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Involvement of oleuropein in (some) digestive metabolic pathways

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Abstract

Olive oil is the principal source of fats in the Mediterranean diet and it has been postulated that the components in olive oil can contribute to a lower incidence of coronary heart disease and cancers (prostate, colon, breast, and skin). The positive effects on human health can be attributed to the high level of phenolic compounds present in olive oil, the major ones being oleuropein, hydroxytyrosol and tyrosol. The aim of the present study was to evaluate the effect of oleuropein on enzymes involved in specific pathways of metabolism of proteins, carbohydrates and lipids.

In particular, the effects of oleuropein on enzymes, such as trypsin, pepsin, lipase, glycerol dehydrogenase, glycerol-3-phosphate dehydrogenase, and glycerokinase, were investigated.

Results demonstrate that oleuropein is able to activate pepsin and shows an inhibitory effect toward all the other enzymes tested, which suggests a new role for this polyphenol. In addition, a new method for lipase activity assay is presented.

Keywords: Oleuropein; Polyphenols; Metabolism regulation

1. Introduction

Since antiquity, oil obtained from the fruit of the olive (*Olea europaea*) has been considered to have many medicinal properties (Holy Bible). In recent years, various researches have shown that polyphenols, or seciridoids, present in olive oil act as antihypertensive, antithrombotic, antibacterial, antiviral, antidepressant, antioxidant (Manna et al., 2002), antiatherosclerotic, hypoglycemic (Le Tutor & Guedon, 1992; Gonzalez et al., 1992; Driss, Duranthon, & Viard, 1996) hypocholesterolemic (Ficarra, Ficarra, de Pasquale, Monforte, & Calabro, 1991) and antiatherogenic (Visioli & Galli, 2001) agents. The constitution of olive oil is primarily triacylglycerols and ~0.5–1.0% non-glyceridic constituents. It is also the source of at least 30 phenolic compounds, its phenolic content depending on a number of factors, the main one being the production and storage of the oil (Brenes, Garcia, Garcia, & Garrido, 2001). The major phenolic compounds in olive oil are oleuropein, hydroxytyrosol and tyrosol. Other polyphenols, such as vanillic acid, p-coumaric acid, vanillin, demethyloleuropein, homoorientin, verbascoside, rutin, luteolin 7-O-glucoside, apigenin 7-O-rutinoside, apigenin 7-O-glucoside, luteolin, oleuropein aglycone, cyanidin 3-O-glucoside, and finally cyanidin 3-O-rutinoside, are present, both in the leaves and in the olive oil (Romani, Mulinacci, Vinceri, & Cimato, 1999). Even if at lower activities, they have the same pharmacological properties as oleuropein (Gonzalez et al., 1992).

Recent studies have demonstrated that the average oleuropein content in olive oil from Apulia varies between 3.1 and 11 mg oleuropein/kg of oil and that it depends on cultivar (Perri, Raffaelli, & Sindona, 1999; Owen et al., 2000).

The aim of the present work is to investigate the possible effects of oleuropein on trypsin and pepsin, lipase, glycerol dehydrogenase, glycerol-3-phosphate dehydrogenase, and glycerolkinase, enzymes involved in glucidic, proteic, and lipidic metabolism.

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2. Materials and methods

2.1. Materials

Oleuropein was supplied from Extrasynthese; 3-Palmitoyl-sn-glycerol and glycerol were from Fluka. All other chemical reagents were from Sigma.

2.2. Enzyme activities assay

Trypsin, glycerol dehydrogenase and glycerol-3-phosphate dehydrogenase activities were calculated by continuous direct spectrophotometer assays, while pepsin activity was measured by a discontinuous direct spectrophotometer assay. All these assays were performed in a thermostatically controlled Varian DMS-90 spectrophotometer.

A discontinuous direct HPLC assay was used for detection of both lipase and glycerokinase activities, using an Alltech apparatus with a RP Supelcosil LC-18 column (5 μm; 4.6×250 mm). All results are the averages of at least four determinations in three different experiments.

2.3. Trypsin (EC 3.4.21.4)

Trypsin activity was calculated by utilizing *N*-benzoyl-DL-arginine *p*-nitroanilide as substrate and measuring ($\lambda = 405$ nm) the *p*-nitroanilide production at 37 °C (Gaertner & Puigserver, 1992). In a final volume of 1 ml, the incubation mixture contained: 0.023 M Tris HCl, pH 8.1, 5.7 mM $CaCl_2$, increasing substrate concentrations (0.057, 0.114, 0.23, 0.45, 0.92, 1.28 mM), 1.6 U of trypsin, with or without increasing oleuropein concentration (0.42, 0.63, 0.84 mM). One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitroanilide per minute at 37 °C and pH 8.1.

2.4. Pepsin (EC 3.4.23.1)

The pepsin activity was assessed by utilizing denatured hemoglobin as substrate, recording the increase of absorbance at $\lambda = 280$ nm due to the peptides obtained from hemoglobin hydrolysis, after 10 min of incubation at 37 °C and subtracting the endogenous pepsinogen activity present in pepsin, according to Kay (1975).

The incubation mixture, in a final volume of 1.6 ml contained: 15 U pepsin, 10 mM HCl and increasing hemoglobin concentrations (0.0019, 0.0038, 0.0075, 0.015, 0.019 mM). In order to evaluate the effect of the polyphenol on this enzymatic activity, the assays were conducted in the presence of 0.42, 0.61, and 0.84 mM oleuropein.

The unit of enzymatic activity was defined as the amount of enzyme that hydrolysed 1 μmol of hemoglobin per minute at 37 °C and pH 2.0.

2.5. Glycerol dehydrogenase (EC 1.1.1.6)

Glycerol dehydrogenase activity was determined by utilizing glycerol and β-NAD as substrates and measuring the NADH production ($\lambda = 340$ nm) at 37 °C. The incubation mixture, in a final volume of 0.5 ml, contains: 50 mM Tris–HCl, pH 8.2, 50 mM KCl, 0.75 mM βNAD, 0.425 U glycerol dehydrogenase, increasing glycerol concentrations (0.3, 0.6, 1.2, 1.8 mM) and increasing oleuropein concentrations (0.41, 0.63 and 0.82 mM). One enzyme unit was defined as the amount of enzyme that oxidized 1 μmol of glycerol to dihydroxyacetone per minute at 37 °C and pH 8.2.

2.6. Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8)

Glycerol-3-phosphate dehydrogenase activity was investigated by utilizing glycerol-3-phosphate and β-NAD as substrates and recording the NADH formation ($\lambda = 340$ nm) at 37 °C.

The incubation mixture in a final volume of 0.5 ml contained: 50 mM TFK, pH 7.7, 0.75 mM β-NAD, 0.19 U glycerol-3-phosphate dehydrogenase, increasing glycerol-3-phosphate concentrations (0.3, 0.6, 1.2, 1.8 mM) with or without increasing oleuropein concentrations (0.27, 0.41, 0.55 μM). One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of NADH per minute at 37 °C at pH 7.7.

2.7. Lipase (EC 3.1.1.3)

Lipase activity was determined by a continuous indirect HPLC assay measuring, at $\lambda = 254$ nm, the time-dependent 3-palmitoyl-sn-glycerol decrease. The incubation mixture, in a final volume of 0.5 ml contained 18.5 mM Tris–HCl, pH 9.25, 0.5 U lipase, 30 mM NaCl, 0.02 mM $CaCl_2$, 3-palmitoylglycerol (3, 4, 5, 6 mM), with or without increasing oleuropein concentrations (0.41, 0.63, 0.82 mM). The reaction mixtures were incubated at 37 °C. Hundred microlitre aliquots of each mixture were stopped at different times (0 and 15 min) by adding 50 μl of 1.2 M $HClO_4$. After 10 min on ice and centrifugation for 5 min at 14,000xg (A.L.C. Int. Eppendorf Centrifuge, mod 4214), 130 μl of each supernatant were neutralized by adding 35 μl of 0.79 M K_2CO_3, kept on ice for 5 min and centrifuged again. Twenty microlitres of each supernatant obtained were injected onto a RP Supelcosil LC-18 column (5 μm; 4.6×250 mm). The amounts of products and residual substrate were determined from the peak areas of the HPLC-separated compounds with reference to appropriate standards. The elution of the samples occurred at 1.3 ml/min in 100 mM TFK, pH 6.0. The unit of enzymatic activity was defined as the amount of enzyme that

hydrolyzed 1 μmol of 3-palmitoyl-sn-glycerol per minute, at 37 °C and at pH 9.25.

2.8. Glycerokinase (EC 2.7.1.30)

The glycerokinase activity was investigated by a discontinuous direct HPLC assay, utilizing glycerol and ATP as substrates, and calculating the time-dependent ATP degradation ($\lambda = 254$ nm). The incubation mixture, in a final volume of 0.5 ml, contained 12.5 mM Tris–HCl, pH 8.5, 1.25 U of glycerokinase, 2 mM $MgCl_2$, 1 mM glycerol, increasing ATP concentrations (0.05, 0.1, 0.5 mM) and increasing oleuropein concentrations (0.42, 0.63, 0.84 mM). The incubation was conducted for 0 and 5 min at 37 °C. Afterwards, the samples were treated as previously described (see lipase) and the elutions were carried out by a 5 min linear gradient (0–15%) of buffer A (100 mM TFK, pH 6.0) and buffer B (100 mM TFK, pH 6.0 containing 30% v/v MeOH). The unit of enzymatic activity was defined as the amount of the enzyme that produced 1 μmol of ATP per minute at 37 °C, pH 8.5.

3. Results

3.1. Effect of oleuropein on protein catabolism

3.1.1. Oleuropein effect on pepsin enzymatic activity

Pepsin shows, for hemoglobin, a $K_m = 9.6$ μM and a $V_{max} = 0.0075$ M min^{-1}. As shown in Fig. 1, oleuropein proves to be an activator of pepsin activity.

3.1.2. Oleuropein effect on trypsin activity

The kinetic parameters of trypsin for the synthetic substrate *N*-benzoyl-DL-arginine *p*-nitroanilide were calculated and resulted in $K_m = 0.434$ mM and a $V_{max} = 0.868$ mM min^{-1}. Furthermore, the effect of oleuropein on trypsin activity was investigated: the phenolic compound proved to be a competitive inhibitor toward trypsin with $K_i = 0.85$ mM (Fig. 2).

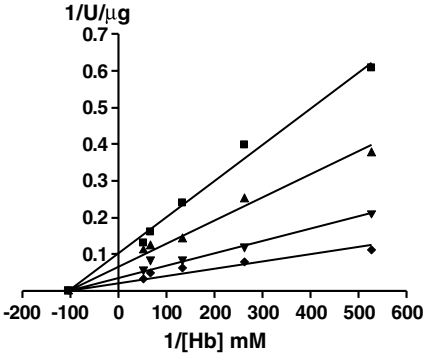

Fig. 1. Effect of oleuropein on pepsin enzymatic activity. Lineweaver–Burk plot: the enzyme activities were measured as described in Section 2 utilizing different hemoglobin concentrations as substrate (■) and in presence of 0.42 mM (▲), 0.61 mM (▼) and 0.84 mM (◆) oleuropein.

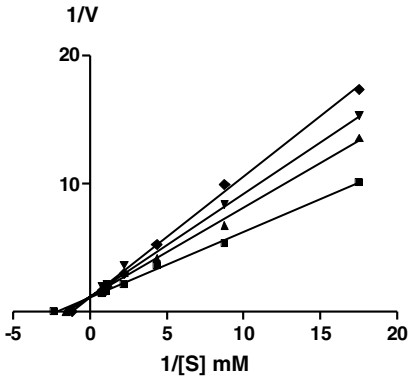

Fig. 2. Effect of oleuropein on trypsin enzymatic activity. Lineweaver–Burk plot: the enzyme activities were measured as described in Section 2 utilizing different *N*-Benzoyl-DL-arginine *p*-nitroanilide concentrations as substrate (■) and in presence of 0.42 mM (▲), 0.63 mM (▼) and 0.84 mM (◆) oleuropein.

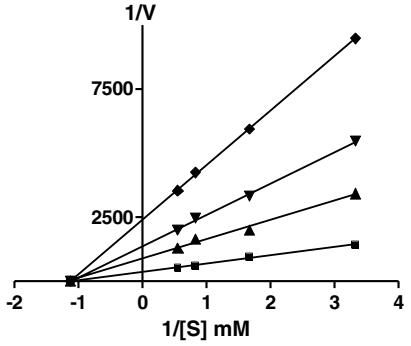

Fig. 3. Effect of oleuropein on glycerol dehydrogenase activity. Lineweaver–Burk plot: the enzyme activities were measured as described in Section 2, utilizing a fixed concentration of β-NAD and variable concentrations of glycerol as substrates (■). The assays were performed in the presence of 0.42 mM (▲), 0.61 mM (▼) and 0.84 mM (◆) of oleuropein.

3.2. Effect of oleuropein on carbohydrates metabolism

3.2.1. Oleuropein effect on glycerol dehydrogenase activity

Glycerol dehydrogenase showed, for glycerol, its natural substrate, a $K_m = 0.89$ mM and a $V_{max} = 2.86$ M min^{-1}. As shown in Fig. 3, oleuropein acted as a non-competitive inhibitor (with $K_i = 0.019$ mM) for glycerol dehydrogenase activity.

3.2.2. Oleuropein effect on glycerol-3-phosphate dehydrogenase activity

Glycerol-3-phosphate dehydrogenase showed, for its natural substrate, glycerol-3-phosphate, a $K_m = 0.48$ mM and a $V_{max} = 7.78$ mM min^{-1}. As shown in Fig. 4, oleuropein showed a non-competitive inhibition with

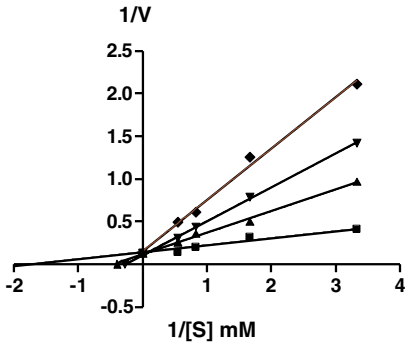

Fig. 4. Effect of oleuropein on glycerol-3-phosphate dehydrogenase activity. Lineweaver–Burk plot: the enzyme activities were measured as described in Section 2, utilizing a fixed concentrations of β-NAD and increasing glycerol-3-phosphate concentrations as substrates (■). The assays were performed in the presence of 0.27 μM (▲), 0.41 μM (▼) and 0.55 μM (◆) of oleuropein.

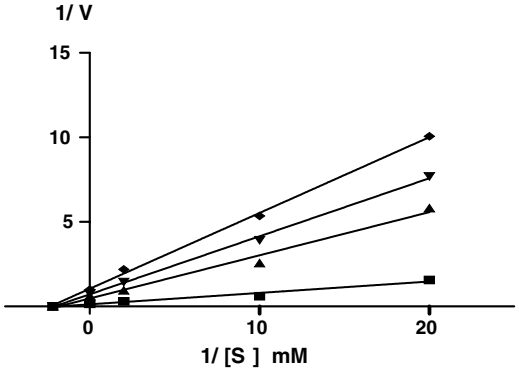

Fig. 5. Effect of oleuropein on glycerokinase activity. Lineweaver–Burk plot: the enzyme activities were measured as described in Section 2, utilizing a fixed concentrations of glycerol and increasing ATP concentrations as substrates (■). The assays were performed in the presence of 0.41 mM (▲), 0.63 mM (▼) and 0.82 mM (◆) of oleuropein.

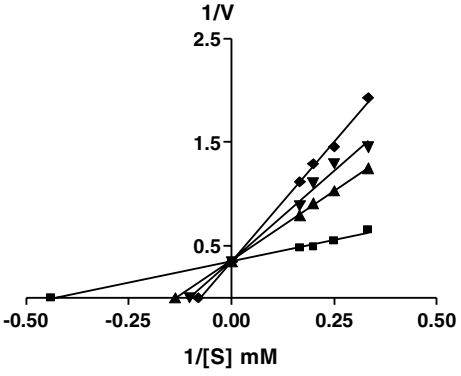

Fig. 6. Effect of oleuropein on lipase activity. Lineweaver–Burk plot: the enzyme activities were measured as described in Section 2 utilizing 3-palmitoylglycerol as substrate (■). The assays were performed in the presence of 0.41 mM (▲), 0.63 mM (▼) and 0.82 mM (◆) of oleuropein.

$K_i = 0.0636$ μM toward glycerol-3-phosphate dehydrogenase.

3.2.3. Oleuropein effect on glycerokinase activity

In the assay condition used, glycerokinase showed for ATP a $K_m = 0.46$ mM and a $V_{max} = 0.018$ M min^{-1}. As shown in Fig. 5, oleuropein is a non-competitive inhibitor with $K_i = 0.05$ mM for glycerokinase activity.

3.3. Oleuropein effect in lipid metabolism

3.3.1. Oleuropein effect on lipase activity

Lipase showed, for 3-palmitoyl-sn-glycerol, a $K_m = 2.27$ mM and a $V_{max} = 0.0029$ M min^{-1}. As shown in Fig. 6, oleuropein proved to be a competitive inhibitor with $K_i = 0.19$ mM for lipase activity.

4. Discussion

In recent years, the number of reports describing the beneficial properties of olive oil has increased. These reports suggest that the components in olive oil may have more health benefits than previously thought and consequently there have been numerous experiments investigating the fate of olive oil constituents. We focussed our attention on oleuropein, one of the major polyphenols present in olive oil. Although many researchers have investigated its positive effects on human health, here we report data regarding the effects that oleuropein has, in vitro, on enzymes involved in proteic, glucidic, and lipidic metabolism. Oleuropein has a positive effect on pepsin activity: in the presence of 0.84 mM oleuropein, an increase of V_{max} value from 0.0075 to 0.016 M min^{-1} was observed. Under these conditions, protein degradation stimulation occurs. On the other hand, oleuropein has an inhibitory effect toward trypsin enzymatic activity. In particular it proved to be a non-competitive inhibitor with a $K_i = 0.85$ mM.

Furthermore, the effect of this polyphenol toward lipase activity was assessed. Oleuropein was a non-competitive inhibitor toward lipase activity with a $K_i = 0.19$ mM. This effect resulted in a lower fatty acid liberation in the cell.

Glycerol, a product of lipase activity, could be transformed into glycerol-3-phosphate by glycerokinase and, moreover, into dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase. Otherwise, or besides, glycerol could be transformed into dihydroxyacetone by glycerol dehydrogenase.

We also investigated the effects that oleuropein showed toward enzymes involved in glycerol metabolism. In particular, its effects on glycerol dehydrogenase, glycerol-3-phosphate dehydrogenase and glycerokinase enzymatic activities were studied.

In all cases analyzed, an inhibitory effect of oleuropein was seen. In addition, oleuropein proved to be a non-competitive inhibitor toward these enzymes, and the inhibition constants were calculated.

Toward glycerol dehydrogenase, glycerokinase, and glycerol-3-phosphate dehydrogenase, oleuropein showed a $K_i = 0.019$ mM, 0.05 mM, and 0.0636 μM, respectively.

From these results, it is evident that oleuropein could be a metabolism modulator. In fact, we have reported that it inhibits the liberation of fatty acid from triglycerides and also has inhibitory effect also on the enzymes involved in glycerol transformation. At the same time, oleuropein stimulates pepsin enzymatic activity, resulting in a higher protein degradation.

The phenomenon is most important for Mediterranean populations, that have a diet with a high concentration of olive oil. The constitution of olive oil is primarily triacylglycerols and ~0.5–1.0% non-glyceridic constituents. Olive oil is also source of at least 30 phenolic compounds, its phenolic content depending on a number of factors but mainly on the production and storage of the oil (Brenes et al., 2001).

The oleuropein content in olive oil varies from 3.1 to 11 μg/g of olive oil, depending on the cultivar (Perri et al., 1999). Considering that these populations consume, in one day, about 35 g of olive oil, the oleuropein ingested is from 109 to 385 μg, a quantity that can have inhibitory or activatory effects on the enzymes assayed, comparable to what we have found in vitro. In particular, oleuropein seems to be an activator of protein digestion and inhibitor of triacylglycerol absorption. This last property could be useful for human health in order to control the body weight, but these results will require in vivo experiments.

Moreover, a relatively simple and new method for assay of the lipase activity was developed. This method could be adopted for daily practice in chemical–clinical analysis laboratories.

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