3BIO: Journal of Biological Science, Technology and Management 5(2): 168-175
e-ISSN: 2655-8777 http://journals.itb.ac.id/index.php/3bio

In Silico Characterization of Lycopene Forming Phytoene Desaturase (CrtI) Protein from Wheat Leaf Rust Fungi (*Puccinia triticina*)

Yehezkiel Vieri Polandos[1], Fenny Martha Dwivany[1], Karlia Meitha[1*]

1) School of Life Sciences and Technology, Institut Teknologi Bandung

*) Corresponding author; e-mail: karliameitha@itb.ac.id
Received: 2023-03-25
Accepted for publication: 2023-11-27

Abstract

Carotenoid is a highly economical compound with a variety of bioactivities. However, 98% of total carotenoid used is still being manufactured by chemical-based synthesis, reducing bioactivities and is not environmentally friendly, hence the use of biofortification approach is sought. Lycopene forming phytoene desaturase (CrtI) is one of the key enzymes with the potential to develop as bioparts in recombinant carotenoid biosynthesis. CrtI from *Puccinia triticina* and *Blakeslea trispora* are considered as promising candidates due to the high amount of carotenoid in the fungi. This research aims to characterize CrtI enzyme from P. *triticina* and B. *trispora* and the interaction with substrate, i.e.,15 cis-phytoene. The results showed that CrtI from P. *triticina* protein has 2 unique motifs, determining the three-dimensional CrtI protein structure. According to docking analysis, CrtI enzyme from P. *triticina* is predicted to bind to the substrate more spontaneously as indicated by the lower energy of affinity (-8.3 kcal mol-1) and more residues interaction compared to CrtI from *Blakeslea trispora*. In conclusion, the CrtI protein from P. *triticina* is suggested as the candidate for further exploration to design expression in a recombinant system.

Keywords: *Carotenoid, bioparts, biofortification, molecular docking, pro-vitamin A*

1. Introduction

Carotenoids are a group of terpenoid derived compounds that can be found in photosynthetic organisms such as algae, bacteria, plants, and in some groups of fungi as color pigments that play a role in photoprotection against photo-bleaching [1]. Among approximately 750 carotenoid compounds that have been identified, there are 50 compounds that have been studied further and are known to have biological activities that are beneficial to humans. Carotenoid compounds have antioxidant, anti-inflammatory, antiobesity, antidiabetic and various other biological functions, but the most known function of carotenoids is as a main source of provitamin A for mammals such as humans. Some carotenoids that have high economic value are α-carotene, β-carotene, lycopene, astaxanthin, lutein and zeaxanthin [2].

Due to the variety of functions of carotenoid that are beneficial to humans, various methods have been developed to produce carotenoids on an industrial scale for pharmaceuticals, nutraceuticals, cosmetics, and animal feed additives purposes. However, 98% of the total commercial carotenoids still come from synthetic carotenoids with physico-chemical based production. The carotenoids produced by these methods are considered to have lower bioactivity than natural carotenoids and still produces waste that can harm the environment [3]. Hence, an approach that is considered more environmentally friendly is intended such as the use of living organisms as natural carotenoid-producing 'factories' known as biofortification.

Biofortification is a method to increase the essential biological value of an organism by selective breeding or genetic engineering [4]. Currently, carotenoid biofortification approach has been attempted in various organisms. The method that has been widely developed is the engineering of metabolic pathways focused on food plants by inserting genes involved in the biosynthesis of carotenoids from carotenoid-producing organisms, in hope that these modified organisms can accumulate high carotenoids [5]. Some of the organisms that have been successfully modified are bananas (golden cavendish) [6], rice (golden rice) [7], tomatoes, eggplant, and various other food crops by inserting key genes from naturally high carotenoid-producing organisms [8]. However, biofortification

DOI: 10.5614/3bio.2023.5.2.1

carried out on higher plants poses several weaknesses, such as the time-consuming growth process to produce organs where carotenoids accumulate, requires a large area of land, and the extraction method used is relatively complicated [9].

Alternative and promising biofortification methods have also been developed by using simpler organisms such as bacteria, algae, and fungi [10]. Fungi are considered as the most suitable group of organisms to be the host cells in producing large amounts of carotenoids. Their metabolic pathways are less complicated than algae and could also grow to higher densities than bacteria [11]. Two key factors that affecting the quality and quantity of carotenoids production in the biofortification process are the expression vector and the similarity of the molecular system (i.e. compatibility between promoter and RNA polymerase, and codon readings) between the host cell and the gene source organism of the carotenoid biosynthetic gene used. [12]. Phytoene desaturase gene is believed to be a key gene in carotenoid biosynthesis because its function is very crucial in the formation of carotenoid-forming precursors, i.e., lycopene [13]. There are 4 types of phytoene desaturase that can produce 4 precursors for different biosynthetic pathways (*neurosporene-forming Phytoene desaturase*, *zeta-karoten*-forming *Phytoene desaturase, lycopene*-forming *Phytoene desaturase,* and *3,4-didehydro lycopene*-forming *Phytoene desaturase*). To produce high value carotenoids such as β-carotene and zeaxanthin, the specific *lycopene*-forming *phytoene-desaturase* is needed to produce lycopene as a precursor which will later enter carotenoid biosynthesis [14].

Phytoene desaturase or CrtI is one of the key enzymes involved in carotenoid biosynthesis which has the function of converting 15-cis-phytoene into all-trans-lycopene compounds. This enzyme has been discovered and isolated from various types of organisms including bacteria, fungi, algae and plants. Phytoene desaturase itself belongs to the o*xidoreductase* or *dehydrogenase* enzyme group which has the function of catalyzing the process of releasing hydrogen and electrons [14]. In catalyzing the dehydrogenation process of the 15-cis phytoene compound, the Phytoene desaturase enzyme has a cofactor in the form of *flavin adenine dinucleotide* (FAD) as an acceptor of hydrogen and electrons which will later turn into a reduced structure, namely FADH2 [13].

Several fungi are known to have high carotenoid accumulation as well as high β-carotene accumulation in the rust fungi group. Wheat leaf rust fungus (*Puccinia triticina*) has 4 times higher β-carotene per gram of dry cells [15] than *Blakeskea trispora*, which is currently considered the gold standard in producing carotenoids in fungi [16]. B. *trispora* is the first natural carotenoid-producing microorganism that was confirmed to produce food-grade standardized food coloring by the European Union in 2000. Due to this, research related to the biofortification of carotenoids in microorganisms especially fungi, is still dominated by B. *trispora* [17]. Unfortunately, the demand for carotenoids continues to increase but carotenoid production from B. *trispora* is barely satisfy the industrial sector [16]. Therefore, improving the quality and quantity of carotenoid production is absolutely essential. The exploration of lycopene forming phytoene desaturase gene from the wheat leaf rust (P. *triticina*) as a key gene for carotenoid biofortification was made. Biofortification itself relies on bioparts, the sequence of genetic material that encodes a specific biological function to construct a biological device [18]. In this study, the characterization of the CrtI protein sequence and the interaction between the CrtI protein from the P. *triticina* with 15-*cis-phytoene* as a candidate for superior biopart in carotenoid biofortification was carried out by in silico approach. Future application would include the insertion of this biopart candidate into the genome of competent host cells from groups of fungi such as *Yarrowia lipolyca* to produce large amounts of natural carotenoids [19].

2. Methodology

2.1 Retrieval of Protein Sequence

The protein sequences of lycopene forming phytoene desaturase (CrtI) from wheat leaf rust fungus or *Puccinia triticina* (OAV98295.1) and oat crown rust fungus or *Puccinia coronata* (PLW53833.1) were retrieved from the NCBI database. For comparison, the CrtI sequences from *Neurospora crassa* (AAA33555.1), *Phaffia rhodozyma* (CAA75240.1), and *Blakeslea trispora* (AAO46894) from NCBI were also retrieved as shown in table 1.

Table 1. CrtI protein sequence status and information from NCBI database.

No.	Organism	Length (aa)	Sequence status	Accession code
1	*P. coronata*	579	Hypothetical	PLW53833.1
2	*P. triticina*	672	Hypothetical	OAV98295.1
3	*B. trispora*	582	Fully annotated	AAO46894.1
4	*P. rhodozyma*	582	Fully annotated	CAA75240.1
5	*P. ananatis*	492	Fully annotated	P21685
6	*N. crassa*	595	Fully annotated	AAA33555.1

DOI: 10.5614/3bio.2023.5.2.1

2.1 Retrieval of Protein Sequence

The protein sequences of lycopene forming phytoene desaturase (CrtI) from wheat leaf rust fungus or *Puccinia triticina* (OAV98295.1) and oat crown rust fungus or *Puccinia coronata* (PLW53833.1) were retrieved from the NCBI database. For comparison, the CrtI sequences from *Neurospora crassa* (AAA33555.1), *Phaffia rhodozyma* (CAA75240.1), and *Blakeslea trispora* (AAO46894) from NCBI were also retrieved as shown in table 1.

2.2 Protein Sequence Alignment and Phylogenetic Tree Construction

Multiple sequence alignment (MSA) was performed on CrtI protein sequences from P. *triticina*, P. *coronata*, N. *crassa*, P. *rhodozyma*, and B. *trispora* with MUSCLE program using the MSA progressive alignment method [15]. a phylogenetic tree was reconstructed base on the results of the previous alignment with the maximum likelihood method with a bootstrap value of 1000 using the MEGA-X software [20].

2.3 Protein Motif and Domain Analysis

NCBI Conserved Domain Search program was used to search for protein domains from all CrtI protein sequences [21]. Domain visualization was performed using Illustrator for Biological Sequences (IBS) software [22]. The CrtI protein motives from the fungi P. *triticina*, P. *coronata*, P. *rhodozyma*, and B. *trispora* were analyzed and compared with CrtI motif from bacteria *Pantoea ananatis* (P21685). MEME Suite 5.4.1 program was used to search for conservative motives [23]. Furthermore, the function of each conservative motif obtained previously by using Interpro 87.0. program was observed [24].

2.4 Three-dimension protein structure prediction

Prediction of three-dimensional structure was carried out in I-TASSER program [25]. In the three-dimensional structure prediction process, CrtI protein sequences from P. *triticina* (OAV98295.1) and B. *trispora* (AAO46894.1) were predicted by multiple threading alignment and iterative structural assembly methods based on the three-dimensional structure of proteins in the Protein Data Bank (PDB) [26]. Out of 5 best models results from I-TASSER prediction, one model with the highest C-score was further used for visualization in PyMol [27]. The three-dimensional predictive structure of the protein was validated using ERRAT with the error value parameter of a sequence relying on the distribution and interactions between non-bonded atoms in the protein model [28].

2.5 Three-dimension protein structure prediction

Protein-ligand docking analysis was carried out on the three-dimensional structure of the CrtI proteins of P. *triticina* and B. *trispora* to see the interaction of the protein with the 15-cis-Phytoene substrate. Docking was done on the active side of the protein in AutoDock Vina v1.1.2 software [29]. Protonation was carried out on the three-dimensional structure of the protein with the add hydrogen feature in the edit menu, the protein document was exported in pdbqt format. A cage or grid box was made that covered the active part or hydrophobic pocket of the protein and then the dimensions parameters of the cage were recorded.

The three-dimensional structure of the 15-cis-phytoene substrate was extracted from the NCBI PubChem database in mol.2 format. In AutoDockTools-1.5.7, the torsion of the substrate was tested then the ligand document was exported in pdbqt format. Ligand-protein docking was carried out between CrtI protein model and 15-cis-phytoene using AutoDock Vina-1.1.2 program used exhaustiveness parameter of

32. Then the energy affinity (kcal mol^{-1}) was analyzed in each pose of the docking ligand with CrtI protein and the interaction between the ligand and amino acid residues was visualized using PyMol software in the 3Å area.

3. Results and Discussion

Two lycopene-forming phytoene desaturase (CrtI) hypothetical protein sequences from rust fungi were retrieved, namely wheat leaf rust fungi (P. *triticina*) and oat crown rust fungi (P. *coronata*), those two species are member of the genus *Puccinia* which have high β-carotene content in spores [30, 31]. In addition, CrtI sequences from B. *trispora* [32], *Phaffia rhodozyma* [33], and *Pantoea ananatis* [14] were also extracted as shown in table 1. The CrtI from these three organisms have been well studied regarding their function in producing essential carotenoids such as β-carotene and astaxanthin, especially the protein from B. *trispora* which is considered as one of the best candidates (gold standard) in producing β-carotene from the fungal group [32]. Furthermore, CrtI sequence from *Neorospora crassa* was included as an out-group, because this fungus naturally accumulates carotenoid precursors in the form of neurosporene, suggesting distinct CrtI structure [15].

The CrtI protein sequences used in this study have varying sequence lengths where the CrtI protein sequence from P. ananatis has the shortest sequence length of 492 residues while the sequence from B. *trispora* is the longest CrtI sequence, with a length of 672 residues. Apart from those two CrtI sequences, other organisms have relatively similar lengths ranging from 579 residues to 595 residues. The CrtI protein sequences of the two rust fungi, P. *triticina* and P. *coronate*, are hypothetical in the NCBI database, while other CrtI sequences have been fully annotated. Fully annotated sequences indicate that functional analysis have been conducted to the proteins, while the hypothetical status indicates that proteins are expected to be expressed from an open reading frame but no experimental evidence of translation yet [5].

According to the phylogenetic analysis between the four naturally occurring essential carotenoid-producing fungi: P.

 DOI: 10.5614/3bio.2023.5.2.1

triticina, P. *coronata*, P. *rhodozyma*, and B. *trispora*, a phylogenetic tree with a topology as shown in Figure 1(a). The phylogetic tree was made by using the maximum likelihood method, it was found that the rust fungi formed clusters consisting of P. *triticina* and P. *coronata*, this was due to the two CrtI protein sequences from this rust fungus having a high percent identity as 88.0% and percent similarity as 93.2%. Rust fungi formed a cluster related to the fungus B. *trispora* (indicated by the red dotted line) where the CrtI protein from B. *trispora* whom known as the gold standard for producing β-carotene in fungi, in other word the rust fungi have a similar primary protein structure with B. *trispora*. Furthermore, these three β-carotene-producing fungi formed an ingroup with the fungus P. *rhodozyma* because they had similarities to form lycopene as the precursors. These four fungi are separated from N. *crassa* because these fungi have CrtI that produce neurosporine precursors. The results obtained confirmed that the rust fungus has a CrtI protein sequence that is similar to B. *trispora* and has the potential to be used as bioparts to produce essential carotenoids such as β-carotene.

Based on the analysis of the CrtI protein sequences of P. *triticina*, P. *coronata*, and B. *trispora*, it was found that they have 3 shared domain families (Figure 1(b)). The first domain is DAO or FAD dependent oxidoreductase family (Pfam id: PF01266) which is marked in green. The next are flavin-containing amine oxidoreductase (Pfam id: PF01593) which is marked in blue, and the transmembrane helix region in red. Meanwhile, the CrtI protein sequence from P. *rhodozyma* only has a flavin-containing amine oxidoreductase domain and a transmembrane helix region. The DAO or FAD dependent oxidoreductase (red parts in figure 1b) is a domain that interacts with co-enzyme [14], the flavin-containing amine oxidoreductase (green parts in figure 1b) serves as the active site or substrate binding region [34], while the transmembrane helix region (blue parts in figure 1b) is the part that regulates the localization of protein. In the CrtI sequences from *Puccinia* and *Blakeslea* it was found to contain flavin-containing amine oxidoreductase that is crucial in psi-ends formation in both lycopene chemical structure terminals or ring-formation in provitamin A [14] that can not be found on the other fungi CrtI protein.

According to the analysis of the CrtI protein sequence motifs carried out using MEME Suite v5.4.1 between four β-carotene producing fungi, 13 sustainable motifs or consensus motifs were found as shown in Table 2. The position of the motif on each CrtI protein is shown in Figure 1(c). Among the total 13 motifs, 5 consensus motifs were found in the CrtI protein sequences fromboth bacteria and fungi, while the other 8 motifs were only found in fungi. Among those 8 consensus motifs, there are 2 consensus motifs found only in rust fungi (Puccinia), namely consensus motifs number 9 and 12. Consensus motifs number 9 and 12 are only found in rust fungi which can change the three-dimensional structure of proteins, so that it can indirectly affect the strength of the interaction between the protein and the ligand. Of the 13 consensus motifs obtained from the CrtI protein, it was successfully grouped into 3 main domains, including the substrate binding domain (SBD), FAD/NAD binding domain (FBD) and also the non-conserved 'helical' structure domain (NHS) [14].

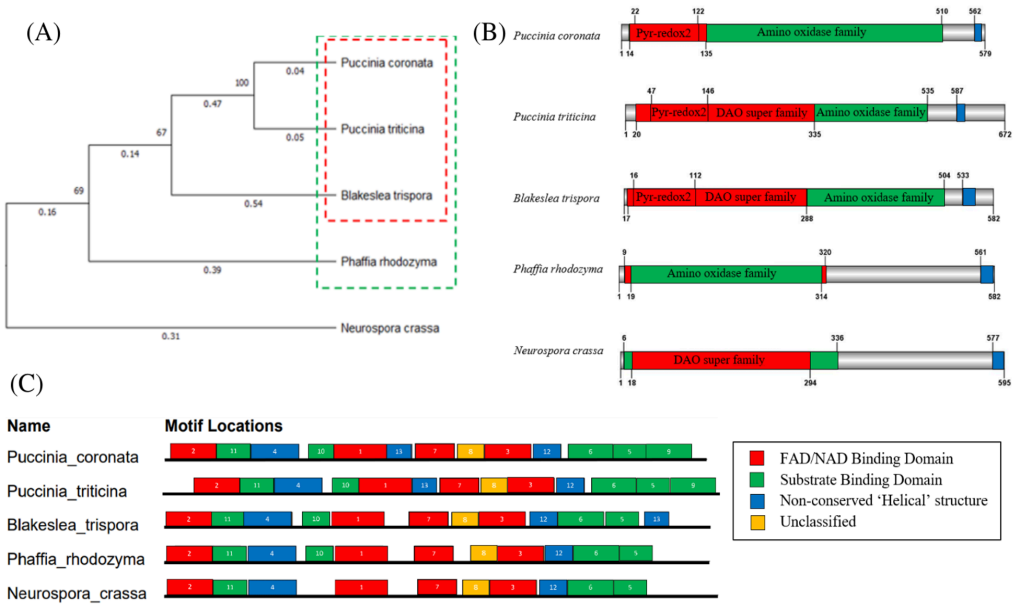

Figure 1. (a) Phylogenetic tree of CrtI protein sequences between P. *triticina*, P. *coronata*, P. *rhodozyma*, and B. *trispora* using the maximum likelihood method, (b) Domains found in the CrtI protein sequences from P. *triticina*, P. *coronata*, P. *rhodozyma*, B. *trispora*, and N. *crassa*., (c) Consensus motifs found in the CrtI protein sequence P. *triticina*, P. *coronata*, P. *rhodozyma*, B. *trispora*, and P. *ananatis*.

DOI: 10.5614/3bio.2023.5.2.1

Table 2. (Sequence and characteristics of each motif of the CrtI protein sequence

No	Motif	e-value	Length (aa)	Domain
1.	YFWSERLRRAFTFSSMYLGMSPYRAPAT YSLLQYTELAQGIWYPRGGFHR	2.0e-077	50	FAD /NAD binding domain
2.	IIGAGIGGLATAARLAKEGFDVTVVEKN DFSGGRCSLIEKDGFRFDRGPS	4.1e-066	50	FAD /NAD binding domain
3.	YRESFDEIFDGHGLPHDPSFYVNVPSRID PSAAPEGKDAIIVLVPIGHL	4.8e-065	49	FAD /NAD binding domain
4.	CDPNYVVHFDDKETVTLSSDMPKLKSEI ERFEGKDGWARFLKFMSEGQTH	1.2e-055	50	non-conserved 'helical' structure
5.	TIDRLYFVGASTHPGTGVPIVLAGSKLTA EKVLK	7.2e-046	34	Substrate binding domain
6.	FSDLIESESMNTPHTWEKDLNLFKGSILG LSHNIFQVJNFRPHTKH	7.2e-044	46	Substrate binding domain
7.	KATGVELENGERLEADVVISNADLVYTY SNLLPQTKYTKK	9.0e-035	40	FAD /NAD binding domain
8.	KLTCSSISFYWSIKRKIPSLVTHNIFLAE	7.0e-027	29	Unidentified
9.	DIPWDLTVRSNRPITVDRATDPSGRTTEL NKMSKPWLLDLKENNW	5.7e-015	45	Substrate binding domain
10.	YEVSIKEVLLKDYPTFWSILKLZLVRMAL KJHVFDKJHRRA	4.8e-013	41	Substrate binding domain
11.	YLMPEJFEDLFDDLGERVDDW	1.8e-011	21	Substrate binding domain
12.	NNLSKNWEKIISHAREFVLHTIENNILQP	9.3e-011	29	non-conserved 'helical' structure
13.	LPRSLEAITKKNGGQVLYSSPVKRIILS	6.3e-003	28	non-conserved 'helical' structure

The results of the three-dimensional predictive structure of the protein are shown in Figure 2. In the three-dimensional structure of the CrtI protein from B. *trispora*, the active site of the protein (indicated by red dots) shifts to the back (relative to the orientation of the display in Figure 2), this is due to the presence of amino acid sequences 528 to 582 with a function as a membrane binding domain. The 528 – 582 residues occupy the substrate binding domain of the CrtI protein, causing a shift in the substrate binding domain of the CrtI protein from B. *trispora* that is predicted to affect the decrease in energy affinity in the interaction process between protein and ligand. In addition, after juxtaposing the two protein models, a Root mean square deviation (RMSD) value of 6.93 was obtained, which indicated that the two protein structures were significantly different [31]. In terms of secondary structure, the CrtI protein of B. *tirspora* consists of 27.66% alpha-helix and 12.54% beta-sheet. This proportion is higher than that of P. triticina which has 25.5% alpha-helix and 8.11% beta-sheet. These differences of alpha-helix and beta-sheet proportion in protein secondary structure may affect the 3D dimensions of the protein especially in their hydrophobic pocket and the difference structure on hydrophobic pocket can affect interaction between the protein with the substrate [35].

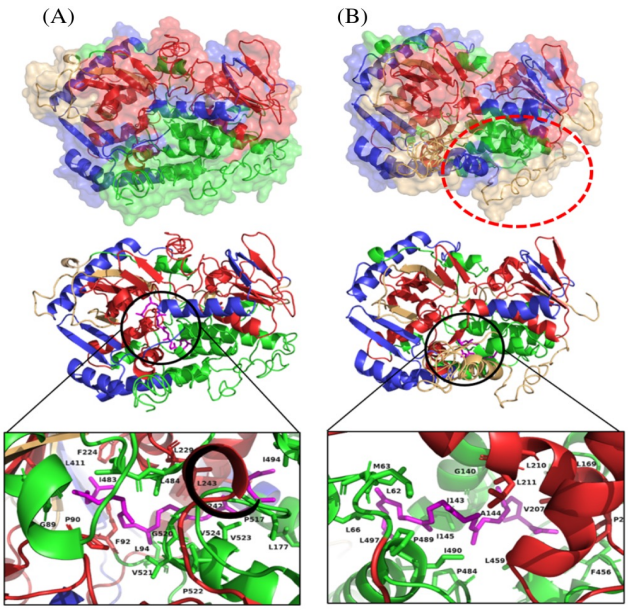

Figure 2. Three-dimensional predictive structure of the CrtI protein (a) P. *triticina* and (b) B. *trispora*. The red part indicates the FAD binding domain, green indicates the substrate binding, Part of the CrtI protein that interacting with the 15-cis-phytoene substrate (shown in pino) from (c) P. *triticina* and (d) B. *trispora*.

DOI: 10.5614/3bio.2023.5.2.1

The interaction of the CrtI protein from 4 species of β-carotene producing fungi on the 15-cis-phytoene substrate with the help of Autodock Vina-1.1.2 obtained the energy affinity values for every ligand pose are shown in table 3. P. *triticina* with the lowest affinity energy of -8.3 kcal mol-1 were found in both the first and second ligand poses, while the third pose had an affinity value of -8.2 kcal mol-1. Where the lower or negative energy affinity value indicates the interaction that occurs between the substrate and the protein occurs more spontaneously. In addition, the energy affinity value of the protein interaction of CrtI from P. *triticina* has a relatively consistent value for the three ligand poses, in contrast to B. *trispora* which has a relatively less consistent affinity value between each ligand pose (in table 3). According to statistical tests using the t-test, it was found that the interaction between CrtI from P. *triticina* is more spontaneous than the interaction between the substrate and CrtI protein from other fungi. In the pose of the CrtI protein ligand from *Puccinia* and *Blakeslea*, it was observed that there was a change in the molecular structure of the substrate which indicated a change in the initial substrate. Cuttriss et. al. [1] showed that the process 15-cis phytoene into the final product of all-trans lycopene with an indication of the formation of 15.9'-discis phytofluene as intermediate compounds.

Table 3. Energy affinity and ligand pose of docking results between CrtI protein and 15-cis-phytoen

Organism	Energy Affinity (kcal mol^{-1})	Ligand Pose
P. coronata	-7,4	
	-7,1	
	-6,9	
P. triticina	-8,3	
	-8,3	
	-8,2	
P. rhodozyma	-7.0	
	-6.6	
	-6.5	
B. trispora	-8,2	
	-8,0	
	-7,9	

After observing the interactions that occurred at a radius of 3Å from the substrate, it was observed that the CrtI protein from P. *triticina* contain 20 residues that interact with the substrate while in the CrtI from B. *trispora* there are 19 residues that interact with the substrate as shown in Figure 2 (in the black box). Amino acid residues that interact with substrates at radius 3Å form an instantaneous dipole or Van der Waals interactions that equivalent to 0.4 Kj mol-1 [36]. According to that assumption, it was estimated that the interaction between CrtI protein from P. *triticina* and 15-cis-phytoene has an interaction strength of 8.0 Kj mol-1 while CrtI from B. *trispora* has an interaction strength of 7.6 Kj mol-1. So, from the results of the docking analysis between the 15-cis phytoene substrate and the CrtI protein, the CrtI protein from P. triticina has a more spontaneous and stronger than the interactions that occur between the CrtI protein from other fungi such as B. *trispora* which is considered as a gold standard for producing essential carotenoids.

Thus, it is suggested that the CrtI protein from P. *triticina* has the potential for further development as bioparts to

DOI: 10.5614/3bio.2023.5.2.1

produce β-carotene. However, in its development as a part of complete synthetic biology system in β-carotenoid production CrtI still requires other bioparts such as CrtB, CrtE, and CrtY. Research on the biofortification of β-carotene in fungi is currently one of the latest and promising studies being developed. One example is the formation of a bioreactor from the fungus Y. lipolytica as a competent host cell that is inserted by genes from other fungi to produce β-carotene on an industrial scale [15].

4. Conclusion

In this study, it was found that CrtI protein from P. triticina has 2 unique motifs and one unique domain from other fungi which will affect the secondary structure and tertiary structure of the protein. Furthermore, docking analysis also suggested that interaction between 15-cis phytoene with CrtI protein from P. *triticina* requires less energy compared to with CrtI protein from other fungi species. Hence, it is proposed that CrtI protein from P. *triticina* has the potential to develop as bioparts to replace CrtI from B. *trispora*, which is currently considered the gold standard for producing carotenoids in fungi.

Acknowledgement

The authors are very grateful to the assistance of Mr. Putu Wirayoga Prakasa for the introduction to the bioinformatic tools.

References

[1.] Cuttriss, A. J., Cazzonelli, C. I., Wurtzel, E. T., & Pogson, B. J. 2011. Carotenoids. https://doi.org/10.1016/B978-0-12-386479-6.00005-6

[2.] Ellison, S. L. 2016. Carotenoids: Physiology. In Encyclopedia of Food and Health. Elsevier. https://doi.org/10.1016/B978-0-12-384947-2.00120-3

[3.] Gong, M., & Bassi, A. 2016. Carotenoids from microalgae: A review of recent developments. Biotechnology Advances, 34(8). https://doi.org/10.1016/j.biotechadv.2016.10.005

[4.] Singh, U., Praharaj, C. S., Chaturvedi, S. K., & Bohra, A. 2016. Biofortification: Introduction, Approaches, Limitations, and Challenges. In Biofortification of Food Crops. Springer India. https://doi.org/10.1007/978-81-322-2716-8_1

[5.] Sandmann, G. 2002.Combinatorial Biosynthesis of Carotenoids in a Heterologous Host: A Powerful Approach for the Biosynthesis of Novel Structures. ChemBioChem, 3(7), 629. https://doi.org/10.1002/1439-7633(20020703)

[6.] Paul, J.-Y., Khanna, H., Kleidon, J., Hoang, P., Geijskes, J., Daniells, J., Zaplin, E., Rosenberg, Y., James, A., Mlalazi, B., Deo, P., Arinaitwe, G., Namanya, P., Becker, D., Tindamanyire, J., Tushemereirwe, W., Harding, R., & Dale, J. (2017). Golden bananas in the field: elevated fruit pro-vitamin A from the expression of a single banana transgene. Plant Biotechnology Journal, 15(4). https://doi.org/10.1111/pbi.12650

[7.] Paine, J. A., Shipton, C. A., Chaggar, S., Howells, R. M., Kennedy, M. J., Vernon, G., Wright, S. Y., Hinchliffe, E., Adams, J. L., Silverstone, A. L., & Drake, R. (2005). Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nature Biotechnology, 23(4). https://doi.org/10.1038/nbt1082

[8.] Mishiba, K.-I., Nishida, K., Inoue, N., Fujiwara, T., Teranishi, S., Iwata, Y., Takeda, S., & Koizumi, N. 2020. Genetic engineering of eggplant accumulating β-carotene in fruit. Plant Cell Reports, 39(8). https://doi.org/10.1007/s00299-020-02546-8

[9.] Nisar, N., Li, L., Lu, S., Khin, N. C., & Pogson, B. J. 2015. Carotenoid Metabolism in Plants. Molecular Plant, 8(1), 68–82. https://doi.org/10.1016/j.molp.2014.12.007

[10.] Wang, L., Liu, Z., Jiang, H., & Mao, X. 2021. Biotechnology advances in β-carotene production by microorganisms. Trends in Food Science & Technology, 111. https://doi.org/10.1016/j.tifs.2021.02.077

[11.] Gao, S., Tong, Y., Zhu, L., Ge, M., Zhang, Y., Chen, D., Jiang, Y., & Yang, S. 2017. Iterative integration of multiple-copy pathway genes in Yarrowia lipolytica for heterologous β-carotene production. Metabolic Engineering, 41, 192–201. https://doi.org/10.1016/j.ymben.2017.04.004

[12.] Li, X.-R., Tian, G.-Q., Shen, H.-J., & Liu, J.-Z. 2015. Metabolic engineering of Escherichia coli to produce zeaxanthin. Journal of Industrial Microbiology and Biotechnology, 42(4). https://doi.org/10.1007/s10295-014-1565-6

[13.] Mlalazi, B., Welsch, R., Namanya, P., Khanna, H., Geijskes, R. J., Harrison, M. D., Harding, R., Dale, J. L., & Bateson, M. 2012. Isolation and functional characterisation of banana phytoene synthase genes as potential cisgenes. Planta, 236(5), 1585–1598. https://doi.org/10.1007/s00425-012-1717-8

[14.] Schaub, P., Yu, Q., Gemmecker, S., Poussin-Courmontagne, P., Mailliot, J., McEwen, A. G., Ghisla, S., Al-Babili, S., Cavarelli, J., & Beyer, P. 2012. On the Structure and Function of the Phytoene Desaturase CRTI from Pantoea ananatis, a Membrane-Peripheral and FAD-Dependent Oxidase/Isomerase. PLoS ONE, 7(6), e39550. https://doi.org/10.1371/journal.pone.0039550

[15.] Wang, E., Dong, C., Park, R. F., & Roberts, T. H. 2018. Carotenoid pigments in rust fungi: Extraction, separation, quantification and characterisation. Fungal Biology Reviews, 32(3), 166–180. https://doi.org/10.1016/j.fbr.2018.02.002

[16.] Papadaki, E., & Mantzouridou, F. T. 2021. Natural β-Carotene Production by Blakeslea trispora Cultivat-

 DOI: 10.5614/3bio.2023.5.2.1

ed in Spanish-Style Green Olive Processing Wastewaters. Foods, 10(2), 327. https://doi.org/10.3390/foods10020327

[17.] Sen, T., Barrow, C. J., & Deshmukh, S. K. 2019. Microbial Pigments in the Food Industry—Challenges and the Way Forward. Frontiers in Nutrition, 6. https://doi.org/10.3389/fnut.2019.00007

[18.] Garner K. L. 2021. Principles of synthetic biology. Essays Biochem. 2;65(5):791-811. https://doi.org/10.1042/EBC20200059

[19.] Larroude, M., Celinska, E., Back, A., Thomas, S., Nicaud, J.-M., & Ledesma-Amaro, R. 2018. A synthetic biology approach to transform Yarrowia lipolytica into a competitive biotechnological producer of β-carotene. Biotechnology and Bioengineering, 115(2), 464–472. https://doi.org/10.1002/bit.26473

[20.] Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Molecular Biology and Evolution, 35(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

[21.] Lu, S., Wang, J., Chitsaz, F., Derbyshire, M. K., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., Marchler, G. H., Song, J. S., Thanki, N., Yamashita, R. A., Yang, M., Zhang, D., Zheng, C., Lanczycki, C. J., & Marchler-Bauer, A. (2020). CDD/SPARCLE: the conserved domain database in 2020. Nucleic Acids Research, 48(D1), D265–D268. https://doi.org/10.1093/nar/gkz991

[22.] Liu, W., Xie, Y., Ma, J., Luo, X., Nie, P., Zuo, Z., Lahrmann, U., Zhao, Q., Zheng, Y., Zhao, Y., Xue, Y., & Ren, J. (2015). IBS: an illustrator for the presentation and visualization of biological sequences: Fig. 1. Bioinformatics, 31(20), 3359–3361. https://doi.org/10.1093/bioinformatics/btv362

[23.] Bailey, T. L., & Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proceedings. International Conference on Intelligent Systems for Molecular Biology, 2, 28–36.

[24.] Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., Nuka, G., Paysan-Lafosse, T., Qureshi, M., Raj, S., Richardson, L., Salazar, G. A., Williams, L., Bork, P., Bridge, A., Gough, J., Haft, D. H., Letunic, I., Marchler-Bauer, A., … Finn, R. D. 2021. The InterPro protein families and domains database: 20 years on. Nucleic Acids Research, 49(D1), D344–D354. https://doi.org/10.1093/nar/gkaa977

[25.] Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., & Zhang, Y. 2015. The I-TASSER Suite: protein structure and function prediction. Nature Methods, 12(1), 7–8. https://doi.org/10.1038/nmeth.3213

[26.] Berman, H.M., Henrick, K., Nakamura, H. (2003) Announcing the worldwide Protein Data Bank Nature Structural Biology 10 (12): 980.

[27.] Schrödinger, L., & DeLano, W. (2020). PyMOL. Retrieved from http://www.pymol.org/pymol

[28.] Colovos, C., & Yeates, T. O. 1993. Verification of protein structures: Patterns of nonbonded atomic interactions. Protein Science, 2(9), 1511–1519. https://doi.org/10.1002/pro.5560020916

[29.] Trott, O., & Olson, A. J. 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. Journal of Computational Chemistry, 31(2), 455–461. https://doi.org/10.1002/jcc.21334

[30.] Giannoukos G, Ciulla DM, Huang K, Haas BJ, Izard J, Levin JZ, Livny J, Earl AM, Gevers D, Ward DV, Nusbaum C, Birren BW, Gnirke A. Efficient and robust RNA-seq process for cultured bacteria and complex community transcriptomes. (2012). Genome Biol. 2012;13(3):R23. doi: 10.1186/gb-2012-13-3-r23

[31.] Miller, M. E., Zhang, Y., Omidvar, V., Sperschneider, J., Schwessinger, B., Raley, C., Palmer, J. M., Garnica, D., Upadhyaya, N., Rathjen, J., Taylor, J. M., Park, R. F., Dodds, P. N., Hirsch, C. D., Kianian, S. F., & Figueroa, M. 2018. De Novo Assembly and Phasing of Dikaryotic Genomes from Two Isolates of Puccinia coronata f. sp. avenae , the Causal Agent of Oat Crown Rust. MBio, 9(1). https://doi.org/10.1128/mBio.01650-17

[32.] Yan, Z., Wang, C., Lin, J., & Cai, J. 2013.Medium optimization using mathematical statistics for production of β-Carotene by Blakeslea trispora and fermenting process regulation. Food Science and Biotechnology, 22(6), 1667–1673. https://doi.org/10.1007/s10068-013-0265-8

[33.] Rodríguez-Sáiz, M., de la Fuente, J. L., & Barredo, J. L. 2010. Xanthophyllomyces dendrorhous for the industrial production of astaxanthin. Applied Microbiology and Biotechnology, 88(3), 645–658. https://doi.org/10.1007/s00253-010-2814-x

[34.] Guo, Z., Li, B., Cheng, L.-T., Zhou, S., McCammon, J. A., & Che, J. 2015. Identification of Protein–Ligand Binding Sites by the Level-Set Variational Implicit-Solvent Approach. Journal of Chemical Theory and Computation, 11(2), 753–765. https://doi.org/10.1021/ct500867u

[35.] Fujiwara, K., Toda, H. & Ikeguchi, M. 2012. Dependence of α-helical and β-sheet amino acid propensities on the overall protein fold type. BMC Struct Biol. 12 (18). https://doi.org/10.1186/1472-6807-12-18

[36.] Göltl, F., Grüneis, A., Bučko, T., & Hafner, J. 2012. Van der Waals interactions between hydrocarbon molecules and zeolites: Periodic calculations at different levels of theory, from density functional theory to the random phase approximation and Møller-Plesset perturbation theory. The Journal of Chemical Physics, 137(11), 114111. https://doi.org/10.1063/1.4750979

 DOI: 10.5614/3bio.2023.5.2.1