In vitro Inhibition of Pancreatic Lipase by Polyphenols: A Kinetic, Fluorescence Spectroscopy and Molecular Docking Study

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Summary

The inhibitory activity and binding characteristics of caffeic acid, p-coumaric acid, quercetin and capsaicin, four phenolic compounds found in hot pepper, against porcine pancreatic lipase activity were studied and compared to hot pepper extract. Quercetin was the strongest inhibitor (IC50=(6.1±2.4) μM), followed by p-coumaric acid ((170.2±20.6) μM) and caffeic acid ((401.5±32.1) μM), while capsaicin and a hot pepper extract had very low inhibitory activity. All polyphenolic compounds showed a mixed-type inhibition. Fluorescence spectroscopy studies showed that polyphenolic compounds had the ability to quench the intrinsic fluorescence of pancreatic lipase by a static mechanism. The sequence of Stern-Volmer constant was quercetin, followed by caffeic and p-coumaric acids. Molecular docking studies showed that caffeic acid, quercetin and p-coumaric acid bound near the active site, while capsaicin bound far away from the active site. Hydrogen bonds and π-stacking hydrophobic interactions are the main pancreatic lipase-polyphenolic compound interactions observed.

Key words: polyphenolic compounds, pancreatic lipase, enzymatic inhibition, molecular docking, anti-obesity effect

Introduction

Obesity is a global public health concern that has been described as a risk factor for diseases such as cardiovascular, hypertension, type II diabetes, and some forms of cancer (1). The seriousness of this concern is evidenced by the warning issued by the World Health Organization that 2.8 million people die every year because of obesity-related diseases (2). Several strategies have been developed to overcome this problem, among them the use of drugs and surgeries, or most frequently changes in alimentary habits. These changes include not only diets low in caloric intake, but also inclusion of foods rich in bioactive compounds that exert specific beneficial effects, such as polyphenolic compounds. Several mechanisms may explain the anti-obesity actions of polyphenolic compounds, including the interaction and inhibition of digestive enzymes like pancreatic...
lipase (EC 3.1.1.3) (3,4), an enzyme responsible for the hydrolisis of 50-70 % of ingested lipids (5).

Spices are examples of bioactive-rich foods that have been identified for their beneficial effects on human health (6). Pancreatic lipase inhibition has been reported by different polyphenolic compound-rich extracts from herbs and spices such as tea (Camellia sinensis) (7), anise myrtle (Syzygium anisatum) (8), Chinese liquorice (Glycyrrhiza uralensis) (9) and muscadine grapes (Vitis rotundifolia) (10). Inhibition of pancreatic lipase by individual polyphenolic compounds has also been reported, mainly as inhibition percentage and inhibition saturation kinetics, showing IC_{50} values (concentration of compound/sample required to inhibit enzyme activity by 50 %) (9,11). Among polyphenolic compounds, quercetin has been reported as one of the best pancreatic lipase inhibitors, while phenolic acids have shown low inhibitory activity (1,10). Sergent et al. (1) obtained IC_{50} values for quercetin and orlistat of (21.5±9.4) and (32.0±8.5) μM, respectively. Polyphenolic compounds are mostly found in food as glycosylated derivatives; however, the relevance of studying pure polyphenolic compounds in their form of aglycones comes from the fact that these compounds interact with the digestive enzymes on the brush border cells of the small intestine mainly as aglycones (12). Polyphenolic compounds extracted from hot peppers are recognized for their antioxidant activity (13), and anti-obesity properties, related to an increase in energy expenditure (14); however, no information about its possible inhibitory activity against pancreatic lipase has been reported. According to Chen and Kang (15), jalapeño hot peppers contain several phenolic acids such as p-coumaric and caffeic acids, and flavonoids like quercetin and luteolin. Hot peppers also contain capsaicin, a compound unique to the Capsicum species, and responsible for their pungency. Capsaicin is known for its anti-inflammatory properties (15), but barely studied for its ability to inhibit digestive enzymes. Interaction of quercetin with pancreatic lipase has been reported, but there are discrepancies on the reported results. Both competitive (10) and non-competitive (16) inhibition modes have been reported. In the case of phenolic acids such as caffeic acid, p-coumaric acid and capsaicin, as far as we know there are no reports describing the mechanism of their inhibition of pancreatic lipase.

The aim of the present study is to determine the inhibition mechanisms of some polyphenolic compounds (caffeic acid, p-coumaric acid and quercetin) present in jalapeño hot peppers towards pancreatic lipase by evaluating their IC_{50} values, kinetic parameters and intrinsic fluorescence, and by performing docking analysis to predict their interactions with residues of pancreatic lipase binding site. The inhibitory effect of a dry smoked jalapeño pepper polyphenolic extract was also analyzed.

Materials and Methods

Materials

Porcine pancreatic lipase type II, p-nitrophenyl laurate (pNPL), caffeic acid, p-coumaric acid, capsaicin, quercetin, Tris, bovine serum albumin (BSA) and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and ethanol were purchased from J.T.Baker (Center Valley, PA, USA). Orlistat capsules from Redustat® (Liomont® Laboratories, Toluca, Mexico) were purchased in a local drugstore.

**Extraction of polyphenolic compounds from dry smoked jalapeño pepper (chipotle)**

Extraction of polyphenolic compounds was carried out according to Moreno-Escamilla et al. (13), the phenolic content was determined by the Folin-Ciocalteu’s method, and results were expressed in μg of gallic acid equivalents (GAE) per g of extract.

**Pancreatic lipase activity assay**

Commercial pancreatic lipase was prepared according to McDougall et al. (17) with some modifications. Pancreatic lipase was dissolved in 20 mM Tris buffer (pH=7.2) at the concentration of 2.0 mg/mL, centrifuged at 1411×g in Eppendorf 5810R centrifuge (Eppendorf AG, Hamburg, Germany) for 20 min, and the supernatant was filtered in a polyvinylidene fluoride (PVDF) membrane filter (0.22 μm; Durapore®, Hercules, CA, USA). Purity of the lipase and co-enzyme B was estimated by SDS-PAGE analysis (93 % purity; EDAS 290, Kodak®, New York, NY, USA). Protein concentration was determined by Bradford method (18), reading the absorbance of cationic form of the dye Coomassie Blue G250 at 595 nm, and results were expressed in mg/mL using bovine serum albumin (BSA; 1 mg/mL) as standard. Pancreatic lipase substrate (pNPL, 0.08 % mass per volume) was dissolved in 5 mM sodium acetate solution (pH=5.0, 1 % Triton X-100). Final pNPL concentrations were 11.7-124.2 μM. Caffeic acid, p-coumaric acid and capsaicin were dissolved separately at three different concentrations (25, 50 and 75 μg/mL) in methanol. Due to its lower solubility, quercetin was dissolved in ethanol at the same concentrations. An Orlistat solution was prepared at 1.2 mg/mL in methanol (19). Stability of the compounds in the solvent was tested for the time of assay by measuring their UV-Vis spectra (Agilent 8453 HP UV-Vis spectrometer G1103A; Agilent Technologies, Santa Clara, CA, USA).

The control assay contained 500 μL of Tris 200 mM working buffer solution (pH=8.2), 260 μL of distilled water, 140 μL of pancreatic lipase solution and 40 μL of substrate solution. For the inhibition studies, 60 μL of each polyphenolic compound (or extract) and 200 μL of distilled water were used. The substrate was added to start the reaction, then the absorbance was recorded at 400 nm during 1000 s at 37 °C. All samples were assayed in triplicate.

The inhibition percentage was calculated according to Dalar and Konczak (20) from the measured absorbances of control and sample (endpoint):

\[
\text{Inhibition percentage} = \left( \frac{A_{bs} - A_b}{A_{bs} - A_s} \right) \times 100
\]

where \(A_{bs}\) is the absorbance of the control blank, \(A_b\) is the absorbance of the control, \(A_{sb}\) is the absorbance of the sample blank and \(A_s\) is the absorbance of the sample. Blanks were prepared without the pancreatic lipase solution and the substrate solution, respectively. IC_{50} was cal-
culated from a substrate concentration versus inhibition percentage plot.

Accurate Kᵢ values of polyphenolic compounds were determined by calculating the apparent maximum reaction rate (vₘₐₓ) and Michaelis-Menten constant (Kᵢ) by both linear (Lineweaver-Burk) and non-linear (Michaelis-Menten) analyses. First, apparent vₘₐₓ and Kᵢ values were obtained from the least-square regression lines by plotting the reciprocal of the substrate concentration [S] against the reciprocal of the enzyme reaction velocity as in the work by Alvarez-Parrilla et al. (21):

\[
\frac{1}{v_o} = \frac{1}{v_{\text{max}} + K_i \cdot [S]} \quad /2/ 
\]

These values were used as initial values for the determination of the parameters by non-linear analysis using Sigma Plot v. 13.0 (22), by fitting the value of the experimental initial reaction rate (vₒ) as a function of the initial inhibitor concentration to the Michaelis-Menten equation (Eq. 3). The vₒ values were obtained from a linear curve fitting (slopes) of a time-course plot of the enzymatic reaction:

\[
v_o = \frac{v_{\text{max}} \cdot [S]}{K_m + [S]} \quad /3/ 
\]

Kᵢ and Kᵢ' (dissociation constant of free enzyme and enzyme-substrate complex, respectively) values for a mixed-type inhibition were obtained from the following equations (23):

\[
\begin{align*}
K_{\text{m',i}} &= \frac{K_m}{v_{\text{max}}'}, \quad /4/ \\
v_{\text{max}}' &= \frac{v_{\text{max}} \cdot [I]}{1 + K_i} \quad /5/ 
\end{align*}
\]

where apparent Kₘ' and vₘₐₓ' are the values in the presence of the inhibitor, and [I] is the inhibitor concentration. These calculations were done for each inhibitor concentration.

Polyphenolic compound-pancreatic lipase interaction measured by fluorescence spectra

Different volumes of caffeic acid, p-coumaric acid, quercetin and capsaicin (or extract) at 0.0125 M were added into a 10-mL flask (final concentrations 0.025-0.25·10⁻⁴ M). A volume of 1.0 mL of the pancreatic lipase solution (10⁻⁴ M) was added, and the solution was diluted to 10 mL with the working buffer and incubated at 37 °C for 1 h in darkness. Emission fluorescence spectra were measured in the 300-550 nm interval, at a fixed excitation wavelength of 280 nm in a RF 5301-PC spectrophotometer (Shimadzu, Columbia, MD, USA). Even though the intrinsic fluorescence of pancreatic lipase is attributed to three amino acid residues, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp), the fluorescence of Phe and Tyr residues at this wavelength is negligible, so intrinsic fluorescence of the enzyme is mainly due to Trp (24). The polyphenolic compounds do not exhibit Förster resonance energy transfer (FRET) at that excitation wavelength (25). The Stern-Volmer equation was used to determine the presence of a quenching mechanism, by plotting F₀/F against [Q]:

\[
\frac{F_0}{F} = 1 + k_q \cdot \frac{[Q]}{[S]} = 1 + K_{\text{sv}} \cdot [Q] \quad /6/ 
\]

where F₀ and F are the fluorescence intensities at the maximum λₘₐₓ in the absence and presence of an interacting molecule (polyphenol), respectively, and [Q] is the molar concentration of the interacting molecule. The kₚ is the bimolecular quenching constant, τ₀ is the fluorescence lifetime in the absence of the quencher, and Kₛ is the Stern-Volmer quenching constant (26). A τ₀ of 1.59 ns was used for pancreatic lipase, according to previously published results (27).

Molecular docking

The crystal structure of pancreatic lipase (Protein Data Bank (PDB) (28) code 1ETH (29)) was used as template for the generation of pancreatic ligand complex models. Ligand structures: caffeic acid, p-coumaric acid and quercetin were obtained from crystallographic complexes with different proteins (PDB codes: 4EYQ (30), 1D7E (31) and 1E8W (32)). Capsaicin structure was generated and minimized using PyMOL (33). The docking analyses were performed with AutoDock Vina using the interface installed in UCSF Chimera (34) run with the default parameters and a search volume of approximately the same size as pancreatic lipase. The crystal structure of pancreatic lipase was considered rigid, and polyphenolic compounds and capsaicin structures were considered flexible during docking. The possible interactions of the best-scored solution of the models for each ligand were analyzed.

Statistical analysis of the enzyme kinetics and the binding experiments

All the experimental assays were run in triplicates. Results are expressed as mean values±standard deviation. Analysis of variance and Fisher’s least significant difference analysis were performed by SPSS v. 20 software (35) for the determination of statistical differences between treatments.

Results and Discussion

Pancreatic lipase activity

The effect of four compounds: caffeic acid, p-coumaric acid, quercetin and capsaicin (Fig. 1), commonly found in hot pepper, and the hot pepper extract, on the activity of pancreatic lipase were monitored at saturation conditions, using 62.1 μM pNPL. Enzyme inhibition percentage increased in a hyperbolic trend, as the concentration of the polyphenolic compounds increased. p-Coumaric acid and capsaicin appear to have weak inhibition power, since lower inhibition was observed even at higher concentrations.
(data not shown). The hot pepper extract showed no pancreatic lipase inhibition (36). To compare the inhibitory capacity of the analyzed compounds, IC\textsubscript{50} values were determined for caffeic acid, p-coumaric acid, quercetin and orlistat (Table 1). Caffeic acid had the highest IC\textsubscript{50}, followed by p-coumaric acid and finally quercetin, which showed an IC\textsubscript{50} statistically similar to that of orlistat. In agreement with these results, You \textit{et al.} (10) observed that quercetin had lower IC\textsubscript{50} (276.8 µM) than ellagic acid (342.4 µM). Both quercetin and ellagic acid have the same molecular mass of 302.2 g/mol, respectively; however, their molecular structures are different (Figs. 1c and d). Quercetin, which is a flavonoid, has two aromatic rings in its structure and one heterocyclic ring in a linear structure, while ellagic acid possesses two aromatic rings and two heterocyclic rings, presenting a more rigid structure. This emphasizes the relevance of the structure of flavonoid in comparison with other polyphenolic structures (37). For example, the differences between ellagic acid and quercetin might be associated with their different inhibitory activities against pancreatic lipase (17). Differences between phenolic acids and flavonoids such as quercetin may be responsible for our obtained different inhibitory capacities. Similar to our results, where phenolic acids had higher IC\textsubscript{50} values than quercetin, Sergent \textit{et al.} (1) observed that quercetin IC\textsubscript{50} (21.5±9.4 µM) was lower than that of ferulic acid.

![Image of chemical structures](image_url)

**Fig. 1.** The chemical structures of polyphenolic compounds: a) caffeic acid, b) p-coumaric acid, c) quercetin, d) ellagic acid, and e) ferulic acid; and the compounds: f) capsaicin, and g) orlistat. Elements (C, H and O) of caffeic acid, p-coumaric acid, quercetin and capsaicin for the molecular docking analysis are indicated.
Table 1. Pancreatic lipase apparent catalytic parameters \((v_{\text{max}}, K_m, K_i, \text{and } IC_{50})\) and IC\(_{50}\) of the hydrolysis of \(p\)NPL in the presence of different polyphenolic compounds, capsaicin and orlistat

| Compound      | \(c/\mu\text{M}\) | \(v_{\text{max}}/(\mu\text{M/s})\) | \(K_m/\mu\text{M}\) | \(K_i/\mu\text{M}\) | \(IC_{50}/\mu\text{M}\) |
|---------------|------------------|-----------------------------------|---------------------|---------------------|---------------------|
| \(p\)NPL (control) | 11.7-124.2        | (0.11±0.03)\(^a\)                 | (1.96±0.20)\(^a\)   | n.d.                | n.d.                |
| Caffeic acid  | 300.21           | (0.10±0.02)\(^a\)                 | (0.30±0.06)\(^a\)   | (370.2±18.4)\(^a\)  | (388.2±30.2)\(^a\)  |
| \(p\)-Coumaric acid | 275.00          | (0.10±0.01)\(^a\)                 | (0.95±0.10)\(^a\)   | (205.7±44.1)\(^b\)  | (290.7±39.7)\(^b\)  |
| Quercetin     | 14.53            | (0.10±0.02)\(^a\)                 | (2.15±0.40)\(^a\)   | (12.0±4.4)\(^a\)    | (17.9±6.9)\(^a\)    |
| Capsaicin     | 40.47            | (0.10±0.02)\(^a\)                 | (1.80±0.19)\(^b\)   | (66.0±14.3)\(^b\)   | (50.7±13.0)\(^b\)   |
| Orlistat      | 9.68             | (0.09±0.03)\(^a\)                 | (0.30±0.05)\(^b\)   | (16.9±4.8)\(^b\)    | (19.0±2.8)\(^b\)    | (4.0±1.0)\(^b\) |

The data are presented as mean values±standard deviation of triplicate analyses. Different letters in the same row indicate statistically significant differences compared to control, or between treatments for dissociation constant of free enzyme and enzyme-substrate complex \((K_i, \text{and } IC_{50})\). \(K_m=\text{Michaelis-Menten constant, n.d.=not determined}\)

\((123.9±13.4) \mu\text{M}; \text{Fig. 1e}) \text{ and not significantly different from Orlistat (32.0±8.5) \mu\text{M}, detected using 4-methylumbelliferone as substrate.} \text{In contrast, no significant effect of the extract on pancreatic lipase activity was observed. Similar results have been reported for black chokeberry (Aronia melanocarpa L.) extracts (38). However, other studies have shown high to moderate pancreatic lipase inhibition by plant extracts such as cumin (Cuminum cyminum) (39), green tea (Camellia sinensis) (40), muscadine grape (Vitis rotundifolia) (10), and other edible plants, for example Beijing grass (Murdannia loriiformis) (41). Here, the lack of pancreatic lipase-inhibitory effect could be explained by the difference in the content of bioactive compounds compared to other extracts with high inhibitory activity, or by the synergistic/antagonistic effects among bioactive compounds in the extracts (38). According to Chen and Kang (15), hot pepper extracts contain high mass fractions (per dry mass) of \(p\)-coumaric acid ((4.56±0.11) mg/g) and capsaicin ((9.05±0.05) mg/g), both of them with low pancreatic lipase-inhibitory activity, which may explain the low effect of the extract. Another phenolic acid mainly found in hot peppers is caffeic acid ((1.32±0.08) mg/g), while quercetin is the major flavonoid ((8.86±1.03) mg/g). The observed \(IC_{50}\) values of \(p\)-coumaric acid, caffeic acid and quercetin were compared to their content found by Chen and Kang (15). We found that only quercetin had a higher content than its \(IC_{50}\) value (almost 5-fold), whereas phenolic acids had lower content than their \(IC_{50}\) values (i.e. almost 6-fold for \(p\)-coumaric acid). In this way, the absence of the inhibitory effect of the hot pepper extract may be explained by the low content of capsaicin and phenolic acids (lower than their \(IC_{50}\) values).

The effects of caffeic acid, \(p\)-coumaric acid, quercetin and capsaicin on the enzyme kinetics of pancreatic lipase using \(p\)NPL as substrate were evaluated, and results are shown in Fig. 2, where the initial velocity \(v_0\) shown as absorbance change \((\lambda=400 \text{ nm})\) in arbitrary units per second, versus substrate concentration (\(\mu\text{M}\)) was plotted. Even though all compounds had a significant effect (p<0.05) on \(v_0\) compared to control, quercetin seemed to be the best inhibitor. To characterize the inhibitory pattern, both linear and non-linear analyses of inhibition curves of each compound and the extract were performed. Linearized plots indicated a mixed-type inhibition, in agreement with other authors (5,10,26), and suggested two inhibition components, competitive and non-competitive. Next, non-linear regression fit of data to Eqs. 3–5 was performed to obtain the catalytic parameters of pancreatic lipase in the presence and absence of polyphenolic compounds (Table 1). Significant differences in apparent \(K_m\) values with respect to control were observed among all compounds. The apparent \(v_{\text{max}}\) values showed no significant differences with respect to control. Similar apparent \(v_{\text{max}}\) and smaller apparent \(K_m\) values of quercetin than of control support the idea that a mixed-type inhibition regulates the process (42). Phenolic acids possessed significantly lower apparent \(K_m\) values than control. Quercetin had an apparent \(K_m\) higher than control, and capsaicin did not exhibit statistically significant differences compared to control. According to Zhang et al. (43), a higher molecular mass structure might be beneficial for decreasing enzymatic activity (higher apparent \(K_m\) value), which can be attributed for example to the increase of hydroxyl group in the structure of quercetin with respect to phenolic acids. Narita and Inouye (42) observed a mixed-type inhibition when using \(p\)-coumaric acid as inhibitor.
of pancreatic α-amylase activity and explained that lower apparent $K_w$ values in the presence of inhibitors meant that the binding affinity of the substrate and the inhibitor for their respective sites increases when both are present ($p$-coumaric acid and substrate).

Lower $K_i$ than $K_i'$ values of the polyphenolic compounds (caffeic acid, $p$-coumaric acid and quercetin) were observed. These lower $K_i'$ values indicate that the inhibitor has more affinity for the enzyme-substrate complex than for the free enzyme in a mixed inhibition mechanism (42). The competitive component of mixed inhibition prevailed in the systems. All tested compounds except capsaicin had $K_i$ values for the polyphenolic compounds was quercetin, caffeic acid, $p$-coumaric acid and capsaicin. Our results agree with those of Li et al. (16), who studied the interaction of quercetin, isoquercetin and rutin with pancreatic lipase and observed that the sequence of $K_w$ values did not correspond to the inhibition sequence. Also, our $K_w$ value $(4.5\pm0.7)\times10^{-4}$ M$^{-1}$ for quercetin was similar to the value obtained by Li et al. (16) of $4.95\times10^{-4}$ M$^{-1}$. Contrarily to the calculated $K_w$ values observed by Gonçalves et al. (48) of pancreatic lipase and procyanidin fractions from grape seed, Li et al. (16) did not find a relation between the polyphenolic structures with higher molecular mass or higher number of hydroxyl groups, and higher $K_w$ values.

The calculated $k_q$ values were higher than $2.0\times10^{-10}$ M$^{-1}$s$^{-1}$, the maximum value for a dynamic mechanism in aqueous medium (16,26). This indicates that the quenching process is an intrinsic and static process, involving only Trp residues (5,16,26). Static quenching has been previously reported for the interaction of pancreatic lipase with flavonoids, e.g. quercetin (16). This type of quenching is characterized by the formation of a non-fluorescent complex between the enzyme and the ligand, in which the enzyme segment contains the Trp residue (46). Thus, the observed changes in the maximum emission intensity reflected the direct interactions of the analyzed compounds with the ar-

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**Fig. 2.** Effect of caffeic acid, $p$-coumaric acid, quercetin and capsaicin on the hydrolysis of p-nitrophenyl laureate (pNPL) catalyzed by pancreatic lipase. Symbols represent experimental initial reaction rate ($v_0$ (expressed as $\Delta A_{405\text{nm}}$ s$^{-1}$))±standard deviation. The lines are Michaelis-Menten curves fitted to experimental data. Asterisk represents statistical difference between treatments (Fisher’s least significant difference analysis, $p<0.05$)
Table 2. Stern-Volmer and bimolecular quenching constant values of the pancreatic lipase interaction with caffeic acid, p-coumaric acid, quercetin and capsaicin

| Compound         | $K_s/(10^{-4}\text{M}^{-1})$ | $R^2$   | $k_q/(10^{-13}\text{M}^{-1}\text{s}^{-1})$ | $\Delta F/\text{AU}$ |
|------------------|-----------------------------|--------|------------------------------------------|----------------------|
| Caffeic acid     | $(3.7\pm1.2)^{ab}$          | 0.9798 | $(2.3\pm0.8)^{ab}$                       | $(19.1\pm1.8)^{a}$   |
| p-Coumaric acid  | $(2.0\pm0.6)^{ab}$          | 0.9089 | $(1.3\pm0.2)^{ab}$                       | $(18.7\pm2.3)^{a}$   |
| Quercetin        | $(4.5\pm0.5)^b$             | 0.9699 | $(2.8\pm0.1)^{a}$                       | $(18.1\pm2.0)^{a}$   |
| Capsaicin        | $(0.40\pm0.01)^c$           | 0.9885 | $(0.25\pm0.03)^{c}$                     | $(6.6\pm2.2)^{c}$    |

$\Delta F$ corresponds to the difference between registered fluorescence intensity of analyzed compounds (caffeic acid, p-coumaric acid, quercetin and capsaicin) at c=1 and 25 µM. The data are presented as mean values-standard deviation of triplicate analyses. Different letters in the same row indicate statistically significant values (Fisher’s least significant difference analysis, p<0.05). $K_s=$Stern-Volmer quenching constant, $k_q=$bimolecular quenching constant, $F=$fluorescence intensity.

**Fig. 3.** Effect of polyphenolic compounds on fluorescence spectra of pancreatic lipase: a) emission fluorescence spectra of pancreatic lipase (2.0 mg/mL) in the presence of different concentrations of caffeic acid (final concentrations: 0, 0.025, 0.05, 0.10, 0.125, 0.15, 0.175, 0.20, 0.225 and 0.25·10^{-4} M), $\lambda_{em}=280$ nm, and b) Stern-Volmer plot (Eq. 6) for the experimental data of pancreatic lipase fluorescence ($\lambda_{em}=354$ nm) at different caffeic acid concentrations as quencher [Q]. $F_0$ and $F=$fluorescence intensities at the maximum of $\lambda_{em}$ in the absence and presence of the polyphenolic compounds.
Fig. 4. Crystal structure of pancreatic lipase (Protein Data Bank code 1ETH (28)) model of the binding sites for caffeic acid, p-coumaric acid, quercetin and capsaicin achieved by docking experiments: a) surface representation of the overall three-dimensional structure of pancreatic lipase (shown in dark grey colour at www.ftb.com.hr) indicating the active site and the potential binding sites for polyphenolic compounds (b-e). Specific interactions of: b) caffeic acid, c) p-coumaric acid, d) quercetin, and e) capsaicin. Proposed binding amino acids are pointed out (shown in green colour at www.ftb.com.hr). Hydrophobic bonds and polar interactions are drawn in dotted lines (shown as blue and green dotted lines, respectively, at www.ftb.com.hr)
near Asp\textsuperscript{80}, arginine\textsuperscript{257} (Arg\textsuperscript{257}), valine\textsuperscript{260} (Val\textsuperscript{260}) and alanine\textsuperscript{261} (Ala\textsuperscript{261}) (Fig. 4d). In the capsaicin-pancreatic lipase complex, capsaicin interacted mainly with leucine\textsuperscript{41} (Leu\textsuperscript{41}), lysine\textsuperscript{2} (Lys\textsuperscript{2}), Arg\textsuperscript{65}, Glu\textsuperscript{371} and Tyr\textsuperscript{404} (Fig. 4e). This different binding region for capsaicin, with respect to the polyphenolic compounds, could be attributed to the different structure of capsaicin (53), shown in Fig. 1. In this way, the non-competitive component of capsaicin could be in this site. Even though the polyphenolic compound binding sites were in the vicinity of the catalytic site, the small fluorescence shift may be attributed to the fact that no significant polarity changes were predicted around Trp residues.

Table 3. Binding of compounds to pancreatic lipase and details of interactions

| Ligand atom | Residue atom | Residue number | Distance/Å | Type      |
|-------------|--------------|----------------|------------|-----------|
| Caffeic acid |              |                |            |           |
| OE1         | NE           | His\textsuperscript{264} | 3.0        | Polar     |
| OE1         | OG           | Ser\textsuperscript{153} | 3.1        | Polar     |
| C3          | C2           | Phe\textsuperscript{78}  | 3.7        | Hydrophobic |
| O1          | C1           | Tyr\textsuperscript{115} | 4.8        | Hydrophobic |
| O1          | C3           | Tyr\textsuperscript{115} | 3.9        | Hydrophobic |
| O1          | C4           | Tyr\textsuperscript{115} | 3.9        | Hydrophobic |
| C1          | C2           | Pro\textsuperscript{101} | 4.1        | Hydrophobic |
| C2          | C2           | Pro\textsuperscript{101} | 4.1        | Hydrophobic |
| C5          | C2           | Phe\textsuperscript{216} | 4.1        | Hydrophobic |
| C4          | C1           |                | 3.8        | Hydrophobic |
| p-Coumaric acid |        |                |            |           |
| OE1         | NE           | His\textsuperscript{264} | 3.3        | Polar     |
| OE1         | NB           |                | 3.3        | Polar     |
| C3          | C2           | Phe\textsuperscript{78}  | 3.5        | Hydrophobic |
| C6          | C1           | Tyr\textsuperscript{115} | 3.6        | Hydrophobic |
| C1          | C5           |                | 4.0        | Hydrophobic |
| C1          | C2           | Phe\textsuperscript{216} | 3.9        | Hydrophobic |
| OE2         | C5           |                | 3.8        | Hydrophobic |
| Quercetin   | O3'          | OD1            | 3.0        | Polar     |
| O3'         | NH2          | Arg\textsuperscript{217} | 3.1        | Polar     |
| O4'         | NH1          |                | 3.1        | Polar     |
| C5'         | CH3          | Val\textsuperscript{26} | 3.8        | Hydrophobic |
| C6'         | CH3          | Ala\textsuperscript{261} | 3.6        | Hydrophobic |
| O3          | NE           | His\textsuperscript{264} | 3.2        | Polar     |
| O4          | C2           | Phe\textsuperscript{216} | 3.7        | Hydrophobic |
| C5          | C1           |                | 3.9        | Hydrophobic |
| C6          | C2           |                | 3.9        | Hydrophobic |
| C7          | C1           |                | 3.9        | Hydrophobic |
| O7          | C3           | Tyr\textsuperscript{115} | 4.1        | Hydrophobic |
| C8a         | C2           | Phe\textsuperscript{78}  | 3.5        | Hydrophobic |
| Capsaicin   | O2           | EO1            | Glu\textsuperscript{371} | 2.9 | Polar     |
| O2          | OG           |                | 2.7        | Polar     |
| O1          | OG           |                | 4.3        | Polar     |
| O1          | NH           | Arg\textsuperscript{65} | 3.8        | Polar     |
| C6          | NH1          |                | 3.8        | Hydrophobic |
| O8          | NE           | Lys\textsuperscript{62} | 3.1        | Polar     |
| C11         | CE1          | Leu\textsuperscript{41} | 3.8        | Hydrophobic |
| C11         | CE2          |                | 3.7        | Hydrophobic |
| C15CH3      | CE2          |                | 3.9        | Hydrophobic |

His=histidine, Ser=serine, Phe=phenylalanine, Tyr=tyrosine, Pro=proline, Asp=aspartic acid, Arg=arginine, Val=valine, Ala=alanine, Glu=glutamic acid, Lys=lysine, Leu=leucine
He et al. (7) proposed that the binding forces between polyphenolic compounds and pancreatic lipase were mainly hydrophobic interactions. These hydrophobic and non-covalent interactions are mentioned in Table 3 and shown in Figs. 4b-e (blue dotted lines; colour version available at www.ftb.com.hr). Among them, π-stacking interactions are formed between aromatic rings from polyphenolic compounds and pancreatic lipase aromatic amino acids, Phe and Tyr. Our results are in agreement with those reported by Kim et al. (54), which indicated that hydrophobic bonds (with residues like Phe) were the main interactions found between polyphenolic compounds and pancreatic lipase. Another hydrophobic bond predicted by the docking analysis was the hydrogen bond between the hydroxyl groups of polyphenolic compounds and polar groups of pancreatic lipase (5). Fewer polar interactions in Fig. 4 (shown as green dotted lines; see www.ftb.com.hr) were observed for the polyphenolic compound-pancreatic lipase complexes. Quercetin-pancreatic lipase complex presented more polar interactions compared to the rest of the polyphenolic compound-pancreatic lipase complexes.

The quercetin size and structural rigidity could be responsible for its binding characteristics (more polar interactions) and consequently its higher pancreatic lipase inhibitory potency. You et al. (10) reported a higher inhibitory potency (lower IC50) of quercetin than of ellagic acid (Fig. 1). Böhl et al. (55) described that quercetin (among other flavonoids) presented higher affinity towards proteins due to structural characteristics. Quercetin possesses characteristics such as catechol structure in B ring and double bond between C2 and C3 in C ring. Other factors such as number of aromatic rings and number of free hydroxyl groups present in their structure are associated with the ability of polyphenolic compounds to interact with proteins (37,56).

ΔF values calculated from fluorescence studies for polyphenolic compound-pancreatic lipase interactions could be associated with their binding with three amino acids close to Trp residues. According to docking results, caffeic acid, p-coumaric acid and quercetin bound with Phe29 and His264, found near Trp residues located at positions 86 and 252. In contrast, the capsaicin-pancreatic lipase complex was located in the vicinity of only one Trp residue (Trp39, which is close to Leu41 and Lys43). This suggested that polyphenolic compounds have a higher effect on protein conformation (Trp environment) than capsaicin and may explain the lower ΔF, Kf, and Ks values obtained for the latter in the fluorescence studies.

Caffeic acid showed high π-stacking interactions with Phe29, Tyr135, Pro138 and Phe216, and two polar bonds with Ser53 and His264. A total of ten possible bonds between this polyphenolic compound and pancreatic lipase were observed. It seems that caffeic and p-coumaric acids showed an ability to form a complex with pancreatic lipase (no significant differences between Kf and Ks values) similar to that observed for quercetin, but through less polar interactions (2 against 4 or 5 observed for the other studied compounds). This higher number of hydrophobic interactions present in the phenolic acid-pancreatic lipase complex, instead of the more polar interactions present in quercetin-pancreatic lipase complex, could explain the lower inhibitory activity of the phenolic acids. Notwithstanding, further studies regarding the phenolic acid-pancreatic lipase binding are needed.

Conclusion

In summary, all the tested polyphenolic compounds showed mixed-type inhibition of pancreatic lipase, quercetin being the strongest inhibitor tested, with IC50 similar to that of orlistat. Phenolic acids showed intermediate inhibitory activity, while capsaicin and the jalepeño pepper extract showed the lowest inhibition. Binding studies showed that polyphenolic compounds might form a complex with pancreatic lipase throughout a static quenching mechanism. Molecular docking analysis showed that all compounds except capsaicin bound close to the active site of pancreatic lipase and that hydrogen bonds and π-stacking interactions were the main polyphenolic compound-pancreatic lipase interactions. This study confirmed stronger inhibitory activity of quercetin than of non-flavonoids, and pointed out that the reported anti-obesity effects of polyphenolic compounds or hot pepper extracts are probably unrelated to pancreatic lipase inhibition. Also, it showed that flavonoids and phenolic acids bind to similar sites in pancreatic lipase but subtle differences in binding may account for their different inhibitory potencies.

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