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Original Article

Two N-terminally derived and expressed fatty acid-binding peptides of DUF538

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ABSTRACT

The fatty acid-binding ability of DUF538 (domain of unknown function 538) protein family in plants was predicted by using molecular docking bioinformatics tools. Two N-terminally derived and engineered peptides (peptide1: KWLVNKIK GKMQ; peptide2: PSICEVGYKDSSVLKFTTKT KVMIWVK) were identified to be the fatty acid-binding elements of DUF538. Using His tag-based recombinant technology, the heterogeneous expression and production of the predicted binding peptides were successfully examined and reported in *Escherichia coli* cells without affecting the recombinant bacterial cell growth. The recombinant extracts containing peptide1 and peptide2 fusion products exhibited palmitic acid-binding abilities of about 64% and 28%, respectively. As a first report, the presently studied peptides were introduced as novel fatty acid-binding peptides that may be used in medical biotechnology and drug development.

KEYWORDS: DUF538, peptide expression, fatty acid, palmitic acid, his tag.

1. INTRODUCTION

DUF (Domain of Unknown Function) protein family comprises so many putative hypothetical proteins sharing one or more highly conserved domains with no clarified functions. To date, more than 4000 DUF families have been identified in Pfam database that include more than 22% of the entire database [1-3]. DUF538 members are among the most studied DUF proteins that have been found in more than 40 plant species, almost exclusively in *Embryophyta* including the wide ranges of monocotyledonous and dicotyledonous plants [3]. They have been often identified from several plants challenged with various abiotic and biotic environmental stresses including drought, elevated temperatures, nutrient deficiency, mixed elicitors, fungal pathogens, nematodes and *Meloidogyne* infection [1, 3-7]. The involvement of DUF538 proteins in plant growth and developmental processes has also been recently investigated, highlighting the importance of DUF538 protein family in higher plants [8]. Their molecular weights are about 19-21 kDa and encode around 170 amino acids. The three-dimensional structures of DUF538 proteins are dominated by ß-strands (PDB ID: D1ydua1). In various structural and functional studies by our research team, DUF538 proteins have been suggested to belong to binding proteins. They have been found to be the structural homologues of bactericidal/permeability-increasing (BPI) proteins in the innate immune system of mammalians. Their potential to bind to the lipid A moiety of lipopolysaccharides (LPS) on the outer leaflet of the bacterial membranes was predicted similar to BPI of human immune system [9]. Later on, the binding abilities of DUF538 proteins towards methylester compounds such as chlorophyll and pectin molecules have also been reported [10-12]. Very recently, the binding properties of DUF538 domain-containing proteins toward lipid/lipid-like compounds such as cholesterol

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molecules were investigated. They have been suggested to play an important role in cholesterol transporting and metabolism [13].

Considering all these together, to increase our knowledge about the binding peptides, our attempt was made to investigate and identify the fatty acid-binding potentials of DUF538 protein toward palmitic acid. Using computational and recombinant-based experimental approaches, the palmitic acid-binding ability of DUF538 protein and its heterogenous expression in *E. coli* system was investigated aimed at contributing to the novel research areas of DUF538 protein family/lipid-binding peptides in plants. As a first report, the presently introduced peptides were predicted as novel binding candidates that may be used in medical biotechnology and drug development.

2. MATERIALS AND METHODS

2.1. Computational analysis

The protein sequence of *Arabidopsis* DUF538 was extracted from protein database, NCBI. The interaction of the ligand (palmitic acid) with the target protein was performed by molecular docking to determine the binding energy and binding sites of the ligands on the test protein. AutoDock software version 4.2 was used for molecular docking analysis. Protein data bank (PDB) for protein and ligand structures were obtained from Rcsb and PubChem databases, respectively. Docking parameters were created and the AutoDock program was run and all the output conformations were saved to a dlg file. After docking, the dlg file was opened and analyzed using AutoDockTools software. The conformations inside this file were ranked based on their binding energy.

2.2. Bacterial strains and experimental materials

Bacterial strains DH5α and BL21 of *E. coli* were provided from laboratory stock and used as the hosts for recombinant expression. DH5α strain was used for cloning and BL21 carrying T7 RNA polymerase gene was used for protein expression and production purpose. pET-26b (+) fusion vector containing His-tag and T7 promoter was used as recombinant expression vector (Cat. No. 69862-3). Gel extraction kit (Cat. No. K3035-1; Bioneer) was utilized for the DNA extraction from gel materials. Plasmid miniprep kit (Cat. No. K3112; Bioneer) was used for plasmid isolation and purification from transformed recombinant host cells. Restriction enzymes *Nde*I and *Xho*I and T4 DNA ligase were purchased from Bioneer company. Palmitic acid (>99%; CAS No. 57-10-3) and vanillin (99 %; CAS No. 121-33-5) were from Sigma. All of the materials used for this research project were of molecular biology grades.

2.3. Cloning and expression of binding peptides

For cloning purpose, the genes of designed binding peptides consisting of 12 and 27 amino acids were synthesized by GENErey biotechnology company (gene-ray.com.cn). Both the synthesized DNA fragments and pET-26b expression vector were double digested by *Nde*I and *Xho*I DNA restriction enzymes located at the ends of fragments of interests for directional cloning. Restrictions were carried out for 1.30 hrs at 37 °C. The restricted products were then analyzed electrophoretically on 0.8% agarose gel stained by red safe dye. The DNA fragments were purified from the gel according to Bioneer gel extraction kit protocol and ligated to each other using T4 DNA ligaze enzyme using 15 µl vector DNA and 5 µl of insert DNA at 22 °C for 1 h. The ligated products were then transformed into the DH5α competent cells by using transformation and storage solution (TSS) containing 50 µg/ml kanamycin. The recombinant plasmid vectors were then isolated from transformed recombinant cells and confirmed by double digestion and analysis on 0.8% agarose gel. In order to express the selected recombinant fusion vectors, they were transformed into BL21 (DE3) *E. coli*-expressing strain [1, 14].

2.4. Bacterial growth assay

To investigate the possible effects of recombinant products' expression on transgenic bacteria, the growth patterns of recombinant and non-recombinant bacteria were analyzed spectrophotometerically. For this, single clones of transgenic and non-transgenic bacteria were separately incubated in 50 ml of luria broth (LB) liquid medium

containing kanamycin held at 180 rpm incubator shaker for 14-16 hrs. In order to induce the expression of recombinant products, isopropyl-β-D-thiogalactoside (IPTG) inducer molecule was added to each sample. Finally, the absorbance of the samples was measured at 600 nm by using Reyleigh type of UV/VIS scanning spectrophotometer.

2.5. Protein extraction and detection

For this purpose, the crude proteins of recombinant and non-recombinant expressing cells were separately isolated after bacterial lysis in TES (tris-ethylenediaminetetraacetic acid-sucrose) buffer containing 20 mM Tris, 5 mM EDTA (ethylenediaminetetraacetic acid) and 10% sucrose through three times homogenization process at 25000 rpm for 30 sec. For high yield protein extraction, the bacterial lysis steps were performed in the presence of SDS (sodium dodecyl sulfate). The amount of crude protein in each sample was approximately analyzed by measuring the absorbance of the samples at 280 nm. Each experiment was carried out with three replications. Data points represented on the graphs are the mean values of three replicates of each test.

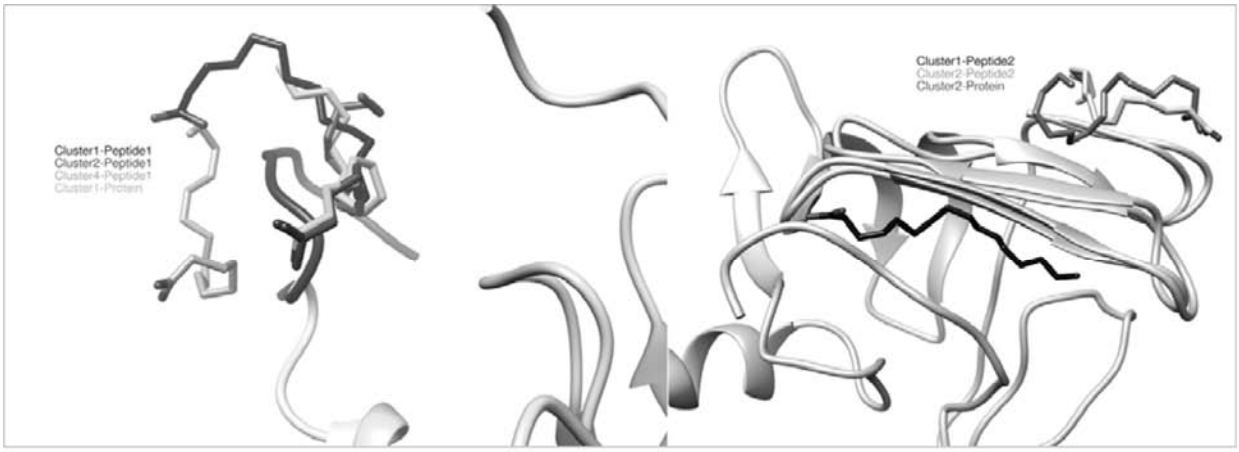
2.6. Fatty acid binding assessment

To analyze the fatty acid binding ability of fused products, the sulfo-phospho-vanillin (SPV), total lipid quantification method was used. Briefly, a specified volume of the recombinant crude extracts were separately passed through immobilized metal ion affinity chromatography (IMAC) column. Then after, a specified amount of palmitic acid was separately passed thought the fused product containing column. The total lipid content of test samples was assessed by using SPV method before and after passing through the column. For SPV assay, an aliquot of solution of palmitic acid (20 µg/ml) was evaporated to dryness on a 37 °C water bath. To the test tube containing palmitic acid, 0.05 ml of water and 0.75 ml of concentrated sulfuric acid was added. The mixture was heated in a boiling water bath for 40 min and then cooled to room temperature. 5 ml of phospho-vanillin reagent (dissolving 50 mg vanillin in 50 ml of 17% phosphoric acid) was added to the tube; after mixing, the color was developed in a 37 °C bath for 15 min. The absorbance of the test sample was then measured at 525 nm and the changes in the absorbance of test sample before and after passing through the IMAC column were estimated and presented as the approximate binding capacity of fused peptide towards palmitic acid. Each experiment was carried out with three replications. Data points represented on the graphs are the mean values ± SD of replicates of each test.

3. RESULTS AND DISCUSSION

3.1. Molecular docking analysis of palmitic acid binding to DUF538

In the present study, the AutoDock server version 4.2 was used to investigate the binding of test ligand to target protein. The protein pdb for DUF538 and the structure of ligand (palmitic acid) were obtained from Rcsb and PubChem databases, respectively. By using AutoDockTools, polar hydrogens and Gasteiger charges were added, and then rotatable bonds were calculated. Grid box with a distance of 0.375 angstroms was selected and protein was placed inside the box with the dimensions of 126 × 126 × 126 and the number of 300 runs. Figure 1 represents the interactive structure of DUF538-palmitic acid complex. As it has been shown, palmitic acid molecule enables to bind to DUF538 protein by two interactive locations named as peptide 1 and peptide 2 which are located at N-terminus and C-terminus parts of DUF538 protein, respectively. The locations and the amino acid sequences of derived interactive peptides (including “KWLVN KIKGKMQ” and “PSICEVGYKDSSVLKFTTK TKVMIWVK”) are represented in Figure 1. The three-dimensional structures of derived peptides are also separately represented in the same figure. Figure 2 shows the number of clusters, the number of structures in each cluster, and the amount of best binding energy for each cluster in DUF538-palmitic acid complexes. According to this figure, cluster 1 in peptide1-palmitic acid complex has 23 structures with the binding energy of -5.1 kcal/mol, while, the cluster 1 has 64 structures with the binding energy of -4.2 kcal/mol in peptide2-palmitic acid complex. To select the best binding mode, binding energy and



MDQIFNKVGSYWLGQKANKQFDSVGNDLNSVSTSIEGGT**KWLVNKIKGKMQ**KPLPELLKE
YDLPIGIFPGDATNYEFDEETKKLTVLI**PSICEVGYKDSSVLKFTT**TVTGHLEKGKLTDV
EGI**KTKVMIWVK**VTSISTDASKVYFTAGMKKSRSRDAYEVQRNGLRVDKF

Peptide1: KWLVNKIKGKMQ

Peptide2: PSICEVGYKDSSVLKFTTKTKVMIWVK

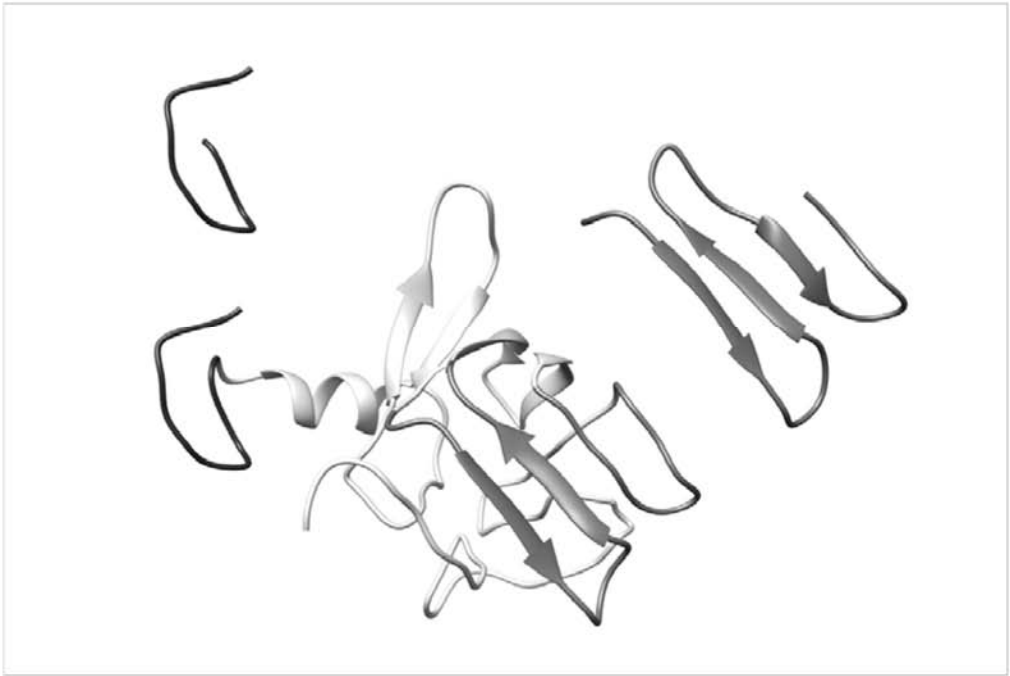


Figure 1. Molecular docking of palmitic acid to DUF538 protein (upper), the amino acid sequences of palmitic acid-binding peptides (middle) and three-dimensional structures of the derived peptides (lower).

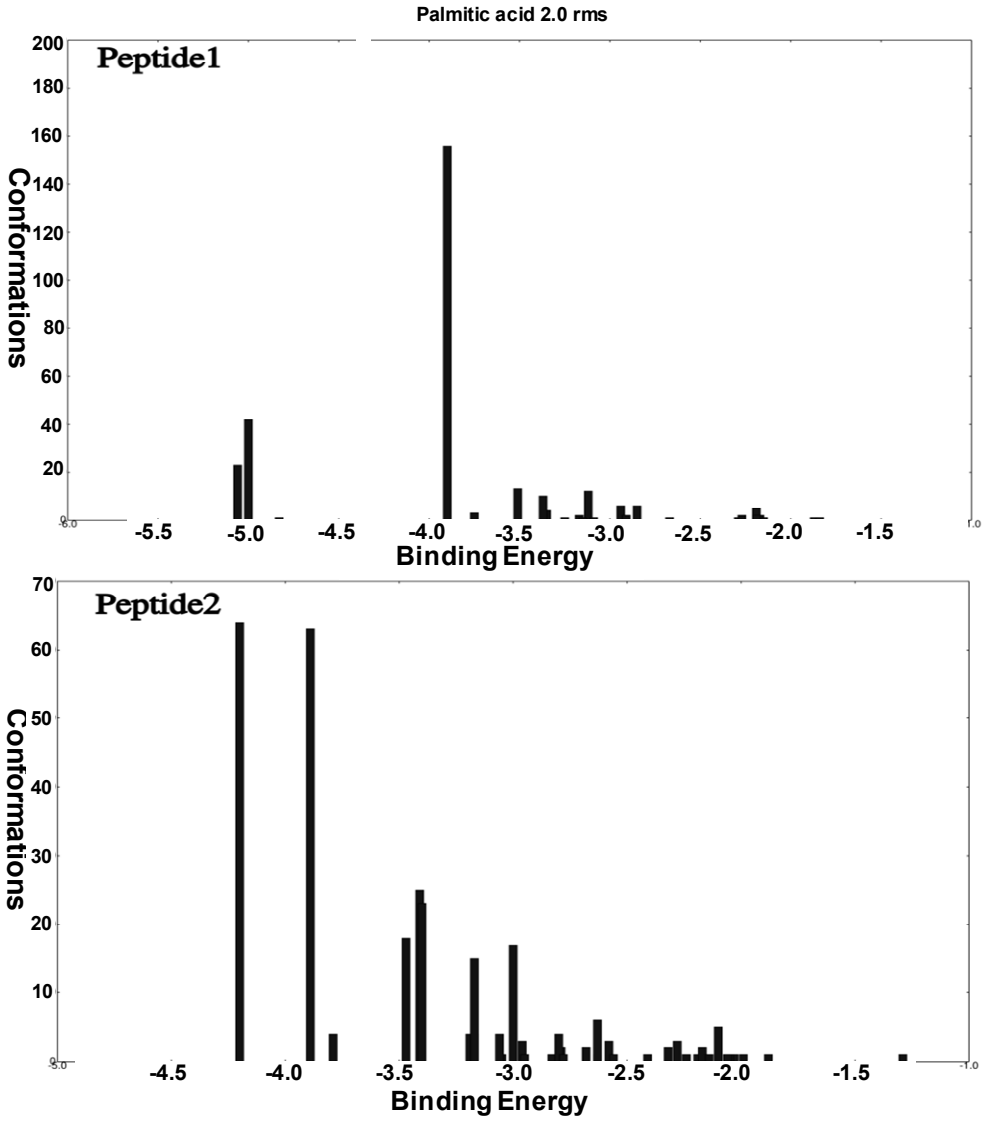


Figure 2. Number of clusters (conformations) and amounts of binding energies of docking of palmitic acid to DUF538 protein.

cluster size are important factors. Binding energy indicates the binding strength of the ligand to the protein and the cluster size shows the frequency and probability of binding of the ligand to different sites on the protein. Table 1 shows the various energies including electrostatic energy, van der Waals energy, and inhibitory constant of palmitic acid-peptide1 and peptide2 complexes for the first clusters. As shown in the table, cluster 1 has 23 members in palmitic acid-peptide1 complex and has a binding energy value of -5.06 kcal/mol, while cluster 2 has 42 members and a binding energy of -5 kcal/mol. According to this table, cluster 4 has 156 structures and a binding energy of -3.59 kcal/mol, with an energy difference of -1.47 kcal/mol, compared to cluster 1. Comparing the binding energies reveals that cluster 1 has more affinity to palmitic acid than other clusters. In addition, the value of ligand efficiency in these three clusters is -0.28, and the inhibitory constant values are 194.55, 216.65 and 1390, respectively. The amounts of intermolecular energy are -9.24, -9.18 and -8.07 kcal/mol, respectively. Evaluation of the van der waals and

Table 1. Energy details of the top clusters obtained by molecular docking of palmitic acid to DUF protein.

Ligand	Cluster	No. of structures	Binding energy	Ligand efficiency	Ki (μM)	Intermol energy	VdW energy	Elec. energy
Pep1	Cluster 1	23	-5.06	-0.28	194.55	-9.24	-7.56	-1.68
	Cluster 2	42	-5	-0.28	216.65	-9.18	-7.3	-1.87
	Cluster 4	156	-3.59	-0.282	1390	-8.07	-5.99	-2.08
Pep2	Cluster 1	64	-4.2	-0.23	832.16	-8.38	-6.82	-1.56
	Cluster 2	63	-3.89	-0.22	1400	-8.07	-6.37	-1.7

electrostatic energies shows that the van der waals energy plays a very important role in binding the palmitic acid molecule to peptide1, while the electrostatic energy values for the these three clusters are very small.

Data analysis related to palmitic acid-peptide2 complex shows that the binding energies for cluster 1 and 2 are -4.2 and -3.89 kcal/mol, indicating that cluster 1 has more affinity for palmitic acid molecule than cluster 2. Also, the ligand efficiency for both clusters is about -0.23. The inhibitory constant for cluster 1 is 832.16 and for cluster 2 is 1400 μM. The amount of intermolecular energy for cluster 1 is equal to -8.38 and for cluster is 2 -8.07 kcal/mol. The amount of van der waals energy for cluster 1 is equal to -6.82 and for cluster 2 is -6.37 kcal/mol. The amount of electrostatic energy for cluster 1 is equal to -1.56 and for cluster 2 is equal to -1.7. Similar to palmitic acid-peptide 1 complex, the results reveals that the van der waals energy plays an important role in binding of palmitic acid molecule to peptide2. The overall data analysis specifically those related to binding energies (-5.06 versus -4.2) and inhibitory constant values (194.55 versus 832.16) indicates that peptide1 has more affinity to palmitic acid compared to peptide2. Also, compared to van der waals energy, the amounts of electrostatic energies (-7.56 versus -1.68 and -6.82 versus -1.56) indicate the absence of a charged functional group in the structure of the palmitic acid. According to the results, comparing the binding potentials (particularly including binding energies and inhibitory constant values) indicates that palmitic acid has more affinity to peptide1 than peptide2. However, both the peptides are able to bind to palmitic acid molecule.

3.2. Expression and bacterial growth analysis of binding peptides

For expression test, the peptide DNA sequences were separately inserted into pET-26b (+) vector, and then were transformed into *E. coli* DH5α competent cells and selected on LB agar bacterial medium containing 50 μg/ml kanamycin. The recombinant transformed bacteria were then characterized by the extraction and double digestion of plasmid DNA using *Nde*I and *Xho*I restriction endonucleases. The restricted products were electrophoretically analyzed on agarose gel (the gel photograph not presented). After that recombinant plasmids were transformed into BL21-expressing *E. coil* cells and the recombinants were selected on LB medium supplemented with kanamycin and IPTG inducer molecule. To analyze the difference between recombinant and non-recombinant cells, the crude proteins were separately isolated from bacterial cells thought the lysis of bacteria in the presence of SDS. The expression and the amount of crude protein in each test sample were approximately analyzed by measuring the absorbance of the samples at 280 nm. The comparison of absorbance data between recombinant and non-recombinant protein samples (1.8 versus 2.2) revealed that the recombinant peptides are successfully expressed in bacterial cells (Figure 3). To evaluate the possible effects of expressed products on *E. coli* cells, the rates and patterns of recombinant and non-recombinant bacterial cell growths were analyzed

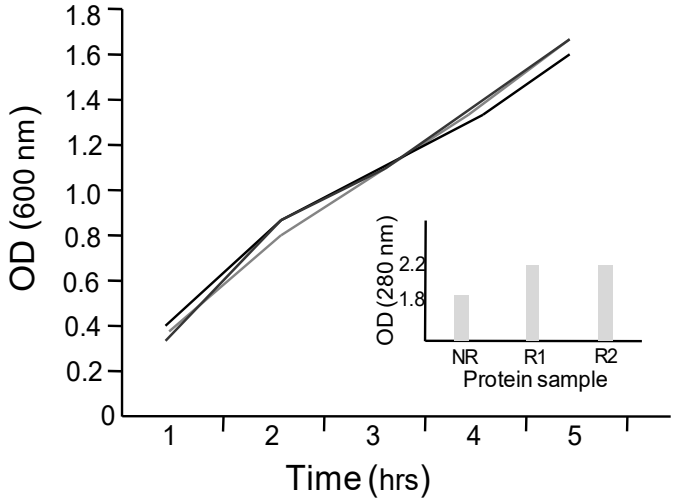


Figure 3. Bacterial growth assessment and relative protein detection by OD measurements at 600 and 280 nm. Upper line: control non-recombinant sample; Middle line: peptide1 recombinant sample; Lower line: peptide2 recombinant sample. NR: non-recombinant control; R1: peptide1 recombinant sample; R2: peptide2 recombinant sample.

under induced conditions using IPTG. The growth of bacteria was separately assessed by measuring the optical density (OD) of bacterial cultures at 600 nm with an incubation time of 5 hrs. Comparison of the results indicated that the growth patterns of recombinant bacteria are similar to that of non-recombinant cells and they are not affected by the induced expression of recombinant binding peptides inside the *E. coli* cells (Figure 3).

The changes in the absorbance (A_{525}) of test samples (before and after passing through the IMAC column) were estimated and presented separately as the approximate binding capacity of fused peptides towards palmitic acid molecules. The results showed that the absorbance of the peptide1 containing test sample was decreased to 0.63 as compared to the absorbance of the fused product-free control sample (1.8). Also, the results confirmed that the absorbance of the peptide2 containing test sample was decreased from 1.8 to 1.2. On the other hand, analysis of the data revealed that about 64% and 28% of the palmitic acid molecules are absorbed by peptide1 and peptide2 fusion products, respectively (Figure 4).

As a first report, the aim of this study was to analyze the binding potential of DUF538 protein to palmitic acid (as a frequent fatty acid in biological world) and to predict the possible binding peptide/peptides. The next experimental step in this study involved expression analysis of predicted peptides in *E. coli* system as fusion products. Based on our recent research results, DUF538 domain-containing proteins were suggested to be able to bind to lipid-like molecules such as chlorophyll and lipopolysaccharides and cholesterol [10, 11, 13]. Thus, DUF538 protein family was proposed to be the structural/functional homologue of lipocalin (lipid-binding anticalin-like proteins) protein family in plants or animals [13]. As part of these studies, we analyzed the binding ability of DUF538 protein to palmitic acid and predicted two separate peptides (namely peptide1 and peptide2) that are able to bind to a test fatty acid molecule using the molecular docking as well as the recombinant fusion protein technology assessments. The fatty-acid-binding proteins are a family of transport proteins for fatty acids or other lipophilic substances such as eicosanoids and retinoids in mammalians [15, 16]. These proteins are thought to facilitate the transfer of fatty acids between extra- and intracellular membranes or to certain intracellular receptors [17, 18]. Fatty-acid-binding protein levels have been shown to be declined with ageing and synaptic activity [19]. We hope the results of the present work will open the gate to specific and interesting studies on

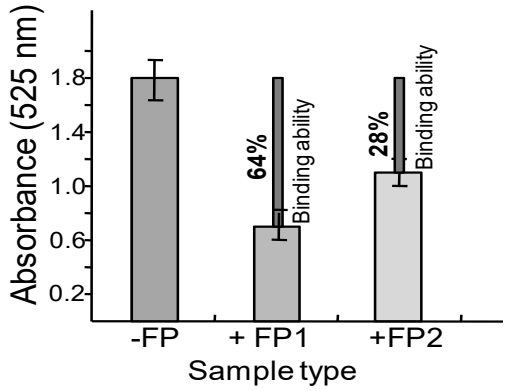


Figure 4. Fatty acid-binding assessment. Binding ability of fused products was measured colorimeterically by absorbance changes at 525 nm. –FP: in the absence of fused product; +FP1: in the presence of fused peptide1; +FP2: in the presence of fused peptide2.

fatty acid-transporting and metabolizing potencies of DUF538 or predicted peptides in plant and animal systems. The predicted peptides were successfully expressed as recombinant fusion products in *E. coli* expression system opening the way for their possible high scale production for further heterogeneous studies or applications.

CONCLUSION

Binding peptides/proteins are essential for most of the cellular events and regulations. Their interaction or binding with different other molecular partners determine their molecular function as well as their mechanisms of action in biological world. Thus, based on the binding abilities of the presently predicted peptides, they could be nominated as a novel binding element and could be used in plant, animal or medical biotechnology and drug development.

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CONFLICT OF INTEREST STATEMENT

The author declares that there are no conflicts of interests.

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