. Accordingly, it can be mentioned that to maintain the structural integrity the presence of disulfide bond in larger phytases is elemental.

Looking over the 3D structure of phytase bearing disulfide bond(s), it can be pointed out that they show related 3D structures which are more extended comparing phytases lack any disulfide bond. Nonetheless, phyatses with no disulfide bond naturally represent more globular structure rather than extended one (Figures **1** and **2**). This can probably be explained by the fact that since for protein conformational stability either the compactness of structure or bearing disulfide bond is essential. Microorganisms fulfill this by either one of them which means in some phytase it is achieved by creating disulfide bond and in others it is accomplished by conforming protein structure into globular structure and compact conformation. Indeed, phytases with more globular structure might do not require disulfide bond to maintain their 3D structure while for larger phytases to support the integrity of their extended structure the presence of disulfide bond is inevitable.

Identical Parameters in Phytases Convey Disulfide Bond

Analyzing protein structural parameters of phytases performed using Prot Param tool and CLC protein workbench software. ProtParam is a tool which allows the computation of the physiochemical parameters based on sequence. CLC protein workbench creates a software environment which enabling users to make a large number of advanced protein sequence analyses [34] . Information obtained from these tools showed some identical parameters in phytases containing disulfide bond (Table **3**). Aliphatic index is a measure of the relative volume occupied by aliphatic side chain of alanine, valine, leucine and isoleucin. Fascinatingly, aliphatic index of phytases with disulfide bond is higher than that of in phytases without disulfide bond. An increase in the aliphatic index increases the thermostability of globular proteins [35]. Phytases that contain more disulfide bond and high aliphatic index are more thermostable than the others. *Escherichia coli* phytase has highest aliphatic index and maximum disulfide bond. It has been demonstrated that it can withstand temperatures up to 100˚ over a period of the initial enzymatic activity [36].

Using ProtParam protein tool percentage of amino acids was calculated and results tabulated in Table **3**. Percentage of glycine in phytases that contain disulfide bond is almost equal while for the rest of phytases variable number of glycine is evident. Glycine is a small amino acid plays a unique role in the structure of folded because of its small size and the minimal steric hindrance of their side chains, which adopt a wide range of conformations [37].

Type of amino acids and their percentage play important role in folding of proteins [38], glycine play an important role in creating turns [37], maybe equal percentage of it gives the phytases a same flexible folding structure which strengthen by disulfide bonds.

Phylogenic Analyses

Phylogenic methods can be used for many purposes, including analysis of morphological and several kinds of molecular data such as comparisons of more than two sequences, estimation of evolutionary relationships among organisms, DNA sequences and protein sequences [39-41]. There is a vast variety of tools for phylogenic analysis. Multiple sequences analysis, superimposition and phylogenic tree are tools for finding out conserved regions, morphological

similarity and evolutionary relationships, respectively [21, 27, 40, 42].

For analysis and comparison it is useful to superimpose related structure [42]. The root mean square deviation (RMSD) is the measure of the average distance between atoms (usually the backbone atoms) of superimposed proteins [43]. Superimposition for phytases with disulfide bonds carried out using SPDBV revealed that folding of these phytases is almost similar and values of RMSD for superimposed proteins is approximately to 2.5 A˚ that confirmed identical folding for phytases with disulfide bond. Existence of conserved regions in these phytases gives them the same conformation in secondary and tertiary structure which most of the secondary structure composed of α-helix. It is believed that α-helix structure provides proteins more stability than other secondary structures such as strand-structures and since most of phytases are extracellular proteins they are adopted to this structure [44]. Although it is not always clear whether protein with the same fold are evolutionary related, they should still be superimposabale, thus multiple sequences alignment is necessary for proving that phytases with disulfide bond have same evolutionary course.

Multiple sequences alignments for phytases constructed using CLC protein workbench. Alignment of these phytaeses showed that phytases possess disulfide bond are identical in sequence and have conserved glycines. Position and percentage of glycine play an important role in protein folding [37]. The folding shape or conformation of a protein depends on linear amino acid of it [45]. It is likely that having conserved glycine amino acids and almost equal percentage of it which endow a protein flexible structure, necessitate having disulfide bond for disulfide-bearing phytases and almost compensate their extended 3D structure and furnish them with a stable conformation. Disulfide bond is necessary for correct function of these phytases which are extracellular or preplasmic. Taken together, number of conserved amino acids among phytases with disulfide bond is significant thus indicates they probably have same evolutionary course (Figure **5**).

Using Genious multiple sequence alignment of phytases carried out and phylogenic tree has been drawn, as well. The phylogenic tree is a diagram for evaluating the evolutionary relationship among organisms, DNA sequences, protein sequences. By interpretation of a phylogenic tree, useful information about evolutionary course of protein sequences could be concluded [30, 31]. The phylogenic tree for phytases revealed 3 nodes that each of them represents a cluster of phytases that have the same ancestor. The first node consists of 2 tips that both of them lack any disulfide bond (3F41 and 303L), second nodes consist of 2 tips (3AMR and 2GFI), the third node consists of phytases with disulfide bond (1DKP, 1SK9, 2WNH, 3K4P). This phylogenic tree showed that all phytases that have disulfide bond except 2GFI place in the same cluster. In other words, phytases with disulfide bond have the same evolutionary course. Phytases bearing disulfide bond cluster has the longer branch. The length of branches represents evolutionary lineage changes over time [31], thus ancestor of this node is prior to other nodes. 2GFI with 4 disulfide bond in each chain and 3AMR that lacks any disulfide bond place in same cluster (second node) that showed phytases without disulfide bond oriented from phytases with disulfide bond over time and obtained some characteristic such as more globular structure which make them stable and to some extent compensate the absence of disulfide bond.

CONFLICT OF INTEREST

None to Declare.

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