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INTERACTION OF HUMAN α-AMYLASES WITH INHIBITORS FROM WHEAT FLOUR

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Summary

The interaction of four purified α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) inhibitors with human salivary and pancreatic α-amylases was investigated. The inhibitory activity of the four proteins towards salivary α-amylase was significantly increased by pre-incubation of the enzyme with inhibitor before adding substrate. This effect was not observed with the inhibition of pancreatic α-amylase by inhibitors 1 and 2. Inhibition of both amylases was affected to different degrees by incubating starch with inhibitor prior to the addition of enzyme. Maltose, at concentrations which only slightly affected amylase activity, prevented the inhibition of both enzymes by all four inhibitors. Gel filtration studies on salivary amylase-inhibitor mixtures showed the formation of EI complexes on a mol-to-mol ratio. A similar complex between pancreatic α-amylase and inhibitor 4 was obserbed, though complex formation between pancreatic α-amylase and the other inhibitors was not clearly demonstrated.

Introduction

The presence in wheat of α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) inhibitors has been well documented and reviewed [1]. O'Donnell and McGeeney [2] described the isolation and purification of one of these inhibitors which exhibited a far greater (100-fold) specificity for human salivary α-amylase than for human pancreatic α-amylase. The use of this inhibitor to distinguish between salivary and pancreatic isoenzymes in human serum has since been reported [3]. We have recently described the isolation from wheat

flour of four α-amylase inhibitors, all of which exhibit differential specificities towards the two human α-amylases [4]. The ratio of inhibitory activity towards human salivary α-amylase relative to human pancreatic α-amylase was 140, 25, 2 3 and 1.4 for inhibitors 1, 2, 3 and 4, respectively. This marked difference in inhibition specificity prompted the investigation of the interaction between the inhibitors and the two human α-amylases The present study deals with the influence of a number of physical and chemical parameters on the enzyme-inhibitor reactions and examines the formation of α-amylase-inhibitor complexes

Experimental methods

Enzyme preparations Saliva, collected from laboratory personnel was pooled and centrifuged. Amylase was separated from the supernatant by gel filtration on Sephadex G-100 in 50 mM phosphate buffer (pH 6.9)/50 mM NaCl/0.5 mM $CaCl_2$ (Buffer 1). The active eluate was dialysed against 10 mM Tris-HCl buffer (pH 8.9)/3 mM $CaCl_2$ (Buffer 2) and applied to a DEAE-Sephacel column (30 × 1 cm). The column was eluted with a 0.0—0.5 M NaCl gradient The active peaks, which represent the isozyme forms of salivary α-amylase [5], were collected.

Human post-mortem pancreatic tissue was homogenised in 4 vol. (w/v) of Buffer 1 and centrifuged. The supernatant was fractionated at 4° C with ethanol and the precipitate which formed at 40—70% ethanol collected by centrifugation, washed with absolute alcohol and dried. The dry powder was dissolved in Buffer 1 and applied to a Sephadex G-100 column. Elution was performed in the same buffer at a flow rate of 12 ml · h^{-1}. The active eluate was dialysed against Buffer 2 and applied to a DEAE-Sephacel column (30 × 1 cm) The column was eluted with a 0.0—0.1 M NaCl gradient and the active peaks, which represent the isozyme forms of pancreatic α-amylase [5], collected.

Inhibitor fractions The inhibitor proteins were purified from wheat flour as described previously [4].

Amylase and inhibitor assay α-Amylase activity was measured by the Phadebas blue starch method [6] incorporating 0.5 mg/ml albumin in the reaction mixture for optimal enzyme activity [7]. The standard inhibitor assay was carried out by preincubating 0.1 ml amylase solution with 10—50 μl inhibitor for 30 min at 20° C in 0.5 ml Buffer 1/4 mg/ml albumin. At the end of the pre-incubation period the volume was adjusted to 4.1 ml with water and the remaining amylase activity assayed by the Phadebas method. To determine the optimum pre-incubation time and pH of the inhibition reactions these assay parameters were adjusted accordingly.

Substrate-inhibitor interaction The effect of insoluble blue starch on the inhibition reaction was studied by incubating samples of each inhibitor (50 μg) with Phadebas blue starch (50 mg) for 30 min at 20° C. The samples were then centrifuged and the supernatant solutions assayed for inhibitory activity.

Effect of maltose on inhibition reactions Two series of experiments were performed to examine the effect of maltose on the interaction of enzyme and inhibitors In one series, enzyme and inhibitor were pre-incubated at 20° C for 30 min and maltose (final concentration 10—100 mM) added The mixtures

were allowed to stand at 20° C for a further 10 min before measurement of the residual α-amylase activity. In the second series, maltose (10—100 mM) was mixed with the inhibitor prior to the addition of enzyme and the entire mixture preincubated for 40 min before amylase assay.

Calcium depletion tests. Enzyme samples were incubated with 5 μmol EDTA before the addition of inhibitor After a further 30 min pre-incubation, enzyme activity was measured using washed blue starch [8] as substrate in Buffer 1.

Gel filtration Examination of enzyme-inhibitor mixtures by gel filtration was performed by applying 1.0 ml of a solution containing known molar quantities of enzyme and inhibitor to a Sephadex G-100 column (68 × 2 cm) Elution was carried out in 10 mM phosphate buffer, (pH 6 9)/10 mM NaCl, at a rate of 10 ml · h^{-1}. Individual samples of the four inhibitors and the two enzymes were also chromatographed on the same column. The enzyme-inhibitor mixtures were preincubated for 1 h at 20° C before application. Column eluates were scanned at 280 nm on an LKB Uvicord II and individual fractions (2 ml) assayed for enzyme and inhibitory activity to determine the exact position of the inhibitor peaks.

Results

Salivary and pancreatic isozymes DEAE-Sephacel chromatography of salivary α-amylase yielded four isozyme peaks with specific activities 33.7 I.U./mg, 311.5 I U./mg, 532 I U./mg and 424 I.U./mg. Each isozyme was assayed for inhibition by the four isolated inhibitors. No difference in susceptibility to inhibition was observed among the isozymes. Pancreatic α-amylase was separated into two isozyme forms by ion-exchange chromatography on DEAE-Sephacel. As with salivary α-amylase, no difference in inhibition susceptibility was observed between the two pancreatic isozymes.

Inhibition of salivary and pancreatic α-amylases The pre-incubation time necessary to achieve maximum inhibition of salivary α-amylase varied with both enzyme and inhibitor concentration (the higher the inhibitor/enzyme ratio the shorter the time). This was observed with all four inhibitors. A 60 min preincubation was sufficient to achieve maximum inhibition at all enzyme concentrations tested. In contrast, no pre-incubation was required to achieve maximum inhibition of pancreatic α-amylase by inhibitors 1 or 2. This was not affected by varying either enzyme or inhibitor concentrations. However, pre-incubation of pancreatic α-amylase with inhibitors 3 or 4 for up to 40 min was required to obtain maximal levels of inhibition. These results indicate that the observed ratio of inhibitory activity towards human salivary α-amylase relative to human pancreatic α-amylase (salivary/pancreatic ratio) is particularly dependent on the time of pre-incubation of inhibitor with enzyme. If the enzyme-inhibitor interaction is not allowed to reach equilibrium before addition of substrate non-maximal inhibition levels will be observed and low salivary/pancreatic ratios calculated.

The relationship between pH of the pre-incubation buffer and inhibitor activity was investigated over a pH range of 4.0—9.0 In all cases, maximum inhibition was observed between pH 5.5 and 7 5. This corresponded to the pH

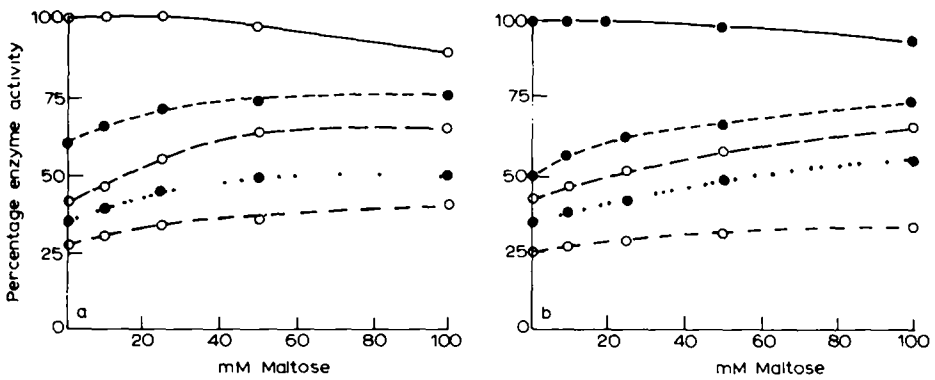

Fig 1 Effect of increasing maltose concentration on the inhibition of (a) salivary α-amylase and (b) pancreatic α-amylase by inhibitors isolated from wheat flour ○———○, salivary α-amylase, ●———●, pancreatic α-amylase, ●·····●, inhibitor 1, ○———○, inhibitor 2, ● ●, inhibitor 3, ○ — — ○, inhibitor 4

range of optimal activity for both salivary and pancreatic α-amylases under the same experimental conditions.

Under optimum pre-incubation conditions the maximum observed reduction in enzyme activity was 94—97%. Inhibitor concentrations up to 50-times greater than those required to reduce enzyme activity by 50% failed to completely inhibit either enzyme. This inability to achieve 100% inhibition of amylase by wheat inhibitors has been noted by previous workers [1].

Substrate-inhibitor interaction. Experiments on the interaction of Phadebas insoluble starch and the inhibitors indicated that this starch does bind significant quantities of inhibitors 1, 2 and 3 (30, 19 and 12 μg of each respective inhibitor bound by 50 mg starch). The potency of inhibitor 4 was not affected by the addition of insoluble starch. A second series of tests were performed to establish if inhibitor bound to insoluble starch could still inhibit α-amylase. In these tests enzyme was added directly to a pre-incubated mixture of starch and inhibitor. Table I summarizes the results obtained It would seem that inhibitor bound to Phadebas starch is not available for interaction with enzyme

Effect of maltose on the inhibition reactions Significant reversal of the inhibition of both human α-amylases by all four inhibitors was observed when maltose was added to a preincubated solution of enzyme and inhibitor (Fig 1) Reversal was most noticeable at low inhibitor concentrations. The highest maltose concentration tested (100 mM) did not completely reverse the inhibition of either enzyme by any of the isolated inhibitors. At this level, maltose itself causes approx. 15% inhibition of enzyme activity. When maltose was included in the system prior to the preincubation of enzyme and inhibitor it was found to prevent the action of the inhibitors. 100 mM maltose could completely block the inhibition of salivary α-amylase by concentrations of inhibitor which normally caused 70% inhibition. This same effect was observed with the inhibition of pancreatic α-amylase by inhibitors 3 and 4 Although some blocking of inhibition of pancreatic α-amylase by inhibitors 1 and 2 was observed, complete prevention of inhibition was not observed with 100 mM maltose.

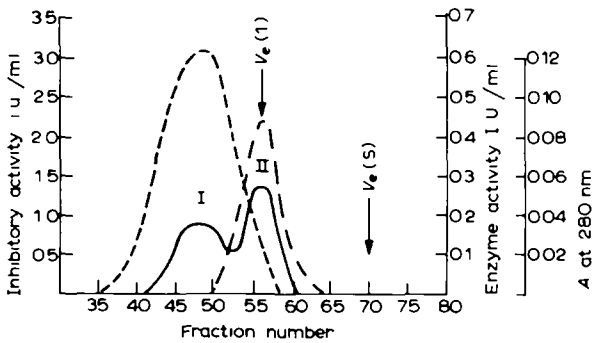

Fig 2 Elution profile of salivary α-amylase-inhibitor 1 mixture on a Sephadex G-100 column (V_0 = 54 ml, V_t = 160 ml) The mixture contained 9 5 nmol inhibitor 1 and 3 15 nmol enzyme Fraction volume = 2 ml. — — —, enzyme activity, — — —, inhibitory activity, ———, protein V_e (1), V_e of inhibitor 1, V_e (S), V_e of salivary α-amylase

Calcium depletion tests Lang et al. [9] have suggested that wheat α-amylase inhibitors may exert their effects simply by chelating Ca^{2+}, an ion which is vital for optimum amylase activity. This possibility was studied by examining the effect of the inhibitors on human salivary and pancreatic α-amylases under conditions where essentially no free Ca^{2+} was available The Ca^{2+} chelator, EDTA, caused substantial loss in activity of both enzymes, reducing salivary α-amylase by 74% and pancreatic α-amylase by 37%. Despite this, it was observed that all four inhibitors still exerted inhibition effects on the Ca^{2+}-depleted enzymes. The potency of the inhibitors was reduced in the Ca^{2+}-deficient system This is probably due to the insensitivity of the enzyme assay under these conditions.

Enzyme-inhibitor complex formation. Complex formation was examined by submitting inhibitor mixtures to gel filtration on a Sephadex G-100 column. Quantities of each inhibitor and enzyme, identical to those used in subsequent experiments, were first examined on the same column to determine their exact elution volumes (V_e). The elution profiles of a mixture containing 9.5 nmol

TABLE I

THE EFFECT OF INHIBITOR BOUND TO BLUE STARCH IN REDUCING THE ACTIVITY OF HUMAN SALIVARY AND PANCREATIC AMYLASES

Inhibitor solutions were pre-incubated with insoluble blue starch and the reaction started by the addition of enzyme In control tests, (without pre-incubation) enzyme and starch were added to inhibitor within a 10 s interval

Inhibitor	Pre-incubation with starch	Percentage enzyme activity	
		Salivary amylase	Pancreatic amylase
Inhibitor 1	—	64	74
	+	100	100
Inhibitor 2	—	86	68
	+	100	100
Inhibitor 3	—	73	89
	+	91	100

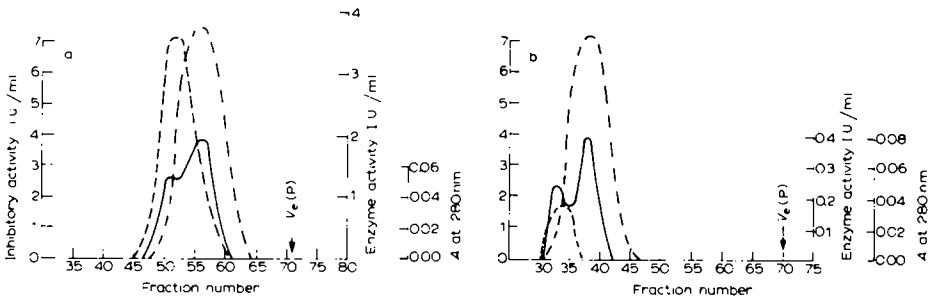

Fig 3 Elution profiles of pancreatic α-amylase-inhibitor mixtures on Sephadex G-100 (a) Inhibitor 1-pancreatic α-amylase 9 5 nmol inhibitor 1 and 2 1 nmol enzyme were present in the mixture applied to the column (vol = 1 ml) (b) Inhibitor 4-pancreatic α-amylase 9 0 nmol of inhibitor 4 and 2 1 nmol enzyme were applied to the column (vol = 1 ml) Fraction volume = 2 ml — — —, enzyme activity, — — , inhibitory activity, ———, protein V_e (P), V_e of pancreatic α-amylase

inhibitor 1 and 3.15 nmol salivary α-amylase is shown in Fig. 2. Two protein peaks were eluted. Peak II had a V_e identical to that of free inhibitor and when assayed was found to display significant inhibitory activity. Peak I was enzymatically active (accounting for 5% of the enzyme applied) even though its elution volume was significantly different from that of the free enzyme. This fraction was collected, concentrated by ultrafiltration (Amicon, UM-2 membrane) and examined by SDS-gel electrophoresis. Two protein bands of equal intensity, corresponding to salivary α-amylase and inhibitor, were observed, indicating that this peak did represent a salivary amylase/inhibitor 1 complex. Since 5.28 nmol free inhibitor were recovered in peak II, it was calculated that 3.15 nmol amylase combined with 4.22 nmol inhibitor, indicating an EI complex composition of 1 mol enzyme/mol inhibitor. The results of similar gel filtration experiments carried out on mixtures of salivary α-amylase and inhibitors 2, 3 and 4 are presented in Table II. All four EI complexes, obtained via gel filtration, were tested for ability to inhibit pancreatic α-amylase but no inhibition was observed This indicated that a tertiary complex, i.e., salivary amylase-inhibitor-pancreatic amylase cannot form. Similarly, when a second inhibitor was added to any EI complex no further inhibition was observed. Experiments in which different combinations of the inhibitors were pre-incubated with enzyme

TABLE II

RECOVERY OF FREE INHIBITOR FOLLOWING GEL-FILTRATION OF SALIVARY α-AMYLASE-INHIBITOR MIXTURES ON SEPHADEX G-100

Each mixture contained 3 15 nmol enzyme and 9 5 nmol inhibitor

Inhibitor	Quantity recovered (nmol)	Quantity bound (nmol)	Stoichiometry of EI complex (E I)	% Active enzyme in EI complex
Inhibitor 1	5 28	4 22	1 1 3	5
Inhibitor 2	4 99	4 51	1 1 4	9
Inhibitor 3	5 5	4 0	1 1 2	12
Inhibitor 4	4 15	5 35	1 1 6	2

and then assayed for amylase activity indicated that the inhibition effects on both enzymes of all four inhibitors were additive.

Mixtures of pancreatic α-amylase and the four inhibitors were also examined by gel filtration. The elution profile obtained with pancreatic α-amylase-inhibitor 1 is shown in Fig. 3a. Although the elution position of the active enzyme peak was shifted downwards, assay of the enzymic and inhibitory activity of the eluted fractions indicated an 85% recovery of both enzyme and inhibitor. Thus, these gel filtration experiments did not clearly demonstrate the formation of an EI complex between inhibitor 1 and pancreatic α-amylase Similar results were obtained with mixtures of pancreatic α-amylase and inhibitors 2 and 3. With inhibitor 4, 2.25 nmol inhibitor were not recovered when a mixture containing 9.0 nmol inhibitor and 2.1 nmol pancreatic α-amylase were applied to the Sephadex G-100 column (Fig. 3b). This would imply a 1 1 complex formation. Only 3% of the added enzyme activity was recovered in the inhibitor 4-pancreatic α-amylase complex peak.

Discussion

The observed inhibition of salivary α-amylase by the four isolated inhibitors was markedly increased by pre-incubation of the enzyme with the inhibitors. This effect has been previously noted [2,10] and has been attributed to the fact that the enzyme-inhibitor interaction is slower than starch hydrolysis [1,11] Thus, when starch is added to a non-equilibrated mixture of enzyme and inhibitor the enzyme will bind preferentially to starch. No preincubation was necessary to achieve maximal inhibition of pancreatic α-amylase by inhibitors 1 and 2. This may mean that the interaction of these inhibitors with pancreatic α-amylase is more rapid than with salivary α-amylase. An alternative explanation is that much larger quantities of inhibitors 1 and 2 are required to inhibit pancreatic α-amylase to the same extent as salivary α-amylase. It can be envisaged that such high levels of inhibitor would drive the reaction $E + 1 \rightleftharpoons EI$ to the right, rapidly 'mopping-up' the relatively small quantity of enzyme present. This would explain the observed results without making any assumptions about the relative binding rates of the inhibitors to the two enzymes. Some authors attribute the importance of pre-incubation to the tendency of the inhibitors to bind starch [2,12]. It is suggested that the substrate, when added to a non-equilibrated mixture of inhibitor and enzyme, binds the free inhibitor, thus making it unavailable for interaction with enzyme. Our studies with Phadebas insoluble starch confirm the observation of O'Donnell and McGeeney [2] that this starch binds significant quantities of inhibitor. It was also found that the bound inhibitor was not able to inhibit α-amylase. Thus, the binding of inhibitor to insoluble starch may be a causative factor in lowering the level of inhibition observed with the Phadebas assay system when non-equilibrated mixtures of enzyme and inhibitor are employed. Maltose was also found to prevent the inhibition of salivary and pancreatic α-amylases by the four isolated inhibitors. Unlike starch, maltose could prevent the inhibition of both enzymes to a similar extent. This points to an interaction between maltose and enzyme rather than maltose and inhibitor. That maltose and enzyme interact is evident from the inhibition of enzyme activity noted at high

maltose concentrations The removal of α-amylase from affinity columns of wheat inhibitor by maltose [13] provides further evidence for a maltose-enzyme interaction. When added to a preincubated mixture of enzyme and inhibitor, maltose caused some reversal of inhibition. Similar observations have been reported for the 0 19 inhibitor-Tenebrio molitor larval (TmL) amylase system [10]. The mechanism through which maltose causes dissociation of the EI complex is not clear Maltose is a product of the hydrolytic digestion of starch and is thought to cause competitive inhibition of α-amylase at high concentrations. Elodi et al [15] have shown that binding of maltose to porcine pancreatic α-amylase takes place at the active site and is accompanied by limited structural changes in the conformation of the enzyme It may be that such conformational changes modify the inhibitor binding site(s) on the enzyme in such a way that inhibitor can no longer interact with the enzyme.

Gel filtration experiments clearly demonstrated the formation of salivary α-amylase-inhibitor complexes The molar combining ratios of the complexes were calculated as being 1 1. This binding ratio is also confirmed by kinetic studies [16] A 1 1 ratio has been reported for the 0.19 inhibitor-Tenebrio molitor larval amylase and 0.19-chick pancreatic α-amylase systems [11,17] These gel-filtration studies, and assays performed in Ca^{2+}-deficient systems, also confirm that the inhibitors do not act by chelating Ca^{2+} or removal of Cl^-

The elution volumes of the salivary α-amylase-inhibitor complexes on Sephadex G-100 did not correspond to the expected V_e values based on the molecular weights of the components Thus, even when bound to inhibitor the enzyme retained a capacity to bind to the Dextran matrix of the gel All the EI complexes isolated by gel filtration displayed some enzymatic activity which could not be further inhibited by the addition of more inhibitor. This, allied to the fact that 100% inhibition of enzyme was not observed at any inhibitor concentration tested, indicates that the enzyme, when bound to inhibitor, still retains a capacity to catalyse a slow rate of starch hydrolysis, as previously suggested [1,14] This indicates that the inhibitor binding site on the enzyme is not the catalytic site. Evidence from kinetic studies [16] also confirm that the mechanism of inhibition is not competitive.

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References

1 Buonocore, V , Petrucci, T. and Silano, V (1977) Phytochemistry 16, 811—820
2 O'Donnell, M D and McGeeney, K F (1976) Biochim Biophys Acta 422, 159—169
3 O'Donnell, M D , FitzGerald, O and McGeeney, K F (1977) Clin Chem 23, 560—566
4 O'Connor, C M and McGeeney, K F (1980) (BBA 69241)
5 Fridhandler, L , Berk, J E and Ueda, M (1972) Clin Chem 18, 1493—1498
6 Ceska, M , Birath, K and Brown, B (1969) Clin Chim Acta 26, 437—444
7 O'Donnell, M D and McGeeney, K F (1974) Enzyme 18, 348—355
8 O'Donnell, M D and McGeeney, K F (1974) Enzyme 18, 356—367
9 Lang, J A , Talley, D J and Saunders, R M (1973) Fed Proc Abst 32, 554
10 Petrucci, T , Rab A , Tomasi, M and Silano, V (1976) Biochim Biophys Acta 420, 288—297

11 Silano, V , Poerio, E and Buonocore, V (1977) Mol Cell Biochem 18, 87—91
12 Shainkin, R and Birk, Y (1970) Biochim Biophys Acta 221, 502—513
13 Buonocore, V , Poerio, E , Gramenzi, F and Silano, V (1975) J Chromatogr 114, 109—114
14 Buonocore, V , Poerio, E , Pace, W , Petrucci, T , Silano, V and Tomasi, M (1976) FEBS Lett 67, 202—206
15 Elodi, P , Mora, S and Krysteva, M (1972) Eur J Biochem 24, 577—583
16 O'Connor, C M , O'Donnell, M D and McGeeney, K F (1980) La Clinica Dieto Logica 7, 443—444
17 Buonocore, V , Gramenzi, F , Pace, W , Petrucci, T , Poerio, E and Silano, V (1980) Biochem J 187, 637—645