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Inhibitory Effects of Tunisian Marine Algal Extracts on Digestive Lipases

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Abstract The lipase inhibitory activity of ethanol extracts obtained from some marine algae collected on the Tunisian coast was evaluated. *Caulerpa prolifera* extract markedly reduced both dog gastric (DGL) and human pancreatic lipase (HPL) activities. Generally, the inhibition reached 100% after 40 to 60 min of incubation depending on lipase types and on substrates used. Moreover, the inhibitory effect of *C. prolifera* extract on lipases appeared to be accelerated by adding bile salts, which likely modified the interface and allowed the inhibitory compound to inactivate the lipase. The separation of *C. prolifera* extract by thin-layer chromatography (TLC) resulted in eight fractions showing efficient inhibition rate against DGL, compared to the crude extract. In the case of HPL, TLC fractionation reduced the inhibitory rates, suggesting that the effect of algal extract on lipases may be caused by a synergetic action of several compounds within the extract. High-performance liquid chromatograph separation resulted in isolation of a major compound displaying high inhibition capacity of HPL activity. *Caulerpa prolifera* extract may therefore be useful in developing antiobesity drugs.

Keywords Algae · Extraction · Inhibition · Lipase · TLC · HPLC

Introduction

The increased human assimilation of lipids is associated with various diseases like obesity, cardiovascular diseases, diabetes, and hypertension [1, 2]. Presently, obesity is an increasing public health problem, and it represented a common nutrition disease affecting the majority of adults in developed countries such as the USA [3]. Hence, it is well known that lipid digestion for humans and most animals successively involves a preduodenal

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(gastric in Human) lipase and a pancreatic lipase associated to its protein cofactor: the colipase [4–6]. Consequently, to limit fat absorption, many researches were conducted to evaluate lipase inhibitor activities from various sources. Recently, ORLISTAT (hydrogenated derivative of lipstatin obtained from *Streptomyces toxitricini*), which inhibits gastric, pancreatic, and carboxyl ester lipase, has been developed as a promising agent for obesity therapy [7–10]. However, it was reported that many natural products may contain lipase inhibitors, which can be used as a treatment for obesity. Proteins from soybean seeds [11, 12] and green tea extract [13] were reported to inhibit lipases. According to Gargouri et al. [12, 14], hydrophobic proteins such as serum albumin and β-lactoglobulin inhibit lipase activity by binding to the surface of the substrate micelle [11, 15]. Furthermore, it was demonstrated that secondary metabolites present in plant extracts such as saponins, flavinoids, and alkaloids can exhibit an inhibitory effect on lipase activity [16, 17]. For example, it was demonstrated that aqueous extracts from three plants (*Eriochloa villosa*, *Orixa japonica*, and *Setaria italica*) exhibited strong in vitro antilipase activity [17]. However, there is a requirement for further studies to elucidate the effect of other purified compounds, from natural resources like marine algae, on lipolytic enzymes to select the most appropriate ones for safe, natural, and cost-effective obesity control treatment. Hence, the aim of this study was to investigate the gastric and pancreatic lipase inhibitory activities of ethanol extracts obtained from marine algae collected from the coastline of Tunisia.

Materials and Methods

Enzymes

Dog gastric lipase (DGL) produced on transgenic corn (Meristem Therapeutics, France) and human pancreatic juice was collected in La Timone hospital (Dr. René Laugier, France).

Algal Material

Seven marine algal species were collected from the Tunisian coast. These included *Caulerpa prolifera*, *Chaetomorpha linum*, *Sargassum vulgare*, *Cystoseira shiffneri*, *Codium bursa*, *Dilophus fasciola*, and *Laurencia papillosa*. After collection, samples were rinsed with water and kept at −20 °C until their use.

Algal Extract Preparation

After pressing briefly between sheets of paper and drying at room temperature, 5 g of marine algae was extracted with 100 ml of ethanol by stirring at 37 °C for 24 h. After centrifugation (30 min, 30,000×*g*), the supernatant was evaporated under vacuo at lower temperature (<45 °C). Organic extract was dissolved in 5 ml of ethanol and used to measure the lipase inhibitory activity.

Measurement of Gastric and Pancreatic Activities

The lipase activity was measured on tributyrin (TC4) using a pH-stat Tittrator at 37 °C as previously described [18]. For DGL, the reaction medium contained 250 μl TC4 in 30 ml of distilled water containing 150 mM NaCl, 2 mM sodium deoxycholate (NaDC), and 1.5 μM of bovine serum albumin. For pancreatic juice, the reaction medium contained

250 μl TC4 in 30 ml Tris–HCl 2 mM pH 8.2, 4 mM NaDC. The lipase activity was determined by measuring the rate of release of butyric acid (titration with NaOH 0.1 N). One lipase unit (IU) was defined as 1 μmol of fatty acid titrated per minute.

Lipase activity was measured on olive oil at 37 °C as previously reported [19]. For pancreatic juice, the reaction medium contains 10 ml of olive oil emulsion (10 ml olive oil and 90 ml of gum arabic), in 20 ml of distilled water, 4 mM NaDC, 1 mM $CaCl_2$, and 2 mM Tris–HCl pH 8.2. For DGL, the reaction medium contains 10 ml of olive oil emulsion, in 20 ml of distilled water, 4 mM NaDC, 1 mM $CaCl_2$, and 2 mM sodium acetate pH 5.5. In the case of DGL, the reaction was run for 5 min, then the pH was shifted to 9. The volume of NaOH added, as compared to a control without enzyme, was used to calculate the rate of released fatty acids [20].

Measurement of Inhibitory Effects of Algal Extracts on Lipases

To measure the inhibitory effects of algal extracts, lipase was preincubated with each algal extract. The reaction medium contained 5 μl of the algal extract and 50 μl of the enzyme mixed in absence or in the presence of 5 mM NaDC. Aliquots of 10 μl from the reaction sample were used to assess the residual lipase activity, as indicated above. A control with ethanol instead of ethanolic extract was run in parallel.

Partial Purification of Inhibitor Compounds

Thin-layer Chromatography Thin-layer chromatography (TLC) of algal extract was performed on silica gel plate. The migration solution contained 78% hexane, 17.5% ethyl

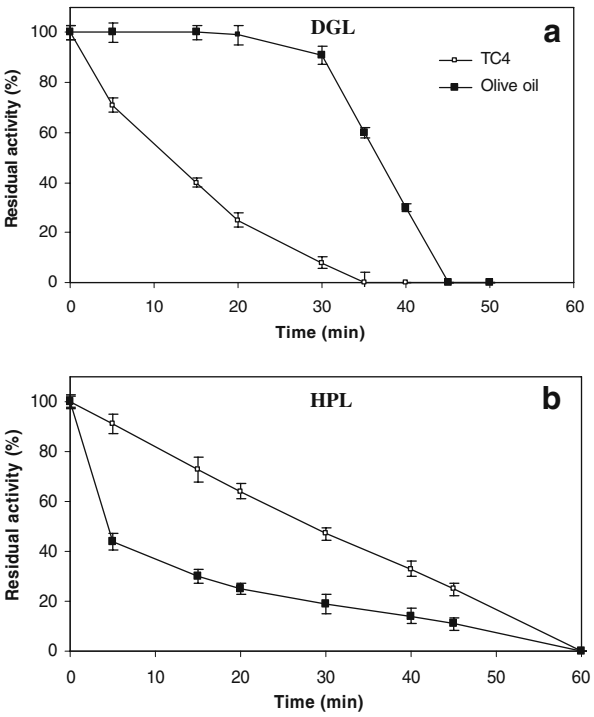

Fig. 1 Effect of *C. prolifera* ethanol extract on DGL (**a**) and HPL (**b**) activities. Lipase activity was determined using tributyrin (*TC4*) or olive oil as substrates, in the presence of 4 mM NaDC

ether, 2.5% methanol, and 2% acetic acid. Spots were visualized by spraying the plate with iodine vapors. The visualized spots were scratched from TLC plates, dissolved each one in 20 μl of ethanol and used to test their effect on gastric or pancreatic lipase activity. The scratched spots (containing lipase inhibitors) were investigated by a high-performance liquid chromatograph (HPLC).

High-performance Liquid Chromatography A HPLC equipped with a constant flow pump and a spectrophotometer was used to separate the algal extract. Separation was accomplished using C-18 reversed-phase column (8×250 mm) equilibrated at a flow rate of 1 ml/min, with a mobile phase consisting of 70% (v/v) acetonitrile in water. The separation was monitored with a variable wavelength UV detector operated at 220 nm.

Results

Effect of Algal Extracts on Lipase Activities

Seven marine algae were extracted using ethanol, and the inhibitory effects of these extracts were screened against DGL and human pancreatic lipase (HPL) (contained in human pancreatic juice) using TC4 or olive oil as substrate. Only *C. prolifera* extract displayed a marked inhibitory activity against the two enzymes (Fig. 1). Depending on the substrate, the DGL inhibition started before the first sampling (at 10 min of incubation) with TC4 and after 20 min with olive oil. Fifty percent of DGL inhibition was observed after 10 and 35 min using TC4 and olive oil as substrates, respectively. However, total inhibition was reached after 35 and 45 min in the presence of TC4 and olive oil, respectively (Fig. 1a). In

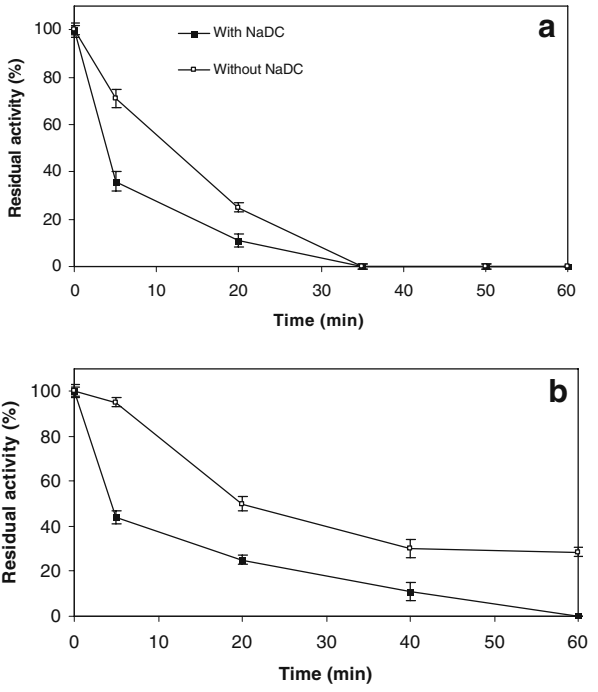

Fig. 2 Effect of *C. prolifera* extract on lipase activities in the absence or in the presence of NaDC. **a** DGL-catalyzed hydrolysis of TC4. **b** HPL-catalyzed hydrolysis of olive oil

the case of the pancreatic juice, the inhibition reached 50% after 30 min of incubation with TC4 and 5 min of incubation with olive oil (Fig. 1b). A total inhibition was observed within 60 min for both substrates.

Effect of Sodium Deoxycholate (NaDC) on Lipase Inhibitory Activity of *C. prolifera* Extract

To mimic the in vivo conditions, TC4 emulsion was incubated with DGL and with a supramicellar concentration (5 mM) of bile salts (NaDC) in the presence of *C. prolifera* extract. As shown in Fig. 2a, as compared to the assay without NaDC, the lipase activity was markedly lower and the inhibitory effect of *C. prolifera* extract on DGL appeared to be accelerated by bile salts addition. However, for the two cases, the total inhibition was attained within 35 min (with and without NaDC). A similar effect of NaDC was observed when using olive oil emulsion as a substrate. In fact, the presence of NaDC allowed a total inhibition after 60 min of incubation (Fig. 2b).

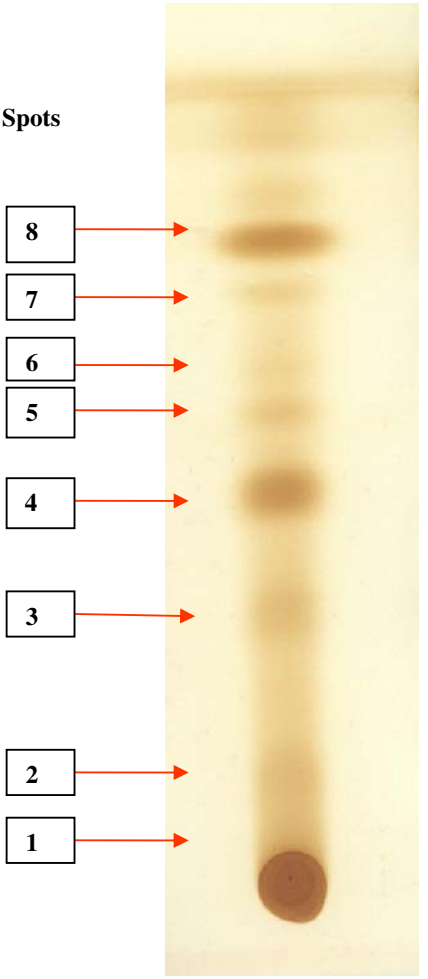

Fig. 3 TLC of *C. prolifera* extract. Migration solution contains 78% hexane, 17.5% ethyl ether, 2.5% methanol, and 2% acetic acid

Partial Purification of *C. prolifera* Extract

Figure 3 showed eight visible spots obtained upon separation of *C. prolifera* extract using a TLC. The test of lipase (DGL and HPL) inhibition was carried out with compounds extracted from each spot of silica gel, using olive oil as substrate. Table 1 shows results of relative mobility (R_f) of each fraction on TLC and their corresponding DGL and HPL inhibition rates. Concerning the DGL activity, all spots had an inhibitory capacity, and the inhibition percentage fluctuated between 56% and 100%. For HPL, only spots 1 and 2 revealed antagonistic effects with inhibition rates of about 46% and 36%, respectively (Table 1). Interestingly, only spots 1 (with R_f=0.06) and 2 (with R_f=0.13) exhibited inhibition for both lipases (DGL and HPL). For this reason, HPLC was used to separate the lipase inhibitor compounds contained in spots 1 and 2. HPLC profiles of each spot showed a major peak and other minor peaks, as indicated in Fig. 4. Fraction corresponding to the major peak 1 (obtained from spot 1) showed only 15% and 5% inhibition of DGL and HPL, respectively (Fig. 5). However, fraction corresponding to peak 1′ (obtained from spot 2) yielded 20% (for DGL) and 95% (for HPL) of inhibition (Fig. 5).

Discussion

The aim of this study was to evaluate the possible inhibitory effect of algal extracts on digestive lipases in vitro. Our findings indicated that, among seven algae tested, only *C. prolifera* extract inhibited both gastric and pancreatic lipases using TC4 as a short-chain triacylglycerol substrate and olive oil as long-chain triacylglycerol substrate. Generally, the inhibition depends on the lipase type and on the used substrate. Experiments showed that activities of both gastric and pancreatic lipases are markedly reduced in the presence of *C. prolifera* extract. Using TC4, DGL was more efficiently inhibited as compared to HPL (half inactivation times were 15 and 30 min for DGL and HPL, respectively). Nevertheless, when olive oil was used as substrate, the HPL was more markedly inactivated than DGL (half inactivation times were 5 and 40 min for HPL and DGL, respectively). The variability of the inhibition rates of DGL and HPL may be attributed to the fact that compounds contained in the extract interacting with each enzyme could not be the same.

Table 1 Inhibition of DGL and HPL by *C. prolifera* ethanolic extract (spots scratched from TLC plates) after 40 min of incubation.

	Relative mobility (R_f)	DGL inhibition (%)[a]	HPL inhibition (%)[a]
Crude extract	–	70±3	86±4
Spot 1	0.06	56±3	46±2
Spot 2	0.13	100±3	36±3
Spot 3	0.36	100±1	4±3
Spot 4	0.50	100±3	0±0
Spot 5	0.59	100±2	4±2
Spot 6	0.66	100±4	0±0
Spot 7	0.75	100±4	0±0
Spot 8	0.81	78±3	4±2

The activities were measured using olive oil as substrate

[a] Mean ± SD

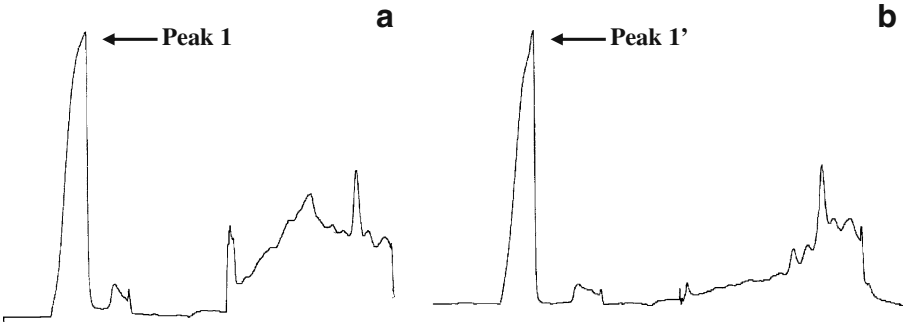

Fig. 4 HPLC peak profiles of the spots 1 (**a**) and 2 (**b**) scratched from TLC plates. Spots 1 and 2 are those from TLC separation (Fig. 3)

For the two enzymes, the addition of bile salts (4 mM NaDC) increased the inhibitory effects of algal extract. The added NaDC is likely to create a mixed bile salts/inhibitory compound interface at which the lipase is adsorbed and subsequently inactivated. According to Tsujita et al. [21], while using ε-polylysine as lipase inhibitor, it was suggested that the inhibition requires bile salts and appears to be associated with the formation of a surface-active ε-polylysine-bile salt complex occupying the interface and, consequently, nonspecifically blocks lipase adsorption [21]. Likewise, some proteins (albumin and β-lactoglobulin) displayed inhibition of the lipase activity [12, 14, 22]. This kind of nonspecific inhibition is usually reversed by a further adding of the substrate. Some compounds, such as tetrahydrolipstatin and diethyl-*p*-nitro phenyl phosphate, covalently modify the lipase molecule by reaction with the catalytic serine [7, 18]. In this case, the inhibition is irreversible. In our study, the inhibition of the lipases by the ethanolic extract of *C. prolifera* could not be reversed by a further addition of the substrate (data not shown). This finding suggests that the lipases were specifically modified by the *C. prolifera* extract.

Our results confirmed what was reported by other authors who found that green, red, and brown algae exhibited inhibitor activities against lipases [23, 24]. However, it is very important to note that variations in the inhibitory capacities of algal extracts could be related to many factors (the species, the biological stage, environmental conditions, the extraction processing conditions, etc.). Hence, studies of chemical defenses in marine organisms suggest that these organisms varied widely in the production of secondary metabolites. This production is associated with physical (light, temperature, etc.) and biological factors (community composition, biological stage, etc.), season, and geographical location [25]. For example, it was reported that the production of phlorotannins by a

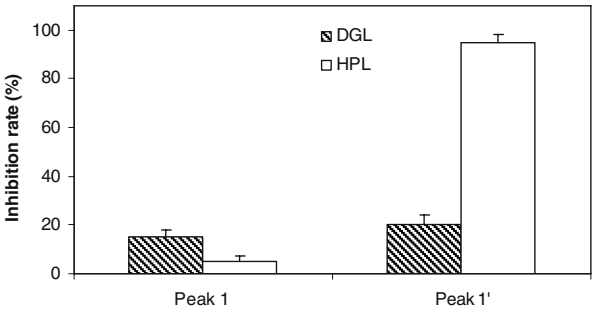

Fig. 5 Inhibition of DGL and HPL after incubation for 40 min with compounds corresponding to peaks 1 and 1′ of HPLC separation (see Fig. 4). Olive oil was used as a substrate

number of brown algae is positively affected by light intensity and fouling pressure [26, 27].

The separation of *C. prolifera* extract by TLC resulted in eight fractions (corresponding to eight spots) showing efficient inhibition rates against DGL. Compared to the crude extract, TLC fractionation enhanced the inhibitory effects, generally in the case of DGL and for the majority of the obtained spots. However, in the case of HPL, TLC fractionation reduced the inhibitory rates. Moreover, only two fractions corresponding to spots 1 and 2 showed inhibitory effects for both lipases with different rates. These results suggest that the effect of algal extract on lipases may be caused by a synergetic action of several compounds within the extract rather than by a single compound [28, 29]. HPLC separation of compounds corresponding to spot 1 confirmed the responsibility of more than one compound for the inhibition of the two enzymes. However, HPLC analysis of spot 2 resulted in purification of a major compound with higher inhibition power of HPL activity as compared to the crude extract. In the present study, only spots 1 and 2 obtained by TLC were examined by HPLC because they exhibited inhibition for both DGL and HPL. Therefore, it would be interesting to investigate fractionation of the remaining spots showing a higher level of inhibition against DGL.

Other studies reported that caulerpenyne was purified from an ethyl acetate extract of *Caulerpa taxifolia*, and this substance may interact directly with pancreatic lipase [23]. In addition, it has been shown in vitro that some condensed polyphenols, such as tannin, had an inhibitory action on different digestive enzymes such as proteases, alfa-amylase, and pancreatic lipase [24]. Our findings suggested that *C. prolifera* may prove to be a good source of effective crude drug for the treatment of obesity. However, further biological investigations are needed, using animal models, to verify the inhibitory activities under in vivo conditions. Further researches must be conducted to identify the compounds responsible for the inhibition of HPL by using infrared and nuclear magnetic resonance spectroscopic and electron-impact-mass spectrometric techniques.

Conclusion

Among seven marine algal species collected from the Tunisian coast, only *C. prolifera* extract displayed a marked inhibitory activity against DGL and HPL. Interestingly, one major compound separated by TLC then HPLC was found to be a powerful inhibitor of the pancreatic lipase. Therefore, this algal extract may be a resource of a potential antiobesity agent. However, more investigations are needed to clarify the compound structure and mechanisms of action and to confirm its pharmacological potential.

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