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THE SPATIAL STRUCTURE OF FICIN: THEORY VS. EXPERIMENT

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Abstract. Ficin is actively used in the food industry like as brewing, baking, in the manufacture of meat and fish products, has potential properties for effective use in medicine and pharmacology, so a deeper study of the structure of the enzyme molecule is needed. Knowledge of the spatial structure of the enzyme makes it possible to understand and predict the behavior of the biocatalyst in various technological processes.

Reconstruction of the spatial structure of the plant protease ficin (EC 3.4.22.3) from *Pisum sativum* (GenBank: AAB41816.1) was performed using high-performance computer modeling. The molecular structure of papain from *Carica papaya* (PDB ID: 9PAP) was used as a model for a similar homologous protein from which the reconstruction was carried out. The reconstructed enzyme model was compared with the deposited ficin structure (PDB ID: 4YYU).

The search for ficin homologs (EC 3.4.22.3) from *Pisum sativum* (GenBank: AAB41816.1) was performed using the FASTA and PSI-BLAST servers in the PDB protein structure database. Multiple alignment was performed using the CLUSTALW server. Alignment calculations and visualization were performed using the UGENE 1.25 bioinformatics package. After processing the initial data, the resulting structure was optimized and relaxed in the AMBER96 force field for 100 picoseconds.

The protocol of the method for obtaining a model is given in detail, which can be used both for scientific and educational purposes.

Keywords: ficin, reconstruction, homology, protein structure bank

Proteolytic enzymes have important medical and industrial value. Cysteine proteases are the most significant of them [1]. These enzymes are also used in the food industry, e.g., brewing [2], bakery [3], meat [4] and fish [5] products manufacturing; chemical industry for synthesis of peptides and amino acids [6, 7], as the active substance of many detergents [8]; in the pharmaceutical industry and medicine as anthelmintic agents, anti-inflammatory agents, an alternative to antibiotics [9, 10].

Cysteine proteases contains ten clans: CA, CD, CE, CF, CH, CL, CM, CN, CO, and C-. A greater part of plant thiol proteolytic enzymes relates to the C1 family, also known as the papain family (CA clan) [11].

The C1 family proteases are synthesized with a signal peptide delivering them to specific cell compartments, and a pro-peptide at the N-terminus, which is subsequently cleaved to activate the enzymes [1]. The enzyme`s active site is located in the pocket between the α-helix and β-sheet and is formed by Cys25 and His159 residues [12]. Residue of Gln19 [13], which precedes Cys25, and Asn175, which orients the His159 imidazole ring [14, 15], are also important for catalysis.

Cysteine proteases are mainly obtained from tropical plants of the genus *Carica* (papain, chymopapain, caricain), *Ficus* (ficin), *Ananas* (bromelain, ananain, etc.) [1]. These thiol proteases are widely used due to their properties: such as abroad substrate specificity, wide boundaries of operating pH (from 5.0 to 8.0) and temperature (the optimum of enzymes lie in the range from 40 to 70 °C). The molecular weight of most thiol proteases is 25-30 kDa [16].

The physiological role of the cysteine proteolytic enzyme family is in their participation in plant growth and development, as well as in cell aging and apoptosis [17]. Thiol proteases are

involved in protein modification, intracellular signal transduction, biotic and abiotic stimuli, and as a defense mechanism [18, 19].

Ficin (EC 3.4.22.3) is cysteine protease isolated from latex plants of the genus *Ficus* [20, 21]. Ficin has several isoforms, and consists of a single polypeptide chain, which is underdoing to autolysis [22, 23].

K.B. Devaraj et al. (2008) received information about the amino acid content of the ficin molecule [24].

M. Azarkan et al. (2011) presented data on the secondary structure of ficin C molecule, which contains 24 ± 4% α-helices, 22 ± 4% β-sheet, 18 ± 2% of chain turns and 36 ± 3% of disordered regions. These values are close to those of the papain secondary structure [25].

To date, the structures of some industrially important thiol proteases, such as papain, bromelain, chymopapain and caricain, are received [26, 27, 28]. 3D surface structure models of several ficin types (PDB IDs: 4YYQ, 4YYR, 4YYV, 4YYW) are showed [29].

Since ficin plays an important role in plant physiology, it is actively used in the food industry, it is promising for medicine and pharmacology application due to the ability to destroys microbial biofilms [30], a more in-depth study of the structure of the enzyme molecule is needed. Knowledge of the tertiary structure of the ficin will help to understand and predict the behaviour of the biocatalyst in the different systems.

There are a number of methods based on computer modeling to obtain a protein structure with an unknown spatial organization, [31]. First of all, it is the folding of a polypeptide chain from a branched conformation into a certain structure with a minimum of energy by calculating the molecular dynamics trajectory or Monte-Carlo calculations. In this method, using significant computational resources of multicore clusters, only a few very small protein structures were successfully obtained, for example, villin [32]. Somewhat later, distributed computing networks, the famous Folding@home and Rosetta@home, appeared. The conformational states from Rosetta's software can be used to initialize a Markov state model as starting points for Folding@home simulations [33]. Conversely, structure prediction algorithms can be improved from thermodynamic and kinetic models and the sampling aspects of protein folding simulations [34]. Thus, Rosetta only tries to predict the final folded state, and not how folding proceeds.

The second widely used approach to obtaining protein structures is to model the folding of a polypeptide chain *de novo* (also known as *ab initio*), i.e. by analyzing the "text" of the amino acid sequence. It is known a tendency for each residue to be part of one or another type of secondary structure [35, 36]. Modern algorithms predict the content of α-helixes, β-sheets and unordered regions using these data [37]. The Jufo 3D Server [38] offers a protein secondary structure prediction from its primary sequence and a three dimensional model. A neural network was learned with low resolution tertiary structure, an amino acid property profile, and the position based scoring matrix of a blast run [39]. It achieves a three state prediction accuracy between 75% and 95% depending on the quality of the structural model.

The third common approach is homology modeling, which we chose in this study. Selection and analysis of proteins of similar sequence [40], for which the spatial structure has already been experimentally established and deposited in ProteinDataBank [41], allows to "tighten" the sequence to the known spatial "frame" of its homologue with a high degree of certainty [42]. The homology modeling process [43, 44] involves several steps, the main ones of which are the search for a structural template and the construction of an amino acid alignment diagram. The decisive factor determining the quality of the models obtained is the degree of homology (or identity) of the sequences of the modeled protein and template (better if > 30%).

The aim of this work is the reconstruction of the spatial structure of the Ficin from *Pisum sativum* (GenBank: AAB41816.1) based on the structure of the papain molecule from *Carica papaya* (PDB ID: 9PAP).

MATERIALS AND METHODS

The search for homologues of the Ficin from *Pisum sativum* (GenBank: AAB41816.1) was performed using the servers FASTA and PSI-BLAST (their analogues) in the base of the PDB proteins structures [45]. Next, we performed multiple alignments using the CLUSTALW server [46]. Calculations and alignment visualization were performed in the UGENE 1.25 bioinformatics package [47].

Researchers often use MODELLER [48, 49], SYBYL [50], Quanta [51], as well as Swiss-Model and Phyre (Protein Homology/analogY Recognition Engine) servers for the final procedure of creating and verifying a model built on the studied protein sequence and the homolog pdb-framework. In this paper, we used the newest version of the Phyre2 package as the main reconstruction tool [52]. Previously, we

also used the MODELLER [53] package for the enzyme reconstruction with the known amino acid sequence (inulinase from *Kluyveromyces marxianus*) by a homolog with the known amino acid sequence and spatial structure (yeast invertase *Saccharomyces cerevisiae*). Our colleagues used the combined approach [54].

RESULTS AND DISCUSSION

The basis for the reconstruction of the ficin protein spatial structure was its sequence (GenBank: AAB41816.1), as well as the sequence and atomic coordinates of the close homologue – papain (PDB ID: 9PAP). The results of multiple alignment of these proteases are shown in Figure 1.

It should be noted that papain has chain folding typical for thiol proteases [26]. The polypeptide chain of papain contains 212 residues and has a molecular weight of 23350 Da. The essential sulfhydryl group places on Cys25, while the other six cysteine residues form three disulfide bridges. Because of its easy availability and its high stability, the enzyme has been the subject of many biochemical and biophysical studies. The three-dimensional structure of the papain molecule was one of the first protein structures to be determined [12, 54]. This structure revealed that the polypeptide chain is folded into two domains of roughly equal size, but completely different conformation. The active site is composed of residues from both domains, as has been found in many enzymes. Subsequent studies included a proposal for the catalytic mechanism for the enzyme [55].

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CLUSTAL O(1.2.4) multiple sequence alignment

9PAP            ------------------------------------------------------------ 0
Pisum_sativum   MASILYSLILFGLITLSLSLDMSSGRSNKEVMTMYEKWLVKHQKVYYGLGEKNQRFQIFK 60

9PAP            ------------------------------------------------------------ 0
Pisum_sativum   DNLIFIDEHNAPNHSYRVGLNEFSDITNKEYRDTYLSRWSNNNIKNKITSVRYAYKAGHN 120

9PAP            --IPEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVTIEGIIKIRTGNLNQYSEQELLDCDR 58
Pisum_sativum   NKLPVSVDWR--GALTPIKNQGSCGACWAFSAVAAVEAINKIVTGSLVSLSEQELVDCDR 178
                  :*  ****  **:**:*******:*******.::*.* ** **.* . *****:****

9PAP            R-SYGCNGGYPWSALQLVA-QYGIHYRNTVPYEGVQRYCRSREKGPYAAKTDGVRQVQPY 116
Pisum_sativum   TKNKGCNGGNQVMAYRFIVENGGLDSQIDYPYLGRQSTCNQAKKNTKVVSINGYKNVQRN 238
                  . *****  .* :::. : *:. :  *** * *  *.. :*.  ... :* ::**

9PAP            NQGALLYSIANQPVSVVLQAAGKDFQLYRGGIFVGPCGNKVDHAVAAVGYGP----NYIL 172
Pisum_sativum   SESALMEAVANQPVSVGIEAYGKDFQLYQSGVFTGSCGTSLDHAVVVVGYGSENGKDYWL 298
                .:.**: ::******* ::* *******:.*:*.* **..:****..****     :* *

9PAP            IKNSWGTGWGENGYIRIKRGTGN-SYGVCGLYTSSFYPVKN------------------ 212
Pisum_sativum   VKNSWGTNWGERGYLKIERNLKNTNTGKCGIAMDATYPTKLRENSEVTNSGYEKLQMLVP 358
                :******.***.**::*:*.  * . * **:  .: **.*

9PAP            ---------   212
Pisum_sativum   VLETPTNVA   367
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Fig. 1. Results of multiple alignment of ficin (GenBank: AAB41816.1) and papain (PDB ID: 9PAP)

After processing the initial data, the resulting structure was optimized, relaxed in the AMBER96 force field for 100 picoseconds what is necessary to relieve the possible core structure strengths of the protein, reconstructed this enzyme according to the coordinates of the high-homologous papain protein from *Pisum sp*.

A protein molecule from *Ficus carica* which is a close homologue of papain (X-ray data from other authors, 4YYU structure, Baldacci-Cresp et al, is available in PDB). For the other ficin that we studied, the original values were obtained – we reconstructed this protein molecule by enzyme sequence not from *Ficus carica*, but *Pisum sp*., while retaining the high homology with papain. We identified that the reconstructed model of the enzyme has a special "pocket" on the surface of the globule, in which the preferred binding sites of large ligands are found, while small crosslinking agents bind without precise localization, being located throughout the enzyme (Figure 2). The procedure was previously tested by us on the inulinase enzyme [53].

We note that the experimentally solved structure of ficin is available on the Protein Data Bank website under the **4YYU** index from *Ficus carica*, added to the bank 2016-04-13 by the group of authors Baldacci-Cresp F., Rodriguez Buitrago J.A., M 'Rabet N., Loris R., Baucher M., Baeyens-Volant D., Azarkan M.

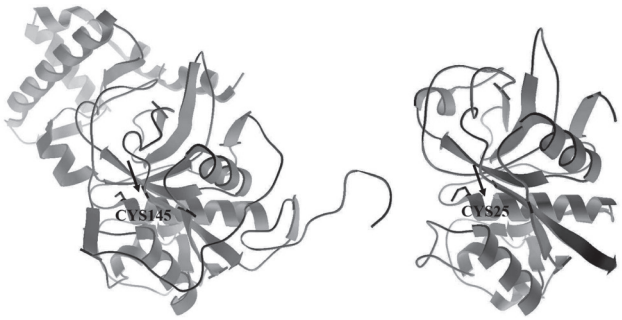

Fig. 2. Ficin [left – is reconstruction from Pisum], here is the active site CYS145. Ficin 4YYU [right], in the active site (catalytic pocket is marked with an arrow) and CYS25

CONCLUSIONS

The spatial full-atom structure reconstruction of the Ficin enzyme industrially significant for the production of pharmaceutical preparations from *Pisum sativum* (GenBank: AAB41816.1) was performed. A methodology specification for obtaining a model that can be used both for scientific and educational purposes are shown.

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ПРОСТРАНСТВЕННАЯ СТРУКТУРА ФИЦИНА: ТЕОРИЯ VS. ЭКСПЕРИМЕНТ

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Аннотация. Фицин активно используется в пищевой промышленности – пивоварении, хлебопекарном деле, при изготовлении мясных и рыбных продуктов, обладает потенциальными свойствами для эффективного применения в медицине и фармакологии, поэтому необходимо более глубокое изучение структуры молекулы фермента. Знания о пространственной структуре энзима позволяют понять и предсказать поведение биокатализатора в различных технологических процессах.

Методами высокопроизводительного компьютерного моделирования выполнена реконструкция пространственной структуры растительной протеазы фицина (КФ 3.4.22.3) из *Pisum sativum* (GenBank: AAB41816.1). В качестве модели близкого по структуре гомологичного белка, по которому была осуществлена реконструкция, была использована структура молекулы папаина из *Carica papaya* (PDB ID: 9PAP). Реконструированная модель фермента была сравнена с депонированной структурой фицина (PDB ID: 4YYU).

Поиск гомологов фицина (КФ 3.4.22.3) из *Pisum sativum* (GenBank: AAB41816.1) производился с помощью серверов FASTA и PSI-BLAST в базе структур белков PDB. Множественное выравнивание проводилось с помощью сервера CLUSTALW. Расчеты и визуализацию выравнивания выполняли в биоинформатическом пакете UGENE 1.25. После обработки исходных данных полученная структура была оптимизирована, отрелаксирована в силовом поле AMBER96 в течение 100 пикосекунд.

Детально приведен протокол методики получения модели, которая может быть использована как в научных, так и в образовательных целях.

Ключевые слова: *фицин, реконструкция, гомология, банк белковых структур*