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Understanding phenolic acids inhibition of α-amylase and α-glucosidase and influence of reaction conditions

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ABSTRACT

Phenolic acids are involved in modulating the activity of starch digestive enzymes but remains unclear if their interaction with enzymes or starch is governing the inhibition. The potential inhibition of nine phenolic acids against α-amylase and α-glucosidase was studied applying different methodologies to understand interactions between phenolic acids and either enzymes or substrates. Vanillic and syringic acids were prone to interact with α-amylase requiring low half-maximum inhibitory concentration (IC_{50}) to inhibit starch hydrolysis. Nevertheless, the initial interaction of phenolic acids with starch somewhat obstructed their interaction with starch, requiring 10 times higher IC_{50}, with the exception of chlorogenic and gallic acid. The study demonstrates that 10% of the phenolic acids were retained during starch gelatinization. Those effects were not really evident with α-glucosidase, likely due to the small molecular size of maltose substrate. Phenolic acids with > 1 hydroxyl group like caffeic and protocatechuic acids showed the lowest IC_{50} against α-glucosidase.

1. Introduction

In the last years, several plant-based products have been reported to inhibit the activity of α-amylase and α-glucosidase, the digestive enzymes that catalyzes the starch breakdown in the digestive tract (Sun, Wang, & Miao, 2020). Vinayagam, Jayachandran, and Xu (2016) reviewed the antidiabetic *in vivo* effect of simple phenolic acids such as gallic, protocatechuic, ellagic, syringic or salicylic acids, owing to their role on glucose and insulin receptor function, both of which play an essential role in diabetes. Extracts from different fruits and vegetables (Papoutsis et al., 2021), teas (Kwon, Apostolidis, & Shetty, 2008) or seaweeds (Lordan, Smyth, Soler-Vila, Stanton, & Ross, 2013) have been investigated for their potential inhibitory action towards α-amylase and α-glucosidase. Most of those studies use crude or purified extracts, and the inhibitory activity has been attributed to polyphenol type compounds. Polyphenols are a large and heterogeneous group of phytochemicals present in plant-based foods and an essential part of human diet (Martinez-Gonzalez et al., 2017). Phenolic acids are one of the most common polyphenols, comprising aromatic phenols of secondary plant metabolites with a carboxylic acid functional group.

Their inhibitory activity against α-amylase and α-glucosidase enzymes has been related with their structure, that allow them to interact with the enzyme or the substrate of the reaction (Sun, Warren, & Gidley, 2019). Numerous studies have described the *in vitro* inhibition of α-amylase and α-glucosidase induced by phenolic compounds, particularly focusing on characterizing new extract sources like seaweed and black legumes (Lordan et al., 2013; Tan, Chang, & Zhang, 2017). For instance, different legume fractions from black soybean were able to inhibit α-amylase and α-glucosidase, with IC_{50} from 0.25 to 2 mg/mL and 0.25 to > 1000 μg/mL, respectively (Tan et al., 2017). It has been previously confirmed the inhibitory action of ferulic acid against α-amylase (IC_{50} of 0.622 mg/mL) and α-glucosidase (0.866 mg/mL) (Zheng et al., 2020), and chlorogenic acid inhibition against α-amylase (IC_{50} of 0.498 mg/mL) (Zheng et al., 2020).

However, rather different conditions have been used for testing their inhibitory activities. Concerning digestive enzymes, some *in vitro* studies try to simulate the *in vitro* starch hydrolysis using human salivary or pancreatic α-amylase, and rat small intestinal α-glucosidase (Tadera, Minami, Takamatsu, & Matsuoka, 2006). However, due to the difficulties to employ these enzymes, porcine pancreatic α-amylase and yeast α-glucosidase are mostly used. As regards the substrate of the reaction, chemical compounds can be used such as p-nitrophenyl maltoheptaoside (BPNPG7) and 4-nitrophenyl-β-D- glucopyranoside (PNPG) for α-amylase and α-glucosidase, respectively (Tadera et al., 2006; Zheng

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et al., 2020). Nevertheless, other compounds like starch and maltose are closely resembling human body reactions (Kato-Schwartz et al., 2020; Sun et al., 2020). In fact, starch is the most extensively used substrate to analyze the *in vitro* inhibition induced by rich polyphenol plant-based extracts, generally mixing the polyphenols with the enzyme solution before the addition of the substrate (Sun et al., 2020). However, considering that polyphenols can either interact with the enzyme or the starch (Zhu, 2015), the order of addition of these components into the reaction might influence the results. Actually, Mkandawire et al. (2013) showed higher inhibitory effect when tannins extracted from sorghum were incubated with α-amylase before adding the substrate, waxy maize starch. Conversely, Camelo-Méndez, Agama-Acevedo, Tovar, and Bello-Pérez (2017) showed greater amylase inhibition when polyphenol rich extracts from blue maize flour were incubated with starch before adding α-amylase. Differences encountered among the reported studies might be ascribed to the experimental conditions used, stressing the importance of evaluating the effect of those conditions on the enzymatic activities.

Previous studies for inhibiting α-amylase or α-glucosidase with polyphenols have been reported either using different substrates or extracts as major polyphenol sources, which does not allow to identify and compare the inhibitory ability of each polyphenol on those enzymes. Therefore, the initial hypothesis is that the polyphenols interaction with substrates or enzymes (α-amylase and α-glucosidase) might be associated with the polyphenol chemical structure. Hence, the objective was to study the *in vitro* inhibitory effect of nine pure phenolic acids with diverse chemical structure, against α-amylase and α-glucosidase. For that purpose, different model systems that included: i. incubation of phenolic acids with the enzymes previous to substrate addition, ii. incubation of phenolic acids with the gelatinized starch, iii. Incubation of phenolic acids with the starch previous gelatinization.

2. Materials and methods

2.1. Materials

Type VI-B α-amylase from porcine pancreas (EC 3.2.1.1) (8 U/mg), type I α-glucosidase from *Saccharomyces cerevisiae* (11 U/mg), D-(+)-maltose, 3,5-dinitrosalicylic acid (DNS), acarbose and native starch from wheat that contained 8.8–11.5% moisture, and < 0.3% protein according to product specifications were obtained from Sigma Aldrich (Sigma Chemical, St.Louis, USA). D-glucose Assay Kit (GOD/POD) was obtained from Megazyme (K-GLUC 08/18, Megazyme International Ireland Ldt., Bray, Co. Wicklow, Ireland). Nine phenolic acids were analyzed: caffeic acid, ferulic acid, gallic acid and protocatechuic acid were from Acros Organic (Acros Organic BVBA, Geel, Belgium); p-coumaric acid and syringic acid were from Alfa Aesar (Alfa Aesar Co., Inc., Ward Hill, USA); sinapic acid and vanillic acid were from Fluka (Fluka Analytical, Buchs, Switzerland) and chlorogenic acid were from Sigma Aldrich (Sigma Chemical, St. Louis, USA). The molecular structures of phenolic acids are represented in Fig. 1. Other chemicals were of analytical grade. Solutions and standards were prepared using deionized water.

2.2. Inhibition assays of α-amylase

The inhibition assay of α-amylase was adapted from different studies (Tan et al., 2017; Zheng et al., 2020). Wheat starch (6.25 mg/mL) was prepared in sodium phosphate buffer (0.02 M, pH 6.9 with 6 mM NaCl), followed by gelatinizing the solution at 100 °C for 20 min. Enzymatic reaction contained: 0.05 mL of phenolic compounds dissolved in ethanol (20%, v/v) at different concentrations, 0.05 mL of α-amylase (50 U/mL) and 0.4 mL of gelatinized wheat starch. Three methodologies were carried out varying the order of substrates addition: ($M1_{AM}$) Enzyme and polyphenol solutions were mixed in a Vortex and preincubated at 37 °C for 10 min. Then, gelatinized starch was added, and the mixture was incubated at 37 °C for 10 min. ($M2_{AM}$) Gelatinized starch and polyphenol were mixed in a Vortex and preincubated at 37 °C for 10 min. Then, enzyme solution was added, and the mixture incubated at 37 °C for 10 min. ($M3_{AM}$) Polyphenol was mixed with granular starch and subjected to heating for starch gelatinization. After cooling down to room temperature, enzyme solution was added, and the mixture was incubated at 37 °C for 10 min.

To stop the reaction, 0.5 mL of 3.5-dinitrosalicylic acid (DNS) color

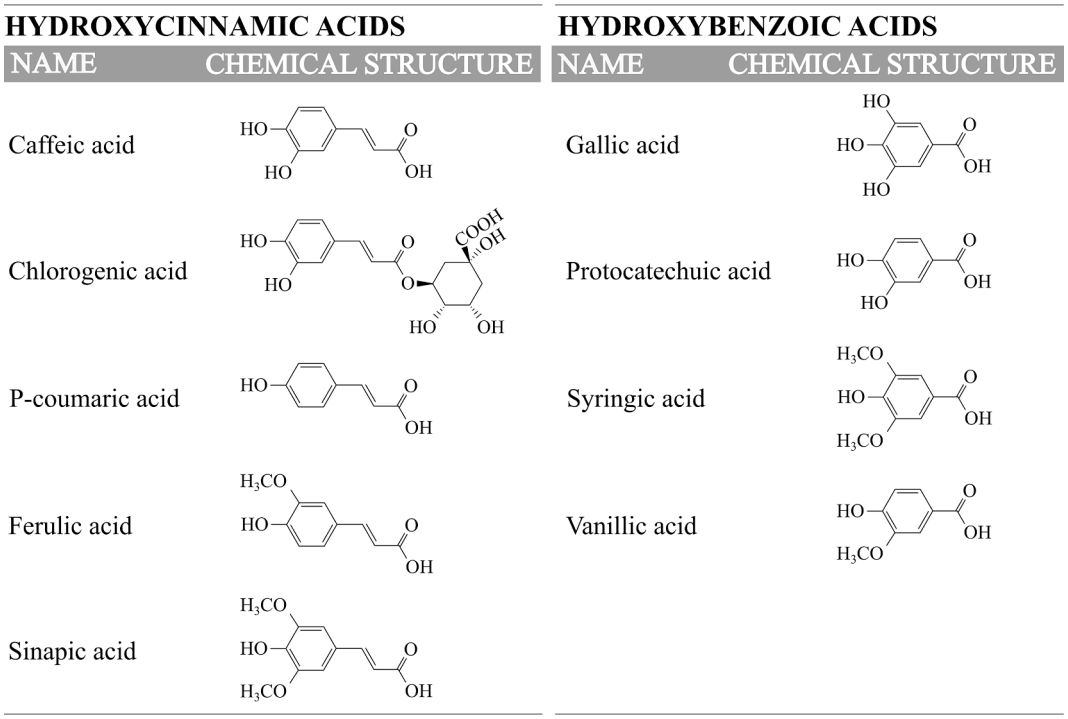

Fig. 1. Molecular structure of tested phenolic acids.

reagent was added and the mixture was incubated in a boiling water bath for 10 min and cooled to room temperature. The reaction mixture was then diluted 1:10 with distilled water, and absorbance was measured at 540 nm in a microplate reader (Epoch Biotek Instruments, Winooski, VT, USA).

2.3. Inhibition assays of α-glucosidase

The α-glucosidase assay was performed using maltose as substrate. Maltose (10 mg/mL) was prepared in sodium phosphate buffer (0.1 M, pH 6.9). Enzymatic reaction contained: 0.05 mL of phenolic compounds dissolved in ethanol (20%, v/v) at different concentrations, 0.05 mL of α-glucosidase (10 U/mL) and 0.4 mL of maltose were used. As in the α-amylase assays, to measure the different effect of incubation in enzyme activity, two methodologies were carried out: ($M1_{AG}$) Enzyme and polyphenol solutions were mixed and preincubated at 37 °C for 10 min. Then, maltose was added, and the mixture incubated at 37 °C for 10 min. ($M2_{AG}$) Maltose and polyphenol were mixed and preincubated at 37 °C for 10 min. Then, enzyme solution was added, and the mixture incubated at 37 °C for 10 min. To stop the reaction, samples were boiled in a water bath for 10 min. Absorbance was measured at 510 nm using a GOD/POD kit. Due to the interference of some colored phenolic acids with the colorimetric GOD/POD method, poly(vinylpolypyrrolidone) (PVPP) was used to remove polyphenols. In that case, 2% m/v of PVPP was added after ending the enzymatic reaction, samples were vortexed for 5 min, and centrifugated at 3000×*g* for 5 min. The absorbance of the supernatant was measured at 510 nm in a microplate reader (Epoch Biotek Instruments, Winooski, VT, USA).

2.4. Percentage of inhibition and IC_{50}

The percentage of enzyme inhibition of phenolic acids was calculated by Eq (1):

$$\%\ enzyme\ inhibition = \left[1 - \left(\frac{Abs_{sample} - Abs_{sample\ blank}}{Abs_{control} - Abs_{control\ blank}}\right)\right] \times 100 \quad (1)$$

where Abs_{sample} is the absorbance value of the sample with substrate solution and enzyme; $Abs_{sample\ blank}$ is the absorbance of sample and substrate without enzymes; $Abs_{control}$ is the absorbance of buffer (instead of sample), substrate solution and enzyme; $Abs_{control\ blank}$ is the absorbance of buffer and substrate without enzyme.

The half maximal inhibitory concentration (IC_{50}) is the concentration of sample required for 50% inhibition of the α-amylase or α-glucosidase activity and was calculated from the concentration-by-inhibition plots.

2.5. High-performance liquid chromatography analysis

Phenolic acids before and after the enzymatic reactions was quantified using high-performance liquid chromatography (HPLC). Samples preincubated with gelatinized starch ($M2_{AG}$) and starch gelatinized in presence of polyphenols ($M3_{AM}$) were dissolved in ethanol, centrifuged and filtered through 0.22 μm mixed cellulose ester filter. The phenolic acid contents were analyzed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a photodiode array detector (DAD) model 2998. Instrument control, data acquisition and data processing were achieved with Waters and Empower software (Waters Corporation, Milford, USA). A C18 column (150 × 4.6 mm, particle size 2.5 μm) (Waters Corporation, Milford, USA) was used. The mobile phases were 0.1 vol% trifluoroacetic acid in acetonitrile (A) and 0.1 vol % trifluoroacetic acid in water (B). Separation was carried out in 27 min under the following conditions: 0 min 5% A; 20 min 50% A; 21 min 100% A; 23 min 100% A; 27 min 5% A. Chromatographic conditions were: injection volume, 20 μL; flow rate 1 mL/min; oven temperature 40 °C, detection wavelengths, 280 and 320 nm. Calibration curves using phenolic acid standards were constructed to calculate their concentration in the samples. The absorption ratio was defined as the quotient between the concentration of free phenolic acids before and after the enzymatic reaction.

2.6. Statistical analyses

The data were expressed as average ± confidence interval of at least three individual measurements and analyzed through one-way analysis of variance (ANOVA) using Statgraphics Centurion XV software (Statistical Graphics Corporation, Rockville, MD, USA). Mean comparison for significant differences was tested using Fisher's least significant differences test at $P < 0.05$.

2.7. Structure-activity relation

To obtain structural parameters that relate molecular structural features and α-glucosidase activity, 3D molecular structure optimization was performed with Allingeŕs MM2 force field method using software Chem3D version 20.1.0.110, (Perkin-Elmer, Madrid, Spain), minimizing the steric energy to RMS Gradient = 0.01.

3. Results and discussion

The inhibitory activity of nine phenolic acids against α-amylase and α-glucosidase, was evaluated using different assay conditions: M1 = preincubation of the polyphenol with the enzyme; M2= preincubation of the phenolic acid with the substrate, M3 = gelatinization of the starch in presence of the phenolic compound. Plots of the enzymatic activities versus concentration of phenolic acids are shown in Figs. 2 and 3.

3.1. Inhibition of α-amylase

Concerning α-amylase inhibition (Fig. 2), as expected, acarbose revealed the highest inhibitory effect. Preincubating acarbose with α-amylase caused enzyme inhibition in the range 3.95 to 67.72%, on varying its concentration from 0.004 to 0.031 mM. Acarbose is a pseudotetra saccharide usually employed as a positive control in inhibition studies of α-amylase and α-glucosidase (Pollini et al., 2020). Also, vanillic and syringic acids showed high inhibitory effect when previously incubated with the α-amylase ($M1_{AM}$). Both polyphenols gave as result a percentage of inhibition of 4.44 to 76.08% when varying its concentration from 1.77 to 2.38 mM. p-Coumaric acid was the polyphenol tested that displayed the lowest inhibitory activity, needing concentrations of 24.37 to 121.83 mM to obtain an inhibition percentage between 4.1 and 88.74%. Hydroxycinnamic acids (caffeic, chlorogenic, ferulic, p-coumaric and sinapic acids) are characterized by a C═C double bond conjugated with a carbonyl group in their structure that stabilizes the binding forces to the active site of the α-amylase (Giuberti, Rocchetti, & Lucini, 2020). Likewise, it has been reported the importance of hydroxyl groups of polyphenols on the interaction with amino acid residues at the active site of α-amylase (Glu233) (Sun et al., 2019). In fact, by molecular docking analysis it was suggested that the removal of hydroxyl groups of polyphenols may decrease the inhibition effect (Sun et al., 2019). Chlorogenic acid has the highest number of OH groups (5), followed by gallic acid (3), and protocatechuic and caffeic (2). Nevertheless, inhibitory effect significantly changed when polyphenols were preincubated with the starch instead of the α-amylase ($M2_{AM}$ and $M3_{AM}$). That change was particularly dramatic in the case of vanillic and syringic acids, which required 10 times higher concentrations to inhibit α-amylase than those obtained when the acids were preincubated with the α-amylase ($M1_{AM}$). In $M2_{AM}$ and $M3_{AM}$ methodologies, phenolic acids with a greater number of hydroxyl groups (chlorogenic and gallic acid) showed higher inhibition effect against α-amylase than vanillic and syringic acids (Fig. 2), which have only one hydroxyl group in their structure.

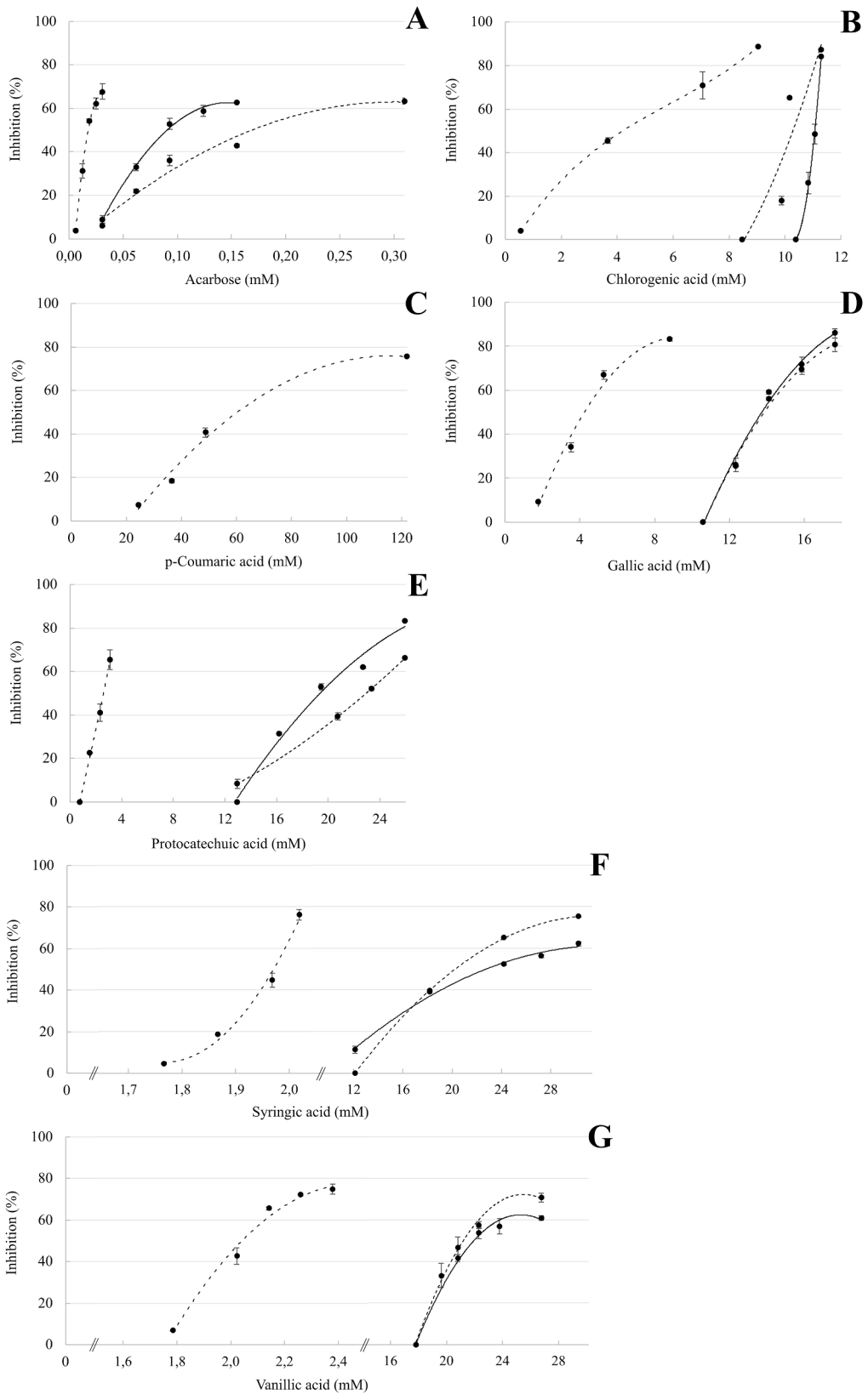

Fig. 2. Inhibitory effect of pure polyphenols against α-amylase. Discontinuous line, $M1_{AM}$ = preincubation PP + enzyme; solid line, $M2_{AM}$ = preincubation PP + substrate; dotted line $M3_{AM}$ = gelatinization PP + starch.

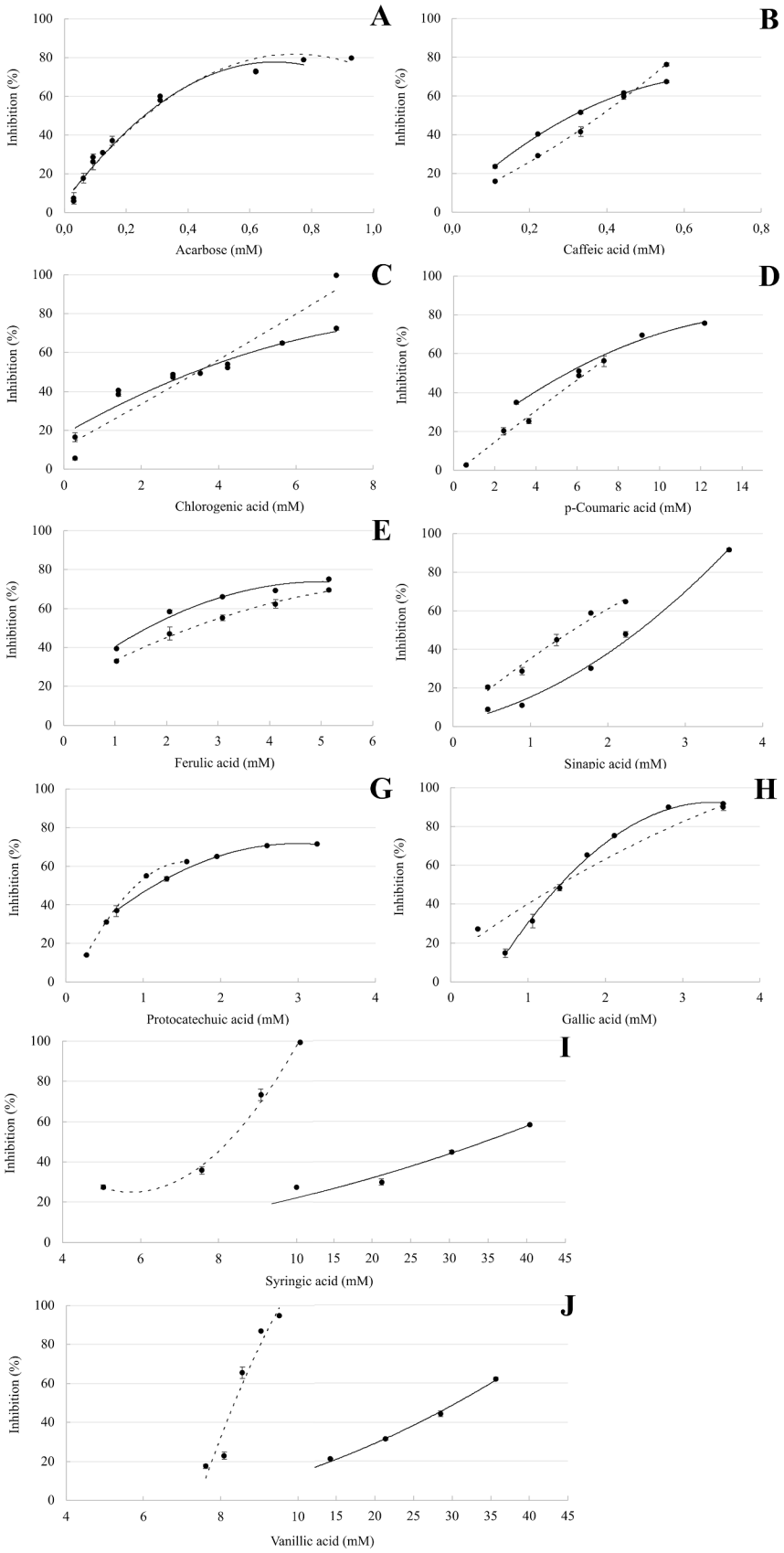

Fig. 3. Inhibitory effect of pure polyphenols against α-glucosidase. Discontinuous line, $M1_{AG}$ = preincubation PP + enzyme; solid line, $M2_{AG}$ = preincubation PP + substrate.

Therefore, results agree with reported statements affirming that phenolic acids and starch digestive enzymes interact by non-covalent interactions, being responsible of their inhibitory activity (Martinez-Gonzalez et al., 2017). Particularly, hydrogen binding, cation-π interactions, salt bridge interactions or electrostatic forces have been described for chlorogenic, caffeic, p-coumaric, vanillic and syringic and the enzyme (Pollini et al., 2020). The differences in the inhibitory concentration observed with the methodologies tested ($M1_{AM}$, $M2_{AM}$, $M3_{AM}$) suggests that starch somewhat hinders the phenolic acids-enzyme interaction, and in consequence greater concentration of phenolic acids are required for the inhibition.

The half-maximum inhibitory concentration (IC_{50}) values of the different analyzed phenolic acids were calculated (Table 1). The ANOVA analysis indicated significant differences ($P < 0.05$) due to the polyphenol acid and the method used. IC_{50} values are specific for the enzyme type, the substrate, and the reaction conditions (Sun et al., 2020), which limit the comparison with other published results. The IC_{50} values of hydroxycinnamic acids such as caffeic, ferulic and sinapic acids could not be determined due to their solubility difficulties (Table 1). There were significant differences in the IC_{50} obtained with $M1_{AM}$ and the other two methodologies: preincubating the phenolic acid with the starch ($M2_{AM}$) and gelatinizing the starch in presence of the polyphenol ($M3_{AM}$). Acarbose, showed the lowest IC_{50} values (0.012 ± 0.001, 0.056 ± 0.003 and 0.194 ± 0.004 mg/mL for $M1_{AM}$, $M2_{AM}$ and $M3_{AM}$) in all the methodologies tested. In $M1_{AM}$ the enzyme was preincubated with the inhibitory substance, encouraging their binding, which causes the inhibition of the enzymatic activity (Sun et al., 2020). Except for p-coumaric acid, the IC_{50} of phenolic acids obtained when preincubating with the enzyme ($M1_{AM}$) were < 1.5 mg/mL. Conversely, in $M2_{AM}$ and $M3_{AM}$ the IC_{50} were about 10 times higher, with the exception of chlorogenic and gallic acid which concentration increased around 3 times. When comparing $M2_{AM}$ and $M3_{AM}$, significantly higher concentrations of phenolic acids were required when they were preincubated with gelatinized starch ($M2_{AM}$), with exception of protocatechuic acid. Chlorogenic, syringic and vanillic acids presented lower IC_{50} values in $M3_{AM}$ (3.57 ± 0.00, 3.98 ± 0.00, 3.55 ± 0.08 mg/mL) than in $M2_{AM}$ (3.92 ± 0.01, 4.50 ± 0.03, 3.65 ± 0.06 mg/mL). Differences might be ascribed to the impact of polyphenols on starch gelatinization and the resulting microstructure. In fact, Han et al. (2020) observed changes in the short-range order microstructure of rice gels when ferulic acid, gallic acid or quercetin were present during starch gelatinization. Similarly, Chai, Wang, and Zhang (2013) obtained gels from high-amylose maize starch with totally different microstructure when adding 2.5% of tea polyphenols.

The higher concentrations of acids required in $M2_{AM}$ and $M3_{AM}$ to inhibit the enzyme might be attributed to interactions of phenolic acids with amylopectin or amylose chains (Mkandawire et al., 2013). Nevertheless, non-covalent interactions between polyphenols and starch can be influenced by the structure of the phenolic compound, the nature of the starch and/or the different experimental conditions (Giuberti et al., 2020). Considering that α-amylase inhibition still occurred at higher concentrations, results suggest the formation of non-inclusion complexes with weaker binding forces (hydrogen bonds, hydrophobic interactions and/or electrostatic and ionic interactions) (Zhu, 2015).

To explain the possible interaction of starch-polyphenols observed in $M2_{AM}$ and $M3_{AM}$ that might explain the higher concentrations required when preincubated polyphenols with starch, the concentration of free polyphenols was quantified by HPLC (Table 1). No statistical differences were observed in the free polyphenol concentration detected at IC_{50} in $M2_{AM}$ and $M3_{AM}$. This confirms that the possible interaction between

Table 1
Effect of different analysis methodologies (M1, M2, M3) on the IC_{50} values of pure phenolic acids (PP) against α-amylase.

		IC_{50} (MEAN ± SD)				Free polyphenols		Absorption ratio
Polyphenol	Treatment	mM		mg/mL		mg/mL		
Acarbose	$M1_{AM}$	0.018 ± 0.001	a	0.012 ± 0.001	a			
	$M2_{AM}$	0.087 ± 0.005	a	0.056 ± 0.003	a			
	$M3_{AM}$	0.300 ± 0.004	a	0.194 ± 0.004	b			
Caffeic acid	$M1_{AM}$	> 5.55		> 1				
	$M2_{AM}$	> 5.55		> 1				
	$M3_{AM}$	> 5.55		> 1				
Chlorogenic acid	$M1_{AM}$	3.90 ± 0.08	d	1.41 ± 0.04	e			
	$M2_{AM}$	11.08 ± 0.04	f	3.92 ± 0.01	j	3.99 ± 0.42	de	1.01
	$M3_{AM}$	10.07 ± 0.00	e	3.57 ± 0.00	hi	3.21 ± 0.30	bcd	0.90
p-Coumaric acid	$M1_{AM}$	52.48 ± 0.89	m	8.62 ± 0.15	l			
	$M2_{AM}$	> 52.48		>8.62				
	$M3_{AM}$	> 52.48		>8.62				
Ferulic acid	$M1_{AM}$	> 5.15		>1				
	$M2_{AM}$	> 5.15		>1				
	$M3_{AM}$	> 5.15		>1				
Sinapic acid	$M1_{AM}$	> 8.92		>2				
	$M2_{AM}$	> 8.92		>2				
	$M3_{AM}$	> 8.92		>2				
Gallic acid	$M1_{AM}$	4.35 ± 0.07	d	0.75 ± 0.01	d			
	$M2_{AM}$	13.53 ± 0.07	g	2.30 ± 0.01	f	2.41 ± 0.19	ab	1.05
	$M3_{AM}$	13.69 ± 0.06	g	2.33 ± 0.01	f	2.30 ± 0.19	a	0.99
Protocatechuic acid	$M1_{AM}$	2.65 ± 0.16	c	0.41 ± 0.02	c			
	$M2_{AM}$	18.84 ± 0.26	h	2.90 ± 0.04	g	3.05 ± 0.52	abc	1.05
	$M3_{AM}$	22.94 ± 0.20	l	3.54 ± 0.03	h	3.12 ± 0.35	abc	0.88
Syringic acid	$M1_{AM}$	1.98 ± 0.01	b	0.39 ± 0.00	c			
	$M2_{AM}$	22.72 ± 0.17	l	4.50 ± 0.03	k	4.80 ± 0.34	e	1.07
	$M3_{AM}$	20.00 ± 0.02	i	3.98 ± 0.00	j	3.35 ± 0.12	cd	0.84
Vanillic acid	$M1_{AM}$	2.05 ± 0.01	b	0.35 ± 0.06	c			
	$M2_{AM}$	21.73 ± 0.33	k	3.65 ± 0.06	i	3.47 ± 0.76	cd	0.95
	$M3_{AM}$	21.10 ± 0.49	j	3.55 ± 0.08	h	3.16 ± 0.06	bcd	0.89
P-value								
Polyphenol		0.0000		0.0000		0.0000		
Method		0.0000		0.0000		0.0265		

$M1_{AM}$ = preincubation PP + enzyme, $M2_{AM}$ = preincubation PP + gelatinized substrate, $M3_{AM}$ = gelatinization (PP + starch). Results were expressed as mean ± SD (n = 2) and values followed by different letters within columns are significantly different ($P < 0.05$).

phenolic acids and starch could only involve weaker bonds. Possible absorption of the polyphenol on the starch gel was evaluated by defining the absorption ratio. This parameter for $M2_{AM}$ (absorption ratio ~ 1) suggested that phenolic acids remained free, but for $M3_{AM}$ the absorption ratio was around 0.9, and even lower for gallic acid. Two plausible explanations for the former reduction when applying $M3_{AM}$ could be the thermal degradation of phenolic acids during the heating treatment or the inclusion of the acids into the starch gel structure. However, the analysis of the amount of phenolic acids in the absence of starch confirmed that heat treatment conditions applied for starch gelatinization did not affect the amount of free phenolic acids. Therefore, the absorption ratio results seem to indicate that 10% of the phenolic acids could have been linked to the gelatinized starch, as it has been suggested previously. Moreover, Igoumenidis, Zoumpoulakis, and Karathanos (2018) described hydrogen bonding or weak van der Waals interactions, no inclusion complexes, between rice starch and caffeic acid after boiling. Similar results were described by Wu, Lin, Chen, and Xiao (2011) and Liu et al. (2020), who reported hydrogen bonding interaction during gelatinization between phenolic compounds (caffeic acid and tea polyphenols) and rice and maize starch, respectively. Betoret and Rosell (2020) viewed an interaction between corn starch and phenolic compounds from *Brassica napobrassica* leaves powder. In addition, they studied the impact of temperature on polyphenols, describing a thermal degradation, but also a protective effect owing to the starch-phenolic compounds interaction. However, the starch and polyphenol working concentrations were considerably higher than in the present study.

Based on the obtained results, most of the phenolic compounds are present in the reaction medium, apart from the 90% in $M3_{AM}$. Therefore, all the inhibition effects registered from the different methods cannot be explained by the interaction between starch and phenolic acids, but by different inhibition mechanism of phenolic acids. In the case of acarbose, different studies describe a mixed-type competitive inhibition of porcine pancreatic α-amylase by phenolic acids like caffeic, chlorogenic or ferulic acids, exhibiting both competitive and uncompetitive mechanisms (Kim et al., 1999; Sun et al., 2019; Zheng et al., 2020). The existence of different inhibitory mechanisms might explain the lower inhibition effect of phenolic acids in $M2_{AM}$ and $M3_{AM}$ methods. In these cases, the inhibitor did not have a preincubation time with the enzyme and therefore had to compete with the substrate for the enzyme active site during incubation or bind the enzyme-substrate complex.

3.2. Polyphenols inhibition of α-glucosidase

Regarding α-glucosidase, two assay conditions ($M1_{AG}$ and $M2_{AG}$) were tested to identify possible impact of phenolic acid preincubation with enzyme. In $M1_{AG}$ the polyphenol was preincubated with the enzyme and in $M2_{AG}$ the phenolic acid was preincubated with the substrate, before the enzymatic reaction. In opposition to α-amylase results, there were minor differences when comparing different methodologies, with exception of syringic and vanillic acids (Fig. 3). Likely, there was some interaction between the maltose and their benzoic structure. Considering the inhibitory ability of the acids, 0.1 to 0.5 mM of caffeic acid induced an inhibition percentage of 16.05 to 76.25%, whereas 0.9 mM acarbose was necessary to reach a 79.54% of inhibition (Fig. 3). Hence, caffeic acid caused greater inhibition than acarbose. Tan et al. (2017) analyzed the effect of commercial polyphenols against α-glucosidase using pNPG as substrate, incubating the polyphenol, substrate, and the enzyme, and also described a high inhibition of caffeic acid compared to other phenolic acids. Syringic and vanillic acids showed the lowest inhibition against α-glucosidase in the $M1_{AG}$ and $M2_{AG}$. >8 mM of these polyphenols were needed to inhibit 50% of the maximum activity of the enzyme. As it was described for α-amylase, hydroxyl groups might play an important role on α-glucosidase inhibition too (Xiao, Kai, Yamamoto, & Chen, 2013). Phenolic acids with > 1 hydroxyl group like caffeic and protocatechuic acids showed higher inhibition effect than some phenolic acids with one hydroxyl group or one hydroxyl group plus one or two methoxy groups in their structure, as p-coumaric, syringic or vanillic acid, which are less polar. A similar result regarding caffeic acid was observed when the activity was on p-nitrophenyl-α-D-glucoside (p-NPG). Oboh, Agunloye, Adefegha, Akinyemi, and Ademiluyi (2015) reported lower enzyme affinity for chlorogenic acid than caffeic acid, when using yeast α-glucosidase and pNPG as substrate, not maltose. Likely, the effect of the substitution of an OH group for quinic acid could produce steric constrains for accommodating the structure to the active site of the enzyme.

The IC_{50} calculated for the α-glucosidase inhibition also revealed differences between the preincubation of the inhibitor with the enzyme or the substrate ($M1_{AG}$ or $M2_{AG}$) lower than those obtained with α-amylase (Table 2). Only ferulic, sinapic, syringic and vanillic acids showed different IC_{50} values depending on the method used, showing higher IC_{50} in $M2_{AG}$ than in $M1_{AG}$, except for ferulic acid. The preincubation of the ferulic acid with the maltose improved its inhibitory effect (0.45 ± 0.05 and 0.29 ± 0.01 mg/mL for $M1_{AG}$ or $M2_{AG}$, respectively). These phenolic compounds (ferulic, sinapic, syringic and vanillic acids) present a methoxy group in the aromatic ring that might be responsible of the differences observed in α-glucosidase inhibition, which agrees with the reported inhibition effect of hydroxycinnamic acids with methoxy groups (Malunga, Joseph Thandapilly, & Ames, 2018).

Although it is known that benzoic acid derivatives are more polar than cinnamic acids, in general, there was not a difference appliable to the two families of compounds. Analysis of calculated LogP values (available at PubChem and https://foodb.ca databases) and first pKa value did not explain the observed differences for all the compounds (data not shown). In search for an explanation to the high difference between vanillic acid (benzoic acid derivative) and syringic acid (cinnamic derivative) and the rest of the compounds, the molecular features were analyzed. Allingeŕs force field method MM2 was applied to optimize the molecular structure in Perkin-Elmer Chem3D. The variable found to explain the high observed differences was the molecular dipole/dipole momentum, which was 1,0107 for syringic acid and 1.744 for vanillic acid, whereas it was in the range 3.658 (p-coumaric acid) to

Table 2
IC_{50} values of pure phenolic acids against α-glucosidase of the different analyzed methodologies.

		IC_{50} (MEAN ± SD)			
Polyphenol	Treatment	mM		mg/mL	
Acarbose	$M1_{AG}$	0.25 ± 0.02	a	0.16 ± 0.01	abcd
	$M2_{AG}$	0.21 ± 0.01	a	0.13 ± 0.01	abc
Caffeic acid	$M1_{AG}$	0.39 ± 0.02	ab	0.07 ± 0.00	ab
	$M2_{AG}$	0.31 ± 0.00	ab	0.06 ± 0.00	a
Chlorogenic acid	$M1_{AG}$	3.19 ± 0.18	e	1.13 ± 0.07	h
	$M2_{AG}$	3.70 ± 0.05	e	1.31 ± 0.02	i
p-Coumaric acid	$M1_{AG}$	6.20 ± 0.04	f	1.02 ± 0.01	gh
	$M2_{AG}$	5.95 ± 0.00	f	0.97 ± 0.00	g
Ferulic acid	$M1_{AG}$	2.32 ± 0.28	d	0.45 ± 0.05	f
	$M2_{AG}$	1.54 ± 0.03	c	0.29 ± 0.01	e
Sinapic acid	$M1_{AG}$	1.48 ± 0.08	c	0.33 ± 0.02	e
	$M2_{AG}$	2.28 ± 0.04	d	0.51 ± 0.01	f
Gallic acid	$M1_{AG}$	1.53 ± 0.05	c	0.25 ± 0.01	de
	$M2_{AG}$	1.44 ± 0.04	c	0.25 ± 0.01	cde
Protocatechuic acid	$M1_{AG}$	0.90 ± 0.01	bc	0.14 ± 0.00	abc
	$M2_{AG}$	1.15 ± 0.06	c	0.18 ± 0.01	bcd
Syringic acid	$M1_{AG}$	8.25 ± 0.09	g	1.63 ± 0.02	j
	$M2_{AG}$	32.36 ± 0.37	i	6.41 ± 0.07	l
Vanillic acid	$M1_{AG}$	8.38 ± 0.01	g	1.41 ± 0.00	i
	$M2_{AG}$	30.90 ± 1.26	h	5.20 ± 0.21	k
P-value					
Polyphenol		0.0000		0.0000	
Method		0.0112		0.0109	

$M1_{AG}$ = preincubation PP + enzyme, $M2_{AG}$ = preincubation PP + substrate. Results were expressed as mean ± SD (n = 2) and values followed by different letters within columns are significantly different ($P < 0.05$).

5.7 (ferulic acid) for the rest of the other compounds, excepting chlorogenic acid (dipole–dipole momentum = 0.03). It seems that an association between two molecules of simple phenolic is necessary to fit or block the active site of α-glucosidase and this situation is more likely to happen when the dipolar moment is high and molecules can form a dipole at the vicinity of active site. The two less polar molecules were less efficient to inhibit the enzyme, probably because this situation does not happen. It seems that chlorogenic acid, which molecular size is similar to that of the natural substrate (maltose), does not need this interaction to fit in the active site. In summary, simple phenolic acids require a dipole–dipole interaction to effectively block the enzyme.

Acarbose was specifically designed to effectively adsorb onto enzyme, but its N—H link cannot be broken, thus being its IC_{50} value much lower than that of phenolics compounds.

4. Conclusions

Different inhibition curves and IC_{50} values were obtained with different phenolic acids, and they were dependent on the previous interaction with the enzyme or the substrate. Results indicated that polyphenol chemical structures affected their capacity to interact with the enzyme. The incubation of the phenolic acid with the enzyme was the most effective way to inhibit α-amylase or α-glucosidase, due to their binding interactions. Conversely, higher concentrations of phenolic acids were needed to inhibit α-amylase when there was previous interaction with starch, which was partially due to the absorption of phenolic acids by starch during gelatinization, but also suggested different inhibition mechanisms. That effect was not observed with small substrates like maltose used for α-glucosidase, in which a dipole–dipole interaction with the phenolic acids is needed to effectively block the enzyme. The presence of methoxyl groups on benzoic acid derivatives showed a high influence in the interaction with the substrates for both α-amylase or α-glucosidase. The present study provides a different approach to explain the α-amylase and α-glucosidase inhibition by polyphenols.

CRediT authorship contribution statement

Andrea Aleixandre: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **José Vicente Gil:** Methodology, Writing – review & editing. **Jorge Sineiro:** Methodology, Funding acquisition, Writing – review & editing. **Cristina M. Rosell:** Conceptualization, Funding acquisition, Investigation, Supervision, Data curation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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