Institut für Pharmazie, Freie Universität, Berlin, Germany

Effect of different phenolic compounds on α-amylase activity: screening by microplate-reader based kinetic assay

I. Funke, M. F. Melzig

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Prof. Dr. Matthias M. Melzig, Institut für Pharmazie, Pharmazeutische Biologie, Königin-Luise-Str. 2+4, D-14195 Berlin
melzig@zedat.fu-berlin.de

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The inhibitory effect of different polyphenolic plant compounds on α-amylase activity was investigated *in vitro*. A kinetic assay was performed using 96-well-plates. Acarbose was used as positive control (IC_{50}: 23.2 μM). Some of the tested compounds, occurring in plants traditionally used in anti-diabetic tea species, showed an inhibition of the enzyme in physiological concentrations, e.g. luteolin, tannic acid, and isochlorogenic acid.

About 100 million people in the world suffer from non-insulin-dependent diabetes mellitus (NIDDM). Many and diverse therapeutic strategies for the treatment of type 2 diabetes are known. Conventional treatment includes reducing the demand for insulin, stimulation of endogenous insulin secretion, enhancing the action of insulin at the target tissues and inhibition of degradation of oligo- and disaccharides (Groop et al. 1997; Perfetti et al. 1998). One group of drugs recently introduced in the management of type 2 diabetes are inhibitors of α-glucosidase. The enzymes summarized as α-glucosidase are responsible for the breakdown of oligo- and/or disaccharides to monosaccharides. Two substances used in therapy are acarbose and miglitol. Both lead to a delay in glucose absorption by inhibiting the breakdown of polysaccharides. The aim of our work was the screening of natural products from plants traditionally used in anti-diabetic treatment. The anti-diabetic potency was defined by the inhibition of α-amylase activity.

Several methods for the determination of α-amylase (EC 3.2.1.1) have been described, whereas the determination of α-amylase using maltooligosaccharides of defined chain length with a 4-nitrophenyl or 2-chloro-4-nitrophenyl group is in current use (Soor and Hincke 1990, Gella et al. 1997; Wallenfells et al. 1980). The substrates are cleaved by α-amylase to yield the free chromophore which can be continuously monitored at 405 nm. We adapted this reaction for a kinetic assay using 96-well-plates in a microplate reader. While most reports in the literature describing investigations regarding to α-amylase activity for diagnostic purpose of pancreatic diseases we established the test for a rapid screening of plant extracts and natural products with a presumed anti-diabetic impact.

The obtained results demonstrate that a variety of phenolic compounds are able to inhibit the activity of α-amylase. They are widely spread in plants which are used as anti-diabetic tea species, spices or food. Mean IC_{50}-values of the investigated natural products are shown in the Table. In 2002, Rohn et al. could show that the ability of phenolic compounds to form quinones increases their reactivity towards enzymes like α-amylase. They assumed that the activity of α-amylase and other enzymes decreases depending on their concentration and on the number and position of hydroxyl groups. Phenolic compounds which are not able to form quinones (as ferulic acid) led to a smaller effect which is explainable by the formation of a semiquinone (Rohn et al. 2002). In 2000 Kim et al. demonstrated the high inhibitory potency of luteolin and luteolin-7-glucoside against α-glucosidase and α-amylase (Kim et al. 2000). The investigations show that substances having the ability to form quinones or lactones or substances with a 4-oxo-pyrane structure induce an inhibiting effect on α-amylase activity. Reactivity against the enzyme of those substances which are not able to form that structures because of methoxy groups (ferulic acid, sinapic acid), steric obstructions (cynarin) or short chain length (quinic acid) were significantly lower. By comparison of IC_{50}-values of chlorogenic acid (1.4 ± 0.03 mM) and isochlorogenic acid (0.56 ± 0.027 mM) (Table) we could show that the steric position of the hydroxyl groups in the molecule is important for the inhibition rate. The hydroxyl groups of the quinic acid rest in the isochlorogenic acid are arranged in the same plane. That presumably leads to a more pronounced effect. The free hydroxyl groups in the molecule seem to be necessary for a more distinguished inhibitory effect on α-amylase (caffeic acid, ferulic acid; Table). Substances with a prevailing quinone structure in the molecule showed the highest inhibition rates in the class of caffeic acid derivatives. The difference between IC_{50}-values of caffeic acid and dihydrocaffeic acid (Table) demonstrates that the double bound in the propionic acid rest seems to be decisive for inhibitory potency. Molecules of more complicated structure and limited free rotation like cynarin are not able to inhibit the enzyme significantly. A glycosidic component is not essential for the inhibiting effect. This fact suggests that the inhibiting mechanism is not based on a competition against the enzyme (as the mechanism of acarbose effect) but a rather specific binding site.

We could show in our studies that the α-amylase inhibition of phenolic compounds is dose-dependent. High concentrations led to inhibition levels up to 90%. Rosmarinic acid is reported to have inhibitory effects on porcine pan-

Table: IC_{50}-values of tested substances

Substance	IC_{50} (mM)
Acarbose	0.023 ± 0.002
Tannic acid	0.14 ± 0.003
Apigenin-7-glucoside	0.17 ± 0.005
Luteolin	0.17 ± 0.026
Luteolin-7-glucoside	0.28 ± 0.002
Fisetin	0.44 ± 0.005
Isochlorogenic acid	0.56 ± 0.027
Rosmarinic acid	1.4 ± 0.008
Chlorogenic acid	1.4 ± 0.03
Cynarin	> 2.0
Caffeic acid	4.8 ± 0.0192
Ferulic acid	> 5.0
Sinapic acid	> 6.7
Quinic acid	> 13.0
Dihydrocaffeic acid	> 14.0

Results are shown as mean ± SD, n = 4

creatic amylase *in vitro* (McCue and Shetty 2004). Herbs containing rosmarinic acid as main phenolic component have been used in traditional medicine for a long time in order to treat diabetes mellitus or cardiac diseases (Eddouks et al. 2002). To discuss the pharmacological significance of the observed IC_{50}-values, the following example is presented: Melissae folium contains app. 4% rosmarinic acid (Ph. Eur. 2002). Preparing a cup of tea with 5 g of the drug and 200 ml water the concentration of rosmarinic acid would be at least 1 mg/ml. We established an IC_{50}-value of rosmarinic acid of 1.4 ± 0.008 mM corresponding to a concentration of 0.5 ± 0.003 mg/ml. An absorption of prospective α-amylase inhibitors is not necessary, because pancreatic α-amylase works in the small intestine.

Experimental

p-Nitrophenyl-α-D-maltopentaoside (PNPG5) was obtained from Megazyme (Bray, Co.Wicklow, Ireland), *p*-nitrophenyl-α-D-maltoheptaoside (PNPG7) from Calbiochem (Schwalbach, Germany). HEPES was purchased from Lancaster (Mühlheim, Germany). α-Amylase (EC 3.2.1.1) from porcine pancreas was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). DMSO was obtained from Merck KgaA, Darmstadt, Germany. Acarbose was a gift from Bayer AG (Wuppertal-Aprath, Germany). Tested substances were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany (ferulic acid, caffeic acid, rosmarinic acid, dihydrocaffeic acid), Carl Roth GmbH & Co., Karlsruhe, Germany (luteolin, luteolin-7-glucoside, apigenin-7-glucoside, isochlorogenic acid), Merck KgaA, Darmstadt, Germany (chlorogenic acid), and Fluka, Buchs SG, Switzerland (fisetin).

The α-amylase assay was performed in 96-well-plates (Greiner). All reagents were dissolved in buffer (HEPES 50 mM, pH = 7.1), DMSO or 5%-(v/v) DMSO-buffer-solutions. Highest concentration of DMSO was 5%. Controls were prepared using the identical solvent to consider the influence of DMSO on the reaction. 50 µl of substrate solution (PNPG5, 25 mM, dissolved in buffer) and solutions of the investigated substances (10–100 µl) were pipetted into the wells. Buffer (HEPES, 50 mM, pH 7.1) was added up to a volume of 150 µl. Reaction was started by rapid addition of the enzyme solution (50 µl porcine pancreatic α-amylase in buffer, 100 U/ml).

Absorption (405 nm) was measured at 3-min intervals for a total period of 90 min at 37 °C using a Tecan Spectra Fluor. The increase of the absorbance was monitored as a function of time to provide a progress curve for the reactions. The curves started in a near-linear manner, decreasing in slope when the enzyme activity was inhibited. The slope of each reaction (time versus optical density at 405 nm) was analysed by linear regression and used for calculation of the inhibition rates, expressed in percent to controls without inhibitors. In each experiment, blind samples without test compounds were measured. All assays were performed at least two times with duplicate samples. IC_{50} values were determined from dose-effect-curves by linear regression using Microsoft Excel. The data were expressed as mean ± SD. As positive control and well established inhibitor of α-amylase activity Acarbose was used.

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