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PURIFICATION AND PROPERTIES OF AN α-AMYLASE INHIBITOR FROM WHEAT

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Summary

Four inhibitors of α-amylase (EC 3.2.1.1) were separated from an alcohol extract of wheat by ion-exchange chromatography on DE52-cellulose. One inhibitor, which showed the greatest specificity for human salivary amylase relative to human pancreatic amylase, has been purified by the following steps: (a) alcohol fractionation (60—90%) of water extract (b) ion-exchange chromatography on QAE-Sephadex A-50; (c) re-chromatography on DE52-cellulose and (d) gel filtration on Sephadex G-50. The purified inhibitor is 100 times more specific for human salivary amylase than for human pancreatic amylase. It shows an electrophoretic mobility of 0.2 on disc gel electrophoresis and a molecular weight of about 21 000. This inhibitor contributes about 16% to the total salivary amylase inhibiting power of the wheat extract.

Introduction

A protein inhibitor of the enzyme α-amylase (α-1,4-glucan-4-glucano-hydrolase, EC 3.2.1.1) was first described in wheat seed in the 1940's [1—4]. Few publications on this subject appeared over the next 25 years. Shainkin and Birk [5] described the isolation and properties of two α-amylase inhibitors from wheat. They differed in electrophoretic mobility and in specificity towards α-amylases of different origins. It is evident from recent investigations [6—10] that there are multiple molecular forms of proteins in wheat seed capable of inhibiting insect and mammalian amylases to various degrees. Since human pancreatic amylase has not been the subject of much study in this context we decided to examine wheat seed for inhibitors of pancreatic amylase and also salivary amylase (unless stated otherwise salivary and pancreatic amylases are from human sources). An inhibitor with much greater specificity for one enzyme than the other would enable the inhibitor to be applied to analysis of mixtures of the two enzymes.

Experimental methods

Extraction of α-amylase

Saliva was collected from laboratory personnel, pooled and centrifuged (8000 × *g*, 1 h). Amylase was separated from the supernatant by gel filtration on Sephadex G-50 at a flow rate of 12—15 ml · h^{-1}. Eluent was 0.1 M NaCl containing 0.5 mM $CaCl_2$ and 10 μM albumin. The source of pancreatic amylase was post mortem pancreatic tissue from patients who had not suffered pancreatic damage or functional disturbance. Tissue samples (1—2 g) were homogenised in ice—cold 0.1 M NaCl containing 5 mM $CaCl_2$ (1 : 4, w/v) using a Mickle homogeniser and centrifuged (5000 × *g*, 15 min, 4° C). Amylase was separated by gel filtration as described above.

Extraction and separation of α-amylase inhibitors

Aqueous extract. Wheat seeds (400 g) of Triticum aestivum species were finely ground and suspended in H_2O (1 : 3, w/v), stirred (1 h) and filtered. The residue was washed again with 1 vol. H_2O, stirred (30 min) and filtered. The clear supernatants were pooled and freeze-dried. The resulting powder (28 g) was stored at room temperature and used as starting material in the purification procedure. An aliquot of this product was dissolved in H_2O (1 : 10, w/v), heated at 70° C (30 min) to inactivate α- and β-amylases, cooled and centrifuged (8000 × *g*, 10 min). The precipitate was discarded.

Alcohol fractionation. The aqueous extract was treated with absolute alcohol to 60% saturation (20° C). The inactive precipitate was separated by centrifugation (8000 × *g*, 30 min) and discarded. The alcohol concentration was raised to 90% saturation and the resulting precipitate, which contained the α-amylase inhibitory activity, was separated by centrifugation as above. This precipitate (referred to hereafter as the alcohol fraction) was washed 3 times with absolute alcohol and allowed to dry overnight at 30° C.

Ion-exchange chromatography. The alcohol fraction was dissolved in Tris · HCl buffer (I = 0.02, pH 9.2 containing 2 mM NaCl) and dialysed overnight against same buffer. Any cloudiness that developed was removed by centrifugation (3000 × *g*, 10 min). The supernatant was applied to a QAE-Sephadex A-50 column (50 cm × 1.5 cm) previously equilibrated with above buffer. 3 column volumes of buffer (pH 9.2) were eluted prior to any change in buffer pH. The pH was reduced stepwise keeping the ionicity constant. 6-ml fractions were collected at a flow rate of 20—24 ml · h^{-1} and analysed for inhibition of both salivary and pancreatic amylases as described below. The fractions containing the first inhibitor peak which emerged from the column at pH 8.9 were pooled, concentrated to 5 ml by ultrafiltration (Amicon UM 2 membrane), adjusted to pH 9.2, diluted with an equal volume of H_2O and applied to a DE52-cellulose column (13 cm × 1 cm) previously equilibrated with Tris-HCl buffer (I = 0.01, pH 9.2 containing 1 mM NaCl). Following application of the inhibitor solution, 5 column volumes of buffer were eluted before starting the NaCl (0—0.1 M) gradient. 2.5-ml fractions were collected. The fractions containing the inhibitor were pooled on analysis and concentrated (Amicon UM 2 membrane) prior to gel filtration. Protein was monitored in all chromatographic fractions by measurement of absorbance at 280 nm on an LKB Uvicord II.

Gel filtration. 2 ml of inhibitor solution from DE52-cellulose peak were loaded on a Sephadex G-50 (62 cm × 2 cm) column and eluted with a 1 mM phosphate buffer (pH 6.9) containing 0.1 M NaCl and 0.5 mM $CaCl_2$ at a flow rate of 12 ml · h^{-1}. 1.9-ml fractions were collected and tested for inhibitor activity. The apparent molecular weight of the inhibitor was determined by comparing its elution volume with those of standard proteins according to Andrews [11]. An aliquot of the purified inhibitor was freeze-dried in 0.03 M ammonium bicarbonate for electrophoretic studies.

Gel electrophoresis

Disc gel electrophoresis was carried out on 80-μg samples of inhibitor using 0.05 M Tris/0.383 M glycine buffer, pH 8.5 according to Davis [12]. Spacer gel and separating gel were 3% and 7.5% acrylamide, respectively. 1 mA of current per tube was applied for the first 15 min followed by 5 mA per tube for a duration of 75 min. The gels were stained for protein with Coomassie Blue [13].

Analytical determinations

Enzyme assay. α-Amylase activity was measured by the Phadebas blue starch method [14] and incorporating albumin (7.4 μM) in the reaction mixture for optimal amylase activity [15]. Since salivary and pancreatic amylases hydrolyse insoluble starches at different rates [16,17] it was necessary to prepare calibration curves for both enzymes. This was done using the prime method of Robyt and Whelan [18] and soluble (Zulkowsky) starch, (E. Merck, A.G., Darmstadt, Germany) as substrate. The amylase unit (I.U.) is defined as that activity which liberates reducing groups corresponding to 1 μmol of maltose/min at 37°C.

Inhibition of α-amylase. The inhibitor solution (10—50 μl) containing 0.1—10 μg protein was added to 0.4 ml of buffer (50 mM phosphate, 50 mM NaCl, 0.5 mM $CaCl_2$, pH 6.9) containing 2 mg albumin, followed by 0.1 ml of amylase solution (42—52 munits) in above buffer. This mixture was incubated at room temperature for 30 min. Enzyme and inhibitor were pre-incubated (30 min) in all assays unless stated otherwise. The volume was made up to 4.1 ml with water, for analysis of remaining amylase activity [14] in a 15 min incubation (37°C) period. A control tube was set up without the addition of inhibitor. The quantity of inhibitor used was that which reduced the amylase activity by 50%. This is because the plot of salivary amylase activity vs. inhibitor concentration is linear in the region of 50% reduction of enzyme activity (see Results). The inhibitor unit is defined as the quantity of inhibitor required to reduce the activity of 2 I.U. of amylase by 50% i.e. 1 unit of inhibitor inhibits 1.0 I.U. of enzyme under these conditions. The ratio of inhibitor activity towards human salivary amylase relative to human pancreatic amylase is referred to as the salivary/pancreatic ratio.

Binding of inhibitor with blue starch. Blue starch tablets (Pharmacia, London, U.K.) were washed free of buffer salts as previously described [19]. Increasing amounts of starch in the range 20—100 mg, were added to 20 μg inhibitor in 1.0 ml of 0.05 M phosphate buffer (pH 6.9) containing 50 mM NaCl and 0.5 mM $CaCl_2$ (20°C). Inhibitor content of supernatant was analysed

after each substrate addition had settled (3—4 min) and the amount of inhibitor bound was calculated.

Protein assay. The Lowry et al. [20] method for protein analysis was used to determine the specific activity of the inhibitor at each step in the purification procedure.

Results

The α-amylase inhibitory activity of the water extract varied with the source of the enzyme. This is in general agreement with the findings of others [6—10,21]. Inhibitory activity (units/mg protein) towards amylases from human saliva, human pancreas, porcine pancreas and Aspergillus oryzae (Sigma Chemical Co., London, U.K.) gave values of 40.0, 10.0, 7.0 and 3.5, respectively. Gel filtration of a water extract or an alcohol fraction on Sephadex G-100 yielded two inhibitor peaks active towards salivary and pancreatic amylases. Both inhibitor peaks showed different salivary/pancreatic ratios, 2.0 for peak 1 and 6.8 for peak 2. Apparent molecular weights of the eluted peaks were 60 000 (peak 1) and 18 000 (peak 2) calculated from the elution volumes according to Andrews [11]. These figures compared well with 66 000 and 22 000, respectively, obtained by Bedetti et al. [10] for the two peaks of salivary amylase inhibitory activity separated from the Triticum aestivum species of wheat.

The chromatographic separation of inhibitors from an alcohol (60—90%) fraction on DE52-cellulose is shown in Fig. 1. At least 4 inhibitor peaks are eluted by a NaCl (0—0.5 M) gradient. All peaks inhibit salivary and pancreatic

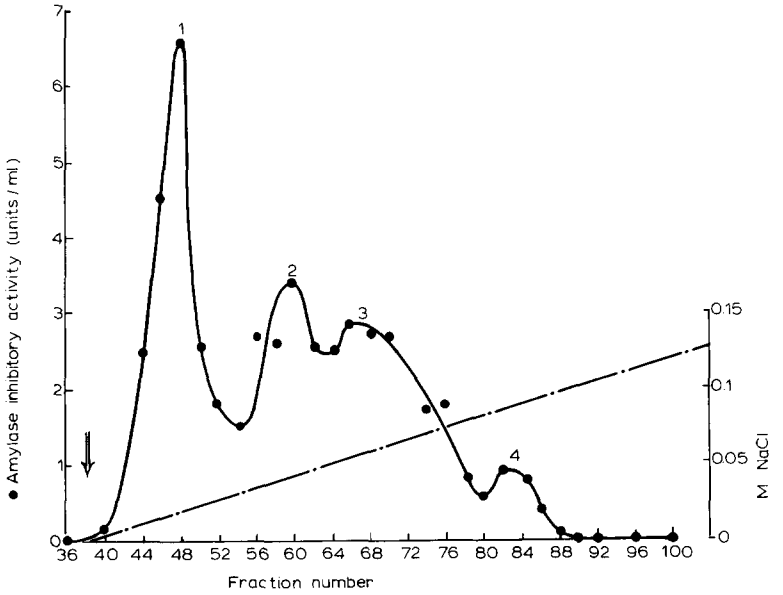


Fig. 1. Separation of α-amylase inhibitors from wheat. Chromatography of 10 ml of alcohol extract (30 mg) on DE52-cellulose column (13 cm × 1 cm). Application buffer was Tris · HCl (I = 0.01, pH 9.2). 4 column volumes of buffer were eluted before start of NaCl gradient (0—0.5 M) at fraction number 38 (indicated by arrow). Fraction volume, 3 ml.

amylases to different degrees. Salivary/pancreatic ratios are 30, 4.1, 2.1 and 1.25 for peaks 1, 2, 3 and 4, respectively. Chromatography of peak 2 inhibitor from Sephadex G-100 column on DE52-cellulose yields two peaks corresponding to peaks 1 and 2 of Fig. 1. It was decided to purify further the inhibitor corresponding to peak 1 (Fig. 1) since it had the greatest salivary/pancreatic ratio.

Isolation and purification of inhibitor

A summary of the procedure is shown in Table I. Chromatographic separation of the alcohol fraction on a QAE-Sephadex anion-exchange column is shown in Fig. 2a. The first inhibitor peak emerges at pH 8.9 with a salivary / pancreatic ratio of 91 and represents 15.6% of the total salivary amylase inhibitory activity of the alcohol fraction (Table I). The subsequent chromatographic pattern on DE52-cellulose of this active fraction from the QAE-Sephadex column as well as Sephadex G-50 gel filtration of the emerging peak are shown in Figs. 2b and c. The salivary/pancreatic ratio of 100, given by the DE52-cellulose peak does not increase further on gel filtration (Table I). Gel filtration achieves a greater degree of purity of the inhibitor as evidenced by the increase in specific activity from 71 to 245 units/mg protein. The apparent molecular weight of 21 000 was calculated from the elution volume on Sephadex G-50 [11].

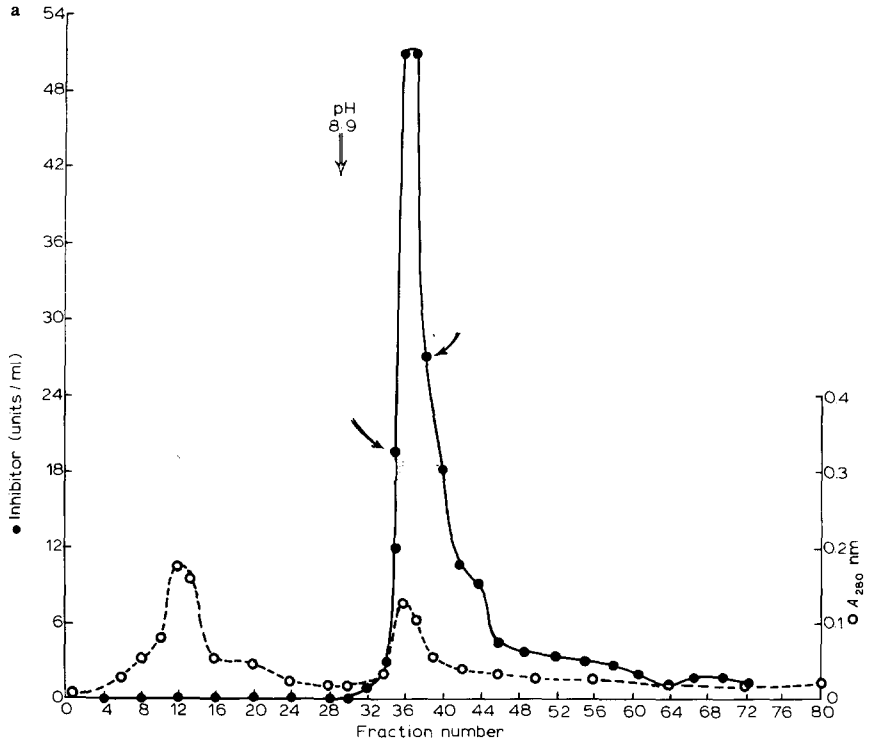
TABLE I

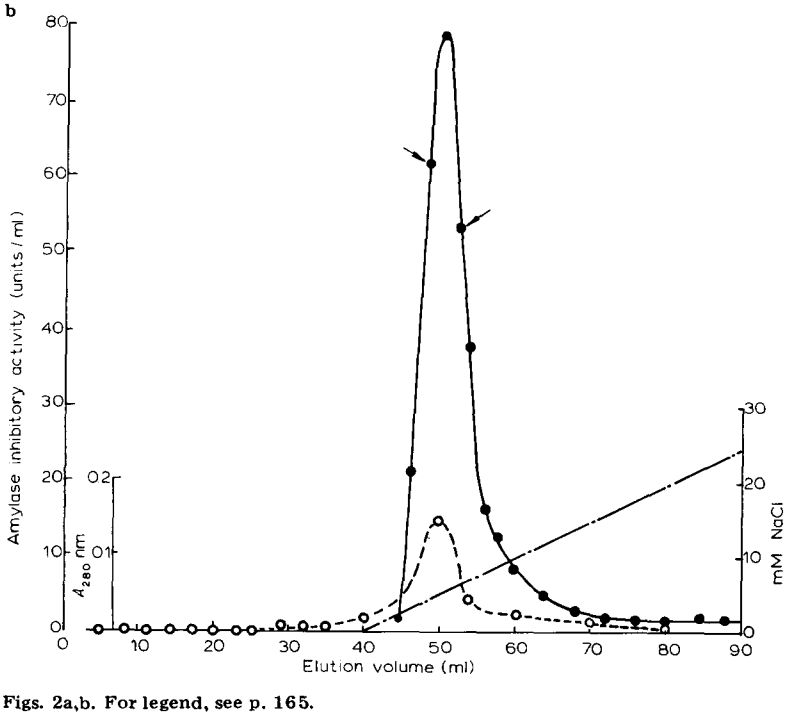
YIELD AND POTENCY OF VARIOUS FRACTIONS OBTAINED DURING THE PREPARATION OF α-AMYLASE INHIBITOR

Purification step	Vol. (ml)	Total salivary amylase inhibitory units	Total protein (mg)	Specific activity (units/mg protein)	HS/HP inhibitor activity ratio**	Recovery from previous step (%)
Aq. extract of wheat seed, freeze-dried 1.9 g	19	17 800	445	40	4.0	—
Aq. extract post heating (70° C, 30 min)	19	17 000	320	55.5	4.0	96
Alcohol fraction (60—90%) 0.5 g	10	8800	156	60	3.7	49.5
Peak 1 Inhibitor from QAE-Sephadex A-50		1375				15.6
Conc. peak fractions	10	800	14.4	55.5	91	9.1
Inhibitor fractions from DE52-Cellulose column		586				42.6*
peak fractions	7.5	407	5.75	71	100	
Sephadex G-50	24	272				19.8*
G-50 peak fractions post Amicon (UM 2) ultra-filtration	4.8	135	0.55	245	100	9.8*

* Calculated on figure of 1375 obtained from QAE-Sephadex peak.

** The inhibition activity of the various fractions against human pancreatic amylase was measured after each step. The human salivary amylase inhibitory units divided by the human pancreatic amylase inhibitory units = HS/HP inhibitor activity ratio.





Figs. 2a,b. For legend, see p. 165.

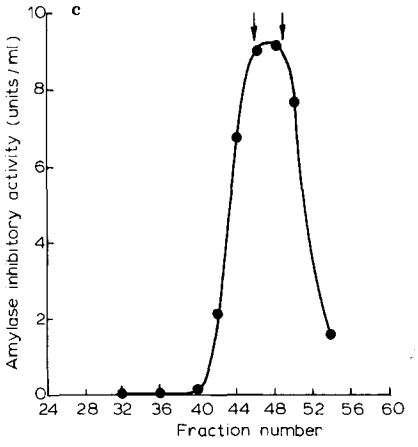


Fig. 2. Procedure for the separation of amylase inhibitor (salivary/pancreatic ratio = 100) by successive column chromatography. ●, salivary amylase inhibitory activity; ○, A_{280nm}, protein content. (a) Chromatography of alcohol fraction (500 mg equilibrated with Tris, I = 0.02, pH 9.2 + 2 mM NaCl) on QAE-Sephadex A-50 column (50 cm × 1.5 cm). Column was washed with 5 column volumes of eluting buffer after application of inhibitor fraction. 6-ml fractions were collected. Elution of inhibitor was performed by a stepwise reduction in pH to 8.9 keeping ionicity (0.02) constant. (b) Chromatography of peak fractions (numbers 36—40) from QAE-Sephadex column (indicated by arrows in Fig. 2a) on a DE52-cellulose column (13 cm × 1 cm). Elution was performed initially with Tris (I = 0.01, pH 9.2, 1 mM NaCl) and then followed by a gradient with respect to increase in concentration of NaCl (0—0.1 M). 2.5-ml fractions were collected. (c) Gel filtration of concentrated inhibitor fractions from DE52-cellulose column (indicated by arrows in Fig. 2b) on Sephadex G-50 column (V_t, 164, V_o, 67 ml, fraction volume, 1.9 ml). Fraction numbers 46—49 pooled and freeze-dried for electrophoretic studies.

Gel electrophoresis

The central fraction of the inhibitor peak of Fig. 2c was submitted to disc gel electrophoresis in a Tris/glycine buffer system. The inhibitor showed essentially a single band of electrophoretic mobility 0.2, and also a minor band. By testing protein fractions eluted with H_2O from slices of the gel for inhibition of both amylases, the minor band was shown to contain inhibitor activity of the same salivary/pancreatic ratio as the major band.

Properties of inhibitor

The inhibition curves (Fig. 3) demonstrate the much greater specificity (100-fold) of the inhibitor towards salivary amylase than towards pancreatic amylase. With salivary amylase, 0.1 μg inhibitor causes 50% inhibition of enzyme activity while 10 μg inhibitor are required to cause the same degree of inhibition of pancreatic amylase. Similar shaped curves are obtained using soluble starch as substrate and a saccharogenic method of analysis [18]. Porcine pancreatic α-amylase and fungal (*A. oryzae*) α-amylase are 600 and 1200 times, respectively less sensitive to the purified inhibitor than is human salivary amylase.

A curve relating the time of pre-incubation of inhibitor with salivary amylase (32 munits) on resulting enzyme activity shows that inhibition increases almost linearly for the first 5—10 min and reaches a plateau within 15—20 min. The time at which the plateau is reached appears to depend on inhibitor concentration.

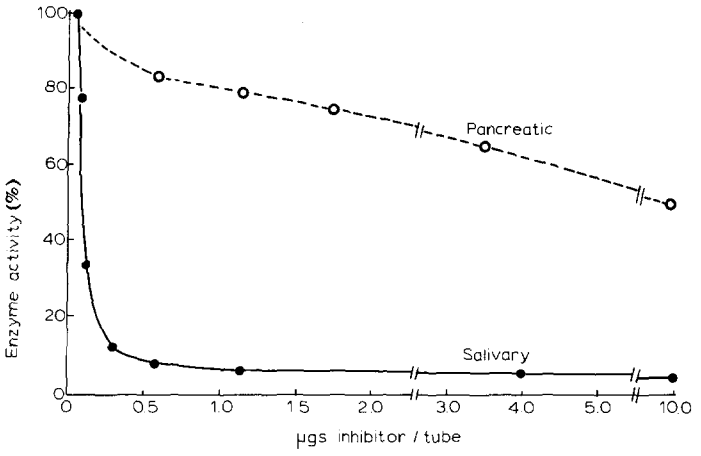


Fig. 3. Effect of inhibitor concentration on human pancreatic amylase (○) and human salivary amylase (●) action on blue starch (44 munits enzyme/tube). Similar curves obtained with soluble starch.

The relationship between the pH of pre-incubation buffer and inhibitor activity was studied using both salivary and pancreatic amylase (Fig. 4). With salivary amylase a pre-incubation pH in the range 6.4—8.0 gave optimal inhibition. A narrower pH range (5.8—7.0) was evident for inhibition of pancreatic amylase. The effect of phosphate and chloride concentration on the potency of the inhibitor showed that increasing concentrations of phosphate buffer (0.1—50 mM, pH 6.9) in the absence of chloride did not effect degree of inhibition of salivary amylase. The addition of 1 mM NaCl to 0.1 mM phosphate buffer, pH 6.9 increased the inhibitor activity from 68 to 85% inhibition.

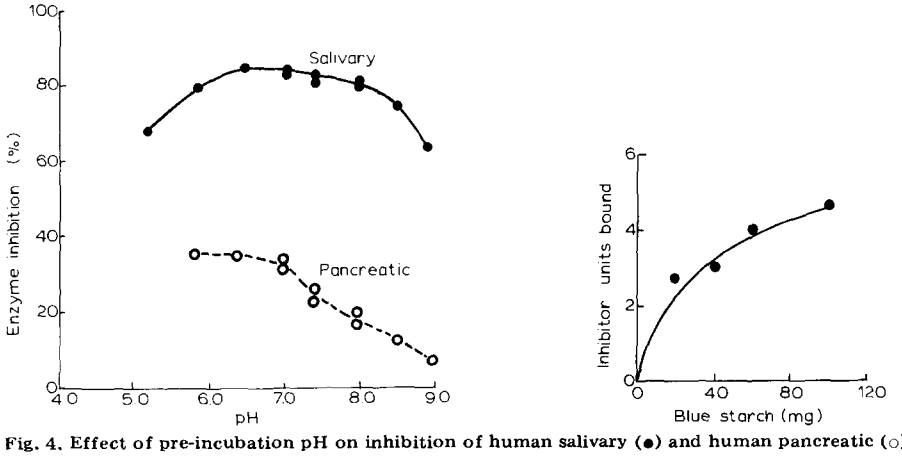


Fig. 4. Effect of pre-incubation pH on inhibition of human salivary (●) and human pancreatic (○) amylase. 0.25 μg of inhibitor protein incubated with salivary amylase and 1.25 μg with pancreatic amylase. Buffers were 50 mM mealate (pH 5.2—5.8), phosphate (pH 6.4—8.0) and Tris (pH 8.0—9.0) containing 50 mM NaCl and 0.5 mM $CaCl_2$.

Fig. 5. Binding of inhibitor with substrate. 20 μg inhibitor (5.4 salivary amylase inhibitory units) added to 1.0 ml of pre-incubation buffer (phosphate, pH 6.9). Increasing amounts of blue starch were added and unbound inhibitor in the supernatant estimated after each substrate addition. Inhibitor units bound to starch were calculated by difference.

TABLE II

EFFECT OF ORDER OF ADDITION OF REACTANTS ON THE ACTIVITY OF INHIBITOR ON SALIVARY AND PANCREATIC AMYLASES

The inhibitor was pre-incubated with either enzyme or substrate and the reaction started by addition of substrate or enzyme accordingly. Units are arbitrary.

Sequence of addition of reactants	Amylase inhibitory units		Salivary/pancreatic ratio
	Salivary	Pancreatic	
(Enzyme + inhibitor) + substrate	50	0.5	100
(Substrate + inhibitor) + enzyme	1.0	0.24	4.1

Further increases of chloride concentration (up to 50 mM) showed no effect even with higher concentrations of phosphate.

The effect of the order of addition of the reactants on inhibition of salivary and pancreatic amylases is shown in Table II. A salivary/pancreatic ratio of 100 is obtained only if enzyme is pre-incubated with inhibitor. The 50-fold lower degree of inhibition of salivary amylase which is obtained if inhibitor is first added to substrate (Table II) is due to a strong binding of inhibitor with substrate. It is apparent from Fig. 5 that 100 mg of blue starch is capable of binding almost 5 units (20 μg) inhibitor. This phenomenon excludes the possibility of studying kinetics of inhibition by classical [22,23] methods.

Discussion

The presence in wheat of an α-amylase inhibitor 30—100 times more active towards human salivary amylase than for porcine pancreatic amylase has been described [2,9,21]. Petrucci et al. [9] reported their inhibitor (peak III) to be equally active towards both human amylases. The present paper illustrates the procedures for the separation of an α-amylase inhibitor which is 100 times more specific for human salivary amylase than for human pancreatic amylase and 600 times more specific for salivary amylase than for porcine pancreatic amylase. This inhibitor which emerges at pH 8.9 from the QAE-Sephadex column represents about 16% of the total salivary amylase inhibitory activity present in the alcohol fraction (Table I). The purified inhibitor lost about 50% activity upon concentration by Amicon ultrafiltration. It is also inactivated by dialysis against de-ionised water. Full activity is retained by dialysis against 5 mM NaCl or 5 mM phosphate buffer pH 6.9. This is similar to the findings of Militzer et al. [3]. Our inhibitor shows an electrophoretic mobility of 0.2 relative to bromophenol blue (=1) which is very close to the figure of 0.19 for the inhibitor of Silano et al. [7] and the peak III inhibitor of Petrucci et al. [9] under identical experimental conditions. Its apparent molecular weight of 21 000 is close to 20 000, 24 000 and 29 650 obtained for inhibitor I [6], inhibitor III [9] and AmI_2 [5], respectively. Silano et al. [7] reported the dissociation of their 0.19 inhibitor into 2 subunits with slightly different molecular weights (13 000 and 14 500) by gel electrophoresis in a dissociating buffer, while a single band was given in non-dissociating solvents. A similar effect was shown by peak III inhibitor of Petrucci et al. [9]. While our

inhibitor migrated essentially as a single band, the minor band, showing slightly greater mobility, may be due to a tendency to dissociate.

The main feature of our inhibitor is its greater (100-fold) specificity for human salivary amylase than for human pancreatic amylase. While it shows many of the physical properties of the 0.19 inhibitor separated by other authors, its differential activity for the two human amylases has not been previously reported. An important factor in the achievement of this difference in specificity is in the order of addition of reactants (Table II). If inhibitor is not pre-incubated with salivary amylase a salivary/pancreatic ratio as low as 4.1 is shown. The influence of the sequence of addition of reactants on extent of inhibition of salivary amylase by various wheat inhibitors has been demonstrated [3,5].

The determination of the dissociation constant K_i of the enzyme-inhibitor complex requires special treatment in this situation [24] and will be dealt with separately. Classical equations [22,23] assume that the inhibitor concentration is not significantly reduced during kinetic treatment. This assumption is invalid in this case due to strong interaction with substrate (Fig. 5). A tendency for the AmI_2 inhibitor [5] to bind with polysaccharides has also been demonstrated.

The use of the inhibitor of high salivary/pancreatic ratio (100) in the quantitative analysis of mixtures of the two enzymes and its application to differential α-amylase assay in blood and body fluids will be reported elsewhere.

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