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Study of the inhibition of α-amylase by the benzo[c]phenanthridine alkaloids sanguinarine and chelerythrine

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Abstract
Inhibition of porcine pancreas and human saliva α-amylase (EC 3.2.1.1) by sanguinarine and chelerythrine was studied. The inhibition of α-amylase was assayed using a biosensor method which utilises a flow system equipped with a peroxide electrode. 250 μM sanguinarine and 250 μM chelerythrine cause complete inhibition of 1.9 nkat α-amylase from porcine pancreas. The same concentration of sanguinarine and chelerythrine caused 23.9% and 7.5% inhibition, respectively, of 1.9 nkat α-amylase from human saliva. Mixed type and partially reversible inhibition was found for both α-amylases treated with either alkaloid.

Keywords: *Sanguinarine, chelerythrine, α-amylase inhibition, amperometric biosensor analyser, peroxide electrode, flow analysis*

Abbreviations: *SA, sanguinarine; CHE, chelerythrine; QBA, quaternary benzo[c]phenanthridine alkaloids; BSA, bovine serum albumin; Buffer A, 20 mM phosphate buffer, pH 6.9 with 5 mM NaCl*

Introduction

The quaternary benzo[c]phenanthridine alkaloids (QBA) have been isolated from some plants of genera from Papaveraceae, Fumariaceae, and Rutaceae families. They are phytoallexins. Sanguinarine (SA) and chelerythrine (CHE), the main alkaloids of *Sanguinaria canadensis* and *Macleya cordata* display multiple biological actions: interact with biopolymers (DNA, proteins), inhibit a wide range of SH-enzymes (e.g. alanine aminotransferase, aspartate aminotransferase, diamine oxidase of *Aspergillus niger*) and tubulin polymerization [1]. QBA show antiinflammatory as well as antimicrobial activity against a wide variety of microorganisms which include *Gram*-positive and *Gram*-negative bacteria, fungi, and protozoa. On the other hand, QBA have been found toxic in many *in vitro* experiments in different kinds of mammalian cells [2]. The iminium bond of QBA (Figure 1) is highly susceptible to nucleophilic attack. In aqueous solutions, there is an equilibrium between the iminium ion form and the hydroxide adducts ("pseudobase" or "alkanolamine"), the equilibrium constant for pseudobase formation, pK_{R+}, in water is 8.1 and 9.2 for SA and CHE, respectively (Ref. [1]).

The medical applications are derived from folk medicine where QBA-containing extracts have been used as expectorant and escharotic agents, for arthritis, warts, haemorrhoids, as well as antiseptics in dermal applications and in otic preparations for fungal infections. Nowadays, preparations containing QBA are used in dental care as active components in dentifrices, gels, and oral rinses with antiplaque effects. As a product for other applications, the veterinary preparation Sangrovit®, manufactured from pure plant

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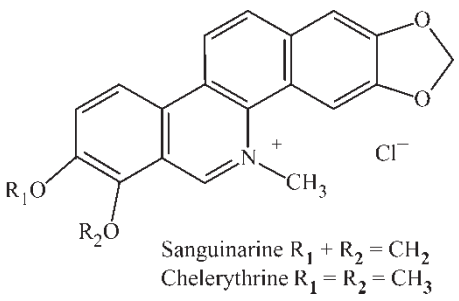

Figure 1. Sanguinarine chloride and chelerythrine chloride structure.

material (Papaveraceae) containing the alkaloids sanguinarine, chelerythrine and chelidonine, is marketed as a weight gain stimulant for livestock [2].

Among QBA-affected enzymes several hydrolases have been found, i.e. lysosomal hydrolases, arylsulphatase, β-galactosidase or acid lipase in homogenates of mouse fibroblast cultures [3], porcine pancreatic and human sputum elastase [4], dipeptidyl peptidase IV in human blood plasma [5], extracellular lipase of microorganism *Candida rugosa* [6]. Astonishingly, in the context of contemporary use, the influence of QBA on α-amylase (salivary or pancreatic) has not been studied yet.

α-Amylase (1,4-α-D-glucan glucanohydrolase; EC 3.2.1.1) hydrolyses starch, glycogen, and related polysaccharides by randomly cleaving internal α-1,4-glycosidic linkages to a significant extent resulting in the release of maltose. It plays an important role in the carbohydrate metabolism of microorganisms, plants and animals. Orthologous enzymes from various sources exhibit marked differences in physical, chemical and catalytic properties. Bacterial amylases and those from porcine pancreas and human saliva have been extensively studied. α-Amylases and related enzymes are widely used in biotechnology for starch degradation and in synthetic chemistry for the production of oligosaccharides by transglycosylation [7].

Human α-amylase is an enzyme produced and stored mainly in the salivary glands and in the pancreas as two distinct isoenzymes, salivary and pancreatic. Porcine pancreatic α-amylase (M_r 52 000) is a glycoprotein [8,9], which consists of two active components (I and II) that have the same molecular weight and optimum pH activity but differ in amino acid composition and isoelectric point [10–12]. It comprises a single polypeptide chain of 475 residues [8,10] with five disulfide bridges and contains one Ca^{2+} ion, bound to two SH-groups [13,14]. Levitzki and Steer [15] report on a binding site for chloride ion that causes a conformational change enhancing activity. Urea and other amide reagents act as inhibitors [16]. α-Amylases are also drug-design targets for compounds for the treatment of diabetes, obesity and hyperlipidaemia [17,18]. Plant α-amylase inhibitors in cereals [19–23] and leguminosae [24–28], have been extensively studied, in part because they play a role in plant resistance to insect and microbial pests [22,29] and also because they are major allergens involved in baker's asthma disease [30].

The affected enzymes in direct contact with QBA-containing products can be α-amylase in saliva as well as the isoenzyme presented in the small intestine. Based on the above described practical applications of QBA, this study was conducted to investigate the effect of QBA on the activities of human salivary and porcine pancreatic α-amylase using a spectrophotometric and a novel instrumental technique. The new method of α-amylase activity determination is based on the measurement of maltose produced using a peroxide electrode equipped with an enzyme membrane located in the flow system. The membrane was obtained by immobilisation of glucose oxidase (EC 1.1.3.4), α-glucosidase (EC 3.2.1.20) and mutarotase (EC 5.1.3.3) on a cellophane membrane, co-crosslinked by gelatin-glutaraldehyde together with bovine serum albumin [31].

Materials

Isolation of alkaloids

The procedure described [32] for isolation of sanguinarine and chelerythrine from *Chelidonium majus* was applied. The QBA extract from *Macleya cordata* (Papaveraceae), purchased from Camas Technologies, Inc., Broomfield, USA, containing sanguinarine and chelerythrine in the ratio 3:1, (1.0 g) was dissolved in water (150 ml), made alkaline with ammonia, extracted ten times with ether (20 ml portions). The organic fractions were pooled, dried with Na_2CO_3, evaporated and the residue dissolved in a mixture of acetic acid–benzene (1: 99) (10 ml) was applied onto an Al_2O_3 (activated by washing with 5% HCl, drying and heating at 170°C for 6 h) column (150 g) and eluted with a gradient of ethanol (1%–6%) in a mixture of acetic acid–benzene (1: 99). Fractions containing sanguinarine and chelerythrine, respectively, were pooled and the evaporation residues dissolved in benzene. Alkaloid chlorides were precipitated by the addition of several drops of hydrogen chloride in diethyl ether. After filtration 148 mg of sanguinarine chloride, mp. 281–285°C, purity 99.3% by HPLC [33] and 47 mg of chelerythrine chloride, mp. 208–210°C, purity 97.2%.by HPLC [33] (lit. [34] mp. sanguinarine chloride 277–280°C, mp. chelerythrine chloride 213–214°C.) were obtained.

Chemicals

Bronopole (2-bromo-2-nitro-1,3-propanediol) and partially hydrolysed starch were purchased from

Pliva-Lachema (Brno, Czech Republic). Bovine serum albumin (BSA), gelatine powder and maltose were purchased from Sigma (St. Louis, MO, USA). Glutaraldehyde (50% solution) was from Fluka Chemika (Buchs, Switzerland). Cellophane membranes were obtained from Inceltech-SGI (Toulouse, France). All other chemicals were of the highest quality commercially available. The spectrophotometric method was based on a kit for α-amylase activity determination; Alfa amylasa (E7, Cat.No. 10253, BioVendor Laboratorní medicína, Brno, Czech Republic) was used.

Enzymes

α-Amylase (EC 3.2.1.1, 400 IU.mg^{-1}, from porcine pancreas) was from Merck (Darmstadt, Germany). α-Glucosidase (EC 3.2.1.20, 66 IU.mg^{-1}, purified from yeast) was purchased from Fluka Chemika (Buchs, Switzerland). Glucose oxidase (EC 1.1.3.4, 100 IU.mg^{-1}, from *Aspergillus niger*) was from Sigma (St. Louis, MO, USA). Mutarotase from hog kidneys (EC 5.1.3.3, 5000 IU.mg^{-1}) was from Roche Diagnostics GmbH (Mannheim, Germany).

Human salivary α-amylase: The unstimulated saliva was obtained from healthy volunteers: 10 ml of distilled water was kept for 2 min in the mouth, then the saliva was collected and filtered through cotton wool. The saliva was then diluted with distilled water for appropriate α-amylase activity before the experiments.

Preparation of samples for spectrophotometric measurements

SA was dissolved in distilled water to prepare 7.41 mM stock solution. The stock solution was diluted with distilled water and the prepared solutions were mixed in a 1:1 ratio with i) saliva solution, diluted to the activity resulting in 12 nkat in the final reaction mixture, or with ii) pancreatic α-amylase solution diluted to the activity resulting in 4.2 nkat in the final reaction mixture. After 1 min the mixture (100 μl) was used for the experiments. Final concentrations of SA in the reaction mixture were 272; 136; 68; 34 and 17 μM, respectively.

Preparation of samples for biosensor assay

SA and CHE were dissolved in distilled water to a final concentration of 10.90 mM and filtered through a nylon mesh cloth. This stock solution was used for the preparations of working solutions, which were mixed with salivary or pancreatic α-amylase solutions to obtain 1.9 nkat.ml^{-1} of pancreatic or salivary α-amylase and 5, 25, 50, 250, 500, 1000, 1500, 2000, and 2500 μM of SA or CHE in the final reaction mixture. After 10 min the mixtures were used for the experiments.

Methods

Implementation of α-amylase activity measurement by a spectrophotometric method (kit E7)

Reagent 1 (1000 μl) was blended with 100 μl of the mixture of saliva with SA (water for blank or saliva with water for sample with uninhibited enzyme), vortexed and incubated at 25°C. After 1 min Reagent 2 (substrate, 250 μl) was added and the reaction mixture stirred at 25°C. Time course of the reaction was measured after 1 min delay on Shimadzu UV-1601 (Shimadzu, Tokyo, Japan) in a 1 cm cuvette at 405 nm. From the linear part of the time course (from 5–10 min) the average ΔA.min^{-1} was calculated. The measurement with porcine pancreatic α-amylase was carried out in the same manner as for human salivary α-amylase.

Enzymes immobilisation procedures and apparatus

Enzymes immobilisation procedures and apparatus (Biosensor analyser, a prototype, M. Jilek Company, Postřelmov, Czech Republic) have been described [31].

Biosensor determination of α-amylase activity

A working solution containing 1% starch in buffer A (20 mM phosphate buffer, pH 6.9 with 5 mM NaCl) was loaded at a flow rate of 1.56 ml.min^{-1} by a peristaltic pump to the flow-through adapter of the enzyme peroxide electrode (cell volume 35 μl). Samples were measured after the electrode current value had become constant.

The working solution (0.8 ml of 1% starch in buffer A) was mixed with 0.2 ml of the mixture (1.9 nkat of human salivary or porcine pancreatic α-amylase and SA or CHE in buffer A) or 0.2 ml of buffer A with α-amylase (for a sample with uninhibited enzyme). After 5 min of incubation at 25°C, the mixture was measured using biosensor analyser with a peroxide electrode equipped with glucose oxidase, α-glucosidase and mutarotase located in the flow system. Peak height of the signal was used for calculating the inhibition where an uninhibited enzyme represented 0% inhibition and a signal with no peak represented 100% inhibition. All solutions (standards and working solutions) were preserved by addition of bronopole (25 mg.l^{-1}). 650 mV was chosen for peroxide electrode measurements because no interference by oxidation products of the substances tested here was observed.

Effect of SA and CHE on the activity of the immobilised enzymes

10 mM maltose in buffer A with 1% starch was assayed by the biosensor with or without 0.5 mM SA or 0.5 mM CHE.

Determination of α-amylase activity

The working solution (0.9 ml) was mixed with 50 μl buffer A and 50 μl of a diluted solution (1:100) of investigated α-amylase (2 mg α-amylase from porcine pancreas was dissolved in 10 ml of buffer A) or 50 μl of a diluted (1:50) α-amylase from human saliva. After incubation for 5 min at 25°C, the mixture was analysed using the biosensor analyser with a peroxide electrode equipped with an enzyme membrane. The solutions with 0.25 – 2 mM maltose in buffer A with 1% starch were analysed by the biosensor and a calibration curve of current responses *vs* concentration of maltose was constructed.

Time-dependent inhibition of α-amylase by SA

The activity of human salivary and porcine pancreatic α-amylase was measured as mentioned above for preincubation times 2, 3, 6, 8, 10, 15, 20, 30, 45, and 60 min. An activity of porcine pancreatic α-amylase of 9.5 nkat and a SA concentration 500 μM were used.

Reversibility of inhibition

The α-amylase from porcine pancreas (9.5 nkat) was dissolved in 5 ml of buffer A containing the inhibitor—SA and CHE, respectively, in a final concentration 2 mM. After standing for 30 min at 25°C, the activity decreased to 0%. The solution was then dialysed against the same buffer at 4°C for 24 h. The activity was assayed by the biosensor analyser with peroxide electrode as described above and a blank sample without the inhibitor was processed in the same way.

Kinetics of the interaction of α-amylase with QBA

The reaction mixture (total volume 1 ml) contained buffer A with 1%, 0.5%, 0.33%, 0.25% and 0.16% starch, 9.5 nkat α-amylase from porcine pancreas and inhibitors SA or CHE in the concentration range of 0–0.1 mM. The mixture (α-amylase and SA or CHE) was preincubated at 25°C for 10 min. The reaction was assayed after 5 min by the biosensor. The obtained values were evaluated on a PC using the program EXCEL.

Statistical analyses

Statistical analyses were performed using Statgraphics software (Statgraphics) by one way analysis of variance (ANOVA) with post hoc Tukey test. Results are presented as mean ± SD.

Results and discussion

Several eukaryotic α-amylases, including human salivary and porcine pancreatic enzyme, in contrast to microbial enzymes, require chloride ions for full catalytic activity [35]. In these α-amylases the removal of chloride resulted in a significant decrease in activity and a shift in pH optimum. Therefore, all determinations were performed in 5 mM NaCl.

The influence of SA and CHE on α-amylase activity from porcine pancreas and human saliva was studied by two different approaches. A method utilising the kit E7, based on a classical detection of a coloured enzyme activity product by spectrophotometry and a new biosensor method were used for α-amylase activity determination [31]. The optimal value of α-amylase activity was chosen for each method to achieve a detectable response.

The spectrophotometric method is based on kinetic measurement of two coupled enzymatic reactions: first α-amylase hydrolyses 4,6-ethylidene-(G7)-4-nitrophenyl(G1)-α-D-maltoheptaoside, then the products are transformed by α-glucosidase to glucose and 4-nitrophenol. The quantity of liberated 4-nitrophenol is proportional to the catalytic activity of α-amylase. This kit is reliable for routine α-amylase activity detection in plasma. Results for the concentration range of 0–272 μM SA are shown for the salivary and pancreatic α-amylase, respectively (Table I and Table II). Because of possible interference of orange coloured SA with the measurement at 405 nm, the data obtained with the above mentioned spectrophotometric method were considered preliminary and the biosensor method was employed in investigating the inhibition of α-amylase by SA and CHE.

First, the influence of the alkaloid on the determination of 10 mM maltose by the biosensor method was studied. Identical current responses were obtained with or without 2 mM QBA (data not shown). None of the immobilised enzymes used in the assay were inhibited by either alkaloid tested (data not shown). Time-dependent inhibition of α-amylase activity was observed for porcine pancreatic α-amylase (9.5 nkat) when a single SA concentration was applied. Figure 2 shows results for porcine pancreatic α-amylase and 500 μM SA. Based on these results, a 10 min preincubation interval was chosen for all further inhibition assays.

Table I. Inhibition of human salivary α-amylase activity by sanguinarine, detected spectrophotometrically.

Sanguinarine [μM]	$\Delta A_{405}.min^{-1}$	Inhibition [%]
Blank	0	–
0	0.072 ± 0.003	0.0
17	0.058 ± 0.001	18.8
34	0.056 ± 0.004	22.8
68	0.052 ± 0.004	26.8
136	0.052 ± 0.002	27.8
272	0.032 ± 0.004	55.3

Final α-amylase activity was 12 nkat. Results are expressed as mean ± SD, n = 3.

Table II. Inhibition of porcine pancreatic α-amylase activity by sanguinarine, detected spectrophotometrically.

Sanguinarine [μM]	$\Delta A_{405}.min^{-1}$	Inhibition [%]
Blank	0	–
0	0.102 ± 0.001	0.0
17	0.103 ± 0.002	0.0
34	0.096 ± 0.003	5.6
68	0.083 ± 0.003	17.8
136	0.064 ± 0.002	36.9
272	0.039 ± 0.002	62.2

Final α-amylase activity was 4.2 nkat. Results are expressed as mean ± SD, n = 3.

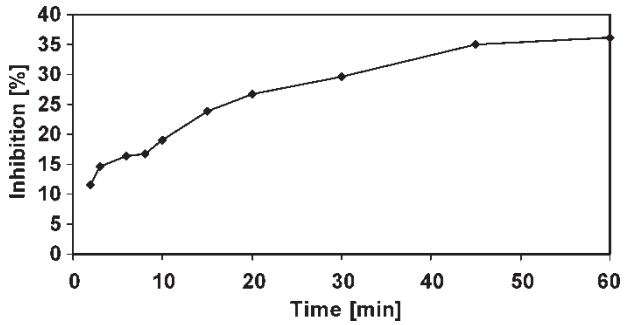

Figure 2. Time-dependent inhibition of porcine pancreatic α-amylase by sanguinarine. Activity of porcine pancreatic α-amylase was 9.5 nkat; sanguinarine concentration was 500 μM. The standard deviations for all presented values were lower than 3.4%, n = 3.

Effects of SA and CHE on the activity of α-amylase from porcine pancreas and human saliva were investigated using a peroxide electrode with enzyme membrane. The results are shown in Tables III and IV. While both alkaloids show essentially the same behaviour towards α-amylase from porcine pancreas, they differ substantially in inhibiting α-amylase from human saliva. IC_{50} values (from Table III) for the alkaloids in the inhibition of porcine pancreatic α-amylase were found: sanguinarine $IC_{50} = 119.4$ μM and chelerytrine $IC_{50} = 111.1$ μM respectively. 50% decrease in the activity of human salivary α-amylase was not observed (Table IV). 2.5 mM sanguinarine causes approximately 48% decrease in human salivary α-amylase activity and 2.5 mM chelerythrine only 23% decrease in human salivary α-amylase activity.

Dialysis of SA-inhibited α-amylase from porcine pancreas restored the activity of the enzyme to 53%. Therefore the inhibition is partially reversible. The same experiment performed with CHE also demonstrated the inhibition to be partially reversible with enzyme activity restored to 79%.

The differences in enzyme inhibition as well as in the reversibility of activity after dialysis may be ascribed to structural features of the two alkaloids that affect their interaction with the enzyme and/or to the differences in the active site of these two enzymes.

The interactions of QBA with α-amylases from porcine pancreas and human saliva were further investigated by kinetic method. For this purpose it was necessary to incubate the alkaloids with the enzyme

Table III. Inhibition of porcine pancreatic α-amylase, detected by biosensor.

	Sanguinarine			Chelerythrine		
Concentration [μM]	I_0 [nA]	I_i [nA]	Inhibition [%]	I_0 [nA]	I_i [nA]	Inhibition [%]
5	0.888 ± 0.013	0.800 ± 0.002	9.9	1.029 ± 0.010	0.948 ± 0.011	7.9
25	0.888 ± 0.013	0.780 ± 0.007	12.1	1.029 ± 0.010	0.833 ± 0.005	19.0
50	0.988 ± 0.005	0.752 ± 0.003	23.9	1.045 ± 0.012	0.750 ± 0.007	28.2
125	0.988 ± 0.005	0.516 ± 0.007	52.2	1.045 ± 0.012	0.576 ± 0.009	55.1
250	0.988 ± 0.005	0.000 ± 0.000	100.0	1.027 ± 0.014	0.000 ± 0.000	100.0

I_0—The current value measured for a sample without inhibitor, I_i—The current value measured for samples with inhibitor. Final α-amylase activity was 1.9 nkat. Results are expressed as mean ± SD, n = 3.

Table IV. Inhibition of human salivary α-amylase, detected by biosensor.

	Sanguinarine			Chelerythrine		
Concentration [μM]	I_0 [nA]	I_i [nA]	Inhibition [%]	I_0 [nA]	I_i [nA]	Inhibition [%]
5	0.602 ± 0.006	0.592 ± 0.007	0.0	0.602 ± 0.006	0.601 ± 0.002	0.0
25	0.602 ± 0.006	0.600 ± 0.003	0.0	0.602 ± 0.006	0.606 ± 0.006	0.0
50	0.568 ± 0.005	0.554 ± 0.002	2.5	0.542 ± 0.001	0.544 ± 0.002	0.0
250	0.588 ± 0.006	0.448 ± 0.007	23.9	0.542 ± 0.001	0.502 ± 0.003	7.5
500	0.588 ± 0.006	0.316 ± 0.009	44.4	0.511 ± 0.004	0.449 ± 0.004	12.2
2500	0.568 ± 0.005	0.308 ± 0.001	47.7	0.511 ± 0.004	0.392 ± 0.004	23.3

I_0—The current value measured for a sample without inhibitor, I_i—The current value measured for samples with inhibitor. Final α-amylase activity was 1.9 nkat. Results are expressed as mean ± SD, n = 3.

for a defined time, 10 min were chosen in our experiments, before the addition of substrate. Kinetic data obtained under these conditions were analysed graphically according to Lineweaver and Burke (1/v versus 1/[S]). The data show that both SA and CHE exhibit a mixed-type inhibition with both the V_{max} and K_m values of the α-amylase-catalysed reactions affected (data not shown).

Conclusions

Investigating the effects of SA and CHE on α-amylase activity by a new biosensor method offers a great advantage over traditional spectrophotometric measurement since there is no interference of the alkaloid absorbance with the assay. While many enzymes are inhibited by both alkaloids, those immobilised on the membrane of the peroxide electrode, and used in experiments described herein, are not. We have found that SA and CHE inhibition of α-amylase depends on the incubation time of the enzyme with the alkaloids, is a mixed-type one, and is partially reversible. The available data essentially rule out an impairment of polysaccharide digestion in humans and farm animals when QBA-containing oral hygiene products are used by the former or supplied in feed additives to the latter because of the minimal effect of QBA to salivary and pancreatic α-amylase on the level of their usual physiological activities.

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