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Research Article

Alpha-amylase inhibition kinetics by caulerpenyne

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Abstract

Many algae have important secretions which are generally used for defensive purposes. These secretions have caught the attention of a number of researchers, as to whether or not these metabolites can be used in medical research. Among these metabolites, caulerpenyne (CYN) which is the main metabolite of the *Caulerpa* species, has had an important place in *Caulerpa* research since the beneficial effects related to its determined properties (such as cytotoxic, antiviral, antiproliferative and apoptotic) have been demonstrated in many scientific reports. In the present study, the inhibitory effect of CYN isolated from *C. prolifera* on alpha-amylase was investigated. The inhibition experiments were done with CYN by a spectrophotometric determination method. In order to evaluate the type of inhibition a Lineweaver–Burk plot was produced. The results obtained from enzyme kinetic studies exhibited a non-competitive type of inhibition, which is characterized by the difference of V_{max} and K_M from those of the free enzyme, of alpha-amylase in the presence of CYN. The present study showed that *Caulerpa* species may be a potential target for the production of diabetic drugs in the light of the results obtained for CYN.

Keywords: Caulerpenyne; Alpha-amylase; Inhibition kinetic.

Introduction

Alpha-Amylase (α-1,-glucan-4-glucanohydrolase; EC. 3.2.1.1) is an enzyme which is related to carbohydrate metabolism and breaks starch down into maltose and dextrin. A metabolic disorder of this system, caused by the lack of insulin secretion or activity, results in illness, mainly diabetes mellitus, which is characterised by an increased blood glucose level (ALI *et al.,* 2006; BHANDARI *et al.,* 2008). An increased level of glucose in the blood can damage some of the body's systems, such as the blood vessels and the nerves (MATSUI *et al.,* 2007). According to a WHO research, 5% of the world population are under threat from this condition (WHO, 2002; BHANDARI *et al.,* 2008). Due to the importance of the problem drawn attention to by the WHO, there has been a great interest among researchers in finding safer

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and more effective drugs. However, the treatment of diabetes without producing any side effects is still a problem in the pharmaceutical industry (CHAKRABORTY AND RAJAGOPALAN, 2002). One of the therapeutic approaches for dealing with diabetes (ALI *et al.,* 2006; BHANDARI *et al.,* 2008; FRED-JAIYESIMI *et al.,* 2009) is decreasing the absorption of glucose from digestive tract. Thus, inhibition of carbohydrate-hydrolysing enzymes such as alpha-amylase is very important for the treatment of diabetes, obesity (OCTIVIO & RIGDEN, 2000; HEIDARI *et al.,* 2005) and the associated secondary symptoms like arterial hypertension and cardiovascular disease (SEGURA & RUILOPE, 2006). Nowadays, acarbose, voglibose and miglitol are generally used for this purpose, but they can cause several side effects, such as abdominal distension, bloating, flatulence and diarrhoea (CHAKRABARTI & RAJAGOPALAN, 2002; KIMMEL & INZUCCHI, 2005; FRED-JAIYESIMI *et al.,* 2009). These side effects can be explained due to the fermentation of carbohydrates in the colon which they are able to reach as a result of the complete inhibition of alpha-amylase (BISCHOFF, 1994). In addition, it is thought that some of these drugs may increase the incidence of renal tumours, hepatic injury and acute hepatitis (SHOBANA *et al.,* 2009).

In the light of the knowledge mentioned above, it is necessary to find efficient inhibitors with minimum side effects, and especially to take them from the natural sources. Various resources have been analysed in order to find a remedy for diabetes (ALI *et al.,* 2006; BHANDARI *et al.,* 2008; CHETHAN *et al.,* 2008; LOIZZO *et al.,* 2008; AGUILAR-SANTAMARÍA *et al.,* 2009; FRED-JAIYESIMI *et al.,* 2009; SHOBANA *et al.,* 2009). It has been reported that some of the compounds related to the defence mechanisms of plants may act as an inhibitors of hydrolytic enzymes such as alpha-amylase (FARIAS *et al.,* 2007).

In the present study, the main secondary metabolite from the *Caulerpa* species, caulerpenyne (CYN), was selected, in order to evaluate its potential for the treatment of diabetes. Several researchers have already focused on observing the chemical effects of CYN: the cytotoxic effects on sea urchins (LEMÉE *et al.,* 1993; GALGANI *et al.,* 1996), antiviral effects on feline immunodeficiency virus (FIV) (NICOLETTI *et al.,* 1999), antiproliferative effects on SK-N-SH cell lines (BARBIER *et al.,* 2001) and on eight human cancer cell lines (FISCHEL *et al.,* 1995) have been reported. The antiproliferative and apoptotic effects were also shown by CAVAS *et al.* (2006). However, there are few studies in the literature in which the enzyme inhibitory potential of CYN and crude extracts of *Caulerpa* species are evaluated. It has been reported that the methanolic extract of *C. taxifolia* and CYN inhibit pancreatic lipase activity (BITOU *et al.,* 1999). The inhibition of lipase activity was also shown by the ethanolic extract of *C. prolifera* (REBAH *et al.,* 2008). The inhibitory activity of CYN purified from *C. prolifera* was revealed by MAYER *et al.* (1993). NINOMIYA *et al.* (1998) have shown that the active metabolite of ethyl acetate extracts of *C. taxifolia,* which is responsible for 12-LOX inhibition is a CYN derivative. Although alpha-amylase inhibition via acetone crude extract of *C. racemosa* has been reported (TEIXERIA *et al.,* 2007), there is no information about the type of inhibition. Because TEIXERIA *et al.* (2007) did not perform any inhibition kinetic experiments, we aimed to elucidate the type of inhibition as a result of kinetic studies.

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Material and Methods

Caulerpenyne determination in C. prolifera

C.prolifera (Forsskål) *J.V. Lamouroux* was collected from the Çeşme coastline described with the coordinates of 38˚ 07' 56 82" N - 26˚ 50' 06 72" E in May 2009. The algae were transported to the laboratory immediately to prevent CYN transformation into aldehydes (JUNG *et al.,* 2002) and were used as a source of CYN. In order to evaluate the amount of CYN in fresh material, the procedures described by BOX *et al.,* (2008) were applied. Preparation of crude methanolic extract was followed by filtering it on SepPack columns. The CYN component was eluted with a mixture of methanol:ethyl acetate 50:50. The concentration of CYN was determined with HPLC by comparing the results with a calibration curve that was obtained by using known concentrations of standard CYN.

Isolation of CYN from C. prolifera

In order to prevent the enzymatic degradation of CYN described in JUNG *et al.* (2002), the fresh alga was extracted with ethyl acetate under liquid nitrogen. The purified CYN was obtained after successive elutings from two silica gel columns using the mixtures of petroleum ether:ethyl acetate (7:3) and petroleum ether:diethyl ether (6:4) respectively as eluents. In order to determine the purity of CYN, it was tested by using reverse phase HPLC and ^{1}H NMR spectroscopy. ^{1}H NMR data were identical with those reported in the literature (AMICO *et al.,* 1978).

Alpha-Amylase activity assay for inhibition tests of CYN

Alpha-Amylase from *bacillus* species (EC. 3.2.1.1, Type II A) and starch were purchased from Sigma Chemical Co., St. Louis, USA and Merck & Co., Inc, respectively. The α-amylase inhibition assay was performed according to the chromogenic method adopted by Sigma–Aldrich, adapted from BERNFELD (1951). The activity was detected by using a starch (1%) solution in a 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride as a substrate. The enzyme solution was pre-incubated with different concentrations of CYN (1:1 ratio) for 10 mins. and 30 mins. at 37˚C to obtain the optimum conditions and 30 mins. was determined as the optimum pre-incubation time for the enzyme-inhibitor mixture. 100 µL of enzyme solution (pre-incubated) was added to 100 µL of starch solution. Then, the reaction was stopped by adding a two-fold dinitrosalicylic acid solution (DNS) and boiled for 5 mins. in a boiling water bath. After cooling the mixture, 2 mL of distilled water was added and the enzyme activity was determined by measuring the maltose equivalents released from starch at 540 nm. All experiments were performed in two replications and the solvent controls were made.

Enzyme inhibition kinetics

The mode of inhibition was determined by using Michaelis-Menten and Lineweaver–Burk equations. Enzyme activities were investigated in the absence or presence of different concentrations of CYN (0.25 mM, 0.75 mM and 1 mM). Various concentrations of starch solution (0.25-2.5 %) were used as a substrate. All experiments were performed in two replications and the results were expressed as mean ± S.D.

Results

In order to determine the amount of the main secondary metabolite of the *Caulerpa* species, CYN, HPLC analyses were per-

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formed with a crude extract of *C. prolifera,* which was prepared according to procedures described by BOX *et al.,* (2008). The CYN content of C. prolifera was found as 6.34 mg/g wet weight of algae by using the HPLC method (Fig. 1).

Since the type of inhibition can be predicted according to the shape of the Lineweaver–Burk plot, this plot was produced (Fig. 2). The results calculated from this plot revealed a non-competitive type inhibition effect on alpha-amylase, due to the different K_M and V_{max} values from those of the control sample (free enzyme). The control sample values were 3333 μmol maltose/mg protein.min and 10 % for V_{max} and K_M, respectively. The counterparts of these values in the presence of different concentrations of CYN are shown in Table 1.

The inhibitory constants (K_I) of CYN on the alpha-amylase enzyme activity were calculated by using the graphs which were plotted according to the two equations below (Fig. 3, Fig. 4):

$$\frac{1}{V_{\max_{app}}} = \frac{1}{V_{\max} \cdot K_1} \cdot [I] + \frac{1}{V_{\max}} \quad (1)$$

$$\frac{1}{K_{m_{app}}} = \frac{1}{K_M \cdot K_1} \cdot [I] + \frac{1}{K_M} \quad (2)$$

where V_{max} represents the maximum velocity of the reaction, K_M is the Michaelis-Menten constant, [I] is the inhibitor concentration, K_I is the dissociation constant for binding of an inhibitor to the enzyme-substrate complex, $V_{max.app}$ and $K_{m.app}$ are the apparent V_{max} and K_M values. K_I values were determined respectively as 0.41 mM and 0.42 mM from these graphs.

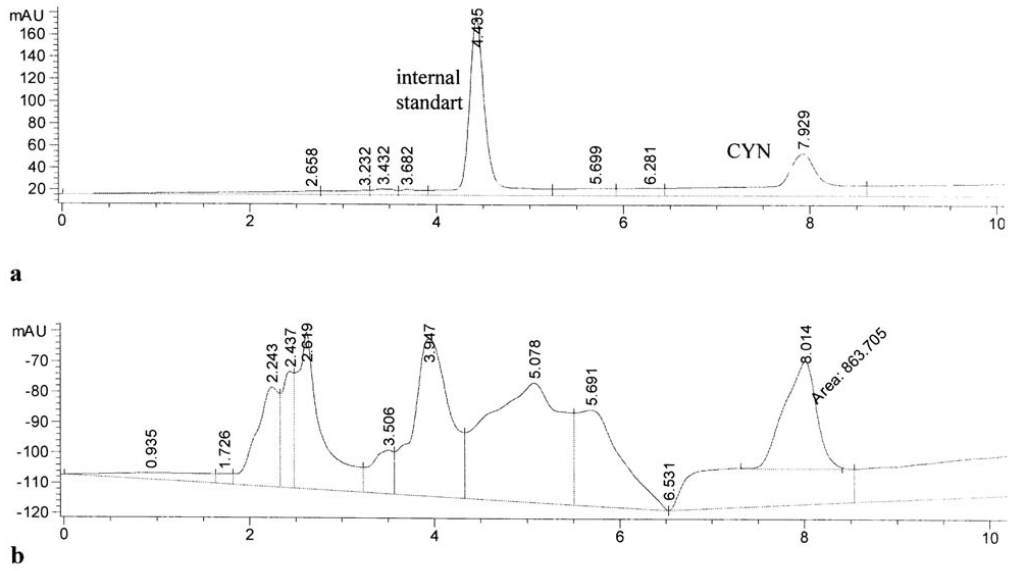


Fig. 1: The chromatograms of HPLC analyses with UV detection of methanolic extracts from *Caulerpa prolifera.* a) 5 ppm CYN with 25 ppm internal standart (4-methoxyacetophenone). b) *C. prolifera* extract. Conditions: HPLC with a reverse-phase (3 μM) column (15 x 0.4 cm) (Teknokroma), eluted with water:metanol (20:80, v:v) at a flow-rate of 0.4 mL/min and with UV detection at 254 nm.

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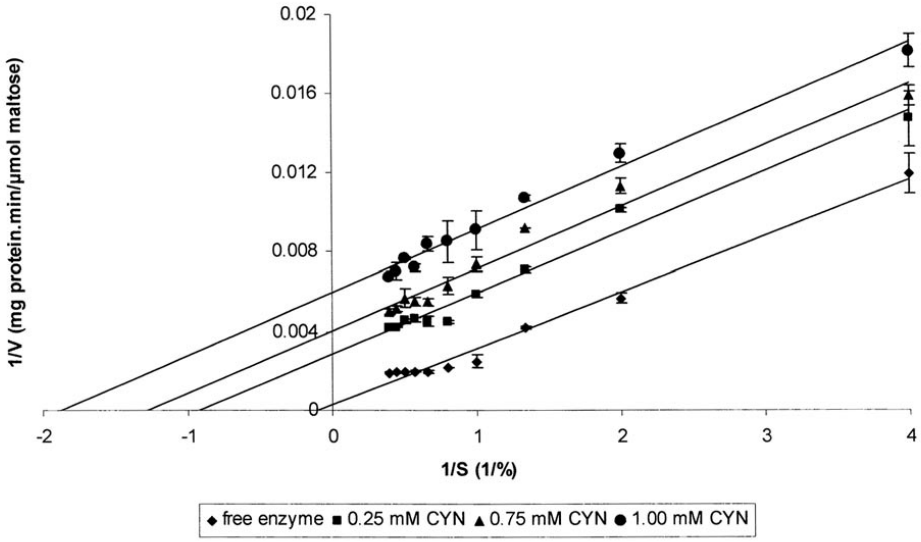


Fig. 2: Lineweaver–Burk plot of alpha-amylase (96 U/mL) reaction with variable starch concentrations in the absence or presence of CYN. Starch concentrations change between 0.25 % - 2.5 %. The values represent the mean ± standard deviation of two independent experiments.

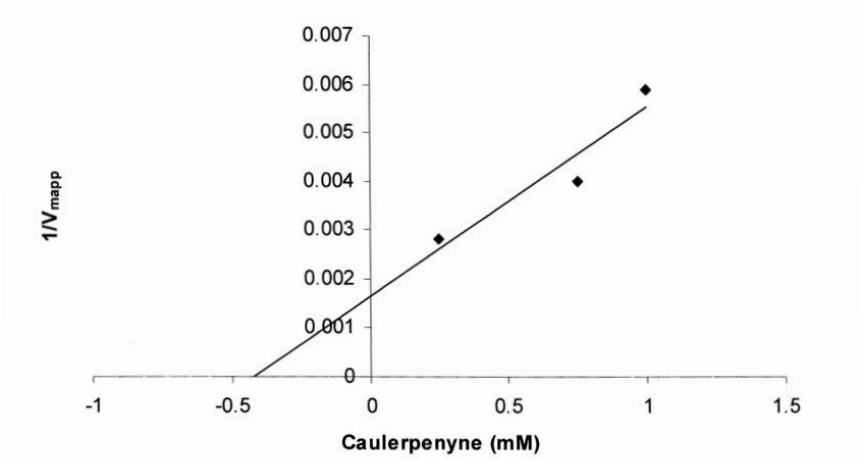


Fig. 3: $1/V_{mapp}$ – Inhibitor concentration plot related to Equation 1.

Table 1
Kinetic properties of alpha-amylase.

CYN concentration	V_{max} (µmol maltose/mg protein.min)	K_M (%)
0 mM	3333	10.0
0.25 mM	357	1.1
0.75 mM	250	0.8
1 mM	169	0.5

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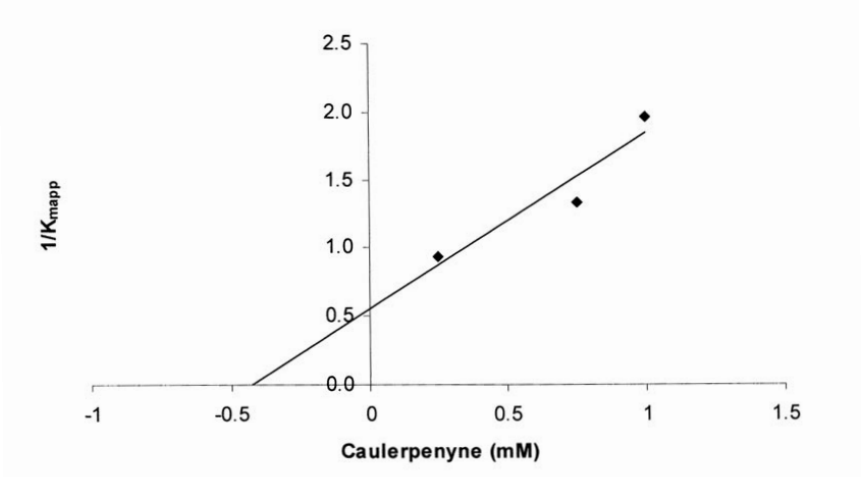


Fig. 4: $1/K_{mapp}$ – Inhibitor concentration plot related to Equation 2.

Discussion

The main secondary metabolite of the *Caulerpa* species was identified as CYN; it is known that the quantity of this metabolite changes depending on many factors, such as season, temperature, depth, stress conditions, etc (AMADE & LEMEE, 1998; SUREDA *et al.,* 2009). As a result of its unique properties, such as antiproliferative and apoptotic effects against some neuroblastoma, tumor and cancer cell lines (CAVAS *et al.,* 2006; FISCHEL *et al.,* 1995; BARBIER *et al.,* 2001) and antiviral properties (NICOLETTI *et al.,* 1999), LOX inhibition (NINOMIYA *et al.,* 1998), PLA2 inhibition (MAYER *et al.,* 1993), lipase inhibition (BITOU *et al.,* 1999; REBAH *et al.,* 2008) and alpha-amylase inhibition (TEIXEIRA *et al.,* 2007) a great deal of research has been done. The CYN content in *C. prolifera* was found to be 6.34 mg/g wet weight of algae by using an HPLC method. This result is well in line with the results described by JUNG *et al.,* (2002). JUNG *et al.* (2002) proposed a new method for determining CYN which underlines that freezing the algae material before extraction is better than freezing after solvent addition to prevent CYN degradation.

There is a great interest in natural sources regarding their use in medical research. Plants in particular have an important position in the area of medical research. More than 1123 plant species have been identified in the treatment of diabetes by GROVER *et al.* (2002), and also more than 200 pure compounds have a significant effect on the lowering of glucose in the blood (MARLES & FARNSWORTH, 1994). The compounds with an inhibitory effect on alpha-amylase enzymic activity are presented in Table 2.

ALI *et al.* (2006) investigated the inhibitory effects of certain Malaysian plants on alpha-amylase and they only found one as promising material for lowering the glucose level. They showed that the hexane extract of *Phyllanthus amarus* caused a significant inhibition of the enzyme with IC50 value of value of 32 Bg/mL. The *in vitro* anti-diabetic effect of the Nepalese medicinal herb Pakhanbhed (*Bergenia ciliata,* Haw.) was investigated, as it has been used to treat diabetes since prehistoric times (BHANDARI *et al.,* 2008). Both the crude methanolic extract of this species and the two active com-

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Table 2
Comparison of compounds with inhibitory effects on alpha-amylase.

Reference	Inhibition compound	Inhibition type	Ki value, % inhibition or IC50	Description-Source (from which source)
Present study	Caulerpenyne	un-competitive	K_i value : 0.41 mM	*Caulerpa prolifera* (marine seaweed)
TEIXERIA *et al.* (2007)	Marine seaweed extracts	-	ED_{50} value : 0.09 mg/mL	acetone crude extract of *Caulerpa racemosa*
ALI *et al.* (2006)	Malaysian plants extracts	-	IC_{50} value : 32 µg/ml	hexane extract of *Phyllanthus amarus*
BHANDARI *et al.* (2008)	Nepalese medicinal herb Pakhanbhed (Bergenia ciliata, Haw.)	-	IC_{50} values :739 µM and 401 µM, respectively	the two active compounds identified as (-)-3-*O*-galloylepicatechin and (-)-3-*O*-galloylcatechin
FRED-JAIYESIMI *et al.* (2009)	*Spondias mombin*	-	73% inhibition at 70 mg/ml	diethyl ether extract of *Spondias mombin*
FRED-JAIYESIMI *et al.* (2009)	Spondias mombin	-	57% inhibition at 20 mg/ml	3β-olean-12-en-3-yl (9Z)-hexadec-9-enoate
LOIZZO *et al.* (2008)	nine plant species, traditionally used against diabetes	-	IC_{50} values : 61.1 µg/mL and 91.2 µg/mL, respectively	*Marrubium radiatum* and *Salvia acetabulosa* methanol extracts
SHOBANA *et al.* (2009)	Eleusine coracana L.	non-competitive type	IC_{50} value : 23.5µg Ki value : 10 µg	isolated phenolic compounds of *Eleusine coracana* L.
CHETHAN *et al.* (2008)	millet polyphenols	mixed non-competitive	Ki value : 66.7 µg	crude extract of millet
CHETHAN *et al.* (2008)	millet polyphenols	un-competitive	67.7% for gallic acid, 71.9% for vanillic acid, 73.5% for quercetin, 79.2% for trans-cinnamic acid	pure compounds isolated from the polyphenol extract such as gallic acid, vanillic acid, quercetin and trans-cinnamic acid

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pounds identified as (-)-3-*O*-galloylepicatechin and (-)-3-*O*-galloylcatechin showed significant inhibition in a dose dependent manner on alpha-amylase. The IC_{50} values were determined as 739 μM and 401 μM for (-)-3-*O*-galloylepicatechin and (-)-3-*O*-galloylcatechin, respectively. The antidiabetic activity of the *Spondias mombin* leaf was demonstrated by FRED-JAIYESIMI (2009). *S. mombin* is traditionally used for the treatment of various diseases such as diabetes, intestinal disorders and fever. In the latter study, it was shown that the most active inhibitory fraction was obtained with diethyl ether and this fraction showed 73% inhibition on *Aspergillus oryzae* alpha-amylase at 70 mg/mL. Moreover, an active compound isolated from this species, 3β-olean-12-en-3-yl (9Z)-hexadec-9-enoate, presented 57% inhibition on alpha-amylase at the concentration of 20 mg/mL. LOIZZO *et al.* (2008) investigated the alpha-amylase inhibitory activities of nine plant species traditionally used against diabetes. It was demonstrated that *Marrubium radiatum* and *Salvia acetabulosa* showed the highest inhibitory activity against alpha-amylase with the IC_{50} values of 61.1 μg/mL and 91.2 μg/mL, respectively. The present investigation took place in the light of the study which was conducted by TEIXEIRA *et al.* (2007) for determining the inhibitory effect of *Caulerpa* extracts on alpha–amylase. They reported that a crude acetone extract of *C. racemosa* has a strong alpha-amylase inhibition effect with an ED50 of 0.09 mg/mL. Although a *Caulerpa* species was used as a source of inhibitory material, there were no kinetic details in this report. According to our results, the alpha-amylase inhibition with CYN represents a non-competitive type of inhibition in consequence of the different K_M and V_{max} values from those of the control sample (free enzyme). SHOBANA *et al.* (2009) investigated the inhibitory effects of phenolic compounds of *Eleusine coracana* L. on alpha-glucosidase and pancreatic amylase. The results obtained from the Lineweaver–Burk plot showed that phenolics exhibited a non-competitive inhibition of pancreatic amylase. This type of inhibition was also reported for *Rhus chinensis* extract (SHIM *et al.,* 2003). CHETHAN *et al.* (2008) determined the effect of millet polyphenols on alpha-amylase. They stated that the type of the inhibition was mixed non-competitive, which was represented by the decrease in V_{max} values while the K_M was constant. Although the polyphenol extracts caused mixed non-competitive inhibition on alpha-amylase, the pure compounds isolated from the polyphenol extract such as gallic acid, vanillic acid, quercetin and trans-cinnamic acid induced non-competitive inhibition. In the light of this study, quercetin was also used as a known inhibitor for alpha-amylase and the non-competitive type of inhibition was determined in the present study (data not shown).

Conclusion

The present study demonstrates that CYN inhibits alpha-amylase activity in a dose dependent manner. According to the results of this study CYN may be used for developing therapeutic agents, but further studies need to be performed. Since amylases play a significant role in diabetes, obesity and other related diseases, *Caulerpa* species-derived metabolites can be promising material for the treatment of these illnesses.

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