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EFEITOS INIBITÓRIOS *IN VITRO* DA ATIVIDADE DA α-AMILASE PANCREÁTICA E SALIVAR E AÇÕES HIPOGLICÊMICAS DE TANINOS CONDENSADOS E HIDROLISÁVEIS

CAMILA GABRIEL KATO

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Dissertação apresentada ao programa de Pós Graduação em Ciências de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de mestre em Ciências de Alimentos.

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Orientadora

Prof.ª Dr.ª Rosane Marina Peralta

BIOGRAFIA

Camila Gabriel Kato nasceu em 08 de dezembro de 1987 na cidade de Maringá, Paraná. Possui graduação em Engenharia de Alimentos pela Universidade Estadual de Maringá. Tem experiência na área de Bioquímica atuando principalmente nos seguintes temas: reaproveitamento de resíduos da indústria alimentícia com a finalidade de se obter um inibidor enzimático.

Dedico

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APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de dois artigos científicos

ARTIGO 1

AUTORES: Camila Gabriel Kato, Adelar Bracht e Rosane Marina Peralta.

TÍTULO: *In vitro* pancreatic α-amylase inhibitory activities and in vivo hypoglycemic actions of commercial condensed and hydrolysable tannins.

REVISTA: PloS One (JCR 3,234)

ARTIGO 2

AUTORES: Camila Gabriel Kato, Verônica Sayuri Nishida, Adelar Bracht e Rosane Marina Peralta

TÍTULO: Inhibitory effects of hydrolysable and condensed tannins on human salivary α-amylase.

REVISTA: LWT – Food Science and Technology (JCR 2,416), submetido em 01/02/2016.

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – Cinco isoamilases têm sido descritas no organismo humano, sendo três amilases salivares e duas amilases pancreáticas. Devido à importância em diversas desordens metabólicas incluindo diabetes e obesidade, a amilase pancreática tem recebido mais atenção que a amilase salivar. Em consequência, uma série de inibidores da amilase pancreática estão disponíveis no mercado, tais como a acarbose, voglibose e miglitol. Entretanto, a amilase salivar humana (HSA), desempenha importantes papéis na boca, incluindo hidrólise do amido da dieta, ligação com a superfície do dente e ligação com bactérias bucais. Todas as três ações contribuem para o processo de formação da placa dental e formação das cáries. Os taninos são moléculas exploradas como inibidores das amilases. Acredita-se que a descoberta de novos materiais ricos em taninos capazes de inibir enzimas possam contribuir para a descoberta de novos medicamentos úteis no controle e tratamento de diabetes, obesidade e outras desordens fisiológicas, tais como as doenças da cavidade bucal, incluindo as cáries. Um dos mais extensivamente estudados taninos condensados (proantocianidina) é o extraído da casca da árvore Acacia negra (*Acacia mearnsii* De Wild.). Este tanino é particularmente rico nos monômeros robinetinidol e fisetinidol. Um dos mais simples e comum tanino hidrolisável é o galotanino com 12 grupos galoil esterificados e um núcleo de glicose. Esta estrutura é particularmente abundante no galotanino da galha de *Rhus chinensis* Mill. (*chinese natural gallnut*). O objetivo do **artigo 1** foi comparar os efeitos inibitórios do tanino condensado de *A. mearnsii* e do tanino hidrolisável da galha de *R. chinensis* sobre as amilase pancreática *in vitro* e ação hipoglicêmica in vivo. Para propósitos comparativos, experimentos similares foram conduzidos utilizando acarbose, eficiente inibidor da amilase pancreática. O objetivo do **artigo 2** foi investigar os efeitos inibitórios dos dois taninos descritos acima sobre a amilase salivar. Em ambos os artigos, nos experimentos *in vitro* foi devotada uma atenção à cinética da inibição, com uma detalhada análise para o modelo que melhor descreve os mecanismos de ação dos inibidores.

MÉTODOS – Amilase salivar humana, amilase pancreática de porco, acarbose e tanino hidrolisável de galha de *Rhus chinensis* foram adquiridos do Sigma-Aldrich Co. Tanino condensado de *Acacia mearnsii* foi adquirido do Labsynth, Brasil. Os experimentos cinéticos com as amilases salivar e pancreática foram conduzidos à 37 °C em tampão fosfato pH 6.9 contendo 6.7 mM NaCl. Amido de batata (Sigma-Aldrich) foi utilizado como substrato. O substrato e um dos três inibidores, acarbose, tanino condensado e tanino hidrolisável foram misturados e a reação foi iniciada pela adição da enzima. A reação ocorreu por 10 minutos. Os açúcares redutores liberados foram quantificados pelo método do ácido 3,5 dinitro-salicílico, utilizando glicose como padrão. Análise estatística dos dados foi realizada por meio do programa *Statistica* (Statsoft, Inc., Tulsa, OK). O ajuste das equações de velocidade inicial aos dados experimentais foi realizada por meio de procedimento iterative não linear de mínimos quadrados utilizando o programa *Scientist* (MicroMath Scientific Software, Salt Lake City, UT). A

decisão pelo melhor modelo (equação) baseou-se no critério de seleção de modelo (MSC) e nos desvios-padrão dos parâmetros otimizados. Ratos machos Wistar com peso entre 200–250 g foram utilizados nos experimentos in vivo. Os animais foram divididos em 9 grupos (n = 4 ratos por grupo). No grupo I (controle positivo) amido comercial de milho (1 g por kg de peso corporal) foi administrado intragastricamente. O grupo II (controle negativo) recebeu apenas água. Grupo III recebeu intragastricamente amido de milho comercial mais acarbose (50 mg/kg). Os Grupos IV, V e VI foi administrado amido de milho comercial e tanino de *A. mearnsii* nas concentrações 100, 250 e 500 mg/kg respectivamente. Finalmente, nos grupo VII, VIII e IX foi dado intragastricamente amido de milho comercial e tanino hidrolisável 100, 250 e 500mg/kg respectivamente. A glicemia foi avaliada antes da administração do amido e inibidores de amilase (tempo 0). Novas avaliações de glicemia foram realizadas após 15, 30, 45 e 60 minutos. Um pequeno corte na cauda do animal foi realizado para obtenção de sangue para avaliação da glicemia utilizando Accu-Chek® Active Glucose Meter.

PRINCIPAIS RESULTADOS, DISCUSSÃO E CONCLUSÕES – No artigo 1, ambos os taninos inibiram a amilase pancreática. Para uma concentração de amido (substrato) de 1 g/100 mL, as concentrações de inibidores capazes de inibir a atividade em 50% (EC_{50}) foram de 240 µg/mL tanino hidrolisável e 200 µg/mL de tanino condensado. Para a acarbose, o EC_{50} foi 2.2 µg/mL. As cinéticas de inibição apresentaram padrões complexos nos quais para ambos os inibidores, mais de uma molécula podem se ligar simultaneamente na enzima livre ou na enzima complexada com o substrato (inibição mista parabólica). O mesmo fenômeno foi encontrado para a acarbose. Isto é revelado a priori pelos plots não lineares 1/v versus [I] e pelos ajustes das equações cinéticas contendo termos de concentração do inibidor elevado ao quadrado (por exemplo, $[I]^2$). Ambos os taninos foram hábeis em inibir a absorção intestinal de amido, como revelado pelos testes de tolerância ao amido em ratos. A inibição pelo tanino hidrolisável foi dependente da concentração, com 53% de inibição na dose de 100 mg/kg e 88% de inibição na dose de 500 mg/kg. Para o tanino condensado, inibição não foi substancialmente diferente para as doses entre 100 mg/kg (49%) e 500 mg/kg (57%). Podemos concluir que ambos os taninos, mas especialmente o tanino hidrolisável pode ser útil no controle dos níveis glicêmicos pós-prandiais em pacientes diabéticos. No **artigo 2**, em experimentos utilizando a amilase salivar humana, foi possível calcular os valores de EC_{50} (concentração do inibidor capaz de produzir uma inibição de 50%) para uma concentração de amido de 1 g/100 mL de 80 µg/mL e 230 µg/mL para o tanino hidrolisável e tanino condensado, respectivamente. A partir da análise dos dados cinéticos, pode-se concluir ambos os taninos causam na HAS uma inibição mista (não competitiva). A HAS livre liga-se ao tanino hidrolisável com maior afinidade, considerando-se o valor de K_{i1} de 22.4 ± 2.9 µg/mL, enquanto K_{i1} para o tanino condensado foi de 157.1 ± 12.6 µg/mL. Levando-se em consideração o importante papel da HAS na formação da placa dentária e subsequente formação da cárie dental, a forte ação inibitória do tanino hidrolisável faz dele um agente útil para a manutenção da saúde bucal.

Palavras-chaves:: inibidores das α-amilases, cáries, diabetes, taninos condensados, taninos hidrolisáveis.

GENERAL ABSTRACT

INTRODUCTION AND AIMS - In the human organism, five isoenzymes of amylase have been described, three salivary amylases and two pancreatic amylases. Due to the importance in several metabolic disorders including diabetes and obesity, the pancreatic-amylase has been more extensively studied than the salivary α-amylase. In consequence, a series of pancreatic α-amylase inhibitors are available in the market, such as acarbose. However, the human salivary amylase (HSA), has important roles in the mouth, including hydrolysis of dietary starch, binding to the tooth surface, and binding to oral streptococci. All three actions contribute to the process of dental plaque and caries formation. Tannins are one of the most extensively studied molecules able to inhibit amylases. It is generally believed that the discovery of new materials rich in tannins with enzyme inhibitory properties can contribute for the discovery of new drugs useful in the control and treatment of diabetes, obesity and other physiological disorders, such as oral diseases, including caries. One of the most extensively studied condensed tannin (proanthocyanidin) is that extracted from the bark of the black wattle tree (*Acacia mearnsii* De Wild.). It is rich in the catechin-like flavan-3-ols monomers robinetinidol and fisetinidol. One of the most simple and common hydrolysable tannin is the gallotannin with up to 12 esterified galloyl groups and a core glucose. This structure is particularly abundant in the gallotannin from chinese natural gallnuts. The aim of the article 1 was to compare the *in vitro* inhibitory effects on the pancreatic α-amylase and the *in vivo* hypoglycemic actions of a condensed tannin from *A. mearnsii* bark and a hydrolysable tannin from Chinese natural gall (*Rhus chinensis* Mill.). For comparative purposes, similar experiments were also run with acarbose, a highly effective inhibitor of pancreatic α-amylase inhibitor. The aim of the article 2 was to investigate the *in vitro* inhibitory effects on the human salivary α-amylase of the same two tannins in the search of new molecules with an increased affinity and specificity for the enzyme. In both articles, in the *in vitro* experiments, especial attention has been devoted to the kinetics of the inhibition, with a detailed search for the model that best describes the mechanism of action.

METHODS – Human salivary α-amylase (HAS), porcine pancreatic α-amylase, acarbose and hydrolysable tannin from Chinese gall nut were obtained from Sigma-Aldrich Co. The *Acacia mearnsii* bark tannin was purchased from Labsynth, Brazil. The kinetic experiments with the HAS and pancreatic α-amylase were carried out at 37 °C in 20 mM phosphate buffer pH 6.9 containing 6.7 mM NaCl. Potato starch (Sigma-Aldrich) was used as substrate. Substrate and one of the three inhibitors, acarbose, condensed tannin or hydrolysable tannin were mixed and the reaction was initiated by adding the enzyme. The reaction was allowed to proceed for 5 min. The produced reducing sugars were assayed by the dinitrosalicylic acid method, using maltose as standard. Statistical analysis of the data was done by means of the Statistica program (Statsoft, Inc., Tulsa, OK). Fitting of the rate equations to the experimental initial rates was done by means of an iterative non-linear least-squares procedure using the Scientist software from MicroMath Scientific Software (Salt Lake City, UT). The decision as to the

most adequate model (equation) was based on the model selection criterion (MSC) and on the standard deviations of the optimized parameters. Male healthy Wistar rats weighing 200–250 g were used in all in vivo experiments. Rats were divided into 9 groups (n = 4 rats per group). To group I (positive control) commercial corn starch (1 g per kg body weight) was administered intragastrically. Group II (negative control) received only tap water. Group III received intragastrically commercial corn starch plus acarbose (50 mg/kg). Groups IV, V and VI received intragastrically commercial corn starch plus *A. mearnsii* tannin 100, 250 and 500 mg/kg respectively. Finally, groups VII, VIII and IX received intragastrically commercial corn starch plus tannic acid extracts 100, 250 and 500 mg/kg respectively. The amounts of inhibitors given to the rats were based on literature data (Ikarashi et al., 2011). Fasting blood glucose levels were determined before the administration of starch and amylase inhibitors (0 time). Later evaluations of blood glucose levels took place at 15, 30, 45 and 60 min. Blood glucose from cut tail tips was determined using Accu-Chek® Active Glucose Meter.

MAIN RESULTS, DISCUSSION AND CONCLUSION – In the **article 1**, both tannins inhibited the enzyme. At a starch (substrate) concentration of 1 g/100 mL the concentrations for 50% inhibition (EC_{50}) were 240 µg hydrolysable tannin per mL and 200 µg condensed tannin per mL. For acarbose the EC_{50} value was 2.2 µg/mL. The kinetics of the inhibition presented a complex pattern in that for both inhibitors more than one molecule can bind simultaneously to either the free enzyme of the substrate-complexed enzyme (parabolic mixed inhibition). The same phenomenon was found for acarbose. This is revealed a priori by the non-linear 1/v versus [I] plots and by the successful fitting of kinetic equations containing squared inhibitor concentration terms (e.g., $[I]^2$). Both tannins were able to inhibit the intestinal starch absorption, as revealed by starch tolerance tests in rats. Inhibition by the hydrolysable tannin was concentration-dependent, with 53% inhibition at the dose of 100 mg/kg and 88% inhibition at the dose of 500 mg/kg. For the condensed tannin, inhibition was not substantially different for doses between 100 mg/kg (49%) and 500 mg/kg (57%). It can be concluded that both tannins, but especially the hydrolysable one, could be useful in controlling the post-prandial glycemic levels in diabetic patients. In the **article 2**, in the experiments using HAS, it was possible to calculate the IC_{50} values (inhibitor concentration producing 50% inhibition) for a starch concentration of 1 g/100 mL of 80 µg/mL and 230 µg/mL for the hydrolysable and condensed tannins, respectively. From the kinetic analysis it can be concluded that inhibition of the HSA by both tannins is of the mixed (or non-competitive) type. The free HSA binds the hydrolysable tannin with higher affinity considering that the K_{i1} value was 22.4±2.9 µg/mL while the K_{i1} value for condensed tannin was 157.1±12.6 µg/mL. Taking into account that HSA has an important role in the dental plaque formation and subsequent dental caries formation, the strong inhibitory action of the hydrolysable tannin could make it an useful agent for oral health.

Keywords: α-amylase inhibitors; caries, diabetes; condensed tannins, hydrolysed tannins.

ARTIGO 1

In vitro pancreatic α-amylase inhibitory activities and *in vivo* hypoglycemic actions of condensed and hydrolysable tannins

Camila Gabriel Kato, Adelar Bracht, Rosane Marina Peralta*

Postgraduate Program in Food Science, State University of Maringá, Brazil,
Department of Biochemistry. University of Maringá, 87020900 Maringá, Brazil

Abstract

The aim of the present study was to compare the *in vitro* inhibitory effects on the pancreatic α-amylase and the *in vivo* hypoglycemic actions of two tannins with well-known chemical structures. The compounds were the hydrolysable tannin from Chinese natural gall (*Rhus chinensis* Mill.) and the condensed tannin from *Acacia mearnsii* De Wild.. For comparative purposes, similar experiments were also run with acarbose, a highly effective inhibitor of pancreatic α-amylase inhibitor. Both compounds inhibited the enzyme. At a starch (substrate) concentration of 1 g/100 mL the concentrations for 50% inhibition (EC_{50}) were 240 μg hydrolysable tannin per mL and 200 μg condensed tannin per mL. For acarbose the EC_{50} value was 2.2 μg/mL. The kinetics of the inhibition presented a complex pattern in that for both inhibitors more than one molecule can bind simultaneously to either the free enzyme of the substrate-complexed enzyme (parabolic mixed inhibition). The same phenomenon was found for acarbose. This is revealed a priori by the non-linear 1/v versus [I] plots and by the successful fitting of kinetic equations containing squared inhibitor concentration terms (e.g., $[I]^2$). Both tannins were able to inhibit the intestinal starch absorption, as revealed by starch tolerance tests in rats. Inhibition by the hydrolysable tannin was concentration-dependent, with 53% inhibition at the dose of 100 mg/kg and 88% inhibition at the dose of 500 mg/kg. For the condensed tannin, inhibition was not substantially different for doses between 100 mg/kg (49%) and 500 mg/kg (57%). It can be concluded that both tannins, but especially the hydrolysable one, could be useful in controlling the post-prandial glycemic levels in diabetic patients.

Key words: tannins, amylases, diabetes, enzyme inhibition, enzyme kinetics.

Introduction

Diabetes is one of the most common metabolic disorders in the world and its prevalence in adults has been increasing in the last decades (Shaw et al., 2010). Diabetes mellitus is a leading cause of morbidity and mortality worldwide, with an estimated 592 million adults being affected in the year 2035 (Guariguata et al., 2014). The greatest increases are expected in low- and middle-income developing countries of the African, Asian, and South American regions. Diabetes is associated with a host of life threatening and potentially disabling macro- and micro-vascular complications (Levin and Pfeifer, 2009). Hence, there is a much larger burden in the form of loss of productivity in consequence of restricted daily activity, which results in high economic costs. A series of recommendations to lifestyle and diet measures associated of using available pharmacological and surgical therapies are available now for reducing the morbidity and mortality related to diabetes (Khavandi et al., 2013). The α-glucosidase inhibitors, which act reducing the rate of polysaccharide digestion from the proximal small intestine and reduce postprandial glucose, are included among oral diabetic agents. Acarbose, voglibose and miglitol are known α-amylase inhibitors available for diabetes treatment. These molecules can be a useful first-line treatment for patients who have a combination of slightly raised basal glucose concentrations and marked postprandial hyperglycemia (Fujisawa et al., 2005, Cai et al., 2013).

The pancreatic α-amylase (α-1,4 glucan-4-glucanohydrolase, EC 3.2.1.1) catalyses the hydrolysis of the α-1,4-glycosidic linkages in starch, glycogen and other oligo- and polysaccharides. Several molecules were reported to possess pancreatic α-amylase inhibitory activity. Among these molecules are flavonoids, polyphenolics, condensed tannins, hydrolysable tannins, terpenes and cinnamic acid derivatives (Silva et al., 2014; Devarajan and Venugopal, 2012; Ikarashi et al., 2011; Miao et al., 2015, Zajacz et al., 2007). Tannins are naturally occurring plant polyphenols. Their main characteristic is that they bind proteins, basic compounds, pigments, large molecular weight compounds, and metallic ions and display antioxidant activities. They are amply distributed in nature and are present in fruits, teas, trees and grasses.

Condensed tannins are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution patterns (Melone et al., 2013). One of the most extensively studied proanthocyanidinis that extracted from the bark of the black wattle tree (*Acacia mearnsii* De Wild.). It is rich in the catechin-like flavan-3-ols monomers robinetinidol and fisetinidol (Figure 1A; Kusano et al., 2011).

Hydrolysable tannins are derivatives of gallic acid (3,4,5-trihydroxyl benzoic acid). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively crosslinked to yield more complex hydrolysable tannins. One of the most simple and common hydrolysable tannin is the gallotannin with up to 12 esterified galloyl groups and a core glucose (Figure 1B). This structure is particularly abundant in the gallotannin from chinese natural gallnuts (Melone et al., 2013).

The aim of the present study was to compare the *in vitro* inhibitory effects on the pancreatic α-amylase and the *in vivo* hypoglycemic actions of two tannins with well-known chemical structures. The first one is the hydrolysable tannin from Chinese natural gall and the second one the condensed tannin from *A. mearnsii*. For comparative purposes, similar experiments were also run with acarbose, a highly effective inhibitor of pancreatic α-amylase inhibitor. In the *in vitro* experiments, especial attention has been devoted to the kinetics of the inhibition, with a detailed search for the model that best describes the mechanism of action.

Material and methods

Materials

Porcine pancreatic α-amylase (Type VI-B), acarbose and the hydrolysable tannin from Chinese natural gallnuts were purchased from Sigma-Aldrich Co. Condensed tannin from *Acacia mearnsii* bark was purchased from Labsynth, Brazil.

Reaction rate measurements

The kinetic experiments with the porcine pancreatic α-amylase were carried out at 37°C in 20 mmol/L phosphate buffer, pH 6.9, containing 6.7 mmol/L NaCl. Both temperature and pH of the assay are close to the optimum values reported in several studies. Potato starch (Sigma-Aldrich) was used as substrate. The substrate (0.05–1.0 g/100 mL) and one of the three inhibitors, acarbose (up to 5 μg/mL), *A. mearnsii* condensed tannin (up to 500 μg/mL) and hydrolysable tannin (tannic acid; up to 500 μg/mL) were mixed and the reaction was initiated by adding the enzyme. The specific activity of the porcine pancreatic α-amylase was 500 units/mg protein. The amount of enzyme added to each reaction system was 1 unit. The reaction was allowed to proceed for 5 min. The reducing sugars resulting from the starch hydrolysis were assayed by the 3,5-dinitrosalicylic acid (DNS) method, using maltose as standard (Miller, 1959). The aldehyde group of reducing sugars converts 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid, which is the reduced form of DNS. The formation of 3-amino-5-nitrosalicylic acid results in a change in the amount of light absorbed at 540 nm. The

absorbance measured using a spectrophotometer is directly proportional to the amount of reducing sugar. The pH of the reaction medium was tested for all situations. No changes were detected during the incubation time.

Animal experiments

Male healthy Wistar rats weighing 200–250 g were used in all experiments. The rats were housed, fed and treated in accordance with the universally accepted guidelines for animal experimentation. Prior to the investigations, the animals were kept for one week under standard environmental conditions. Throughout the experimentation period, the rats were maintained in single cages and had access to standard pelleted diet and water *ad libitum*. Food was withdrawn 18 h before the experiments. All experiments involving rats were done in accordance with the worldwide accepted ethical guidelines for animal experimentation and previously approved by the Ethics Committee for Animal Experimentation of the University of Maringá (Protocol no. 067/2014-CEUA-UEM).

Effects of α-amylase inhibitors on glycemic levels of rats after starch administration

Rats were divided into 9 groups (n = 4 rats per group). To group I (positive control) commercial corn starch (1 g per kg body weight) was administered intragastrically. Group II (negative control) received only tap water. Group III received intragastrically commercial corn starch plus acarbose (50 mg/kg). Groups IV, V and VI received intragastrically commercial corn starch plus *A. mearnsii* tannin 100, 250 and 500 mg/kg respectively. Finally, groups VII, VIII and IX received intragastrically commercial corn starch plus tannic acid extracts 100, 250 and 500 mg/kg respectively. The amounts of inhibitors given to the rats were based on literature data (Ikarashi et al., 2011). Fasting blood glucose levels were determined before the administration of starch and amylase inhibitors (0 time). Later evaluations of blood glucose levels took placeat 15, 30, 45 and 60 min. Blood glucose from cut tail tips was determined using Accu-Chek® Active Glucose Meter.

Calculations and statistical criteria

Statistical analysis of the data was done by means of the Statistica program (Statsoft, Inc., Tulsa, OK). Fitting of the rate equations to the experimental initial rates was done by means of an iterative non-linear least-squares procedure using the Scientist software from MicroMath Scientific Software (Salt Lake City, UT). The decision about the most adequate model (equation) was based on the model selection criterion (MSC) and on the standard

deviations of the optimized parameters.The model selection criterion, which corresponds to the normalized Akaike Information Criterion (Akaike, 1973), is defined as:

$$MSC = \ln\left[\frac{\sum_{i=1}^{n} w_i (Y_{obs_i} - \overline{Y}_{obs})^2}{\sum_{i=1}^{n} w_i (Y_{obs_i} - Y_{cal_i})^2}\right] - \frac{2p}{n} \quad (1)$$

Where: Y_{obs} are the experimental reaction rates, $\overline{Y}_{obs}$ the mean experimental reaction rate, Y_{cal} the theoretically calculated reaction rate, w_i the weight of each experimental point, n the number of observations and p the number of parameters of the set of equations. In the present work, the model with the largest MSC value was considered the most appropriate, provided that the estimated parameters were positive. When the MSC values differed by less than 5%, the one yielding the smallest standard deviations for the estimated parameters was considered the most appropriate model.

Results

Concentration dependences of the α-amylase inhibition

The inhibitor concentration dependences were measured at a fixed starch concentration of 1 g/100 mL and with varying concentrations of the condensed and hydrolysable tannins both in the range up to 500 µg/mL. The results of these measurements are shown in Figures 2 (hydrolysable tannin) and 3 (condensed tannin). All rates were expressed in µmol reducing sugars per minute (v). Figure 2 shows a clear concentration-dependent inhibition of the α–amylase by the hydrolysable tannin. The concentration for 50% inhibition was around 240 µg/mL and at the concentration of 500 µg/L the inhibition reached 82%. Figure 2 also shows the representation of the inverse rates against the inhibitor concentration, which is clearly parabolic and represents a characteristic that has to be taken into account by the subsequent kinetic analyses.

Figure 3 shows the results obtained with the condensed tannin, which also inhibits the enzyme in a concentration-dependent manner. The concentration for 50% inhibition was around 200 µg/mL and at the concentration of 500 µg/L de inhibition reached 83%. The 1/v versus concentration plot also produced a parabola.

For comparative purposes experiments were also done with acarbose, the classical inhibitor of α-amylases. The concentration dependence is shown in Figure 4 from which a

concentration for 50% inhibition equal to 2.2 μg/mL can be derived. At the concentration of 5 μg/mL inhibition reached 76%. The 1/v *versus* concentration plot was not linear, although the concave up curvature became evident only at the highest concentrations.

Kinetics of the α-amylase inhibition by the hydrolysable tannin

When investigating the kinetic mechanism of the inhibitions caused by the hydrolysable and condensed tannins it is indispensable to take into account the 1/v *versus* [I] plots. The parabolic relationships reveal that more than one inhibitor molecule can bind to the at least one enzyme form. There are several mechanistic possibilities. The best way of investigating this is to measure the reaction rates by varying simultaneously the substrate concentration and the inhibitor concentration with subsequent model analysis in order to find out the mechanism that gives the best description of the experimental data. The results of the experiments that were done with the hydrolysable tannin are shown in Figure 5A. Increasing the hydrolysable tannin concentration diminished progressively the amplitude of the saturation curves. Simple inspection already excludes competitive inhibition as there was no tendency for convergence at high substrate concentrations. In the search for the best mechanism that describes the set of data in Figure 5A the equations corresponding to 9 different mechanisms were fitted to the experimental data. Fitting was simultaneous with two independent variables ([S] and [I]), including the rate versus inhibitor concentration data shown in Figure 5B. The best fit was given by the mechanism shown in Figure 6A, in which a single molecule of the inhibitor binds to the free enzyme (E), but two inhibitor molecules bind almost simultaneously to the substrate complexed enzyme (ES). The equation that describes the mechanism in Figure 6A is given below:

$$v = \frac{V_{max}[S]}{K_M\left(1+\frac{[I]}{K_{i1}}\right)+[S]\left(1+\frac{[I]^2}{(\overline{K}_{i2})^2}\right)} \qquad (2)$$

In equation (2) V_{max} is the maximal reaction rate, K_M the Michaelis-Menten constant, [S] the substrate concentration and [I] the inhibitor concentration. The dissociation constants are K_{i1} and $\overline{K}_{i2}$ for the EI and for the ESI_2 complexes, respectively. It must be noted that the squared inhibitor concentration ($[I]^2$) accounts for the parabolic inhibition. Fitting of a model describing linear inhibition, i.e., when only complexes with one inhibitor molecular are allowed (EI and ESI), resulted in particularly poor fits. The optimized parameters are listed in Table 1. The continuous lines in Figure 5A and 5B were calculated substituting the parameters listed in Table 1 into equation (2). As can be seen, the calculated curves agree

pretty well with the experimental ones with no systematic deviations at the extremes of both substrate and inhibitor concentrations. This is valid for both the v *versus* [S] relationships and the v *versus* [I] relationship. The decision why the mechanism in Figure 6A is the best description of the data was based on the combination of the smallest sum of squared deviations with the greatest model selection criterion. This combination also yielded the smallest standard deviations of the optimized K_{i1} and $\overline{K}_{i2}$ values.

Kinetics of the α-amylase inhibition by the condensed tannin

The results of the experiments that were done by varying simultaneously the concentrations of the condensed tannin and the substrate are shown in Figure 7A. Increasing the condensed tannin concentration diminished progressively the amplitude of the saturation curves. As it was the case with the hydrolysable tannin, inspection already excludes competitive inhibition as there was no tendency for convergence at high substrate concentrations. After fitting 9 different models to the experimental data, the best fit was given by the mechanism shown in Figure 6B. The decision was based here again on the combination of the smallest sum of squared deviations with the greatest model selection criterion. The mechanism in Figure 6B is described by the following equation:

$$v = \frac{V_{max}[S]}{K_M\left(1+\frac{[I]}{K_{i1}}+\frac{[I]^2}{K_{i1}K'_{i1}}\right)+[S]\left(1+\frac{[I]^2}{(\overline{K}_{i2})^2}\right)} \qquad (3)$$

In this mechanism the free enzyme binds sequentially two inhibitor molecules forming complexes EI and EI_2. To the enzyme-substrate complex two molecules of the inhibitor bind simultaneously forming complex ESI_2. The dissociation constants for these complexes are K_{i1}, K'_{i1} and $\overline{K}_{i2}$, respectively. In Figure 7A and 7B the continuous lines were calculated with the optimized parameters listed in Table 2. Agreement between theory and experiment was good, with no systematic deviations at the extremes. The K_M and V_{max} values obtained when fitting equation (3) to the condensed tannin data and those obtained when equation (2) was fitted to the hydrolysable tannin data were practically the same (compare Tables 1 and 2). This is expected because the data were obtained with the same enzyme, but agreement speaks in favour of the correctness and reliability of the numerical analyses.

Kinetics of the α-amylase inhibition by acarbose

Figure 8A shows the results obtained in reaction rate measurements where the starch and acarbose concentrations were varied simultaneously. Simple inspection already rules out

competitive inhibition. Model analysis, performed as described above for the tannins, revealed mechanism C (Figure 6C) as the best description of the experimental data. This mechanism is described by the following equation:

$$v = \frac{V_{max}[S]}{K_M\left(1+\frac{[I]^2}{(\overline{K}_{i1})^2}\right)+[S]\left(1+\frac{[I]}{K_{i2}}\right)} \quad (4)$$

The optimized parameters are shown in Table 3. They reveal V_{max} and K_M values very close to those obtained by fitting the hydrolysable and condensed tannin data (Tables 1 and 2). The continuous lines in both panels of Figure 8 were calculated by substituting the optimized parameters listed in Table 3 into equation (4). It is apparent that all curves are satisfactorily described by equation (4). The parabolic nature of the 1/v versus [I] relationship (Figure 4) is accounted for by the squared inhibitor concentration in equation (4). The fact that $\overline{K}_{i1} > K_{i2}$ is consistent with the observation that the curvature in the 1/v *versus* [I] relationship appears only at the highest inhibitor concentrations.

***In vivo* inhibition of α-amylase**

For testing *in vivo* the inhibition caused by both the hydrolysable and condensed tannin, starch was administered to rats and the glycemic levels were followed during 60 minutes. The basis for these experiments is given by the well-established notion that hydrolysis of intragastrically administered starch is a prerequisite for the entrance of the derived glucosyl units into blood. Figure 9 shows the time course of the experiments that were done by administering various doses of the hydrolysable tannin. When an aqueous solution of starch was administered alone, the glycemic levels raised producing a concave down curve with a peak increment of 85% at 30 minutes after administration. When water was administered the glycemic levels remained relatively constant. Administration of starch in combination with various doses of hydrolysable tannin produced increases in the glycemic levels that were less pronounced than those found when starch was administered alone. A dose-dependent effect is apparent. In all cases, however, concave down curves were obtained. Starch plus acarbose administration, the positive control experiment, also diminished the increase in blood glucose concentration, especially during the first 30 minutes, with a peak at 45 minutes.

Figure 10 shows the results of the experiments done with the condensed tannin. The control curves are the same shown in Figure 9. Co-administration of starch and condensed tannin resulted in diminished increases in the glycemic levels. However, the five-fold increase

in the administered dose (100 to 500 mg/kg) did not result in a pronounced enhancement of the effect. This phenomenon can be best appreciated by comparing the areas under the glycemic curves in Figure 11. The areas were computed numerically and subtracted from the area under the curve obtained when water was administered alone. This area can be regarded as a measure of the extra glucose in the circulating blood during the first 60 minutes following starch and tannin administration. Figure 11A shows that the action of the hydrolysable tannin shows a well defined dose-dependent action. The dose of 100 mg/kg already diminished the glycemic response by 53%; with the 500 mg/kg dose the diminution reached 88%. The action of the condensed tannin was similar to that of the hydrolysable tannin at the dose of 100 mg/kg (49%), but further increases in the administered dose were poorly effective, as the 500 mg/kg dose reduced the glycemic response by not more than 57% (Figure 11B).

Discussion

Inhibition of the pancreatic α-amylase by both the hydrolysable and condensed tannin presents several complexities in that for both inhibitors more than one molecule can bind simultaneously to the enzyme (Cleland, 1963; Plowman, 1972). The same phenomenon was found for acarbose. This is revealed a priori by the non-linear 1/v versus [I] plots and confirmed by the numerical analysis in which attempts of fitting an equation describing linear inhibition always produced unfavourable results. Due to the possible heterogeneity of the preparations that were used, it must be remarked that the presence of more than one inhibitor molecules does not invalidate equations (2) and (3), provided that their concentrations are kept at constant ratios as it occurs when different amounts of the same preparation are added (Cleland, 1963; Plowman, 1972). In the latter case, however, the inhibition constants are no longer true dissociation constants but rather complex functions of several individual dissociation constants. They remain, not with standing a measure of the potency of a given inhibitor (Cleland, 1963; Plowman, 1972). Parabolic inhibition is a common phenomenon among phenolics and tannins. The inhibition of α-amylases by a pinhão coat tannin (Silva et al., 2014) and by the *Phaseolus* protein inhibitorα-AI (Desseaux et al., 2002), has been reported to be parabolic. Inhibition of the pancreatic lipase by a *pinhão* coat tannin is also of the parabolic type (Oliveira et al., 2015). Furthermore, the fact that the same phenomenon occurs with a pure and well defined substance such as acarbose, depending on the substrate (Desseaux et al., 2002), is a proof that it is not generated by an eventual heterogeneity of the inhibitor. On the other hand, on some occasions the phenomenon has been neglected. For example, the inhibition of the human α-amylase by a gallotannin was analyzed as being of the

linear type even though the Dixon plots (1/v versus [I]) that were presented are clearly indicating parabolic inhibition (Kandra et al., 2004).

A characteristic of the inhibitory action of both the condensed and the hydrolysable tannin is the observation that they are bound more tighly to the free enzyme (E) than to the enzyme-substrate complex (SE), whereas the opposite occurs with acarbose. This is revealed by the observation that for the hydrolysable and condensed tannins the k_{i1}values are smaller than the $\overline{K}_{i2}$. For the hydrolysable tannin the difference is more pronounced, more precisely 4.5-fold. It could be that binding of the substrate to the enzyme produces structural modifications that make binding of the inhibitor more difficult (tannins) or easier (acarbose). For the tannins it is noteworthy that the simultaneous binding of two inhibitor molecules to the enzyme-substrate complex occurs with both inhibitors (i.e., ESI_2 is formed with both inhibitors), strongly suggesting that a different binding environment is created once the enzyme binds the substrate. The complex EI_2 was detected only in the case of the condensed tannin and acarbose. If it is also formed with the hydrolysable tannin no sufficient information is available in the v versus [S] and [I] curves. It should be noted that in the experiments in which the substrate concentration was varied, the maximal tannin concentrations were equal to 300 μg/mL whereas the v versus [I] relationship was extended to 500 μg/mL. Similarly, the maximal acarbose concentration in the [S] varying experiments was equal to 3 μg/mL, whereas the v versus [I] relationship was extended to 5 μg/mL. Even so the description of the v versus [I] relationships was very good, with minimal deviations at the highest inhibitor concentrations.

From the values of the inhibitor constants it is obvious that acarbose is a much better inhibitor of the pancreatic α-amylase than the tannins. The question of which tannin is a more effective, however, cannot be unambiguously answered. In terms of their masses and at the substrate concentration of 1 g/100 mL the condensed tannin was a slightly better inhibitor because 50% inhibition occurred at the concentration of 200 μg/mL compared to the 240 μg/mL required for the same degree of inhibition by the hydrolysable tannin. However, the inhibition degree will vary with the substrate concentration and at low substrate concentrations the hydrolysable tannin will be a better inhibitor because of the smaller value of its inhibition constant K_{i1} (see Tables 1 and 2). The difference, however, will never be very pronounced. In molar terms analysis is difficult due to the uncertainties involving the molecular weights of both preparations.

Binding to the α-amylases has been regarded as an essential and necessary phenomenon for the inhibitory activity of both the hydrolysable and condensed tannin. Demonstrations of

this phenomenon by methods that do not involve kinetics have been presented for several combinations of tannins and proteins, including enzymes(Frazier et al., 2003; Cala et al., 2010; Barrett et al., 2013). It can be deduced from these studies that binding of tannins to proteins involves both hydrophilic and hydrophobic interactions. It is non-specific in some cases and specific with a certain degree of cooperativity in others (Frazier et al., 2003). In the case of the hydrolysable and condensed tannins used in the present study, binding is certainly a complex phenomenon, as indicated by the parabolic inhibition kinetics and probably facilitated by the numerous hydroxyl groups present in these molecules (see Figures 1A and 1B). These groups are probably responsible for the most important interactions at low concentrations (Cala et al., 2010). At high concentrations, however, random hydrophobic stacking of the planar rings may occur between tannin and protein, as deduced for the wine tannins binding to saliva proteins based on nuclear magnetic resonance and molecular modeling (Cala et al., 2010).

Inhibition of α-amylase (as well as α-glucosidase) results in delayed carbohydrate digestion and glucose absorption with attenuation of post prandial hyperglycemic excursions. The diminution of hyperglycemia in rats to which starch was administered by both the hydrolysable and condensed tannin is thus an expected phenomenon. Demonstration of the phenomenon also proofs that both tannins are able to exert α-amylase inhibition under in vivo conditions and not only in the test tube. In spite of their similar inhibitory activity towards the enzyme, however, the hydrolysable tannin was more effective in lowering hyperglycemia when compared to the condensed tannin. The response practically ceased to increase with condensed tannin doses above 100 mg/kg, whereas the effects of the hydrolysable tannin increased progressively until the dose of 500 mg/kg. The reasons for this behavior are not clear. One possible reason is that the hydrolysable tannin is active on other enzymes equally involved in starch digestion as the α-glucosidases and invertases, for example, whereas the condensed tannin is inactive or less active (Laube, 2002, Ikarashi et al., 2012). Such a phenomenon would diminished the effectiveness of the condensed tannin. Another possible reason, which does not exclude the former, is that the two types of tannins could be suffering the consequence of different gastric events and movements able to affect their effectiveness as inhibitors.

In conclusion, both tannins, but especially the hydrolysable one, are potentially useful in controlling the post-prandial glycemic levels in diabetic patients. Clinical studies are evidently indispensable for evaluating the viability and safety of the use of preparations containing the hydrolysable or even condensed tannins.

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References

Akaide, H. (1973). A new look at statistical model identification. IEEE Transactions on Automatical Control , 19 ,716–723

Barrett, A., Ndou, T., Hughey, C.A., Straut, C., Howell, A., Dai, Z., Keletunc, G. (2013). Inhibition of _-amylase and glucoamylase by tannins extracted from cocoa, pomegranates, cranberries, and grapes. Journal of Agricultural and Food Chemistry, 61, 1477–1486.

Cai, X., Han, X., Luo, Y., Ji, L. (2013). Comparisons of the efficacy of alpha-glucosidase inhibitors on type 2 diabetes patients between asian and caucasian. PloS One, 8, e79421

Cala, O., Pinau, N., Simon, C., Fouquet, E., Laguerre, M., Dufourc, E.J., Pianet, I. (2010). NMR and molecular modeling of wine tannins binding to saliva proteins: Revisiting adstringency from molecular and colloidal prospects. FASEB Journal, 24, 4281–4290.

Cleland, W. W.(1963). The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: Nomenclature and theory. Biochimica et Biophysica Acta, *67*, 173–187.

Desseaux, V., Koukiekolo, R., Moreau, Y., Santimone, M., Marchis-Mouren, G. (2002). Mechanism of porcine pancreatic α-amylase: inhibition of amylose and maltopentose hydrolysis by various inhibitors. Biologia (Bratislava), *57*, 163–170.

Devarajan S., Venugopal S. (2012). Antioxidant and α-amylase inhibition activities of phenolic compounds in the extracts of Indian honey. Chinese Journal of Natural Medicines, 10, 255-259.

Frazier, R.A., Papadopoulou, A., Mueller-Harvey, I., Kissoon, D., Green, R.J.(2003). Probing protein-tannin interactions by isothermal titration micro-calorimetry.Journal of Agricultural and Food Chemistry, 51, 5189–5195.

Fujisawa, T., Ikegami, H., Inoue, K., Kawabata, Y., Ogihara, T. (2005). Effect of two α-glucosidase inhibitors, voglibose and acarbose, on post-prandial hyperglycemia correlates with subjective abdominal symptoms. Metabolism, 54,387-90

Guariguata, L., Whiting, D.R., Hambleton, I., Beagley, J., Linnenkamp, U., Shaw, J.E. (2014). Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Research and Clinical Practice, 103, 137-149

Ikarashi, N., Sato, W., Toda, T., Ishii, M., Ochiai, W., Sugiyama, K. (2012). Inhibitory effect of polyphenol-rich fraction from the bark of *Acacia mearnsii* on itching associated with allergic dermatitis. Evidence-based Complementary and Alternative Medicine. doi:10.1155/2012/120389 (Article ID 120389, 9 pages).

Ikarashi, N., Takeda, R., Ito, K.,Ochiai, W., Sugiyama, K. (2011). The inhibition of lipase and glucosidase activities by *Acacia* polyphenol. Evidence-based Complementary and Alternative Medicine, http://dx.doi.org/10.1093/ecam/neq043 (Article ID 272075, 8 pages).

Kandra, L., Gyémánt, G., Zajácz A., Batta, G. (2004). Inhibitory effects of tannin on human salivary α-amylase. Biochemical and Biophysical Research Communications, 319, 1265-1271.

Khavandi, K., Amer, H., Ibrahim, B., Brownrigg, J. (2013). Strategies for preventing type 2 diabetes: an update for clinicians. Therapeutic Advances in Chronic Disease, 4, 242-261

Kusano, R., Ogawa, S., Matsuo, Y., Tanaka, T., Yazaki, Y., Kouno, I. (2011).Alpha-amylase and lipase inhibitory activity and structural characterization of acacia bark proanthocyanidins. Journal of Natural Products, 74(2), 119–128.

Laube, H. (2002). Acarbose.Clinical Drugs Investigation, 22, 141–156.

Levin M, Pfeifer M (2009). Uncomplicated Guide to Diabetes Complications. 3rd edition. Alexandria, Virginia: American Diabetes Association.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31, 426–428.

Miao, M., Jiang, B., Jiang, H., Zhang, T., Li, X. (2015). Interaction mechanism between green tea extract and human a-amylase for reducing starch digestion. Food Chemistry, 186, 20-25

Oliveira, R.F., Gonçalves, A.G., Dorneles, F.I., Koehnlein, E.A., Marques de Souza, C.J., Bracht, A., Peralta, R.M. (2015). Inhibition of pancreatic lipase and triacylglycerol intestinal absorption by a pinhão coat (*Araucaria angustifolia*) extract rich in condensed tannin. Nutrients, 7, 5601-5614

Plowman, K. M. *Enzyme kinetics*. McGraw-Hill Book Company: New York, USA, 1972.

Shaw, J.E., Sicree, R.A., Zimmet, P.Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Research and Clinical Practice, 87, 4–14

Silva, S. M., Koehnlein, E. A., Bracht, A., Castoldi, R., Morais G. R., Baesso, M. L., Peralta, R. A., Souza, C. G. M., Sá-Nakanishi, A. B., Peralta, R. M. (2014). Inhibition of salivary and pancreatic α-amylases by a pinhão coat (*Araucaria angustifolia*) extract rich in condensed tannin. Food Research International, 56, 1-8.

Table 1

Kinetic analysis and kinetic parameters of the inhibitory action of the hydrolysable tannin. The parameters were obtained by the simultaneous fitting of equation (2) to the experimental data shown in Figure 5 by means of a non-linear least-squares procedure.

Parameter	**Equation (2) fitting optimization**
K_M (g/100 mL)	0.207± 0.017
V_{max} (μmol min^{-1})	0.839± 0.020
K_{i1} (μg/mL)	64.2 ±6.4
$\overline{K}_{i2}$ (μg/mL)	288.6±18.1
Sum of squared deviations	0.0101
Model selection criterion	4.545
Correlation	0.996

Table 2

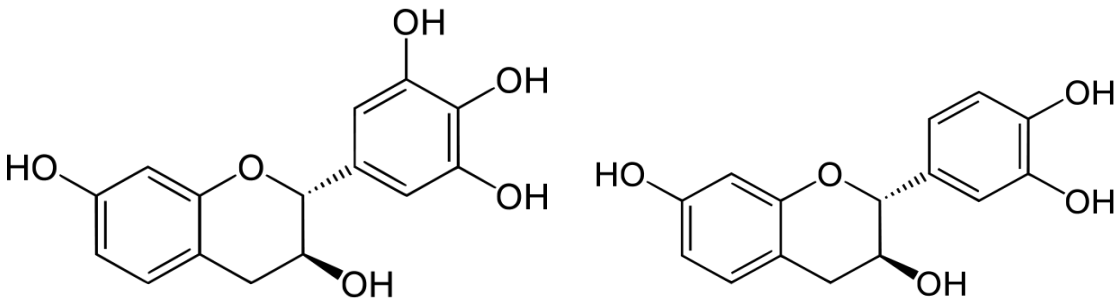
Kinetic analysis and kinetic parameters of the inhibitory action of the condensed tannin. The parameters were obtained by the simultaneous fitting of equation (3) to the experimental data shown in Figure 7 by means of a non-linear least-squares procedure.

Parameter	**Equation (3) fitting optimization**
K_M (g/100 mL)	0.201± 0.015
V_{max} (µmol min^{-1})	0.832± 0.018
K_{i1} (µg/mL)	89.4±13.1
K'_{i1} (µg/mL)	403.2±210.0
$\overline{K}_{i2}$ (µg/mL)	268.5± 23.0
Sum of squared deviations	0.00786
Model selection criterion	4.793
Correlation	0.997

Table 3

Kinetic analysis and kinetic parameters of the inhibitory action of acarbose. The parameters were obtained by the simultaneous fitting of equation (4) to the experimental curves shown in Figure 9 by means of a non-linear least-squares procedure.

Parameter	**Equation (4) fitting optimization**
K_M (g/100 mL)	0.187± 0.020
V_{max} (μmol min^{-1})	0.790± 0.024
$\overline{K}_{i1}$ (μg/mL)	3.18±0.71
K_{i1} (μg/mL)	1.89±0.17
Sum of squared deviations	0.0113
Model selection criterion	3.803
Correlation	0.992



Robinetinidol Fisetinidol

Figure 1A. Chemical structure of the main catechin-like flavan-3-ols found in the condensed tannin from *Acacia mearnsii*.

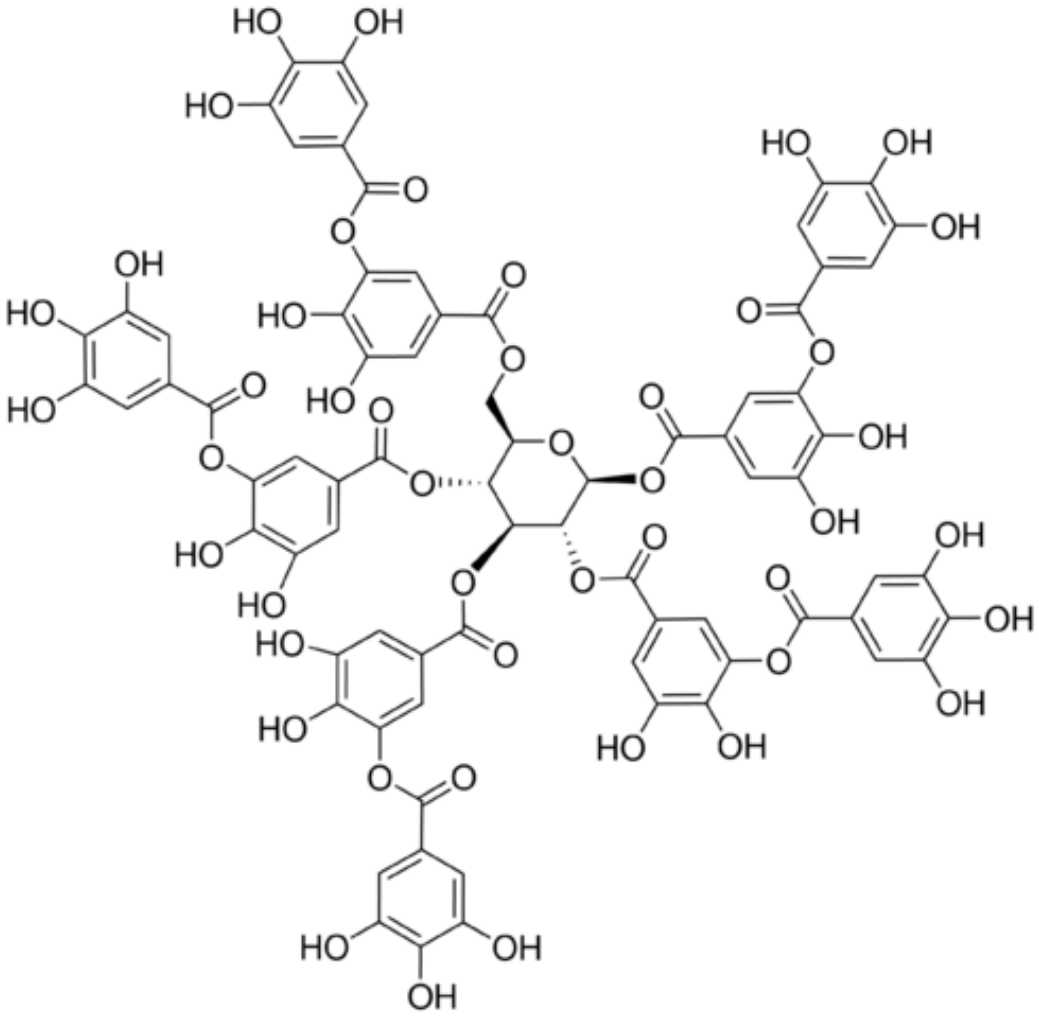


Figure 1B. Chemical structure of a hydrolysable tannin

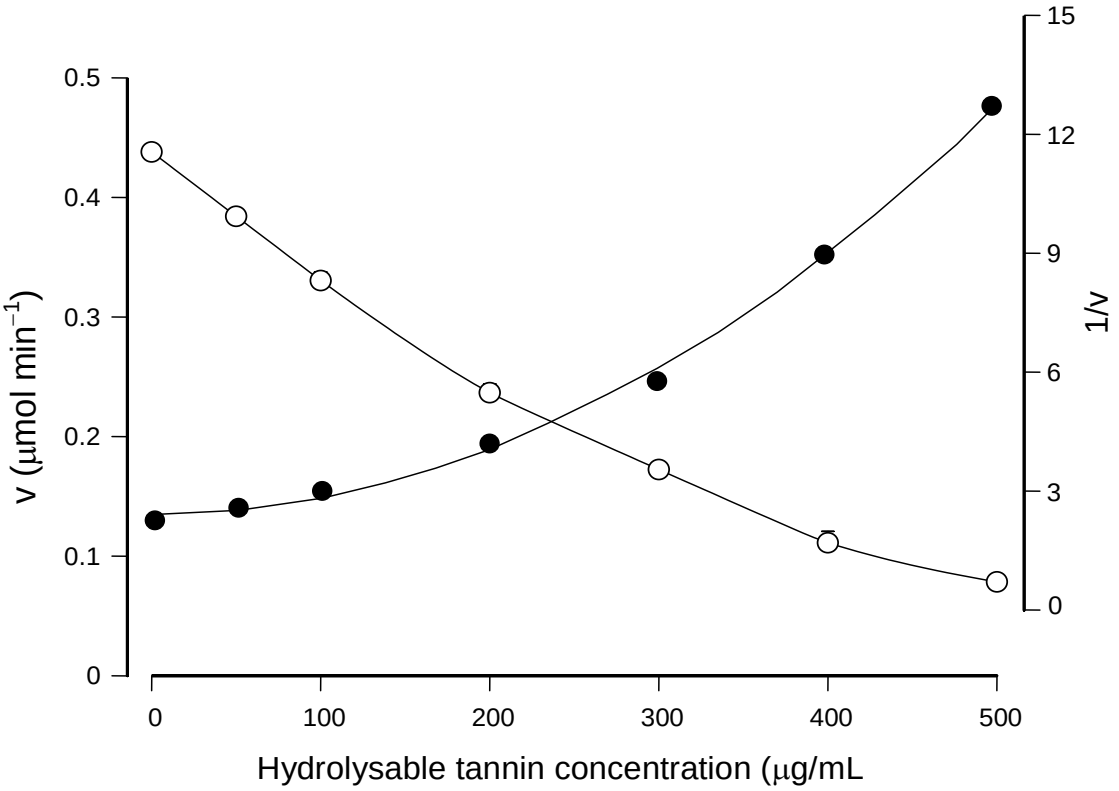


Figure 2. **Concentration dependence of the α-amylase inhibition caused by the hydrolysable tannin.** Each datum point is the mean of three determinations. Reaction rates (v, —○—) and reciprocals of the reaction rates (1/v, —●—) were represented against the hydrolysable tannin concentrations.

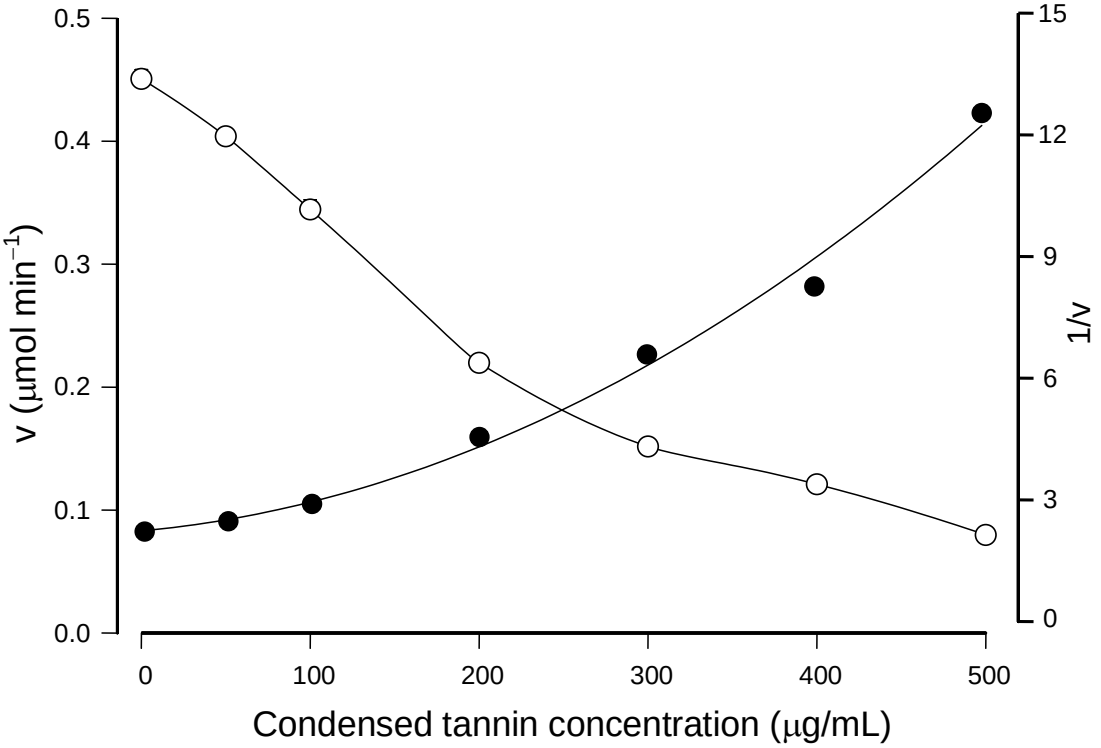


Figure 3. **Concentration dependence of the α-amylase inhibition caused by the condensed tannin.** Each datum point is the mean of three determinations. Reaction rates (v, —○—) and reciprocals of the reaction rates (1/v, —●—) were represented against the condensed tannin concentrations.

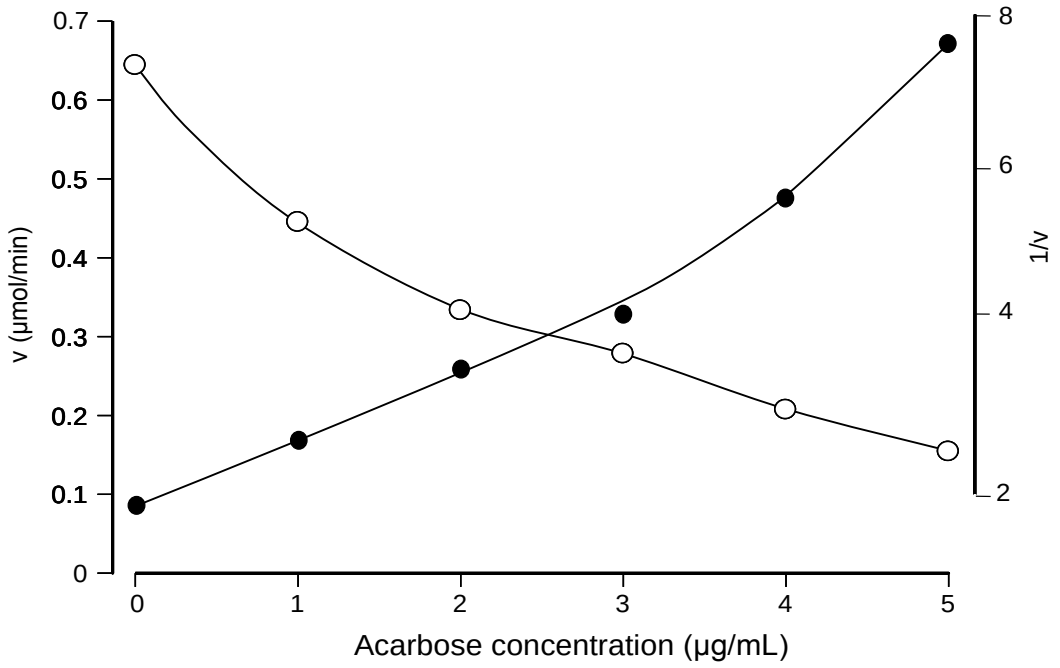


Figure 4. **Concentration dependence of the α-amylase inhibition caused by acarbose.** Each datum point is the mean of three determinations. Reaction rates (v, —○—) and reciprocals of the reaction rates (1/v, —●—) were represented against the acarbose concentrations.

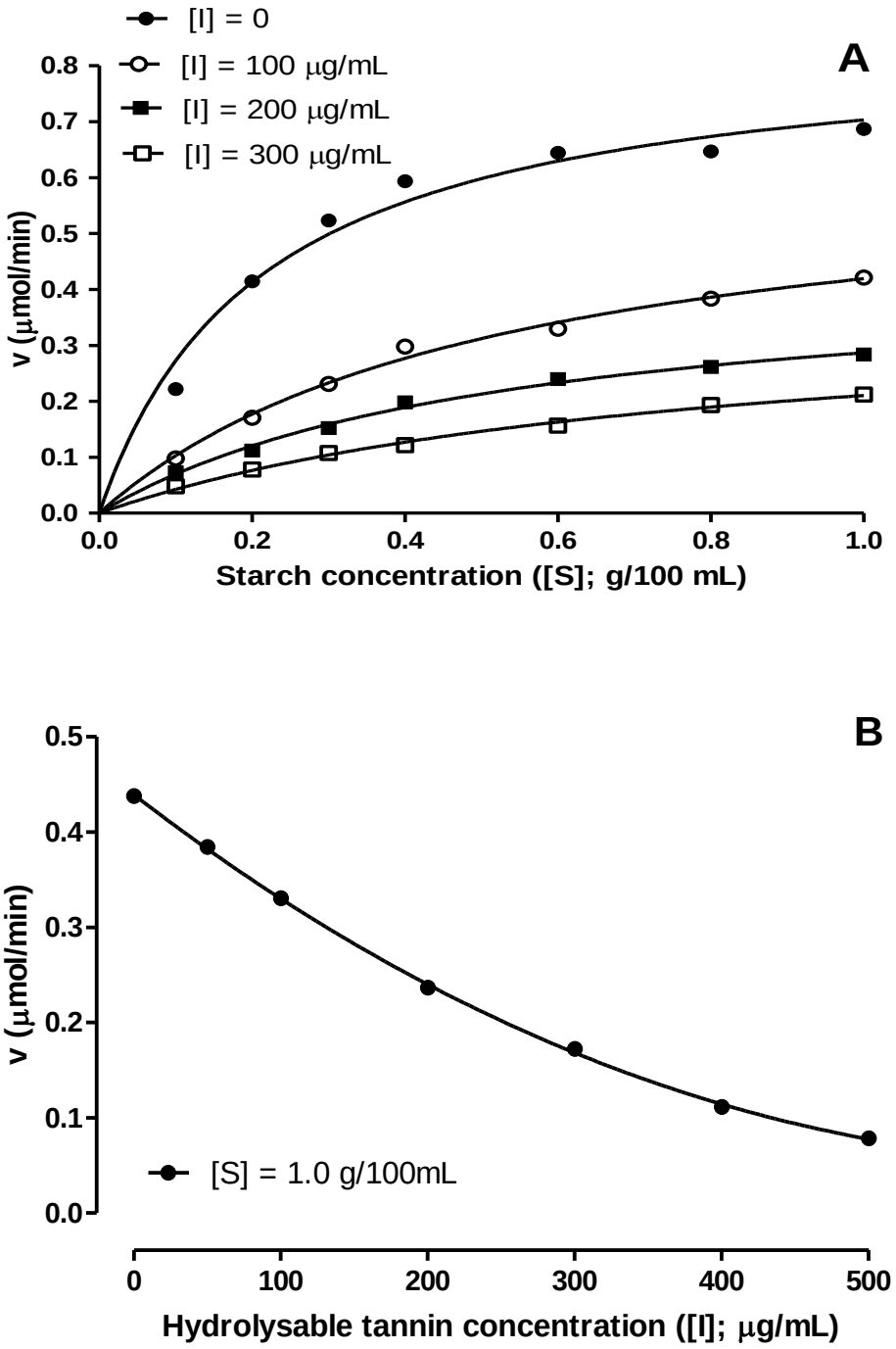


Figure 5. **Reaction rates obtained by varying simultaneously the substrate (starch; panel A) and the hydrolysable tannin (panel B) concentrations.** Each datum point is the mean of three determinations. The lines running through the experimental points were calculated using optimized parameters obtained by fitting equation (2) to the experimental data by means of a non-linear least-squares procedure. Values of the optimized parameters and goodness of fit indicators are listed in Table 1.

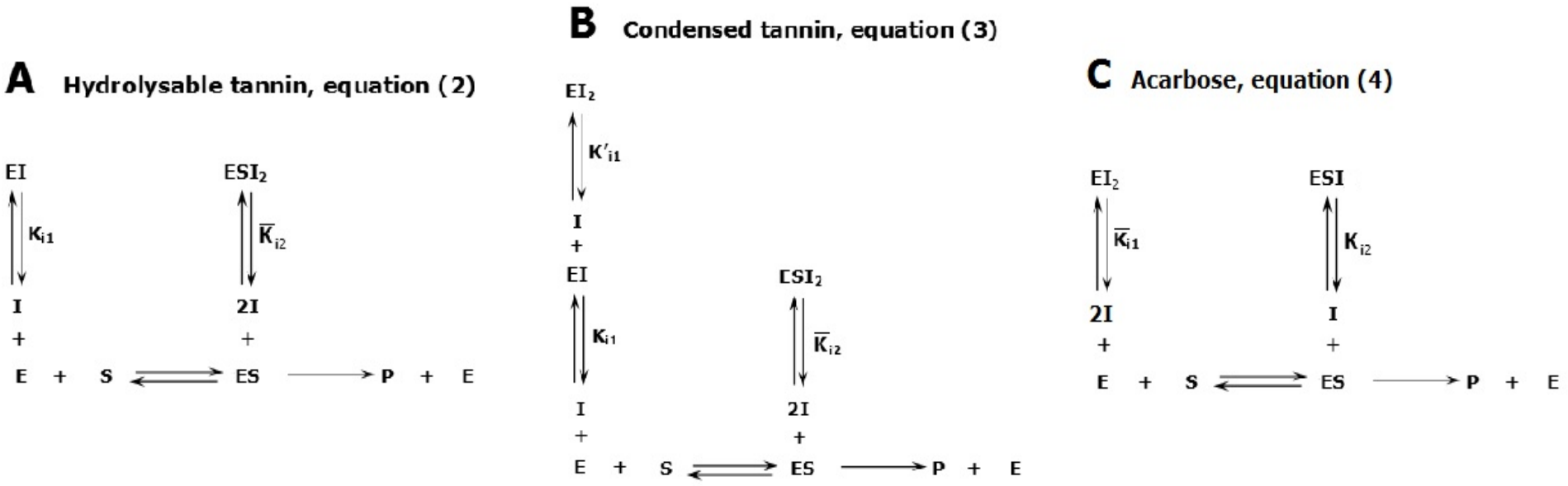


Figure 6. **Schematic representations of the mechanisms described by equations (2), (3) and (4).**

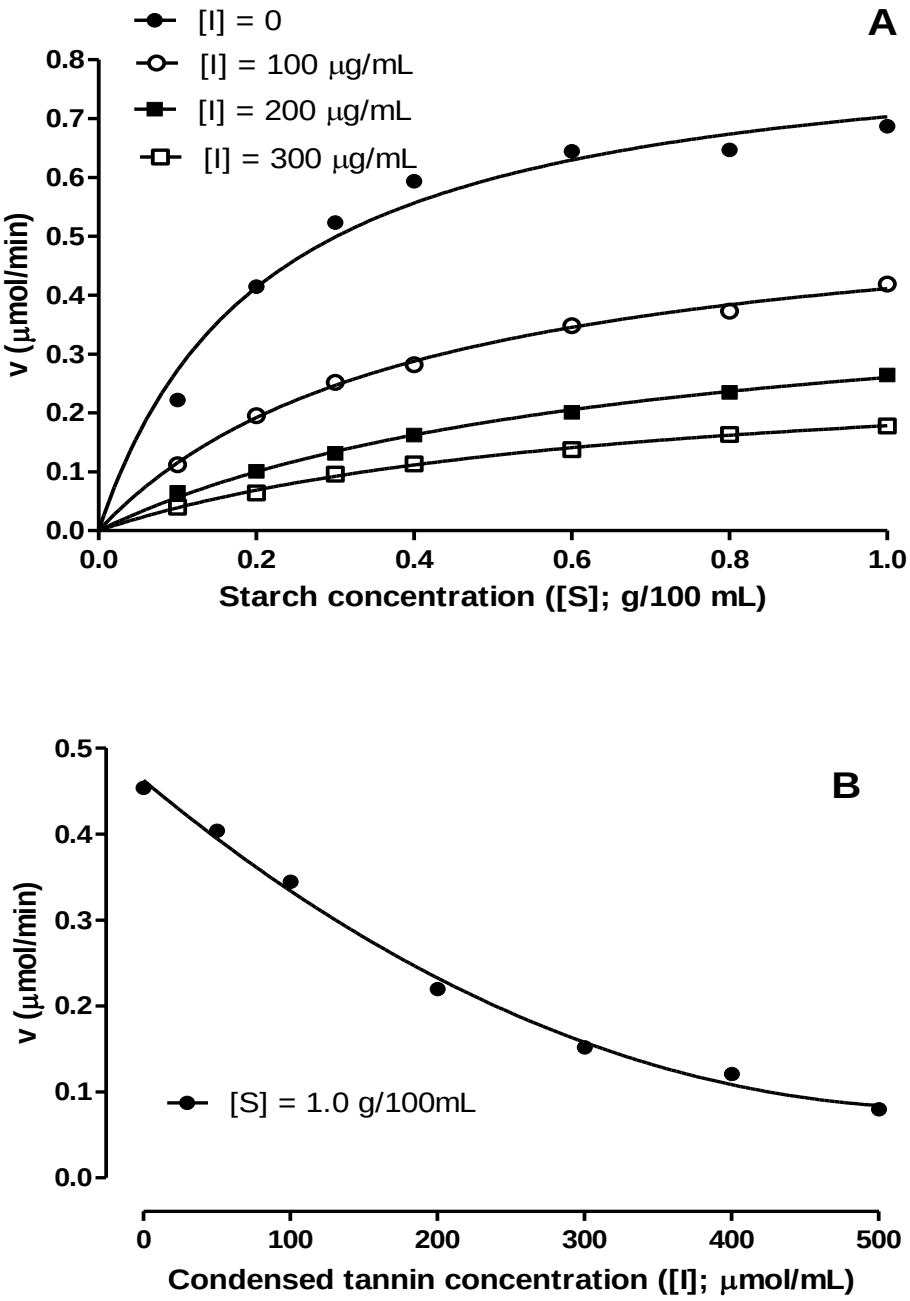


Figure 7. **Reaction rates obtained by varying simultaneously the substrate (starch; panel A) and the condensed tannin (panel B) concentrations.** Each datum point is the mean of three determinations. The lines running through the experimental points were calculated using optimized parameters obtained by fitting equation (3) to the experimental data by means of a non-linear least-squares procedure. Values of the optimized parameters and goodness of fit indicators are listed in Table 2.

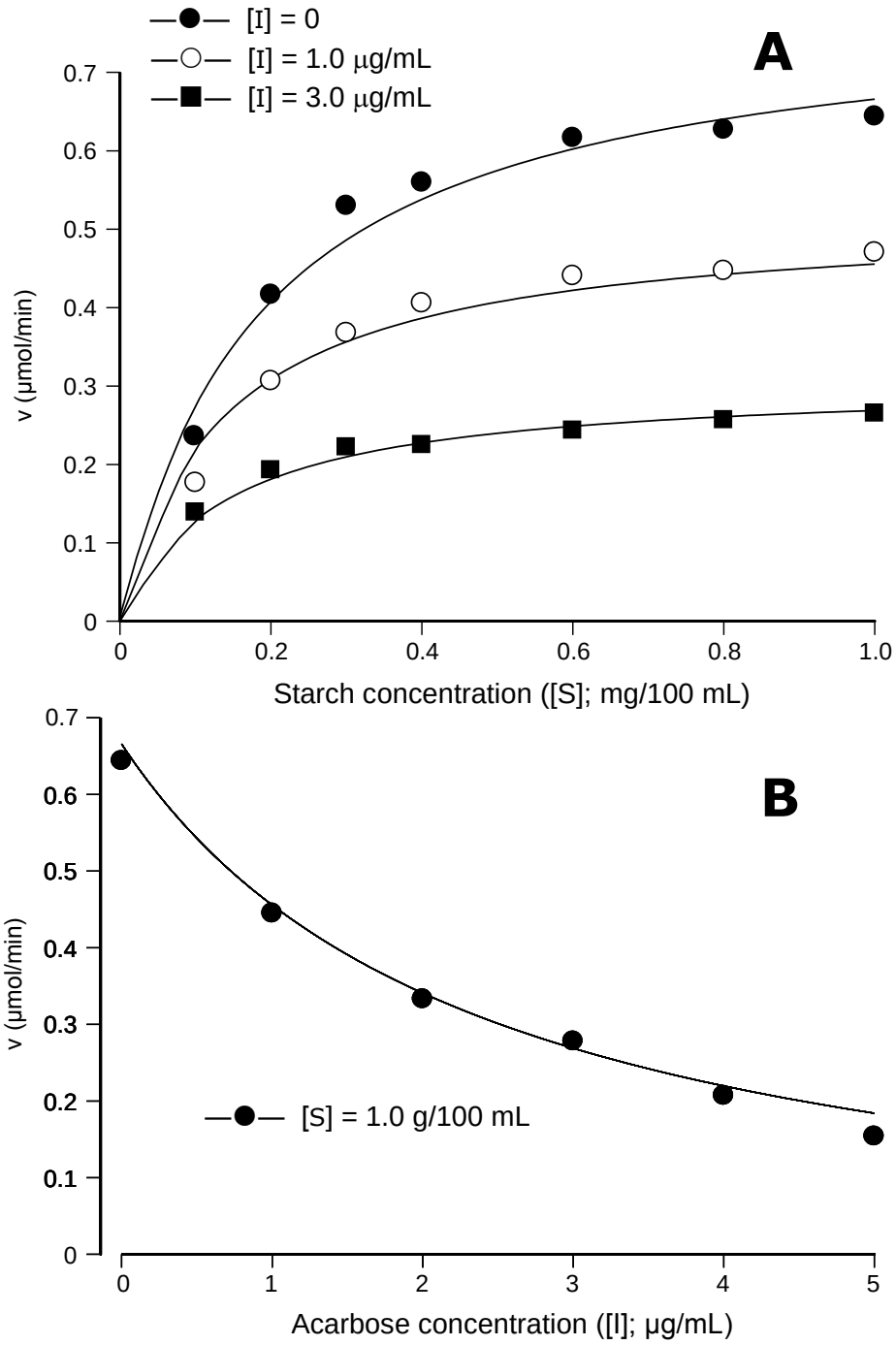


Figure 8. **Reaction rates obtained by varying simultaneously the substrate (starch; panel A) and acarbose (panel B) concentrations.** Each datum point is the mean of three determinations. The lines running through the experimental points were calculated using optimized parameters obtained by fitting equation (4) to the experimental data by means of a

non-linear least-squares procedure. Values of the optimized parameters and goodness of fit indicators are listed in Table 3.

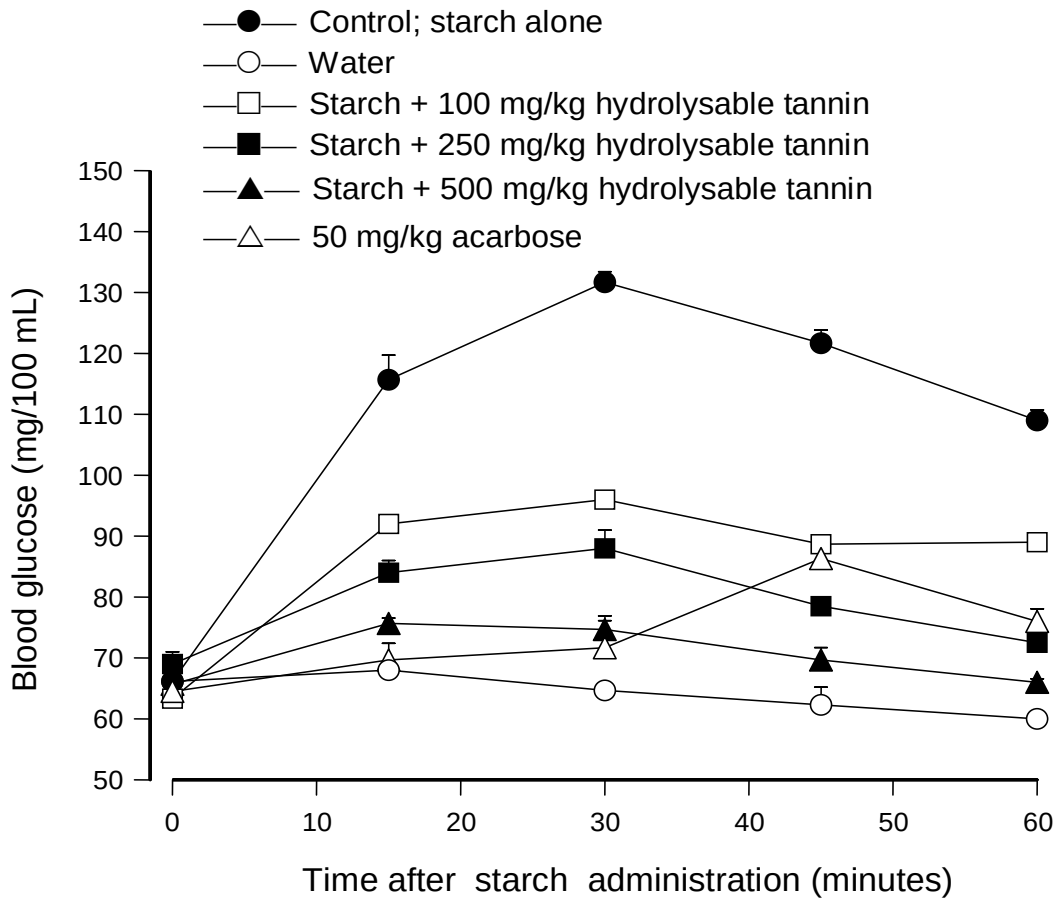


Figure 9. **Influence of the hydrolysable tannin and acarbose administration on the glycemic levels of fasted rats during 60 min following starch administration.** Blood samples from the tail vein were analyzed by means of a glucometer after intragastric starch administration (1 g per kg body weight). The hydrolysable tannin and acarbose were administered intragastrically at the doses given on the top. Each datum point represents the mean ± mean standard errors of three experiments. Experimental details are given in the Materials and methods section.

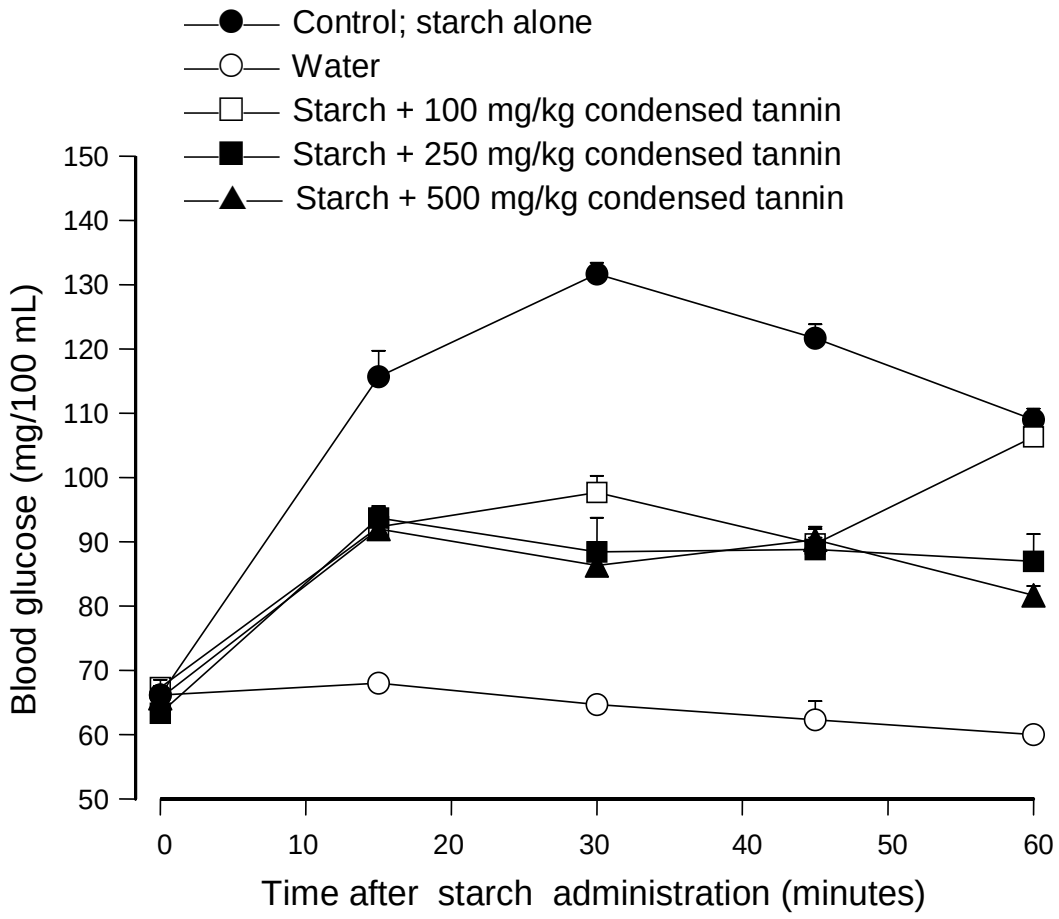


Figure 10. **Influence of the condensed tannin administration on the glycemic levels of fasted rats during 60 min following starch administration.** Blood samples from the tail vein were analyzed by means of a glucometer after intragastric starch administration (1 g per kg body weight). The condensed tannin was administered intragastrically at the doses given on the top. Each datum point represents the mean±mean standard errors of three experiments. Experimental details are given in the Materials and methods section.

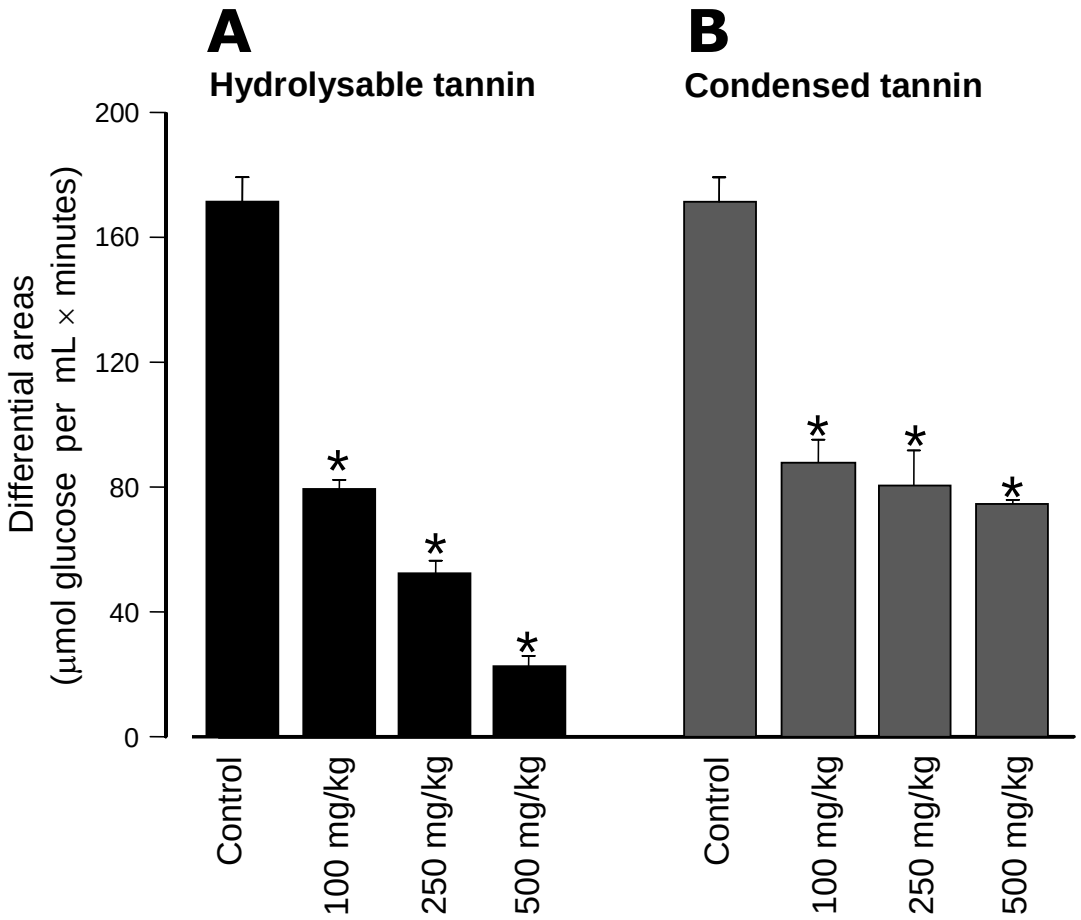


Figure 11. **Areas between the glycemic curves after starch administration (starch alone or starch + α-amylase inhibitors) and the glycemic basal levels.** The areas were determined using the numerical integration procedures of the Scientist software from MicroMath Scientific Software (Salt Lake City, UT). The error terms correspond to standard errors of the means. Asterisks indicate statistical difference relative to the control experiment according to ANOVA followed by post-hoc Student-Newman-Keuls testing ($p \leq 0.05$).

ARTIGO 2

Inhibitory effects of hydrolysable and condensed tannins on human salivary α-amylase

Camila Gabriel Kato[a,b], Verônica Sayuri Nishida[a,b], Adelar Bracht[a,b], Rosane Marina Peralta[a,b*].

[a]Postgraduate Program in Food Science, State University of Maringá, Brazil,

[b]Department of Biochemistry, University of Maringá, 87020900 Maringá, Brazil

*Corresponding author. E-mail: rosanemperalta@gmail.com

Abstract

The purpose of the present work was to investigate the possible inhibitory effects of the condensed tannin from *Acacia mearnsii* de Wild. bark and the hydrolysable tannin from chinese natural gall nuts on the activity of human salivary α-amylase (HAS). The IC_{50} values (inhibitor concentration producing 50% inhibition) for a starch concentration of 1 g/100 mL were 80 µg/mL and 230 µg/mL for the hydrolysable and condensed tannins, respectively. From the kinetic analysis it can be concluded that inhibition of the HSA by both tannins is of the mixed (or non-competitive) type. The free HSA binds the hydrolysable tannin with higher affinity considering that the K_{i1} value was 22.4±2.9 µg/mL while the K_{i1} value for condensed tannin was 157.1±12.6 µg/mL. Taking into account that HSA has an important role in the dental plaque formation and subsequent dental caries formation, the strong inhibitory action of the hydrolysable tannin could make it an useful agent for oral health.

Key words: human salivary amylase, enzyme inhibition, biofilm, dental caries.

1. Introduction

In humans five isoenzymes of amylase (α-1,4 glucan-4-glucanohydrolase, EC 3.2.1) have been described. The three isoforms of salivary amylase and the two isoforms of pancreatic amylase are classified as two different families of isoenzymes. The three-dimensional structures of the α-amylases from human pancreas and saliva and from porcine pancreas have already been determined by X-ray crystallography (Brayer, Luo & Wither, 1995, Ramasubbu, Paloth, Luo, Brayer & Levine, 1996, Qian, Haser & Payan., 1993). Structurally these enzymes are all very closely related. Due to its importance in several metabolic disorders including diabetes and obesity, the pancreaticα-amylase has been more extensively studied than the salivary α-amylase. In consequence, a series of pancreatic α-amylase inhibitors are available in the market, such as acarbose, voglibose and miglitol (Fujisawa, Ikegami, Inoue, Hawabata & Ogihara, 2005; Dabhi, Bhatt & Shah, 2013; Lee et al., 2015). The administration of these molecules can be a useful first-line treatment for patients who have a combination of slightly raised basal plasma glucose concentrations and marked postprandial hyperglycemia. The human salivary amylase (HSA), the most abundant enzyme in human saliva, catalyses the hydrolysis of α-1,4 glycosidic linkages of polysaccharides initiating the digestion of complex carbohydrates in the human oral cavity, where especially starch is partly digested into oligosaccharides, maltose and glucose (Boehlke, Zierau & Hannong, 2015).

A series of molecules have been reported to possess inhibitory activity against α-amylases as for example tannins (Lavelli, Harsha & Fiori, 2015, de Sales, Souza, Simeoni, Magalhães & Silveira, 2012, Zhang & Kashket, 1998, Aizawa et al., 2008, Kim, Jeong, Wang, Lee & Rhee, 2005, Yilmazer-Musa, Griffth, Michels, Schneider & Frei, 2012, Zajácz, Gyémánt, Vittori & Kandra, 2007, Kashket & Paolino, 1988, Gyémán, Kandra, Nagy & Somsák, 2003). Tannins are naturally occurring plant polyphenols. Due to the enormous

structural diversity of the tannins, they are classified into hydrolysable tannins and non-hydrolyzable tannins also known as condensed tannins. The latter are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution patterns (Melone, Saladino, Lange & Crestine, 2013). One of the most extensively studied proanthocyanidins is that one extracted from the bark of the black wattle tree (*Acacia mearnsii* De Wild.). It is rich in the catechin-like flavan-3-ol monomers robinetinidol and fisetinidol (Kusano et al., 2011). Hydrolysable tannins are derivatives of gallic acid (3,4,5-trihydroxyl benzoic acid). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively crosslinked to yield more complex hydrolysable tannins. One of the most simple and common hydrolysable tannin is the gallotannin with up to 12 esterified galloyl groups and a core glucose. This structure is particularly abundant in the gallotannin from chinese natural gallnuts (*Rhus chinensis* Mill.) (Melone et al., 2013). The aim of the present study was to investigate the *in vitro* inhibitory effects on the human salivary α-amylase of these two tannins in the search of new molecules with an increased affinity and specificity for the enzyme.

2. Material and Methods

2.1. Materials

Human salivary alpha-amylase and the hydrolysable tannin from chinese natural gall nuts were obtained from Sigma-Aldrich Co. The condensed tannin from *A. mearnsii* bark was purchased from Labsynth, Brazil.

2.2. Reaction rate measurements

The kinetic experiments with the HSA were carried out at 37 °C in 20 mmol/L phosphate buffer, pH 6.9, containing 6.7 mmol/L NaCl. Both temperature and pH of the assay

are close to the optimum values reported in several studies. Potato starch (Sigma-Aldrich) was used as substrate. The substrate (0.05–1.0 g/100 mL) and one of the two inhibitors, *A. mearnsii* condensed tannin (up to 500 μg/mL) and hydrolysable tannin (tannic acid) (up to 500 μg/mL) were mixed and the reaction was initiated by adding the enzyme. The specific activity of the human salivary α-amylase was 500 units/mg protein. The amount of enzyme added to each reaction system was 1 unit. The reaction was allowed to proceed for 5 min. The produced reducing sugars from the hydrolysis of starch were assayed by the 3,5 dinitrosalicylic acid (DNS) method, using maltose as standard (Miller, 1959). The aldehyde group of reducing sugars converts 3,5-dinitrosalicylic acid into 3-amino-5-nitrosalicylic acid, which is the reduced form of DNS. The formation of 3-amino-5-nitrosalicylic acid results in a change in the absorbance at 540 nm which is directly proportional to the amount of reducing sugar. The pH of the reaction medium was tested in all situations. No changes were detected during the incubation time.

2.3. Calculations and statistical criteria

Statistical analysis of the data was done by means of the Statistica program (Statsoft, Inc., Tulsa, OK). Fitting of the rate equations to the experimental initial rates was done by means of an iterative non-linear least-squares procedure using the *Scientist* software from MicroMath Scientific Software (Salt Lake City, UT). The decision as to the most adequate model (equation) was based on the model selection criterion (MSC) and on the standard deviations of the optimized parameters.

The model selection criterion, which corresponds to the normalized Akaike Information Criterion (Akaike, 1973), is defined as:

$$MSC = \ln\left[\frac{\sum_{i=1}^{n} w_i (Y_{obs_i} - \overline{Y}_{obs})^2}{\sum_{i=1}^{n} w_i (Y_{obs_i} - Y_{cal_i})^2}\right] - \frac{2p}{n} \quad (1)$$

Where: Y_{obs} are the experimental reaction rates, $\overline{Y}_{obs}$ the mean experimental reaction rate, Y_{cal} the theoretically calculated reaction rates, w the weight of each experimental point, n the number of observations and p the number of parameters of the set of equations. In the present work the model with the largest MSC value was considered the most appropriate, provided that the estimated parameters were positive (Akaike, 1973). When the MSC values differed by less than 5%, the one yielding the smallest standard deviations for the estimated parameters was considered the most appropriate model.

3. Results

3.1. Concentration dependences of the human salivary α-amylase inhibition

The inhibitor concentration dependences of the reaction rates were measured at a fixed starch concentration (1 g/100 mL). The results of these measurements are summarized in Figure 1. In Figure 1A, the rates were represented against the inhibitor concentration. It is apparent that both condensed and hydrolysable tannins inhibit the enzyme with a clear concentration dependence. From the graph it is also apparent that the hydrolysable tannin is a stronger inhibitor than the condensed tannin. The IC_{50} value (the concentration of inhibitor required to reduce the rate of the enzymatic reaction by 50%) allows a quantitative evaluation of the effectiveness of each compound: it is equal to 80 μg/mL for the hydrolysable tannin, and 230 μg/mL for the condensed tannin. This means that at the starch concentration of 1 g/100 mL the hydrolysable tannin is 2.9 times more efficient as an inhibitor of the α-amylase than the condensed tannin.

In Figure 1B the reciprocals (1/v) of the reaction rates were represented against the corresponding concentrations. In both cases the relationship is parabolic even though this is less evident for the inhibition caused by the condensed tannin. This occurs because a single

1/v scale was used for both inhibitions and the inhibition degree with the hydrolysable tannin is much more pronounced, what causes a much more evident upward concavity.

3.2. Kinetics of human salivary α-amylase inhibition by the hydrolysable tannin

When investigating the kinetic mechanism of the inhibitions caused by the hydrolysable and condensed tannins it is indispensable to take into account the 1/v versus [I] plots shown in Figure 1B. The parabolic relationships reveal that more than one inhibitor molecule can bind to the at least one enzyme form. There are several mechanistic possibilities. The best way of investigating this is to measure the reaction rates by varying simultaneously the substrate concentration and the inhibitor concentration with subsequent model analysis in order to find out the mechanism that gives the best description of the experimental data. The results of the experiments that were done with the hydrolysable tannin are shown in Figure 2A which shows saturation curves that were progressively lowered as the tannin was added at progressively increased concentrations. The saturation curves do not show any tendency of convergence at high substrate concentrations, which excludes the possibility of competitive inhibition. In the search for the best mechanism that describes the set of data in Figure 2A the equations corresponding to 9 different mechanisms were fitted to the experimental data (Cleland, 1963; Plowman, 1972). Fitting was done simultaneously with two independent variables ([S] and [I]), including the rate versus inhibitor concentration data shown in Figure 1. The best fit was given by the mechanism shown in Figure 3A. In this mechanism the first binding of the inhibitor to the free enzyme (E) can be followed by the binding of a second inhibitor molecule, so that the complexes EI and EI_2 can be distinguished. Additionally, one inhibitor molecule can bind to the ES complex producing the ESI complex. The equation that describes the mechanism in Figure 3A is given below:

$$v = \frac{V_{max}[S]}{K_M\left(1+\frac{[I]}{K_{i1}}+\frac{[I]^2}{K_{i1}k'_{i1}}\right)+[S]\left(1+\frac{[I]}{K_{i2}}\right)} \quad (2)$$

In equation (2) V_{max} is the maximal reaction rate, K_M the Michaelis-Menten constant, [S] the substrate concentration and [I] the inhibitor concentration. The dissociation constants are K_{i1}, K'_{i1} and K_{i2} for the EI, EI_2 and ESI complexes, respectively. It must be noted that the squared inhibitor concentration ($[I]^2$) accounts for the parabolic inhibition. Fitting of a model describing linear inhibition, i.e., when only complexes with one inhibitor molecule are allowed (EI and ESI) resulted in particularly poor fits. The decision about the mechanism giving the best description of the data was based on the combination of the smallest sum of squared deviations with the greatest model selection criterion. This combination also yielded the smallest standard deviations of the optimized parameters, even though there is considerable uncertainty in the determination of K_{i2}. The goodness of the fit of equation (2) to the data can be evaluated by examining panels A and B in Figure 2 and by analysing the optimized parameters listed in Table 1. The continuous lines in Figure 2A and 2B were calculated by substituting the optimized parameters in equation (2). The calculated curves agree pretty well with the experimental ones. Only in the v versus [I] relationship shown in Figure 2B there is a small systematic deviation between calculated and experimental data at the highest inhibitor concentrations. This may be indicating binding events that could not be detected in the v versus [S] experiments, which were done with inhibitor concentrations up to 200 µg/mL due to the fact that it is very difficult to measure accurately initial reaction rates at low substrate concentrations when the inhibition degree exceeds 85%. On the other hand, the deviation may bear some relation to the uncertainly in the determination of K_{i2} already mentioned above. In any case, the mechanism in Figure 3A is perfectly valid for inhibitor concentrations up to 200 µg/mL, which corresponds to an inhibition degree of at least 75%.

Comparison of the optimized inhibition constants listed in Table 1 reveals that K_{i1} is much smaller than K'_{i1}, most specifically the k_{i1} value corresponds to only 3.8% of the k'_{i1} value. On the other hand, k_{i2} is relatively large, so that it is correct to conclude that at low hydrolysable tannin concentrations the inhibitory activity depends largely on the formation of the EI complex.

3.3. Kinetics of human salivary α-amylase inhibition by the condensed tannin

The results of the experiments that were done by varying simultaneously the concentration of the condensed tannin and the substrate are shown in Figure 4A. Increasing the condensed tannin concentration diminished progressively the amplitude of the saturation curves. As it was the case with the hydrolysable tannin, inspection already excludes competitive inhibition as there was no tendency for convergence at high substrate concentrations. After fitting 9 different models to the experimental data, the best fit was given by the mechanism shown in Figure 2B. The decision was based here again on the combination of the smallest sum of squared deviations with the greatest model selection criterion. The mechanism in Figure 2B is described by the following equation:

$$v = \frac{V_{max}[S]}{K_M\left(1+\frac{[I]^2}{(\overline{K}_{i1})^2}\right)+[S]\left(1+\frac{[I]}{K_{i2}}+\frac{[I]^2}{K_{i2}K'_{i2}}\right)} \qquad (3)$$

In this mechanism the free enzyme binds simultaneously (or nearly so) to two inhibitor molecules forming the complexes EI_2. To the enzyme-substrate complex two molecules of the inhibitor bind successively forming complexes ESI and ESI_2. The dissociation constants for these complexes are $\overline{K}_{i1}$, K_{i2} and K'_{i2}, respectively. In Figures 4A and 4B the continuous lines were calculated with the optimized parameters listed in Table 2. Agreement between theory and experiment was good. The K_M and V_{max} values obtained when fitting equation (3) to the

condensed tannin data and those obtained when equation (2) was fitted to the hydrolysable tannin data were practically the same (compare Tables 1 and 2). This is expected because the data were obtained with the same enzyme, but agreement speaks in favour of the correctness and reliability of the numerical analyses. The predictability of the rate *versus* inhibitor concentration relationship can be evaluated by comparing the experimental rates measured at various condensed tannin concentrations and the calculated rates in Figure 2B. Comparison reveals a good agreement between theory and experiment with no systematic deviations.

4. Discussion

It can be concluded that inhibition of the salivary α-amylase by both the hydrolysable and condensed tannin is of the mixed (or non-competitive) type and that it is likely to involve the binding of more than one molecule to the enzyme (Cleland, 1963; Plowman, 1972). This is revealed not only by the non-linear 1/v versus [I] plots but especially by the numerical analysis in which attempts of fitting equations describing linear inhibition always produced unfavourable results. A possible heterogeneity of the tannins that were used does not invalidate the analysis. When more than one inhibitor is present at constant ratios, what was undoubtedly the case of the preparations used in the present study, equations like (2) and (3) are still valid in its general form (Cleland, 1963; Plowman, 1972). Although the inhibition constants are no longer true dissociation constants but rather complex functions of several individual dissociation constants, they are still a measure of the potency of the inhibitors (Cleland, 1963; Plowman, 1972). Parabolic inhibition is a common phenomenon among phenolics and tannins although the phenomenon has been neglected in some studies (Kandra, Gyémánt, Zajácz & Batta, 2004). In others, however, it has been correctly identified. For example, the inhibition of α-amylases by a pinhão coat tannin and by the *Phaseolus* protein inhibitor α-AI (Silva et al., 2014; Desseaux, Koukiekolo, Moreau, Santimone & Marchis-Mouren, 2002), has been reported to be parabolic. The inhibition of the pancreatic lipase by a

pinhão coat tannin is also parabolic (Oliveira et al., 2015). Furthermore, the fact that parabolic inhibition occurs with a pure and well defined substance such as acarbose, depending on the substrate (Desseaux et al., 2002; Oliveira et al., 2015), is compatible with the notion that it is not generated by an eventual heterogeneity of the inhibitor. In the study of Kandra et al. (2004) with the human salivary α-amylase, the same commercial preparation of the hydrolysable tannin from chinese natural gall nuts was used, but the substrate was 2-chloro-4-nitrophenyl-4-O-β-D-galacto-pyranosyl-maltoside (GalG2CNP) instead of starch. The hydrolysable tannin inhibits the hydrolysis of this substrate, but surprisingly the authors analysed this inhibition as if it were of the linear type, neglecting the fact that the Dixon plots (1/v versus [I]) are clearly non-linear (Kandra et al., 2004). These particularities of the latter study make it difficult to find a basis for comparisons with the results of the present study. However, the results of Kandra et al. (2004) indicate that the hydrolysable tannin inhibits the hydrolysis of 2-chloro-4-nitrophenyl-4-O-β-D-galacto-pyranosyl-maltoside in a non-competitive manner and that this inhibition is probably also of the parabolic type.

Several studies have indicated the participation of HSA in at least three important functions in the oral cavity: (1) hydrolysis of dietary starch, (2) binding to the tooth surface, and (3) binding to oral streptococci (Hannig, Hannig & Attin, 2005, Nikitkova, Haase & Scannapieco, 2013, Zhang and Kashket, 1998). All three actions contribute to the process of dental plaque and caries formation. Binding to the enzyme is likely to restrict these three activities. A number of studies have shown that tea extracts (*Camellia sinensis* (L.) Kuntze) reduce dental caries (Rosen, Elvin-Lewis, Beck & Beck, 1984, Narotzki, Reznick, Aizenbud & Levy, 2012, Goenka et al., 2013, Hara et al., 2012). Based on the α-amylase inhibitory activity of tea extracts, the hypothesis has been raised that this activity could be involved in the reduction of the cariogenicity of starch-containing foods (Kashket & Paolino, 1988). For this reason, the hydrolysable tannin, which is bound very strongly by the enzyme, as indicated by the K_{i1} value of 22.4 μg/mL, can be regarded as an useful agent for oral health. More

studies are necessary to evaluate the possibility of incorporating this tannin into dental products such as dentifrices, mouthwashes, dental flosses, and chewing gums that could be helpful in the prevention of dental caries.

Acknowledgments

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References

Aizawa, S., Miyasawa-Hori, H., Nakajo, K., Washio, J., Mayanagi, H., Fukumoto, S. & Takahashi, N. (2008). Effects of α-amylase and its inhibitors on acid production from cooked starch by oral streptococci. *Caries Research, 43,* 17-24.

AKAIKE, H. (1973). A NEW LOOK AT STATISTICAL MODEL IDENTIFICATION. *IEEE TRANSACTIONS ON AUTOMATIC CONTROL, 19,* 716–723.

Boehlke, C., Zierau, O. & Hannong, C. (2015). Salivary amylase. The enzyme of unspecialized euryphagous animals. *Archives of Oral Biology, 60,* 1162-1178.

Brayer, G.D., Luo, Y. & Withers, S.G. (1995). The structure of human pancreatic alpha-amylase at 1.8 Angstrom resolution and comparisons with related enzymes. *Protein Science, 4,* 1730–1742.

Cleland, W. W. (1963). The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: Nomenclature and theory. *Biochimica et Biophysica Acta, 67,* 173–187.

Dabhi, A.S., Bhatt, N.R. & Shah, M.J. (2013). Voglibose: an alpha glucosidase inhibitor. *Journal of Clinical and Diagnostic Research, 7,* 3023-3027.

de Sales, P.M., Souza, P.M., Simeoni, L.A., Magalhães, P.O. & Silveira, D. (2012). α-amylase inhibitors: a review of raw material and isolated compounds from plant source. *Journal of Pharmacy and Pharmaceutical Sciences, 15,* 141-183.

Desseaux, V., Koukiekolo, R., Moreau, Y., Santimone, M. & Marchis-Mouren, G. (2002). Mechanism of porcine pancreatic α-amylase: inhibition of amylose and maltopentose hydrolysis by various inhibitors. *Biologia (Bratislava), 57,* 163–170.

Fuzisawa, T., Ikegami, H., Inoue, K., Kawabata, Y. & Ogihara, T. (2005). Effect of two alpha-glucosidase inhibitors, voglibose and acarbose, on postprandial hyperglycemia correlates with subjective abdominal symptoms. *Metabolism, 54*, 387-390.

Goenka, P., Sarawgi, A., Karun, V., Nigam, A.G., Dutta, S. & Marwah, N. (2013). *Camellia sinensis* (Tea): Implications and role in preventing dental decay. *Pharmacognosy Review, 7*, 152–156.

Gyémánt, G., Kandra, L., Nagy, V. & Somsák, L. (2003). Inhibition of human salivary α-amylase by glucopyranosylidene-spiro-thiohydantoin. *Biochemical and Biophysical Research Communications, 312*, 334-339.

Hannig, C., Hannig, M. & Attin, T. (2005). Enzymes in the acquired enamel pellicle. *European Journal of Oral Science, 113*, 2-13.

Hara K, Ohara M, Hayashi I, Hino T, Nishimura R, Iwasaki Y, Ogawa T, Ohyama Y, Sugiyama, M. & Amano, H. (2012). The green tea polyphenol (-) epigallocatechin gallate precipitates salivary proteins including alpha-amylase: biochemical implications for oral health. *European Journal of Oral Science, 120,* 132–139.

Kandra, L., Gyémánt, G., Zajácz A., Batta, G. (2004). Inhibitory effects of tannin on human salivary α-amylase. *Biochemical and Biophysical Research Communications, 319,* 1265-1271.

Kashket, S. & Paolino, V.J. (1988). Inhibition of salivary amylase by water-soluble extracts of tea. *Archives of Oral Biology, 33,* 845-846.

Kim, Y-M, Jeong, Y-K, Wang, M-H, Lee, W-Y, Rhee, H-I (2005). Inhibitory effect of pine extract on α-glucosidase activity and postprandial hyperglycemia. *Nutrition, 21*, 756-761.

Kusano, R., Ogawa, S., Matsuo, Y., Tanaka, T., Yazaki, Y., Kouno, I. (2011).Alpha-amylase and lipase inhibitory activity and structural characterization of acacia bark proanthocyanidins. *Journal of Natural Products, 74(2),* 119–128.

Lavelli, V., Harsha, P.S.C., & Fiori,, L. (2015. Screening grape seeds recovery from wine making by-products as sources of reducing agents and mammalian α-glucosidase and α-amylase inhibitors. *International Journal of Food Science and Technology, 50,* 1182-1189.

Lee, E.Y., Kaneko, S., Jutabha, P., Zhang, X., Seino, S., Jomori, T., Anzai, N. & Miki, T. (2015). Distinct action of the α-glucosidase inhibitor miglitol on SGLT3, enteroendocrine cells, and GLP1 secretion. *Journal of Endocrinology, 224,* 205-214.

Melone, F., Saladino, R., Lange, H. & Crestine, C. (2013). Tannin structural elucidation and quantitative ^{31}P NMR analysis. 1. Model compounds. *Journal of Agricultural and Food Chemistry, 61,* 9307-9315.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31,* 426–428.

Narotzki, B., Reznick, A.Z., Aizenbud, D. & Levy, Y. (2012). Geen tea: A promising natural product in oral health. *Archives of Oral Biology, 57,* 429-435.

Nikitkova, A.E., Haase, E.M. & Scannapieco, F.A. 2013. Taking the starch out of oral biofilm formation: molecular basis and functional significance of salivary α-amylase binding to oral streptococci. *Applied and Environmental Microbiology, 79,* 416-423.

Oliveira, R.F., Gonçalves, A.G., Dorneles, F.I., Koehnlein, E.A., Marques de Souza, C.J., Bracht, A. & Peralta, R.M. (2015). Inhibition of pancreatic lipase and triacylglycerol intestinal absorption by a *pinhão* coat (*Araucaria angustifolia*) extract rich in condensed tannin. *Nutrients, 7*, 5601-5614.

Plowman, K.M. (1972).Enzyme Kinetics; McGraw-Hill Book Company: New York, NY, USA.

Qian, M., Haser, R. & Payan, F. (1993) Structure and molecular model refinement of pig pancreatic a-amylase at 2.1 Angstrom resolution. *Journal of Molecular Biology, 231,* 785–799. 11.

Ramasubbu, N., Paloth, V., Luo, Y., Brayer, G.D. & Levine, M.J. (1996) Structure of human salivary a-amylase at 1.6 Angstrom resolution: implications for its role in the oral cavity. *Acta Crystallographica. D52*, 435–446.

Rosen S., Elvin-Lewis M., Beck F. M. & Beck E. X. (1984). Anticariogenic effects of tea in rats. *Journal of Dental Research, 63,* 658-660.

Silva, S. M., Koehnlein, E. A., Bracht, A., Castoldi, R., Morais G. R., Baesso, M. L., Peralta, R. A., Souza, C. G. M., Sá-Nakanishi, A. B. & Peralta, R. M. (2014). Inhibition of salivary and pancreatic α-amylases by a pinhão coat (*Araucaria angustifolia*) extract rich in condensed tannin. *Food Research International, 56,* 1-8.

Yilmazer-Musa, M., Griffith, A.M., Michels, A.J., Schneider, E. & Frei, B. (2012). Inhibition of α-amylase and α-glucosidase activity by tea and grape seed extracts and their catechins. *Journal of Agricultural and Food Chemistry, 60,* 8924-8929.

Zajácz, A., Gyémánt, G., Vittori, N. & Kandra, L. (2007). Aleppo tannin: structural analysis and salivary amylase inhibition. *Carbohydrate Research, 342,* 717-723.

Zhang, J. & Kashket, S. (1998). Inhibition of salivary amylase by black and green teas and their effects on the intraoral hydrolysis of starch. *Caries Research, 32,* 233-238.

Table 1

Kinetic analysis and kinetic parameters of the inhibitory action of the hydrolysable tannin. The parameters were obtained by the simultaneous fitting of equation (2) to the experimental curves shown in Figure 2 by means of a non-linear least-squares procedure.

Parameter	**Equation (2) fitting optimization**
K_M (g/100 mL)	0.290± 0.027
V_{max} (µmol min^{-1})	0.553± 0.018
K_{i1}(µg/mL)	22.4±2.9
K'_{i1} (µg/mL)	584.3±203.4
K_{i2}(µg/mL	1037.2 ± 1848.9
Sum of squared deviations	0.00489
Model selection criterion	4.535
Correlation	0.996

Table 2

Kinetic analysis and kinetic parameters of the inhibitory action of the condensed tannin. The parameters were obtained by the simultaneous fitting of equation (3) to the experimental curves shown in Figure 4 by means of a non-linear least-squares procedure.

Parameter	**Equation (3) fitting optimization**
K_M (g/100 mL)	0.281± 0.024
V_{max} (µmol min^{-1})	0.548 ± 0.016
$\bar{K}_{i1}$ (µg/mL)	157.1±12.6
K_{i2} (µg/mL)	569.0±155.2
K'_{i2} (µg/mL)	297.9±188.3
Sum of squared deviations	0.00381
Model selection criterion	4.493
Correlation	0.996

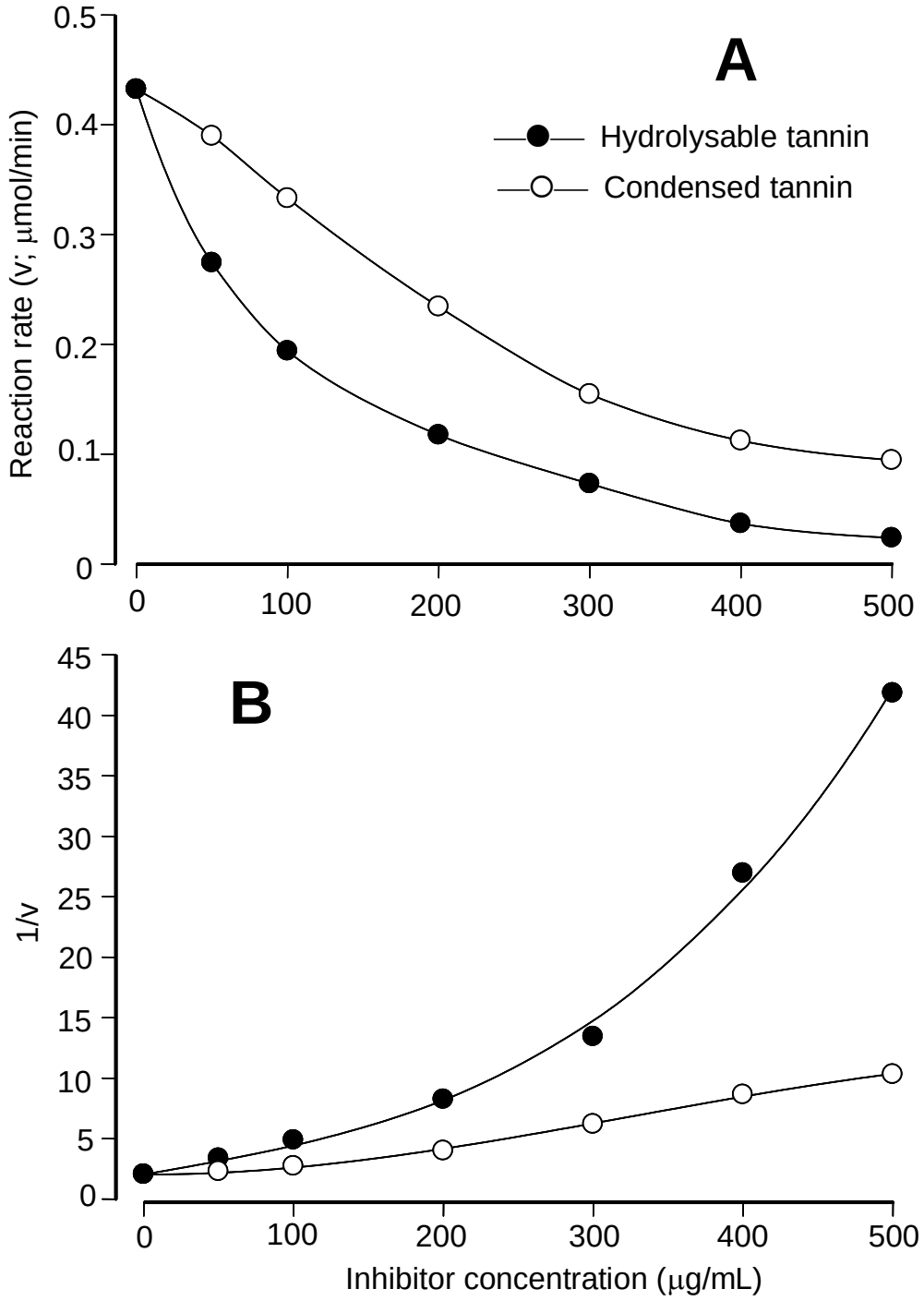

Figure 1. Inhibition of human salivary α-amylase by condensed and hydrolyzable tannins: concentration dependences. Initial reaction rates were measured as described in the material and methods. Each datum point represents the mean of four independent determinations. (A) Reaction rates (v); (B) inverse reaction rates (1/v).

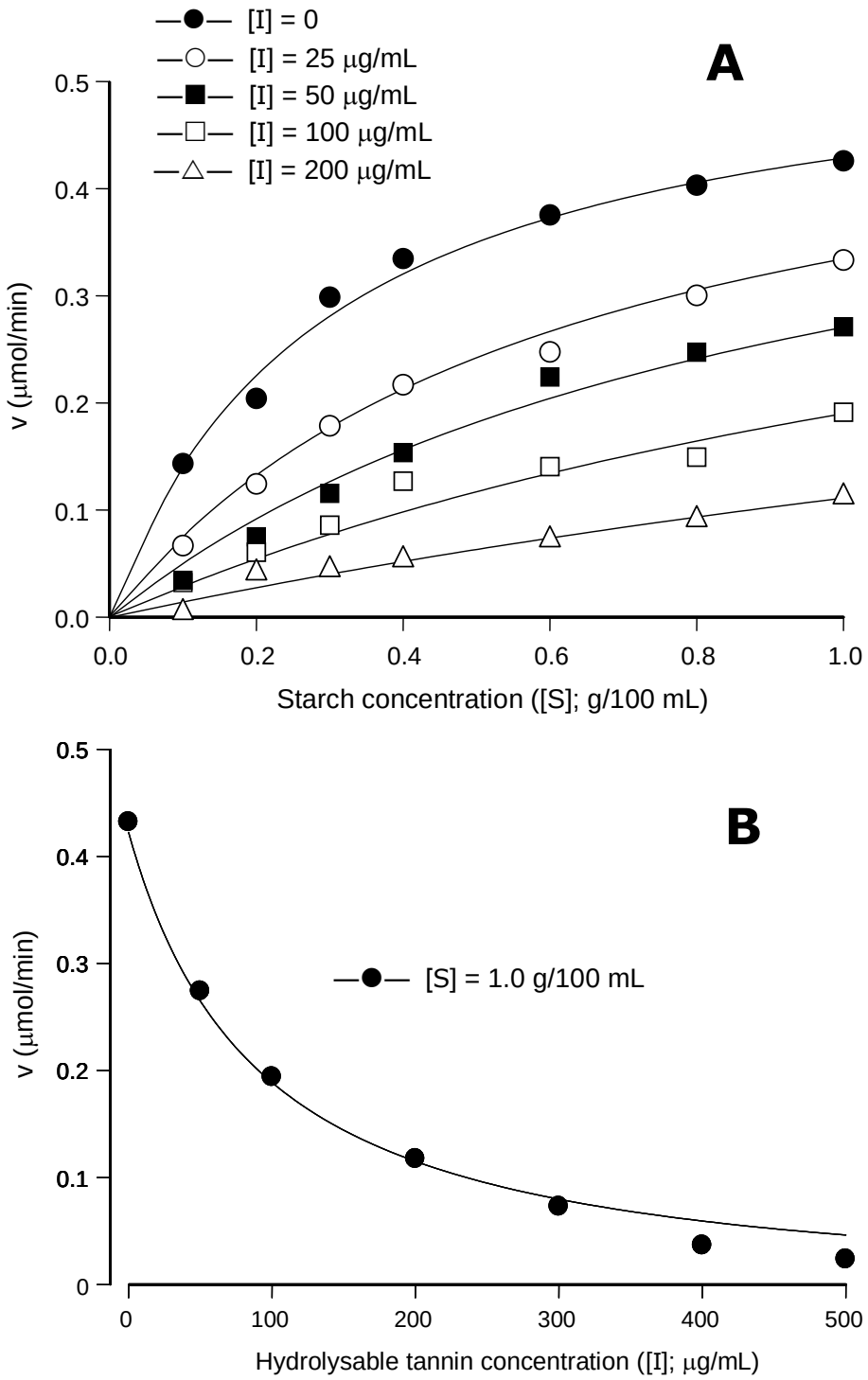

Figure 2. **Reaction rates obtained by varying simultaneously the substrate (starch; panel A) and the hydrolysable tannin (panel B) concentrations.** Each datum point is the mean of four determinations. The lines running through the experimental points were calculated using optimized parameters obtained by fitting equation (2) to the experimental data by means of a non-linear least-squares procedure. Values of the optimized parameters and goodness of fit indicators are listed in Table 1.

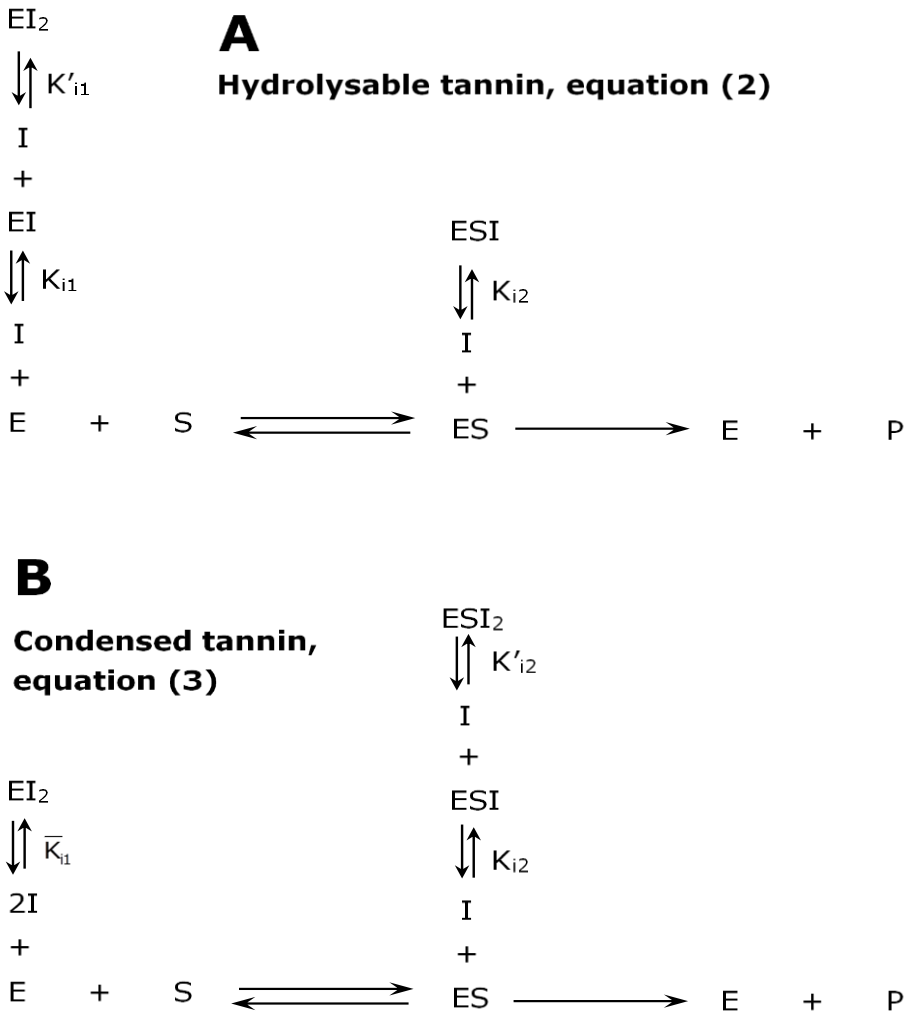

Figure 3. **Schematic representations of the mechanisms described by equations (2) and (3).**

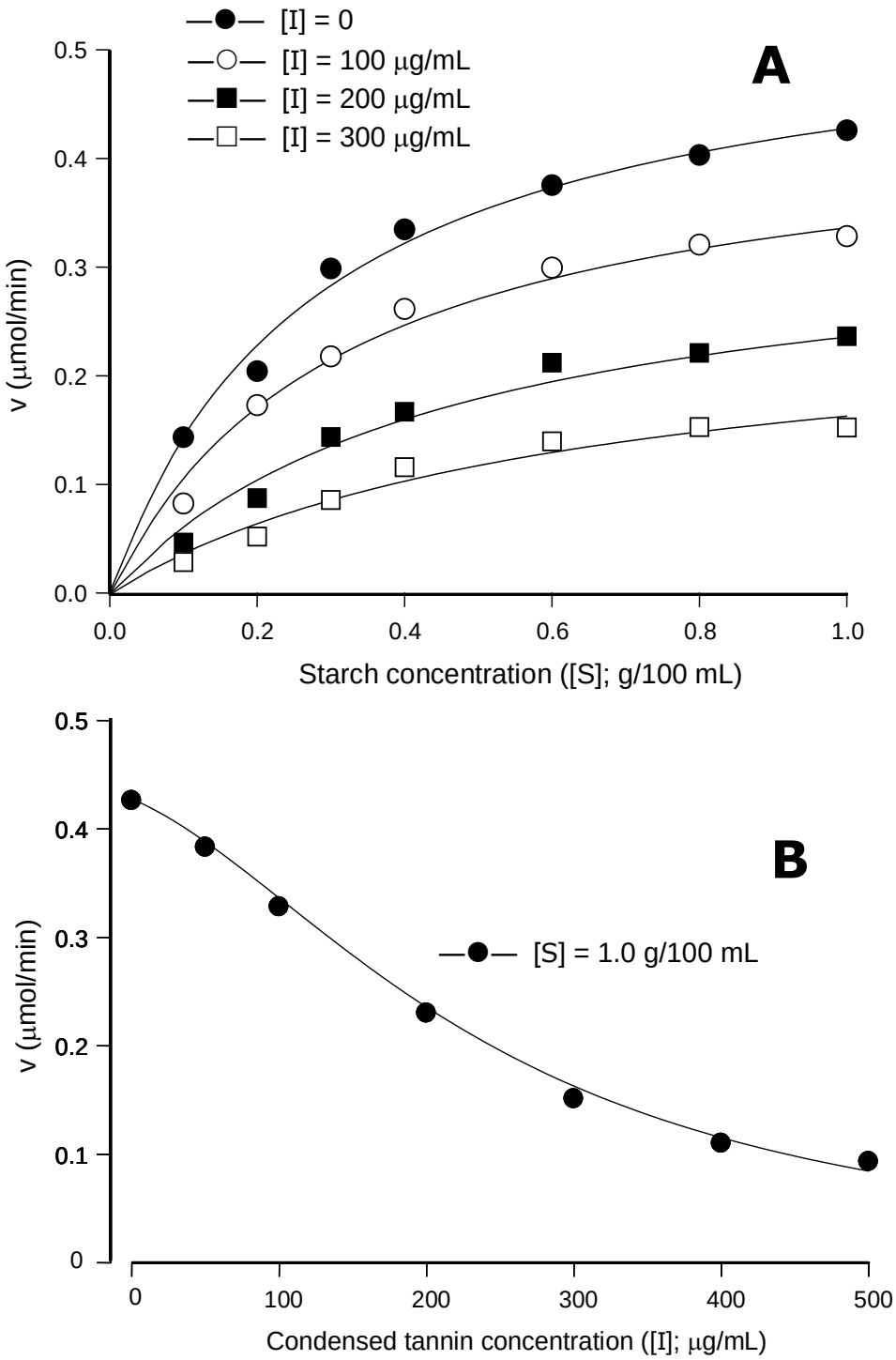

Figure 4. **Reaction rates obtained by varying simultaneously the substrate (starch; panel A) and the condensed tannin (panel B) concentrations.** Each datum point is the mean of four determinations. The lines running through the experimental points were calculated using optimized parameters obtained by fitting equation (3) to the experimental data by means of a non-linear least-squares procedure. Values of the optimized parameters and goodness of fit indicators are listed in Table 2.