Chapter IV

Evaluation of biological activities of purified biomolecules for angiotensin-1 converting enzyme, α-glucosidase and lipoxygenase inhibitory activities, antioxidant and antimicrobial activities
Abstract

Phycocyanin was extracted from *Spirulina* by a freeze thaw method and purified by ammonium sulphate precipitation (20-75%). Enzymatic hydrolysis of phycocyanin with trypsin, chymotrypsin, pepsin, pancreatin and fungal protease. Fungal protease hydrolysate showed highest angiotensin-1converting enzyme (ACE) inhibitory activity (95%) compared to other hydrolysates. Further fungal protease digest separated on sephadex G-25 column. Among the three major peak fractions, F3 fraction showed highest inhibition, this fraction was further fractionated by RP-HPLC on a C-18 column using a binary gradient of 0.1 % TFA and 70 % acetonitrile in water containing 0.05 % TFA at a flow rate of 1mL/min. Among the ten major fractions P7 fraction showed highest inhibition which was further purified by RP-HPLC ion pair chromatography to a single peak. Peptide sequence was identified as AEQP by Edman degradation. Further AEQP was synthesized in the laboratory and found to have potent inhibitory activity against ACE and α-glucosidase with an IC$_{50}$ value of 80.0 ± 3.1 μM and 122 ± 7.5 and $K_i$ value of 25.0 ± 0.7 μM and 51.0 ± 2.7. Lineweaver-burk plot analysis shown that the mode of inhibition of peptide against ACE was competitive and non-competitive against α-glucosidase. In addition studies on synthesized peptide had shown antioxidant activity with different hydroxyl free radicals. The molecular docking studies indicate that the tetra peptide (AEQP) on catalytic sites of both ACE and α-glucosidase provides a better platform to design novel peptide inhibitors and therefore the peptide can be used in therapeutic blood pressure and diabetes management.
Introduction

Hypertension is one of the most common cardiovascular diseases found in adults and is affected around 15–20% of world population. World Health Organisation (WHO) is estimated that the number of adults with hypertension may reach 1.56 billion by 2025 (Zhao et al 2015). It causes serious high risk factor for arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (Ko et al 2015). Angiotensin –1 converting enzyme (EC 3.4.15.1 ACE), a dipeptidyl-dipeptidase, is the key enzyme that functions in the rennin-angiotensin system. ACE increases blood pressure by both converting the inactive decapeptide angiotensin-1 to the potent vasoconstrictor angiotensin-II and inactivating the vasodilator bradykinin.

Many studies have been directed towards the synthesis of ACE inhibitors, such as captopril, enalapril, alacepril, and lisinopril, which are presently used extensively in the treatment of blood pressure. Synthetic ACE inhibitors are remarkably effective, but they cause adverse side effects therefore, the trend has been toward developing safer, natural ACE inhibitors (Onuh et al 2015).

Enzymatic protein hydrolysates containing short-chain peptides with characteristic amino acid composition and defined molecular size are desirable for specific formulations (Mallikarjun Gouda et al 2006).

In recent years peptides derived from functional foods have been shown to possess many biological activities including antihypertensive, antidiabetic, immunomodulatory, antimicrobial, anticancer, anticarcinogenic, antioxidant stress, anti-inflammatory, cholesterol lowering, growth enhancing, mineral binding, radical scavenging, regulation of glucose and insulin homeostasis, antifungal, antiobesity, antiallergic, antiageing (Li Chan 2015).
Bioactive peptides

*Spirulina platensis* is a microscopic, filamentous, spiral shaped, photosynthetic blue green alga belongs to cyanobacteria. Owing to high protein content (65-70 %) and other nutrients. *Spirulina* was used for human nutrition in several countries (Dillon et al 1995). The phycobiliproteins consists of protein moiety and linear tetrapyrrole prosthetic group (phycobilins) attached to the polypeptide through thioether bonds. Based on spectroscopic properties these proteins are broadly classified into 3 groups- allophycocyanin (650-655 nm), C-phycocyanin (610-620 nm) and R-phycoerythrin (540-570 nm). Phycocyanin and related phycobiliproteins are utilised in a number of applications in food, cosmetics, diagnostics and medicine. Phycocyanin is water soluble, strongly fluorescent and having antioxidant, anti-inflammatory, neuroprotective (Romay et al 2003), Cyclo-oxygenase-2 inhibition (Reddy et al 2000), anticancer activities (Pardhasaradhi et al 2003), prevention from renal (Farooq et al 2004) and induced apoptosis (Subashini et al 2004).

Among the different groups of bioactive peptides the antihypertensive inhibitory peptides have received great attention due to their potential beneficial effects related to hypertension.

There is growing interest in using functional foods or nutraceuticals for the prevention and treatment of hypertension or high blood pressure. In the present investigation, ACE inhibitory property of enzymatic hydrolysates of purified phycocyanin, the major pigment protein of *Spirulina* has been demonstrated. The peptide purified by chromatographic methods was identified as tetra peptide (AEQP). Further studies on synthetic peptide AEQP exhibited antioxidant, α-glucosidase and lipoxygenase inhibitory activities.
Results and Discussion

Many ACE inhibitory peptides have been discovered from enzymatic hydrolysates of different food proteins and the present investigation focused on *Spirulina* which consists of over 70 % protein content. In this study the phycocyanin pigment protein hydrolysates were prepared by digest with different proteases. The obtained protein hydrolysate was a rich source of ACE inhibitory peptides.

**Extraction of phycocyanin**

Phycobiliproteins are soluble in water, therefore they can be easily isolated as protein pigment complexes. Thus, the purification procedure of phycobiliproteins is facilitated, allowing one to separate these molecules from other lipid soluble pigments. These molecules have been extracted from cyanobacteria by different methods but appropriate control of pH and ionic strength during the extraction procedure is crucial for complex stability. Several factors can influence the phycocyanin extraction (Silveira 2007). The most important method of extraction of phycocyanin from *Spirulina* was freezing and thawing at two different temperature (Sarada et al 1999). Freezing–thawing is a mild, non-denaturing condition for the extraction of phycobiliproteins as compared to other methods. So this method was opted for extraction of phycocyanin from *Spirulina*.

**Purification of C-PC**

The data of the purification of C-PC from *Spirulina platensis* was summarized in Table 4.1. The amount of phycocyanin was estimated by using formula as mentioned in material and method section (2.2.4e). The sequential treatment of crude extract by fractional precipitation with ammonium sulphate and gel filtration chromatography on sephadex G-150 column refined the purity ratio (A620/A280) > 3.0 with an overall
recovery of about 45–50% and purity of protein was analysed by SDS-PAGE as results indicates purified protein consist of two sub units α and β and their molecular weight were found to be 16 and 17 kD, respectively as shown in Figure 4.1.

![SDS-Profile (12.5%) of purified phycocyanin.](image)

Lane M. Molecular weight markers β-galactosidase (116,000 Da), Bovine serum albumin (66,000 Da), Ovalbumin (45,000 Da), Lactate dehydrogenase (35,000 Da), REase Bsp98I (25,000 Da), β-lactoglobulin (18,000 Da), Lysozyme (14,000 Da). Lane 1. Phycoeyanin subunits

**Table 4.1 Determination of spectrophotometric purity of C-PC as compared to total protein at each stage of purification**

<table>
<thead>
<tr>
<th>Stages of purification</th>
<th>Purity ratio ($A_{620}/A_{280}$)</th>
<th>C-PC (mg/ml)</th>
<th>Protein concentration (Lowry method) (mg/10gdry biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing and thaw</td>
<td>0.91</td>
<td>27</td>
<td>45.76 ± 1.37</td>
</tr>
<tr>
<td>Ammonium sulphate fractionation</td>
<td>2.05</td>
<td>15</td>
<td>36.87 ± 0.37</td>
</tr>
<tr>
<td>Sephadex G-150 column</td>
<td>3.25</td>
<td>17</td>
<td>27.24 ± 1.76</td>
</tr>
</tbody>
</table>

Values are mean ± SD of triplicates
During the process of extraction, cyanobacterial cells were lysed by freezing at -20°C and thawing at 4°C, which also allows APC and PE with other proteins and nucleic acids to be extracted along with CPC.

The designed two-step ammonium sulphate fractionations, was found to be useful in salting out to remove unwanted proteins. Ammonium sulphate was used as a precipitating agent because it maintained the integrity of protein, readily precipitated phycobiliproteins, helped to reduce the quantity of sample handling, highly water soluble at low temperatures and had bacteriostatic effect and helpful in purification of proteins (Englard and Seifter 1990).

The fractional precipitation with ammonium sulphate involves two steps: the first with 25 % saturation of ammonium sulphate that precipitates other proteins, while C-PC remains soluble and further 75 % saturation precipitates C-PC. The resulting C-PC fraction was dissolved in sodium phosphate buffer and dialyzed overnight against same buffer and then loaded on to sephadex G-150 column the blue fraction was eluted and was monitored at 280 and 620 nm and the purity ratio was increased as shown in (Figure 4.2 and Table 4.1).
Figure 4.2. Elution profile of phycocyanin fraction on Sephadex G-150 column.

ACE Inhibition of Phycocyanin Digests.

The phycocyanin extracted from *Spirulina* was digested with the use of five commercially available enzymes and their digestion conditions were mentioned in material and methods section (Table 2.2). ACE inhibitory activity of the acetone powder extract of rat lung was 11.5 units/mg, respectively. ACE inhibitory activity of the crude enzyme digest was determined and fungal protease digest exhibited the most potent ACE inhibitory activity with 98% of inhibition as shown in Table 4.2.
The suite of peptides obtained following fungal protease digest was used in all further studies. Fungal protease act as endo- and exopeptidases and are able to hydrolyze proteins in a more efficient way than an endoprotease alone (In et al. 2002). The production of protein hydrolysates by sequential action of endoprotease and exopeptidase coupled with the development of post-hydrolysis procedures is considered the most effective way to obtain protein hydrolysates with defined characteristics (Kong et al. 2008). Mallikarjun Gouda et al 2006, Shin et al 2001 and Mizuno et al 2004 reported that fungal protease is capable of producing bioactive peptides of shorter length with potent ACE inhibitory activity.

Bioactive peptides are encoded within the primary structure of food proteins where they remain inactive until released by enzymatic hydrolysis. Once released from the parent protein, certain peptides have the ability to modulate the renin-angiotensin system because they decrease activities of renin or angiotensin converting enzyme (ACE), the two main enzymes that regulate mammalian blood pressure. These

Table 4.2 Different enzymatic hydrolysis of phycocyanin on ACE inhibition

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>57</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>72</td>
</tr>
<tr>
<td>Fungal protease</td>
<td>98</td>
</tr>
<tr>
<td>Pepsin</td>
<td>61</td>
</tr>
<tr>
<td>Pancreatin</td>
<td>69</td>
</tr>
</tbody>
</table>

The suite of peptides obtained following fungal protease digest was used in all further studies. Fungal protease act as endo- and exopeptidases and are able to hydrolyze proteins in a more efficient way than an endoprotease alone (In et al. 2002). The production of protein hydrolysates by sequential action of endoprotease and exopeptidase coupled with the development of post-hydrolysis procedures is considered the most effective way to obtain protein hydrolysates with defined characteristics (Kong et al. 2008). Mallikarjun Gouda et al 2006, Shin et al 2001 and Mizuno et al 2004 reported that fungal protease is capable of producing bioactive peptides of shorter length with potent ACE inhibitory activity.

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antihypertensive peptides can also enhance the endothelial nitric oxide synthase pathway to increase nitric oxide levels within vascular walls and promote vasodilation. The peptides can block the interactions between angiotensin II (vasoconstrictor) and angiotensin receptor, which can contribute to reduced blood pressure. (Aluko 2015).

**ACE inhibition of phycocyanin fungal protease digests**

Purified phycocyanin was digested with different concentrations of fungal protease from the range of 0.5% to 6% (w/w). The results indicate the maximum digestion was accomplished with 2% (w/w) of enzyme. A further increase in the enzyme substrate ratio had no effect on digestion profile as shown in Figure 4.3.

![Figure 4.3 Effect of different concentrations fungal protease on phycocyanin digestion.](image-url)
Further the digestion pattern was confirmed on SDS-PAGE loaded with 2% and 4% (phycocyanin digest with fungal protease) (w/w) as shown in Figure 4.4. So therefore 2% (w/w) enzyme substrate ratio used for further purification studies.

Figure 4.4. SDS-Profile (12.5%) of purified phycocyanin digested with fungal protease. Lane M. Molecular weight (Mr) markers. Bovine serum albumin (66,000 Da), Ovalbumin (45,000 Da), carbonic anhydrase (29,000 Da), soybean trypsin inhibitor (20,000 Da), (Lysozyme (14,000 Da). Lane 1-2. Phycocyanin digested with fungal protease with 2% and 4% respectively.
Purification of ACE inhibitory peptide by Sephadex G-25 Column

**Figure 4.5.** Elution profile of fungal protease digest of phycocyanin on sephadex G-25 column.

Fungal protease hydrolysate was fractionated on a sephadex G-25 glass column using size exclusion chromatography as shown in Figure 4.5. There were three major peak fractions (F1–F3) at 230 nm which were pooled and lyophilized and screened for ACE inhibitory activity. Among the three major fractions, F3 exhibited the highest ACE inhibitory activity with more than 90%. The lyophilized active F3 fraction was further separated by RP-HPLC on an ODS column with a linear gradient of acetonitrile containing 0.1% TFA (RP-HPLC run program was mention in material methods section Table 2.3). The elution profiles of the peaks are shown in Figure. 4.6.
Bioactive peptides

There are ten major peaks and each peak fraction (F1-F10) was pooled and lyophilized and screened for ACE inhibitory activity.

![Graphical representation of HPLC separation of fungal protease digest of phycocyanin on C18 column.]

**Figure 4.6.** RP-HPLC separation of fungal protease digest of phycocyanin on C18 column using gradient solvent system.

The F7 fraction (Figure 4.6) showed the most effective ACE inhibitory activity with more than 80% inhibition. Further peptide was purified by RP-HPLC using ammonium acetate buffer system ((RP-HPLC run program was mentioned in material methods section Table 2.4) and finally homogeneity of the peptide was confirmed on same RP-HPLC C18 column (Figure 4.7). (RP-HPLC run program was mentioned in material methods section Table 2.3).
The amino terminal sequence of the peptide was determined by Edman degradation on an automated gas-phase sequencer. The detailed methodology was mentioned in material methods section (2.2.4u). Amino terminal sequence analysis of the peptide by Edman degradation on an automated gas-phase sequentator. The sequence of the peptide was found to be NH₂-Ala-Glu-Gln-Pro-COOH (AEQP) and correspond to Ala⁶¹-Pro⁶⁴ of the β-subunit of phycocyanin of Spirulina platensis as shown in Figure 4.8.

Figure 4.7. RP-HPLC separation of purified peptide fraction using ammonium acetate buffer using C-18 column.
Figure 4.8. Primary amino acid sequence of β-subunit of phycocyanin from *Spirulina Platensis* (UniprotKB: P7250)

At the N-terminal branched chain aliphatic amino acids and at the C-terminal tryptophan, tyrosine, proline and phenylalanine were found to bind to active site of ACE and behaves like a competitive inhibitor (Cushman and Cheung (1971). Ko et al (2012) reported that hydrophobic amino acids such as valine and leucine amino acids at the N-terminal plays an important role in binding to the active site of ACE. These observations suggest that position of amino acid at the N-terminal and at the C-terminal may significantly influence the inhibition of ACE.

Twelve kinds of marine protein hydrolysate had showed ACE inhibitory activity including *Spirulina* (He et al 2007). Microalgae have attracted considerable attention in the recent years on an expression platform for the production of bioactivity of intact proteins as well (Saadi et al 2015).

**Peptide synthesis**

The tetra peptide containing the obtained sequence AEQP was synthesized. The detailed methodology was mentioned in material methods section (2.2.4v). Synthesis of the amide form of the peptide was accomplished using Rink amide MBHA resin and FMOC strategy and purified by analytical and preparative RP-HPLC (The RP-
HPLC linear gradient program was mentioned in material and method section Table 2.3 and 2.4) and single peak was obtained, indicating that the peptide was pure (Figure 4.9). The mass of the peptide was confirmed on ESI positive mode and found to be 443.38 (m/z).

![Figure 4.9](image)

Figure 4.9. RP-HPLC profile indicating the homogeneity of the synthetic peptide (AEQP). A. Analytical. B. Preparative. The peptide was analysed using C18 column.

![Figure 4.10](image)

Figure 4.10. Mass spectrum analysis of synthetic peptide (AEQP) on ESI-positive mode.
ACE Inhibition Kinetics.

The mode of inhibition was evaluated by a Lineweaver-Burk (1934) plot. The synthetic peptide was a potent inhibitor of lung ACE with an IC\textsubscript{50} value of 80.0 \(\mu\)M. The effect of inhibitor concentration on the varying substrate concentration indicated that the synthetic peptide was a competitive inhibitor as shown in Figure 4.11. Bioactive peptides usually contain 3–20 amino acid residues and low MW (molecular weight) peptides are more potent as bioactive peptides than high MW peptides (Lee et al 2010).

![Lineweaver Burk plot of lung ACE inhibition by the synthetic peptide AEQP](Figure 4.11)

**Figure 4.11.** Lineweaver Burk plot of lung ACE inhibition by the synthetic peptide AEQP

**Ki constant**

Kinetic analysis of the tetra peptide with lung ACE inhibition was consistent with a reversible and competitive mechanism of inhibition with respect to the substrate HHL. Representative Michaelis menten and Dixon plots of AEQP are shown in Figure 4.12. The binding affinity (\(K_i\)) of AEQP for ACE was calculated from Dixon the plot and found to be 30.5 \(\mu\)M as shown in Figure 4.12. The AEQP peptide also showed
inhibition against yeast α-glucosidase with an IC$_{50}$ value of 109 ±7.4 µM with $K_i$ value of 42.0 ± 3.7 µM.

Figure 4.12 Kinetic analysis of AEQP with lung ACE was determined by with and without indicated peptide concentrations.
A. Effect of various substrate concentrations on ACE activity presence of different concentrations of AEQP. B. Dixon plot to determine $K_i$ of AEQP.

Effect of AEQP on α-glucosidase and lipoxygenase inhibitory activity.

Different micro molar (µM) concentrations of tetra peptide (AEQP) was screened for α-glucosidase and lipoxygenase inhibitory activity and AEQP was found to inhibit α-glucosidase and lipoxygenase with an IC$_{50}$ values of 109 ± 7.4 and 130.5 ± 5.1. The results are shown in Figure 4.13.
Figure 4.13. Effect of synthetic peptide (AEQP) on α-glucosidase and lipoxygenase inhibitory activity

The inhibition of α-glucosidase activity was considered to be an effective strategy for the control of diabetes by diminishing the absorption of glucose. Studies reported on bioactive peptides purified from albumin, hemp (seed protein), silkworm, spent grain protein hydrolysate, egg white were found to have inhibitory activity against α-glucosidase enzyme. (Yu et al. 2012, Ren et al. 2016, Zhang et al. 2016, Connolly et al. 2014, Yu et al. 2011). A water soluble extract of gouda type cheese was reported to contain an active octapeptide (LPQNIPPL) which could improve glucose tolerance in type 2 diabetes with an effect on plasma glucose levels in rat model (Uenishi et al. 2012). Nongonierma and Fitz Gerald (2013) reported to contain a tetra peptide (FLQP) and the peptide act as antidiabetic agent and thus involved in glycaemic management. A pentapeptide (IPAVF) derived from tryptic digest of β-lactoglobulin of whey protein was involved in postprandial hyperglycemia have emerged as new and promising oral agent for the treatment of type 2 diabetes (Silveira et al. 2013). Our
result suggested that the bioactive peptide from the phycocyanin protein that exhibited the \( \alpha \)-glucosidase inhibitory activity could be considered as a potential AGI agent.

Lipoxygenase catalysed reactions are responsible for variety of human disorders such as atherosclerosis, allergy, inflammation, asthma and hypersensitivity (Steinburg 1999, Prigge et al., 1997, Funk and Colin 2006). The inhibition of 5-lipoxygenase pathway is considered to be interesting in the treatment of a variety of inflammatory diseases. Recent studies reported that microalgae also found to have lipoxygenase inhibitory activity such as eckol, dieckol, 6, 6–bieckol from *Ecklonia cava* and fucophlorethol C from *Colpomenia bulbosa* (rich in brown alga) and were reported to have significant 5-lipoxygenase inhibitory activity (Shibata et al 2003, Vo et al 2012, Kurihar et al 2015). Bioactive peptides extracted and purified from tryptic \( \beta \)-casein digest was shown to have 5-lipoxygenase inhibitory activity (Rival et al 2001a, Rival et al 2001b). So therefore the purified tetrapeptide (AEQP) could plays an important role in regulating the enzymes which are linked to hypertension, postprandial hyperglycemia and inflammatory disorders.

**In Vitro Stability of Synthetic ACE Inhibitory Peptide.**

In order to evaluate the stability of the peptide, the tetra peptide (AEQP) was digested with gastric proteases such as pepsin, pancreatin and combination of both the enzymes. The results indicated that there was no change in the ACE inhibitory activity of the synthetic peptide after treatment with pepsin, pancreatin, and also in combination of both as shown in Table 4.3. These results demonstrate that the peptide is resistant to digestion by enzymes present in the gastrointestinal tract. Especially, the proline residue in the C-terminal end strongly enhanced ACE inhibition. (Mallikarjun Gouda et al 2006, Miyoshi et al 1991).
The discovery of peptides as inhibitors of ACE in food proteins has drawn considerable attention to identify and purify ACE inhibitors from various foods for therapeutic intervention in hypertension (Ariyoshi 1993). In vitro gastric incubation provides easy practical mimics of the fate of such peptides after oral administration. The reduced hypotensive activity of several food-derived peptides following oral administration is due to the fact that they are hydrolysed in the GI tract (Wu and Ding 2002). The low susceptibility of the peptide AEQP to hydrolysis by gastric proteases suggests compositional stability of the peptide (Table 4.3). Fungal protease have endo-peptidase characteristics and bioactive peptides produced by fungal protease are resistant to digestive enzymes such as pepsin, trypsin and chymotrypsin, which would allow for absorption of peptides contained in these sort of hydrolysates (Mallikarjun Gouda et al 2006, Li et al., 2004).

**Antioxidant activity of synthetic peptide (AEQP).**

The synthetic peptide AEQP was evaluated for antioxidant activity in different in vitro systems and the results obtained are summarised in Table 4.4.
Bioactive peptides

The synthesized peptide (AEQP) was shown to have antioxidant activity on DPPH, hydroxyl radical, nitric oxide and reducing power with an effective IC$_{50}$ values (Table 4.4). Recently the interests in peptides from various kinds of food as well as by-products of food processing have been intensified. These peptides are recognized to be composed of functional amino acid sequences with easy adsorption and low molecular weight. (Gu et al 2015). Sheih et al (2009) reported that the isolated peptide (VECYGPNRPQF) derived from pepsin hydrolysate of Chlorella vulgaris protein waste had shown potent antioxidant activity against different varieties of free radicals. Porphyra columbina is a red seaweed which has a high protein content (approximately 30% dry weight) was able to produce a good amount of peptides with various biological activities (Cian et al 2012). Many synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) have been used to retard the oxidation process; however, the use of synthetic antioxidants must be under strict regulation due to potential health hazards (Park et al., 2001).

Table 4.4. Antioxidant activity of synthetic peptide (AEQP)

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH</th>
<th>Reducing power</th>
<th>Hydroxyl radical</th>
<th>Nitric oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEQP</td>
<td>62.0 ± 2.5</td>
<td>57.5 ± 2.8</td>
<td>75.0 ± 3.6</td>
<td>69.0 ± 4.1</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>35 ± 1.8</td>
<td>10.3 ± 0.3</td>
<td>-</td>
<td>42.0 ± 1.7</td>
</tr>
<tr>
<td>BHA</td>
<td>23 ± 2.0</td>
<td>33.4 ± 2.1</td>
<td>17.3 ± 2.1</td>
<td>51.0 ± 3.3</td>
</tr>
</tbody>
</table>

*IC50 value: concentration at which the DPPH and Hydroxyl radicals and nitric oxide were scavenged by 50%; Absorbance was 0.5 for reducing power respectively. Each value is expressed as mean ± SD.

**Table 4.4. Antioxidant activity of synthetic peptide (AEQP)**
Enzymatic hydrolysates of casein derived peptides were reported to have antioxidant activity (Rival et al 2001a and Rival et al 2001b).

Some microalgae have GRASS status and are presently used as edible food in most of the world. Although algae produce good amount proteins and are underutilized and using these proteins can be produce bioactive peptides by enzymatic digestion processes. Sheih (2009) reported that industrial algal waste biomass can be good source for the production of antioxidant and other bioactive peptides. Also these natural peptides exhibited different models of free radical scavenging abilities in addition to nutritive value without significant side-effects. Generally, antioxidant peptides are usually constituted by 3–20 amino acid residues per chain, and the antioxidant activities ascribed to the inherent composition and sequence of amino acids. In this sense, enzymatic hydrolysis could be employed to obtain antioxidant peptides due to its advantages over chemical hydrolysis, such as environmental friendliness, specificity and safety (Gu et al 2015).

Molecular docking studies

Preparation of ligands

The structures of the ligand molecules such as acarbose and lisinopril was drawn using Chem office package V.6.0 and they were assigned with proper 2D orientation (Figure 4.14. A and B). 3-D geometrical optimization was done using Chemsketch V.12.01 software. Openbabel, a standalone tool was used to obtain 3D coordinates for all the ligand candidate.
Figure 4.14. Structure of the ligand molecules A. Acarbose B. Lisinopril

Target preparation

The crystal structure Angiotensin Converting Enzyme (PDB ID: 1O86) and α-glucosidase (PDB ID: 2QMJ) (Figure 4.15 A and B) was retrieved from PDB (www.rcsb.org/pdb) and is edited to remove the hetero atoms. Later it was added with C-terminal oxygen, polar hydrogen and Gasteiger charges.
Figure 4.15. Crystal structure of A. Human Angiotensin-1converting enzyme (PDB ID: 1086), B. α- Glucosidase (PDB ID:2QMJ)
Figure 4.16. Binding sites of enzymes
A. PDB code 1086 (Active pocket of ACE with lisinopril complex and
B. PDB code 2QMJ (Active pocket of α-glucosidase with acarbose complex)

Figure 4.17. Docking studies interaction between  A. ACE with AEQP (PDB:1086)
B. ACE with lisinopril
The binding site information is obtained from ligplot and the residues forming the pocket were identified (Figure 4.16 A and B). Protein–ligand interactive visualization and analysis was carried out in Pymol viewer V.1.5.4.

Automated docking was used to study the interactions of ligand molecules to the binding pocket of the macromolecule. A genetic algorithm method implemented in the AutoDock V.4.2 was employed to study appropriate binding modes of the ligand in different conformations. For the ligand molecules, all the torsions were allowed to rotate during docking. The grid map was set around the residues forming the active pocket, which was retrieved from ligplot. Grid file was generated using AutoGrid program and lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for energy minimization using default parameters.

Figure 4.18. Docking studies interaction between A. α-glucosidase (PDB:2QMJ) with AEQP synthetic peptide B. α-glucosidase (PDB:2QMJ) with standard acarbose
Results

Studies docking the peptide to the active site of angiotensin-1 converting enzyme (PDB ID: 1086) as shown in Figure 4.17A. Studies indicates that a common amino acid residue forming H-bond was Gln\textsuperscript{281} and Lys\textsuperscript{511} and studies docking the peptide to the active site of $\alpha$-glucosidase enzyme (PDB ID: 2QMJ) as shown in Figure 4.18A which showed a common amino acid was Arg\textsuperscript{334}. The results indicated that the peptide (AEQP) could be a critical target for ACE and $\alpha$-glucosidase inhibitory peptide. The best poses of docking studies of tetra peptide (AEQP) at catalytic site of ACE and $\alpha$-glucosidase was shown in (Figure 4.17 and Figure 4.18). The AEQP bind to these catalytic sites was stabilized by H-bonds and hydrophobic interactions with enzyme amino acid residues. (Table 4.5).

**Table 4.5. Docking of AEQP to catalytic sites of ACE and $\alpha$-glucosidase with hydrophobic interactions with enzyme amino acid residues.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding energy (kJ mol\textsuperscript{-1})</th>
<th>Intermolecular energy (kJ mol\textsuperscript{-1})</th>
<th>Total internal energy (kJ mol\textsuperscript{-1})</th>
<th>Torsional energy (kJ mol\textsuperscript{-1})</th>
<th>RM</th>
<th>H-bond</th>
<th>Residue forming H bond</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lisinopril</td>
<td>-6.37</td>
<td>-9.95</td>
<td>-1.47</td>
<td>3.58</td>
<td>00</td>
<td>03</td>
<td>GLN281, TYR520, LYS511, THR282, TYR250</td>
</tr>
<tr>
<td>AEQP</td>
<td>-5.81</td>
<td>-9.39</td>
<td>-0.74</td>
<td>3.58</td>
<td>00</td>
<td>05</td>
<td>ARG334, ARG526, HIS600, ARG334, TYR299</td>
</tr>
<tr>
<td>Acarbose</td>
<td>-1023</td>
<td>-12.91</td>
<td>-1.43</td>
<td>2.68</td>
<td>00</td>
<td>04</td>
<td>ARG334, ARG334, GLN603</td>
</tr>
<tr>
<td>AEQP</td>
<td>-3.88</td>
<td>-7.46</td>
<td>-0.47</td>
<td>3.58</td>
<td>00</td>
<td>04</td>
<td>GLN281, TYR520, LYS511, THR282, TYR250</td>
</tr>
</tbody>
</table>

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Bioactive peptides

The H-bond interaction between Gln$^{281}$ and Lys$^{511}$ of ACE and Arg$^{334}$ of $\alpha$-glucosidase provide a solitary and significant mode of inhibitory properties.

Molecular docking studies of peptides derived from silkworm pupae was reported to inhibit $\alpha$-glucosidase using (PDB ID: 2QMJ model (Zhang et al 2016). In addition, the atoms coordinating the catalytic site is similar to the tetrahedral coordination geometry of lisinopril and acarbose. Possibly the tetrapeptide AEQP like lisinopril and acarbose is a transition state analog and inhibits ACE and $\alpha$-glucosidase through a similar mechanism to that of the drug as shown in Figure 4.17B and Figure 4.18B. These results revealed that the tetrapeptide AEQP could be a potential drug for treating diabetes. The enzyme $\alpha$-glucosidase is of interest to the pharmaceutical research community. Inhibition of the catalytic activity of $\alpha$-glucosidase could slow the absorption of glucose and decrease glucose levels in postprandial hyperglycemia. Therefore molecular docking studies indicate that the tetra peptide inhibitory activity on ACE and $\alpha$-glucosidase provides a better platform to design novel peptide inhibitors and therefore the peptide can be used in therapeutic blood pressure and diabetes management.

Conclusion

The present studies have provided evidence that the purified peptide (AEQP) derived from phycocyanin (fungal protease digest) of *Spirulina platensis* found to have ACE, $\alpha$-glucosidase, lipoygenase and antioxidant activity. Therefore the phycocyanin, a major storage pigment protein of *Spirulina* that is known for its health promoting bioactive molecule is a potential starting material for enzyme-mediated production of bioactive peptides that can be used in health-enhancing functional food/formulations.