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Research Article
RuBisCO of Microalgae as Potential Targets for Nutraceutical Peptides: A Computational Study

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Abstract

Background and Objective: Microalgae are autotrophic organisms abundantly occur in the aquatic environment. Microalgal biomass is used as an economical precursor for biodiesel feedstock and therapeutic bioactive constituents. The RuBisCO is a souvenir in photosynthetic organisms. However, the information about the biomedical application of RuBisCO and its peptides is insufficient. Therefore, this study aimed to evaluate the therapeutic potential of RuBisCO and its peptides through the computational tools. **Materials and Methods:** Fourteen RuBisCO large subunit sequences of algae were retrieved from UniProt and assessed in the BIOPEP server. The biological activity, catalyst action and calculation of bioactive peptides tools were accustomed to verify the frequency of incidence of fragments, proteolytic cleavage and the incidence of elite enzymes with the specified action. The physiochemical parameters of the chosen sequences were performed with Protpram tool. **Results:** The outcome demonstrated *Chaetoceros calcitrans* exhibits the most effective prospect as a supply of DPP-IV inhibiting peptides, *Chlorella pyrenoidosa* for antihypertensive and *Aphanizomenon flos-aquae* for antioxidative, activating ubiquitin and anti-amnestic peptides. High range of bioactive peptides with biological activity in the RuBisCO sequences of *Aphanizomenon flos-aquae*, *Dunaliella salina*, *Chlorella pyrenoidosa* and *Chlorella vulgaris* related to a high content of glycine and proline. Papain and proteinase K, a catalyst with wide specificity release significant active fragments than bromelain and chymotrypsin. The RuBisCO in selected microalgae showed potential for bioactive peptides linked with an elevated level of glycine and proline that are most rich in biologically active peptides. **Conclusion:** Further, experimental studies support the utilization of microalgae RuBisCO as a conventional economical source of bioactive peptides for a human.

Key words: BIOPEP server, ProtParam, RuBisCO, neutraceutical peptides, DPPIV inhibition action

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase/oxygense (RuBisCO) is heterohexadecamer structured protein (L8S8, 540 kDa) made up of eight large subunits and present in land biota including plants and autotrophic organisms like microalgae[1]. It is actively involved in conversion of atmospheric carbon dioxide into organic carbon through calvin cycle[2]. Both large and small subunits of RuBisCO possess functionally bioactive peptides for treatment of cardiovascular diseases, diabetes, neurodegenerative disorders and oxidative stress[3]. Large subunit of RuBisCO is homologically diverse in many photosynthetic organisms like cyanophyta, chemolitho-autotrophs and phototropic bacteria[4]. Especially, the carboxylase enzyme is actively involved in atmospheric carbon accumulation and the oxygenase responsible for biosphere fixed carbon loss by photo-respiratory pathway. However, the ratio of catalytic reaction of carboxylation and oxygenation depend on the nature of the species. The activation of RuBisCO protein maintained by RuBisCO activase a soluble protein in chloroplast[5]. In particular, bacillariophyceae, chloropyceae, cryptophyceae, dinophyceae, euglenophyceae and rhodophyceae exhibited RuBisCO localization in pyrenoid complex. It is containing species viz., *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa*, *Coleochate scutata*, *Dunaliella salina* and *Phorphyridium cruentum*[6,7]. However, in non-pyrenoid organisms, RuBisCO is distributed throughout the chloroplast region[8]. Under nitrogen in deprived condition there was decrease in the RuBisCO content of green microalgae *Chlorella emersonii* and *Gloeomonas* sp.[9].

Bioactive peptides contain 5-20 amino acid residues, which are active at intestinal sites and inactive within the parent protein sequences. Proteins are liberated during physiological digestion, by endogenous and exogenous proteases and peptidases[10]. Recent, studies reported the unicellular and multi-cellular marine algae for bioactive peptides production due to their considerable amount of protein content ~15-47% of dry weight[11]. Notably, the algal biomass possesses a large number of essential amino acids and non-protein coding amino acid residues. Therefore, efforts have been made to isolate definite protein without any change in their molecular structure to enhance their application in pharma and fermentation process[12]. Nevertheless, most microalgae posses rigid cell wall which un-facilitate the isolation of intracellular proteins. Therefore, various physical and chemical methods have been followed such as alkali agent mediated cell dissolution, extraction using various organic solvents, high-pressure homogenization etc. However, there are still many challenges raised over the application of bioactive peptides before getting into clinical trials. Elaborative computational modeling is required to explore structure and functional relationships of peptide fragments form microalgae. Therefore, the present study aimed to identify bioactive peptides from large subunit of RuBisCO protein of *Chaetoceros calcitrans* (Paulsen) Takano, *Chlamydomonas rheinhardtii* P.A. Dang., *Chlorella pyrenoidosa* H. Chick, *Chlorella vulgaris* Beyerinck (Beijerinck), *Dunaliella salina* (Dunal) Teodoresco, *Euglena gracilis* Klebs, *Haematococcus pluvialis* Flotow, *Isochrysis galbana* Parke, *Porphyridium cruentum* (S.F.Gray) Nägeli, *Spirogyra porticalis* (O.F.Müller) Dumortier, *Spirulina maxima* (Setchell and N.L.Gardner) Geitler, *Spirulina platensis* (Gomont) Geitler, *Synechococcus* sp. Nägeli and *Aphanizomenon flos-aquae* Ralfs ex Bornet and Flahault through computational methods. Exclusively, multiple sequence analyses of RuBisCO of selected microalgae were completed. In addition, *in silico* proteolysis of elite proteins was performed. Bioactive peptides within RuBisCO sequences were contrasted with available reports and the fragments ranked accordingly.

MATERIALS AND METHODS

Tools and web servers: UniProt databases[13], ExPASy portal (http://www.expasy.org/proteomics)[14], BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep)[15], ProtParam (http://web.expasy.org/protparam/)[16] and algal database (http://www.algaebase.org/)[17,18] tools were used in the study during March, 2016 in Istanbul Medeniyet University, Turkey.

Sequence information: The primary sequences of RuBisCO protein of *C. calcitrans* (K4ES21), *C. rheinhardtii* (P00877), *C. pyrenoidosa* (A8TKR2), *C. vulgaris* (P12466), *D. salina* (D0FXZ7), *E. gracilis* (P00878), *H. pluvialis* (B7U6F7), *I. galbana* (Q9GFX7), *P. cruentum* (W0RYV8), *S. porticalis* (Q85X88) *S. maxima* (B5VXI0), *S. platensis* (D4ZVW7), *Synechococcus* sp. (P00880), A. flos-aquae (Q934E6) and for reference *O. sativa* (P0C510), *T. aestivum* (P08823), *Z. mays* (P00874), *G. max* (P13917), *G. gallus* (P01012) and *B. taurus* (P02666) were obtained from UniProt database and used for further analysis. Multiple sequence alignments were performed with ClustalW2 (http://www.clustal.org/clustal2/)[19]. The homology of sequence consequences was attained from UniProtKB/Swiss-Prot. The phylogenetic tree was constructed by using ClustalW2.

Determination of frequency analysis of bioactive peptides: Bioactivity possible of microalgal and cyanobacterial RuBisCO calculated by the frequency of occurrence of bioactive peptide (A) using the BIOPEP server. BIOPEP demonstrated the quantity of cryptic bioactive peptides in the protein sequences. A = a/N, where, "a" indicated the quantity of bioactive peptides and N is the total quantity of amino acid residues within a protein sequence[3,20]. Their features as ACE inhibitor, ubiquitin activation, antiamnestic, antioxidative and antithrombotic and DPP-IV inhibitor determined bioactive peptide fragments of each protein. Bioactivity, values of A for different microalgae RuBisCO proteins were compared to values for commonly consumed food protein sources of *Oryza sativa*, *Triticum aestivum*, *Zea mays*, *Glycine max*, *Gallus gallus* and *Bos taurus*.

Analysis of proteolysis and amino acid composition: The proteolysis of RuBisCO protein of selected microalgae species was conducted by using the BIOPEP web server on enzyme action tool[3,21]. The server contain more than fifteen proteolytic enzymes. Food-processing enzymes such as bromelain, chymotrypsin, Proteinase K and papain enzymes selected based on their unique cleavage patterns. Two parameters were determined: (1) The frequency of discharge of bioactive peptides with selected activity by specific enzyme (A_E), which was calculated as follows $A_E = d/N$, where, d is the quantity of peptides with specified activity, that could be discharged by enzyme and N is the quantity of amino acid residues in the protein chain and (2) The relative frequency of discharge of bioactive peptides with specified activity by the selected enzyme (W), which was calculated as follows $W = A_E/A$, where, A_E is the frequency of discharged bioactive peptides with specified activity by specific enzyme and A is the frequency of occurrence of bioactive peptides in the protein sequence. Amino acid composition of proteins was determined by using the ProtParam program based on the RuBisCO sequence of selected microalgae and cyanobacteria[16].

RESULTS

The RuBisCO subunits are estimated rich in microalgae and cyanobacteria and not frequently consumed as primary human food. Cultivation of microalgae in developing countries is a considerable policy to support rural economies. The increasing growth of underutilized algal biomass that can readily be used in human food system as a source of RuBisCO. Sequence alignment of the microalgal RuBisCO subunits in UniProt indicated that they have highly homologous regions in their sequences with 90-100% similarity among the selected algal species. However, *A. flos-aquae* protein has less conserved regions in its sequences with 6.57% similarity among the selected algae. This difference can direct to prominent variations in their respective peptide profiles depending on location of occurrence. Results in Fig. 1 represented multiple sequence alignment of microalgal RuBisCO subunit. The genetic relationship of microalgae and cereals compared through phylogentic tree construction. The result indicated the similarity of microalgae and cereals according to their sequence homology (Fig. 2).

Different types of bioactive peptides in microalgae and cyanobacterial RuBisCO with more than 3000 bioactive peptides were found in BIOPEP. The study was completed in February, 2016. Data in Table 1 shown the frequency of occurrence of the bioactive peptides within microalgae and cyanobacterial RuBisCO subunits for the ACE inhibitor, activating ubiquitin, antiamnestic, antioxidative and antithrombotic and DPP IV inhibitory activities. Most bioactive peptides in the RuBisCO subunits were defined as DPP-IV inhibitors and had DPP-IV inhibiting value of A which was similar to that of cereal plants but lower than bovine milk protein, which has the highest value of A compared to other proteins of microalgae and cereal plants. In addition, the frequencies of occurrence of DPP-IV inhibiting peptides in the microalgae and cyanobacteria were predominantly same, which was unusual considering their highly homologous primary structures. Conversely, RuBisCO subunits had remarkable variations in their values of A with *C. calcitrans* exhibiting the best prospects as sources of DPP-IV inhibiting peptides (Appendix S1) and *Chlorella pyrenoidosa* for ACE inhibitor (Appendix S2) whereas, *A. flos-aquae* showed the lowest value of A, while it is comparable with A values for other microalgae and cereal plant proteins. The RuBisCO sequences of *A. flos-aquae* showed highest A value on antioxidative, activating ubiquitin and antiamnestic frequencies of peptide fragments when compared to other microalgae and cereal crops. The antioxidant peptide sequence of *A. flos-aquae* was shown in Appendix S3.

Data in Table 2 and 3 demonstrated the values of factor describing the predicted efficiency of release of DPP-IV inhibitor and ACE inhibitor peptide fragments from microalgal and cyanobacterial RuBisCO proteins. These two activities were selected as models since fragments showing these activity peptides were most rich in the sequences of all proteins. Mapping of *Aphanizomenon flos-aquae* RuBisCO showed the presence of more than 20 bioactive peptide fragments in its sequences stimulated by the selective proteolytic enzymes (Fig. 3).

```
K4ES21|Chaetoceros      SSTATWTVVWTDLLTACERYRAKAYRVDPVPNSTDSYFAFIAYECDLFEEGSLANLTASI
D0FXZ7|Dunaliella       SSTGTWTTVWTDGLTSLDKYKGRCYDLEPVPGEENQYIAYVAYPIDLFEEGSVTNLFTSI
A8TKR2|Chlorella        SSTGTWTTVWTDGLTSLDRYKGRCYDIEPVPGEENQYIAYIAYPLDLFEEGSVTNLFTSI
P12466|Chlorella        SSTGTWTTVWTDGLTSLDRYKGRCYDIEPVPGEENQYIAYIAYPLDLFEEGSVTNLFTSI
                        ***.***.**** **: ::*:.:.* ::***.. :.*:*::**  *******::** :**

K4ES21|Chaetoceros      IGNVFGFKAVAALRLEDMRIPHSYLKTFQGPATGIVVERERLNKYGTPLLGATVKPKLGL
D0FXZ7|Dunaliella       VGNVFGFKALRALRLEDLRISPAYVKTFVGPPHGIQVERDKLNKYGRGLLGCTIKPKLGL
A8TKR2|Chlorella        VDNVFGFKALRALRLEDLRIPPAYVKTFQGPPHGIQVERDKLNKYGRGLLGCTIKPKLGL
P12466|Chlorella        VGNVFGFKALRALRLEDLRIPPAYVKTFQGPPHGIQVERDKLNKYGRGLLGCTIKPKLGL
                        :.*******: ******:**  :*:*** **  ** ***::*****  ***.*:******

K4ES21|Chaetoceros      SGKNYGRVVYEGLKGGLDFLKDDENINSQPFMRWRERFLYCMEGINRASAATGEVKGSYL
D0FXZ7|Dunaliella       SAKNYGRAVYECLRGGLDFTKDDENVNSQPFMRWRDRFLFVAEAIYKAQAETGEIKGHYL
A8TKR2|Chlorella        SAKNYGRAVYECLRGGLDFTKDDENVNSQPFMRWRDRFLFVAEAIYKSQSETGEIKGHYL
P12466|Chlorella        SAKNYGRAVYECLRGGLDFTKDDENVNSQPFMRWRDRFLFVAEAIYKSQAETGEIKGHYL
                        *.*****.*** *:***** *****:*********:***:  *.* ::.: ***:** **

K4ES21|Chaetoceros      NVTAATMEEVYKRSEYAKAVGSVIIMIDLV-MGYTAIQSIAYWARENDMLLHLHRAGNST
D0FXZ7|Dunaliella       NATAGTAEGMLQRAQCAKELGVPIIMHDYLTGGFTANTSLAHYCRDHGLLLHIHRAMHAV
A8TKR2|Chlorella        NATAATAEEMLKRAECAKDLGVPIIMHDYLTGGFTANTSLAHYCRDNGLLLRIHRAMHAV
P12466|Chlorella        NATAATAEAMMQRAECAKDLGVPIIMHDYLTGGFTANTSLSHYCRDNGLLLHIHRAMHAV
                        *.**.* * : :*:: ** :*  *** * :  *:**  *::::.*::.:**::*** ::.

K4ES21|Chaetoceros      YARQKNHGINFRVICKWMRMSGVDHIHAGTVVGKLEGDPLMIKGFYDVLRLTTLDVNLPY
D0FXZ7|Dunaliella       IDRQRNHGIHFRVLAKTLRMSGGDHLHSGTVVGKLEGEREVTLGFVDLMRDNFVEKDRSR
A8TKR2|Chlorella        IDRQRNHGIHFRVLAKALRLSGGDHLHSGTVVGKLEGEREVTLGFVDLMRDDYVEKDRSR
P12466|Chlorella        IDRQRNHGITFRVLAKALRLSGGDHLHSGTVVGKLEGEREVTLGFVDLMRDDYIEKDRSR
                          **:**** ***:.* :*:** **:*:*********:  :  ** *::*   :: :

K4ES21|Chaetoceros      GIFFEMDWASLRKCMPVASGGIHCGQMHQLIYYLGDDVVLQFGGGTIGHPDGIQAGATAN
D0FXZ7|Dunaliella       GIYFTQDWCSMPGVMPVASGGIHVWHMPALVEIFGDDACLQFGGGTLGHPWGNAPGAVAN
A8TKR2|Chlorella        GIYFTQDWVSLPGTMPVASGGIHVWHMPALVEIFGDDACLQFGGGTLGHPWGNAPGAAAN
P12466|Chlorella        GIYFTQDWVSLPGTMPVASGGIHVWHMPALVEIFGDDACLQFGGGTLGHPWGNAPGAAAN
                        **:*  ** *:   *********  :*  *:  :***. *******:*** *   **.**
```

Fig. 1: Multiple sequence alignment between RuBisCO of *Chaetoceros calcitrans*, *Chlorella pyrenoidosa*, *Dunaliella salina* and *Chlorella vulgaris*

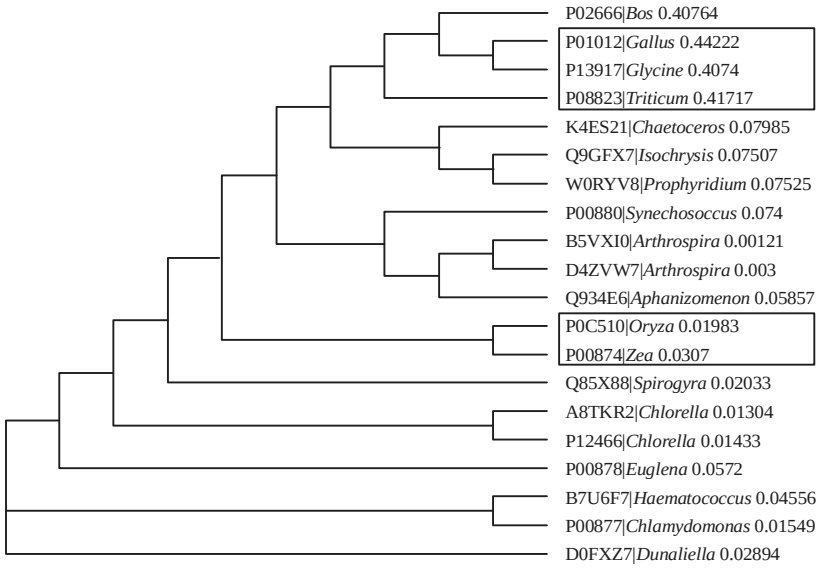

Fig. 2: Phylogenetic tree showing the genetic relationship among microalgae and cyanobacteria RuBisCO and cereal crops RuBisCO *Triticum aestivum* (P08823), *Oryza sativa* (P0C510), *Zea mays* (P00874), *Glycine max* (P13917) and cattles *Gallus gallus* (P01012), *Bos taurus* (P02666)

```
          10          20          30          40
IFGDDSVLQF GGGTLGHPWG NAPGATANRV ALEAVVQARN
          50          60          70          80
EGRNLAREGN DIIREAAKWS PELAVACELW KEIKFEFEAM DTV
```

Ile Phe Gly Asp Asp Ser Val Leu Gln Phe

Gly Gly Gly Thr Leu Gly His Pro Trp Gly

Asn **Ala Pro Gly** Ala Thr **Ala Asn** Arg Val

Ala Leu Glu Ala Val Val Gln Ala **Arg Asn**

Glu Gly Arg Asn Leu Ala **Arg Glu** Gly Asn

Asp Ile Ile Arg Glu Ala Ala **Lys Trp Ser**

Pro Glu Leu **Ala Val Ala Cys Glu Leu** Trp

Lys Glu Ile **Lys Phe Glu Phe** Glu Ala Met

Asp Thr Val

Fig. 3: Mapping of *Aphanizomenon flos-aquae* RuBisCO showed the presence of bioactive peptide fragments in its sequences stimulated by the selective proteolytic enzymes

Appendix S1: DPP-IV inhibitory peptides in the RuBisCO protein of *Chaetoceros calcitrans*

Peptide ID sequence, number of peptide, Sequences location
3169 GP 3 [162-163], [462-463], [476-477]
3171 MP 1 [392-393]
3172 VA 4 [65-66], [140-141], [394-395], [441-442]
3173 MA 1 [448-449]
3174 KA 3 [91-92], [138-139], [272-273]
3175 LA 2 [45-46], [123-124]
3176 FA 1 [108-109]
3179 PA 1 [163-164]
3180 LP 1 [375-376]
3181 VP 1 [100-101]
3182 LL 3 [81-82], [181-182], [305-306]
3183 VV 5 [76-77], [168-169], [200-201], [348-349], [417-418]
3184 HA 2 [10-11], [344-345]
8498 GQ 1 [402-403]
8501 IP 1 [150-151]
8503 TP 3 [51-52], [179-180], [501-502]
8519 KP 1 [187-188]
8520 HP 1 [428-429]
8522 GPA 1 [162-164]
8524 GA 3 [183-184], [435-436], [453-454]
8525 IA 2 [111-112], [295-296]
8526 RA 4 [27-28], [89-90], [241-242], [310-311]
8528 WA 2 [298-299], [385-386]
8530 NP 1 [458-459]
8531 TA 8 [71-72], [83-84], [127-128], [257-258], [290-291], [437-438], [480-481], [497-498]
8532 QP 2 [53-54], [223-224]
8555 FL 2 [211-212], [232-233]
8557 HL 1 [307-308]
8559 AL 5 [22-23], [46-47], [142-143], [442-443], [481-482]
8560 SL 2 [122-123], [387-388]
8561 GL 3 [191-192], [204-205], [208-209]
8595 WRE 1 [228-230]
8637 AA 8 [35-36], [61-62], [62-63], [63-64], [141-142], [244-245], [258-259], [471-472]
8638 PL 3 [180-181], [356-357], [477-478]
8675 WR 1 [228-229]
8676 WK 1 [485-486]
8682 WM 1 [332-333]

Appendix S1: Continue

Supplementary files

8685 WT 2 [74-75], [78-79]
8688 LW 1 [484-485]
8696 YT 3 [37-38], [289-290], [492-493]
8757 AD 2 [454-455], [498-499]
8758 AE 1 [11-12]
8759 AF 1 [109-110]
8760 AG 4 [66-67], [311-312], [345-346], [434-435]
8762 AS 4 [128-129], [242-243], [386-387], [395-396]
8763 AT 7 [72-73], [164-165], [184-185], [245-246], [259-260], [436-437], [504-505]
8764 AV 3 [64-65], [139-140], [273-274]
8765 AY 4 [36-37], [92-93], [112-113], [296-297]
8767 DP 3 [57-58], [98-99], [355-356]
8770 EG 5 [120-121], [203-204], [237-238], [353-354], [452-453]
8773 ES 2 [4-5], [68-69]
8774 ET 1 [12-13]
8775 EV 2 [248-249], [263-264]
8777 EY 1 [269-270]
8778 FN 2 [457-458], [490-491]
8779 FQ 1 [160-161]
8780 FR 2 [48-49], [326-327]
8781 GE 2 [67-68], [247-248]
8782 GF 2 [136-137], [361-362]
8783 GG 4 [207-208], [397-398], [422-423], [423-424]
8784 GH 1 [427-428]
8785 GI 6 [166-167], [238-239], [323-324], [378-379], [398-399], [431-432]
8786 GV 2 [55-56], [337-338]
8788 GY 1 [288-289]
8793 HI 1 [340-341]
8794 HR 1 [309-310]
8795 HS 1 [152-153]
8800 IH 1 [399-400]
8801 II 2 [130-131], [278-279]
8804 IN 3 [220-221], [239-240], [324-325]
8805 IQ 2 [292-293], [432-433]
8810 KG 3 [206-207], [250-251], [360-361]
8814 KR 1 [266-267]
8815 KS 1 [473-474]
8816 KT 1 [158-159]
8818 KW 1 [331-332]
8819 KY 1 [176-177]
8820 LH 2 [306-307], [308-309]
8821 LI 1 [409-410]
8822 LM 1 [357-358]
8823 LN 2 [174-175], [254-255]
8824 LT 3 [82-83], [126-127], [368-369]
8825 LV 1 [285-286]
8826 ME 2 [236-237], [261-262]
8828 MG 1 [287-288]
8829 MH 1 [406-407]
8830 MI 2 [282-283], [358-359]
8832 ML 1 [304-305]
8836 MR 3 [148-149], [226-227], [333-334]
8837 MV 1 [446-447]
8840 ND 1 [302-303]
8841 NE 1 [451-452]
8842 NF 1 [325-326]
8844 NH 1 [321-322]
8845 NL 2 [125-126], [374-375]
8849 NR 2 [240-241], [439-440]
8851 NV 2 [133-134], [255-256]
8853 NY 2 [196-197], [491-492]

Appendix S1: Continue

8854 PF 1 [224-225]
8855 PG 1 [54-55]
8856 PH 1 [151-152]
8858 PK 1 [188-189]
8860 PN 1 [101-102]
8861 PQ 2 [52-53], [459-460]
8862 PS 1 [502-503]
8864 PV 4 [58-59], [99-100], [393-394], [463-464]
8866 PY 1 [376-377]
8867 QA 1 [433-434]
8870 QF 1 [420-421]
8871 QG 1 [161-162]
8874 QL 1 [408-409]
8877 QS 1 [293-294]
8878 QT 1 [479-480]
8879 QV 1 [460-461]
8884 RI 2 [49-50], [149-150]
8885 RK 1 [389-390]
8886 RL 3 [144-145], [173-174], [367-368]
8887 RM 1 [334-335]
8888 RN 1 [450-451]
8890 RW 1 [227-228]
8891 SF 1 [489-490]
8893 SI 2 [129-130], [294-295]
8895 SV 1 [276-277]
8897 SY 3 [106-107], [153-154], [252-253]
8898 TD 4 [42-43], [79-80], [104-105], [495-496]
8900 TF 1 [159-160]
8901 TG 2 [165-166], [246-247]
8903 TI 1 [425-426]
8905 TL 1 [370-371]
8906 TM 1 [260-261]
8909 TR 1 [26-27]
8910 TS 2 [493-494], [505-506]
8911 TT 1 [369-370]
8912 TV 4 [38-39], [75-76], [185-186], [347-348]
8913 TW 1 [73-74]
8914 TY 1 [315-316]
8915 VD 3 [56-57], [97-98], [338-339]
8916 VE 2 [59-60], [169-170]
8917 VF 1 [134-135]
8918 VG 3 [274-275], [349-350], [461-462]
8920 VI 3 [277-278], [328-329], [464-465]
8921 VK 3 [39-40], [186-187], [249-250]
8922 VL 3 [44-45], [365-366], [418-419]
8923 VM 2 [286-287], [447-448]
8924 VN 1 [373-374]
8927 VT 1 [256-257]
8928 VW 1 [77-78]
8929 VY 2 [201-202], [264-265]
8932 YA 2 [270-271], [316-317]
8933 YD 1 [363-364]
8934 YE 2 [113-114], [202-203]
8935 YF 2 [107-108], [456-457]
8936 YG 3 [177-178], [197-198], [377-378]
8939 YK 1 [265-266]
8940 YL 3 [154-155], [253-254], [412-413]
8944 YR 1 [88-89]
8947 YW 1 [297-298]
8948 YY 1 [411-412]

Appendix S2: ACE inhibitor peptides in the RuBisCO protein of *Chlorella pyrenoidosa*

Peptide ID sequence, number of peptide, sequences location
3257 RL 3 [53-54], [170-171], [361-362]
3258 IR 1 [491-492]
3349 IKP 1 [210-212]
3380 RY 1 [113-114]
3383 IY 2 [263-264], [406-407]
3384 VF 1 [160-161]
3386 KW 1 [496-497]
3486 VW 3 [103-104], [428-429], [507-508]
3489 RF 1 [255-256]
3492 VY 1 [227-228]
3494 HY 2 [276-277], [322-323]
3518 VAA 1 [89-91]
3522 IPP 1 [176-178]
3524 VPP 1 [80-82]
3539 LAA 2 [69-71], [501-503]
3547 IRA 1 [491-493]
3550 YL 2 [277-278], [309-310]
3551 LF 3 [141-142], [150-151], [257-258]
3553 YG 2 [201-202], [223-224]
3563 AY 3 [133-134], [136-137], [179-180]
3666 YP 1 [137-138]
7490 GTW 1 [98-100]
7511 LPG 1 [415-417]
7512 GP 1 [186-187]
7513 PL 1 [138-139]
7556 YPLDL 1 [137-141]
7557 YPLDLF 1 [137-142]
7558 VK 2 [49-50], [181-182]
7562 IA 2 [132-133], [135-136]
7580 RW 1 [251-252]
7581 IP 1 [176-177]
7583 AF 1 [71-72]
7584 AP 2 [34-35], [458-459]
7585 LA 6 [16-17], [69-70], [320-321], [356-357], [483-484], [501-502]
7586 KR 2 [5-6], [292-293]
7587 VP 3 [80-81], [124-125], [302-303]
7588 RA 6 [40-41], [167-168], [225-226], [293-294], [335-336], [492-493]
7590 AA 9 [70-71], [87-88], [90-91], [284-285], [461-462], [462-463], [493-494], [502-503], [503-504]
7591 GF 4 [44-45], [162-163], [313-314], [386-387]

Supplementary files

7592 FR 2 [72-73], [353-354]
7593 IF 1 [437-438]
7594 VG 1 [374-375]
7596 GI 4 [190-191], [350-351], [405-406], [425-426]
7598 GA 3 [42-43], [86-87], [460-461]
7599 GL 5 [107-108], [204-205], [215-216], [234-235], [328-329]
7600 AG 4 [41-42], [43-44], [47-48], [85-86]
7601 GH 2 [275-276], [452-453]
7602 HL 2 [10-11], [367-368]
7603 GR 4 [116-117], [202-203], [224-225], [480-481]
7604 KG 2 [115-116], [274-275]
7605 FG 3 [161-162], [438-439], [446-447]
7606 DA 1 [441-442]
7607 GS 1 [145-146]
7608 GV 3 [48-49], [79-80], [301-302]
7611 GK 1 [375-376]
7612 GT 4 [98-99], [371-372], [417-418], [449-450]
7613 WG 1 [455-456]
7614 HG 2 [189-190], [349-350]

Appendix S2: Continue

7615 GE 3 [126-127], [271-272], [379-380]
7616 GG 7 [233-234], [312-313], [364-365], [424-425], [447-448], [448-449], [487-488]
7617 QG 1 [185-186]
7618 SG 3 [363-364], [370-371], [423-424]
7619 LG 5 [206-207], [214-215], [300-301], [385-386], [451-452]
7620 GD 3 [365-366], [439-440], [488-489]
7621 TG 3 [97-98], [270-271], [311-312]
7622 EG 4 [144-145], [378-379], [479-480], [486-487]
7623 EA 3 [84-85], [261-262], [471-472]
7624 NG 1 [327-328]
7625 PG 4 [78-79], [125-126], [416-417], [459-460]
7639 IFG 1 [437-439]
7656 IYK 1 [263-265]
7681 DG 1 [106-107]
7682 NY 1 [222-223]
7691 KY 1 [200-201]
7692 KF 1 [512-513]
7693 KL 3 [197-198], [213-214], [376-377]
7696 AIYK 1 [262-265]
7697 YK 2 [114-115], [264-265]
7698 NK 1 [199-200]
7742 AR 2 [476-477], [484-485]
7743 KA 3 [46-47], [164-165], [358-359]
7746 LVE 1 [434-436]
7810 KP 1 [211-212]
7820 GPP 1 [186-188]
7826 EI 3 [272-273], [436-437], [510-511]
7827 IE 1 [121-122]
7828 EV 2 [382-383], [506-507]
7829 VE 3 [193-194], [396-397], [435-436]
7830 TE 1 [37-38]
7831 LQ 1 [444-445]
7832 LN 1 [198-199]
7834 TQ 2 [409-410], [474-475]
7835 AH 1 [321-322]
7836 PP 3 [81-82], [177-178], [187-188]
7837 PQ 2 [35-36], [76-77]
7840 EK 1 [397-398]
7841 KE 1 [509-510]
7842 HP 1 [453-454]
7843 PH 1 [188-189]
7859 IEP 1 [121-123]
8185 TF 1 [183-184]
8193 AI 1 [262-263]
8951 AV 3 [88-89], [226-227], [339-340]

Appendix S3: Antioxidative peptides in the RuBisCO protein of *Aphanizomenon flos-aquae*

Peptide ID sequence, number of peptide, sequences location
7886 AH 1 [43-44]
7888 EL 2 [114-115], [120-121]
8012 LWK 1 [121-123]
8022 PHA 1 [10-12]
8042 PWG 1 [68-70]
8139 PEL 1 [113-115]
8190 PW 1 [68-69]
8215 IR 1 [103-104]
8462 LW 1 [121-122]

Supplementary files

Table 1: Frequency of occurrence (A) of bioactive peptide fragments in microalgae and cyanobacterial RuBisCO protein

Name of the species	ACE inhibitor	Ubiquitin-med.prot.	Antiamnestic	Antioxidative	Antithrombotic	Dipeptidyl peptidase IV inhibitor	Hypotensive	Immuno-modulating	Immuno-stimulating	Neuropeptide inhibitor
Chaetoceros calcitrans	0.3909	0.0151	0.0086	0.0756	0.0086	0.6523	***	0.0065	***	0.0065
Chlamydomonas rheinhardii	0.4403	0.0189	0.0084	0.0776	0.0084	0.6247	0.0063	0.0042	0.0021	0.0042
Chlorella pyrenoidosa	0.4423	0.0231	0.0105	0.0776	0.0105	0.6394	0.0063	0.0042	***	0.0042
Chlorella vulgaris	0.4382	0.0189	0.0105	0.0776	0.0105	0.6289	0.0063	0.0042	***	0.0042
Dunaliella salina	0.4214	0.0189	0.0105	0.0776	0.0105	0.6268	0.0063	0.0042	0.0021	0.0042
Euglena gracilis	0.4319	0.0168	0.0084	0.0797	0.0084	0.6415	0.0063	0.0042	***	0.0042
Haematococcus pluvialis	0.4163	0.0209	0.0105	0.0795	0.0105	0.6423	0.0021	0.0042	0.0021	0.0042
Isochrysis galbana	0.3666	0.0174	0.0108	0.0694	0.0108	0.6269	0.0022	0.0022	0.0043	0.0022
Porphyridium cruentum	0.3694	0.0143	0.0082	0.0633	0.0082	0.6449	0.002	0.002	0.0041	0.002
Spirogyra sp.	0.4349	0.0177	0.0088	0.0684	0.0088	0.6291	***	0.0044	***	0.0044
Spirulina maxima	0.4111	0.0167	0.0105	0.0753	0.0105	0.6234	0.0084	0.0042	0.0021	0.0042
Spirulina platensis	0.4121	0.0167	0.0105	0.0753	0.0105	0.6213	0.0084	0.0042	0.0021	0.0042
Synechococcus sp.	0.4114	0.0169	0.0084	0.0738	0.0084	0.6203	0.0105	0.0042	***	0.0021
Aphanizomenon flos-aquae	0.4118	0.0235	0.0118	0.0941	0.0118	0.5529	0.0353	***	***	***
Oryza sativa	0.4322	0.0209	0.0084	0.0711	0.0084	0.6211	0.0063	0.0042	***	0.0042
Triticum aestivum	0.3891	0.0147	0.0055	0.0367	0.0092	0.6477	0.0037	0.0018	0.0055	0.0018
Zea mays	0.4331	0.0167	0.0084	0.0607	0.0084	0.6131	0.0021	0.0042	***	0.0042
Glycine max	0.3521	0.0186	0.0093	0.0443	0.0093	0.6551	0.0023	0.0023	0.0023	***
Gallus gallus	0.3582	0.0077	0.0026	0.0696	0.0026	0.6134	0.0077	***	***	0.0077
Bos taurus	0.6018	0.0133	0.0442	0.0841	0.0265	0.8053	0.0044	0.0177	0.0044	0.0133

***Indicates there is no database information, ACE: Angiotensin-converting-enzyme, RuBisCO: Ribulose-1,5-bisphosphate carboxylase

Table 2: Bioactive fragments from RuBisCO protein for ACE inhibitory action

	Bromelain		Proteinase K		Chymotrypsin		Papain	
Name of the species	A_E	W	A_E	W	A_E	W	A_E	W
Chaetoceros calcitrans	0.0252	0.0679	0.0377	0.1016	0.0314	0.0846	0.0314	0.0846
Chlamydomonas reinhardtii	0.0304	0.0731	0.0365	0.0828	0.0345	0.0830	0.0426	0.1025
Chlorella pyrenoidosa	0.0325	0.0774	0.0346	0.0821	0.0365	0.0869	0.0385	0.0917
Chlorella vulgaris	0.0305	0.0731	0.0367	0.0879	0.0345	0.0830	0.0385	0.0926
Dunaliella salina	0.0326	0.0817	0.0346	0.0867	0.0346	0.0867	0.0387	0.0969
Euglena gracilis	0.0325	0.0793	0.0326	0.0792	0.0401	0.0989	0.0448	0.1089
Haematococcus pluvialis	0.0283	0.0721	0.0283	0.0721	0.0305	0.0774	0.0407	0.1032
Isochrysis galbana	0.0294	0.0840	0.0335	0.0957	0.0252	0.0720	0.0294	0.0840
Phorphyridium cruentum	0.0256	0.0739	0.0374	0.1079	0.0257	0.0739	0.0296	0.0851
Spirogyra sp.	0.0299	0.0734	0.0385	0.0941	0.0299	0.0734	0.0384	0.0943
Spirulina maxima	0.0203	0.0526	0.0344	0.0894	0.0263	0.0684	0.0325	0.0842
Spirulina platensis	0.0205	0.0524	0.0367	0.0942	0.0265	0.0680	0.0367	0.0942
Synechococcus sp.	0.0230	0.0606	0.0115	0.0303	0.0113	0.0302	0.0345	0.0910
Aphanizomenon flos-aquae	0.0242	0.0590	0.0343	0.0836	0.0263	0.0664	0.0304	0.0738
Oryza sativa	0.0284	0.0780	0.0265	0.0730	0.0336	0.0926	0.0460	0.1268
Triticum aestivum	0.0202	0.0492	0.0344	0.0837	0.0324	0.0789	0.0304	0.0740
Zea mays	0.0113	0.0336	0.0315	0.0941	0.0180	0.0538	0.0180	0.0538
Glycine max	0.0199	0.0611	0.0250	0..0763	0.0174	0.0534	0.0299	0.0593
Gallus gallus	0.0256	0.0440	0.0342	0.0568	0.0726	0.1249	0.0214	0.0368
Boss taurus	0.0211	0.0370	0.0338	0.0593	0.0169	0.0295	0.0212	0.0371

A_E: Frequency of release of fragments with given activity by selected enzymes, W: Relative frequency of release of fragments with given activity by selected enzymes, RuBisCO: Ribulose-1,5-bisphosphate carboxylase

Table 3: Bioactive fragments of RuBisCO protein for DPP-IV inhibitory action

	Bromelain		Proteinase K		Chymotrypsin		Papain	
Name of the species	A_E	W	A_E	W	A_E	W	A_E	W
Chaetoceros calcitrans	0.0314	0.0503	0.713	0.1141	0.0335	0.0536	0.0335	0.0536
Chlamydomonas reinhardtii	0.0325	0.0547	0.0669	0.1126	0.0446	0.0750	0.0609	0.1025
Chlorella pyrenoidosa	0.0365	0.0600	0.0692	0.1133	0.0467	0.0767	0.0609	0.1001
Chlorella vulgaris	0.0305	0.0508	0.0713	0.1187	0.0467	0.0780	0.0548	0.0916
Dunaliella salina	0.0407	0.0680	0.0692	0.1156	0.0448	0.0748	0.6312	0.1054
Euglena gracilis	0.0345	0.0565	0.0652	0.1064	0.0509	0.0830	0.0692	0.1129
Haematococcus pluvialis	0.0364	0.0595	0.0648	0.1060	0.0386	0.0629	0.0610	0.0994
Isochrysis galbana	0.0335	0.0559	0.0524	0.0874	0.0356	0.0594	0.0440	0.0734
Phorphyridium cruentum	0.0276	0.0452	0.0559	0.0903	0.4354	0.0710	0.0514	0.0839
Spirogyra sp.	0.0320	0.0538	0.0664	0.1111	0.0362	0.0609	0.0554	0.0931
Spirulina maxima	0.0305	0.0512	0.0648	0.1093	0.0344	0.0580	0.0528	0.0887
Spirulina platensis	0.0225	0.0379	0.0571	0.0965	0.0367	0.0620	0.0551	0.0931
Synechococcus sp.	0.0460	0.0870	0.0460	0.0870	0.0112	0.0217	0.0920	0.1740
Aphanizomenon flos-aquae	0.0283	0.0480	0.0687	0.1165	0.0364	0.0617	0.0568	0.0959
Oryza sativa	0.0391	0.0634	0.0478	0.0778	0.0354	0.0576	0.0602	0.0980
Triticum aestivum	0.0263	0.0451	0.0628	0.1077	0.0385	0.0660	0.0567	0.0973
Zea mays	0.0226	0.0363	0.0652	0.1051	0.0472	0.0761	0.0360	0.0580
Glycine max	0.0149	0.0254	0.0475	0.0805	0.0348	0.0593	0.0348	0.0593
Gallus gallus	0.0256	0.00331	0.0726	0.0939	0.1325	0.1713	0.0342	0.0442
Boss taurus	0.0211	0.0276	0.0717	0.0939	0.0381	0.0497	0.0297	0.0387

A_E: Frequency of release of fragments with given activity by selected enzymes, W: Relative frequency of release of fragments with given activity by selected enzymes, RuBisCO: Ribulose-1,5-bisphosphate carboxylase, DPP: Dipeptidyl peptidase

ProtParam calculated the physio-chemical properties such as the absorbance coefficient, aliphatic index, grand average hydropathy (GRAVY) index, isoelectric point, *in vivo* half-life, instability index and molecular weight of selected RuBisCO sequences. As shown in Table 4, the RuBisCO sequences of *C. reinhardtii*, *C. pyrenoidosa*, *C. vulgaris*, *D. salina*, *E. gracilis*, *H. pluvialis*, *Spirogyra* sp. and *A. flos-aquae* have extremely high quantity of glycine and proline amino acids. The incidence of bioactive peptides in RuBisCO sequences-related with the substance of glycine

Table 4: Physiochemical properties of microalgae and cyanobacterial RuBisCO proteins, results obtained using the ProtParam program

Name of the species	Number of AA in residues	Glycine (%)	Proline (%)	Isoelectric point (Pi)	GRAVY index	Aliphatic index
Chaetoceros calcitrans	461	8.9	3.9	28.27	-0.065	85.73
Chlamydomonas reinhardtii	475	10.3	4.4	38.32	-0.226	79.28
Chlorella pyrenoidosa	475	9.9	4.8	40.07	0.317	78.93
Chlorella vulgaris	475	9.9	4.8	40.32	0.310	78.72
Dunaliella salina	475	10.5	4.4	39.38	0.243	78.76
Euglena gracilis	475	10.5	4.2	40.81	0.313	73.98
Haematococcus pluvialis	476	10.3	4.8	42.38	0.212	79.31
Isochrysis galbana	459	9.4	3.9	28.78	0.128	85.69
Phorphyridium cruentum	488	8.8	3.9	31.71	0.162	85.59
Spirogyra sp.	451	10.4	4.7	41.56	0.291	82.00
Spirulina maxima	476	9.5	4.8	46.09	0.322	74.41
Spirulina platensis	476	9.5	4.8	45.65	0.308	74.62
Synechococcus sp.	472	9.5	4.7	42.36	0.270	79.62
Aphanizomenon flos-aquae	83	10.8	3.6	32.37	0.209	82.41
Oryza sativa	477	9.6	4.8	43.50	0.283	77.34
Triticum aestivum	543	7.7	3.3	27.40	0.034	104.86
Zea mays	476	9.9	4.6	42.57	0.258	77.92
Glycine max	427	7.3	6.3	36.64	0.030	84.92
Gallus gallus	386	4.9	3.6	37.11	0.001	89.65
Boss taurus	224	2.2	15.6	94.12	0.154	97.37

AA: Amino acids, GRAVY: Grand average of hydropathy, RuBisCO: Ribulose-1,5-bisphosphate carboxylase, %: Percentage

and proline. The aliphatic index indicated the amount of alanine, isoleucine, leucine and valine occupied in the RuBisCO sequences of selected microalgae. All the selected sequences represent thermo-stability of RuBisCO proteins. The GRAVY value for a peptide of RuBisCO specifies the total of hydropathy values of all the amino acids, separated by the quantity of peptides in the sequence.

DISCUSSION

A number of researchers broadly investigated the applications of microalgal species, as a predominant source for bioactive constituent synthesis, biodiesel feedstock production, feed additives, nanoparticles and nutraceuticals[22-24]. No data was available about the microalgae RuBisCO as a potential source for bioactive peptides. The BIOPEP, a database of biologically active peptide sequences was employed to find out the potential proteins candidates and its frequency of occurrence of bioactive peptides prediction, respectively. Earlier studies reported that the bioactive peptides from seaweeds *Caulerpa* sp. and oats *Avena sativa*[25-27]. Therefore, BIOPEP server for the prediction of bioactive peptides from fourteen microalgae and cyanobacterial species was interpreted. The DPP-IV (E.C. 3.4.14.5) is liable for conversion of Glucagon-like peptide-1 (GLP-1), into an inactive form. It is well known, the GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) both are essential for the increased level of insulin secretion[28]. The inhibition of DPP-IV would be useful for the treatment of diabetes mellitus (DM). Early studies reported DPP-IV inhibitors from microalgae and cyanobacteria active metabolites using high throughput screening, molecular docking and pharmacophore modeling approaches[29,30]. The DPP-IV inhibiting five different peptides Trp-Lys, Trp-Arg, Trp-Leu and Ile-Pro-Ile-Gln-Tyr was isolated from *Amaranthus hypochondriacus*, *Hordeum vulgare*, *Zea mays*, *Chenopodium quinoa*, *Avena sativa*, *Oryza sativa* subsp. Japonica, *Sorghum vulgare*, *Triticum aestivum* large subunit of RuBisCO[31]. The present *in silico* findings specified that some other RuBisCO sequences of microalgae can inhibit DPP-IV activity. Thus, utilization of microalgae and cyanobacterial RuBisCO will offer alternative resources of producing DPP-IV inhibitory peptides, thereby reducing serious stress on costly protein precursors, e.g., dairy milk, which leftovers a popular raw material for the production of the DPP-IV inhibitory peptides.

The ACE (EC 3.4.15.1) obliquely raises blood pressure by changing angiotensin I to angiotensin II, which tighten the blood vessels. It is secreted in the lungs and kidneys by cells in the endothelium of blood vessels[32]. The ACE inhibiting four different peptides MRWRD, MRW, LRIPVA and IAYKPAG were isolated from spinach RuBisCO[33]. High frequency of occurrence of ACE-inhibiting peptides in microalgal and cyanobacterial RuBisCO sequences provide a promising platform for the sustainable production of potent ACE inhibitor peptide-based anti-hypersensitive drugs. Reactive oxygen species (ROS) are obvious metabolic derivatives that can attack macromolecules such as nucleic acids, proteins and lipid membrane leading to many health disorders[34]. Antioxidants may have a constructive result on human diseases as they can reduce metabolic

damages caused by ROS. Metabolic enzymes in the human body, that regulate all the metabolites from liver function to the immune system, are mostly proteolytic enzymes. Proteolytic enzymes are facilitating the chemical breakdown of proteins by the hydrolysis. There are six different types of proteolytic enzymes (aspartate protease, cysteine protease, glutamic acid protease, metalloproteases, serine protease and threonine protease) which are classified according to sites in which they catalyze the cleavage of proteins[35-37]. Serine proteases (chymotrypsin and proteinase K) are responsible for managing different physiological roles including blood coagulation, digestion, immune response, inflammation and reproduction[38]. In humans, cysteine proteases (bromelain and papain) are responsible for cell aging and cell death by attacking collagen and elastin at sites of inflammation. Recently, Doneva *et al.*[39] reported that higher concentration of the papain and bromelain enzyme proteases cause deep hydrolysis destroys the overall exterior and the gustatory behavior of raw Turkey meat proteins. Thereby, in the present study four different proteases, bromelain, chymotrypsin, papain and proteinase K were chosen for proteolytic cleavage. These four enzymes hydrolyze bonds formed by the carboxyl groups of arginine, alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline tyrosine, tryptophan and valine[40].

The AE value in the proteolytic cleavage indicates the precise of the enzyme fit for the discharge of peptides with C-terminal proline in *D. salina*, *E. gracilis*, *P. cruentum*, *C. vulgaris* and *Synechococcus* sp. RuBisCO protein. Conversely, W indicates that the consistency with the type of amino acid residues forming C-terminal and neighboring peptides with DPP-IV and ACE inhibitor activity. Vercruysse *et al.*[41] reported the perspective and obstacles of in silico examination in calculating the chance of flow of bioactive peptides. The competence of *in silico* studies possibly condensed by following factors such as deficient peptide databases and complex specificity of the creating molecule, could not possible to perform computational analysis. The association of hydrolysates from identical substrates utilizing identical chemical may differ when response circumstances are modified[42]. With the above perspective, this study possibly employed effectively to test protein sequences to discover the potential impact of chosen bioactive peptide and release them.

CONCLUSION

In silico analysis of microalgae and cyanobacterial, RuBisCO provides strong projection as pioneer of bioactive peptide fragments when compared to commonly consumed food proteins of cereal crops, except for milk proteins. The prospects square measure is sturdy considering that the property and natural wealth of RuBisCO in algae can scale back the serious dependence on expensive characteristic food proteins and promote future food security. Moreover, the frequency of bioactive peptide fragments increases in the free form during the chemical action compared to frequency in the protein precursor. Papain and proteinase K exhibited the simplest potential for emotional bioactive peptides from RuBisCO. These results can promote new insights into the employment of underutilized biomass from microalgae and cyanobacteria as sources of RuBisCO for production of peptide-based nutraceuticals for human consumption.

SIGNIFICANCE STATEMENT

This study discovers the potential anti-oxidative, activating ubiquitin and anti-amnestic effective peptides derived from RuBisCO large subunits of microalgae viz., *Chaetoceros calcitrans*, *Chlorella pyrenoidosa*, *Aphanizomenon flos-aquae*, *Dunaliella salina* and *Chlorella vulgaris*. This study will facilitate the researcher to utilization of algal biomass on experimentally unexplored biomedical and nutraceutical applications. Moreover, the computational study will provide the alternative eco-friendly source to the Pharmaceutical industry to produce peptides (AN, RE, APG, AVACEL and KFEF) to human nutraceutical value.

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