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ISOLATION AND CHARACTERIZATION OF FOUR INHIBITORS FROM WHEAT FLOUR WHICH DISPLAY DIFFERENTIAL INHIBITION SPECIFICITIES FOR HUMAN SALIVARY AND HUMAN PANCREATIC α-AMYLASES

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Summary

Four α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) inhibitors were isolated from an albumin fraction of wheat flour by ion-exchange and gel-filtration chromatography. The purified inhibitors were characterized according to their electrophoretic mobilities, molecular weights, carbohydrate content, sulphydryl content, susceptibility to proteolytic digestion and specificities in inhibiting human salivary and pancreatic α-amylases. The properties of these inhibitors are compared to similar proteins isolated by other workers

Introduction

In 1946, when Militzer and co-workers [1] extracted and characterized an α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) inhibitor from wheat, they assumed they were dealing with a single protein It is now well established that the inhibitor preparation used by these early investigators contained a mixture of inhibitors [2—7]. The majority of wheat albumins are, in fact, α-amylase inhibitors, capable of inhibiting α-amylases from various sources [8,9]. These albumins can be divided into three heterogeneous groups of molecular weights 12 000, 24 000 and 60 000 [2]. The proteins in the 12 000 molecular weight group, which inhibit a variety of insect α-amylases [9], have

Abbreviation DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

been extensively studied by Silano and co-workers, [5,10—12] and designated the '0.28-isoinhibitor family' (from the electrophoretic mobility of the main component of the group). The 24 000 molecular weight group has been shown to consist of ten proteins, all active inhibitors of α-amylase [2]. Unlike the lower molecular weight albumins, these proteins are potent inhibitors of the human α-amylases The major protein of the group (coded the 0.19 inhibitor) has also been well characterized [5,10—13]. The AmI_2 inhibitor of Shainkin and Birk [3], inhibitors I and II of Saunders and Lang [4] and the inhibitor purified by O'Donnell and McGeeney [7] all display properties characteristic of this group. As yet, little attention has been given to the proteins of the 60 000 molecular weight group, which are also potent inhibitors of the human α-amylases [2].

In this paper we report the isolation and characterization of four α-amylase inhibitors from wheat albumin and compare the properties of the isolated proteins with those of other inhibitors described in the literature.

Experimental methods

Materials. Sephadex G-50 and G-100, QAE-Sephadex A-50, DEAE-Sephacel, CM-Sepharose CL-68, Dextran blue and Phadebas α-amylase test tablets were obtained from Pharmacia (London) Tris-HCl, bovine serum albumin, pepsin (porcine), trypsin (bovine pancreas), α-chymotrypsin (bovine pancreas), Coomassie blue R, 2-mercaptoethanol, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and α-amylases from *Aspergillus oryzae*, *Bacillus subtilis* and hog pancreas were obtained from Sigma Chemical Co., U K. Acrylamide and *N,N'*-methylene-bisacrylamide were Eastman products, *N,N,N',N'*-tetramethylethylenediamine (TEMED) was purchased from G.M B.H. and Co. Ltd., Munich; riboflavin from B.D.H. Ltd. (Poole), bromophenol blue and sodium dodecyl sulphate (SDS) from Polysciences, Inc. (Warrington, PA). Molecular weight protein standards for SDS-gel electrophoresis were from Bio-Rad laboratories (Hertfordshire). All other reagents were Analar grade.

Enzyme preparation Saliva from laboratory personnel was pooled and centrifuged (10 000 × *g*; 30 min, 4°C). 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 fractions containing α-amylase activity were pooled and bovine serum albumin added to a final concentration of 10 μM. Aliquots of this preparation were stored at —20°C. Post mortem pancreatic tissue from patients who had not suffered pancreatic damage or functional disturbance was used as a source of human pancreatic α-amylase. Tissue samples (1—2 g) were homogenised in 4 vol. (w/v) ice-cold Buffer 1 using an Ultra-Turrax homogenizer. The homogenate was centrifuged (10 000 × *g*, 30 min; 4°C) and amylase separated from the supernatant by gel filtration as described above 10 μM bovine serum albumin were added to the eluted amylase and the preparation stored in aliquots at 4°C. Wheat α-amylase was purified from germinated wheat seeds as described by Kruger and Tkachuk [14].

Amylase and inhibitor assay. α-Amylase activity was measured by the Phadebas blue starch method [15] incorporating 0.5 mg/ml albumin in the

reaction mixture for optimal enzyme activity [16]. Inhibitory activity was assayed as described by O'Donnell and McGeeney [7]. The ratio of inhibitory activity towards human salivary α-amylase relative to human pancreatic α-amylase is referred to as the salivary/pancreatic ratio

Extraction and fractionation· 3 kg wheat flour (variety, conditioned Manitoba) were extracted in 0.1 N NaOH (1 : 3 w/v, pH 9.0) for 1 h at 20°C. The suspension was centrifuged at 10 000 × *g* for 30 min and the residue re-extracted in a further vol. of 0.1 N NaOH. The yellow-brown supernatants were pooled and fractionated with $(NH_4)_2SO_4$. The precipitate which formed at 0.4—2.0 M $(NH_4)_2SO_4$ was collected, dialysed and equilibrated with 115 mM Tris-HCl buffer (pH 9.2, $I = 0.01$). The solution was applied to a DEAE-Sephacel column (1.6 × 100 cm) and washed with 2 column vol. Tris-HCl (pH 9.2, $I = 0.01$) before eluting with increasing concentrations of NaCl 6 ml fractions were collected and analysed for inhibition of human salivary and pancreatic α-amylases. The column effluent was continuously monitored at 280 nm on an LKB Uvicord II. Four inhibitor peaks were eluted, collected and lyophilised. The resulting powders were used as starting material for further purification.

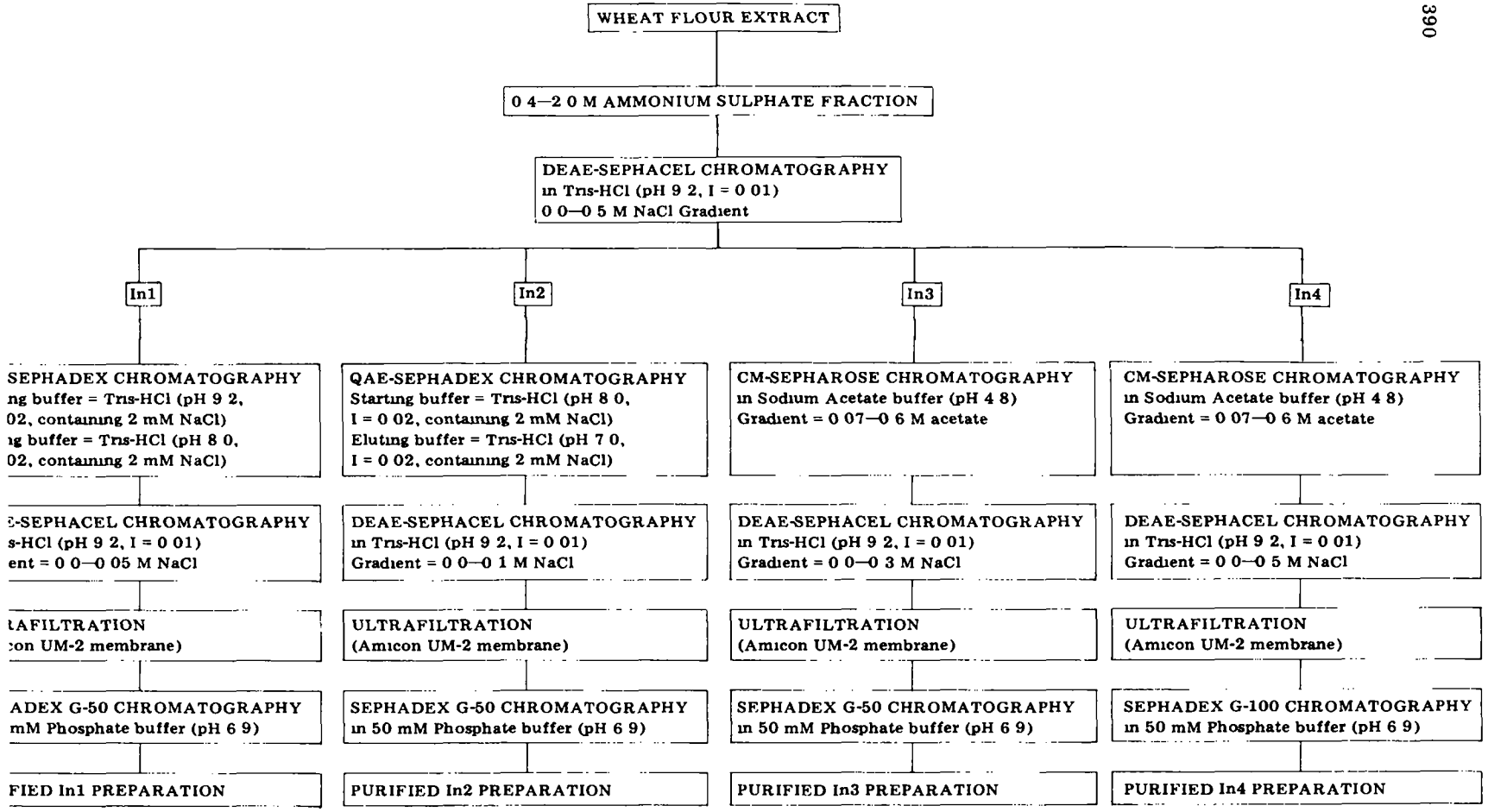
Purification of inhibitor fractions The major inhibitor proteins in each of the four lyophilised fractions were purified by a series of ion-exchange and gel-filtration steps as shown in Fig. 1. At each chromatographic step only the major eluting inhibitor peak was collected and further purified. Both human salivary and human pancreatic α-amylases were used for the detection of inhibitory activity throughout the purification procedures. The Hartree [17] method for protein analysis was used to determine the specific activity of the inhibitor preparations at each stage in the purification process.

Gel electrophoresis Polyacrylamide gel electrophoresis was carried out in a 0.05 M Tris/0.383 M glycine buffer (pH 8.5) according to Davis [18]. Following electrophoresis the gels were stained with Coomassie blue [19]. Electrophoresis in SDS was performed in a continuous buffer system on 10% polyacrylamide gels. The gel and electrophoresis buffers contained 0.025 M Tris/0.19 M glycine/0.1% SDS/0.001 M EDTA, pH 8.3. Protein samples were prepared as described by Laemmli [20].

Molecular weight estimation The apparent molecular weights of the purified inhibitors were determined by comparing their elution volumes with those of standard proteins on a Sephadex G-100 column as described by Andrews [21]. Molecular weights were also determined under dissociating conditions by SDS-polyacrylamide gel electrophoresis according to Weber and Osborn [22].

Treatment with dissociating agents. Electrophoresis grade SDS was added to inhibitor solutions (200—700 μg protein) to a final concentration of 1%. After 24 h, the SDS was removed as described by Lenard [23]. 2-Mercaptoethanol (5 mM), when present, was removed by dialysis. Treatment with 6 M urea was performed in a similar manner and the urea removed by dialysis against Tris-HCl buffer (pH 7.6, $I = 0.05$) for 24 h.

Deamidation. Inhibitor samples, at a protein concentration of 500 μg/ml were dialysed against 0.2 M Na_3PO_4 (pH 9.9) for 6 days at 37°C with several changes of dialysis buffer These are essentially the conditions described by McKerrow and Robinson [24] which cause hydrolysis of amide side chains. At



g 1 Scheme for the purification of four α-amylase inhibitors from wheat flour Four inhibitor fractions (abbreviated In1, In2, In3 and In4) were separated from $(NH_4)_2SO_4$ extract of wheat flour by chromatography on DEAE-Sephacel These were purified by ion-exchange and gel-filtration chromatography as indicated each chromatographic step only the major eluting inhibitor was collected and further purified

the end of this period the samples were dialysed against water for 24 h and freeze-dried. The resulting products were assayed for inhibitory activity and examined by gel electrophoresis.

Proteolysis. Samples of the inhibitors (200 μg protein) were incubated at 37°C with 0.3 ml trypsin (103 units/ml) or α-chymotrypsin (94 units/ml) in Tris-HCl buffer (pH 7.6). Incubation with pepsin (0.3 ml, 110 units/ml) was carried out in 0.02 M HCl (pH 2.5).

Heat treatment Inhibitor samples (100 μg) in 10 mM phosphate buffer (pH 6.9) were incubated at 37, 60 and 100°C for 60 min Samples were removed at 10 min intervals and assayed for inhibition of human salivary and pancreatic α-amylases.

Chemical analyses The number of sulphydryl groups present in the inhibitor proteins were determined before and after reduction by the method of Cavallini et al. [25]. The carbohydrate content of the inhibitors was measured as described by Krystal and Graham [26].

Activity of α-amylases from different sources. In addition to human salivary and human pancreatic α-amylases the effect of the inhibitors on the activity of hog pancreatic, *A. oryzae*, *B subtilis* and wheat α-amylases was examined. To assay wheat α-amylase, Phadebas blue starch tablets were washed free of buffer salts as described by O'Donnell and McGeeney [27]. The assay was performed in 0.1 M sodium acetate buffer (pH 5.6)/40 mM $CaCl_2$/50 mg washed starch as substrate. The other enzymes were assayed under the same conditions as described for the human α-amylases.

Results

Extraction and purification of inhibitor proteins. The chromatographic separation of α-amylase inhibitors from an $(NH_4)_2SO_4$ fraction of wheat flour on DEAE-Sephacel is shown in Fig. 2. Four inhibitor peaks were eluted by a

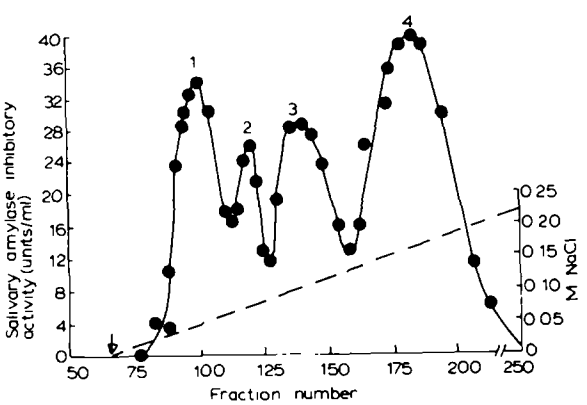


Fig 2 Fractionation of α-amylase inhibitors from an $(NH_4)_2SO_4$ extract of wheat flour by chromatography on a DEAE-Sephacel column (1 6 X 100 cm). Application buffer was Tris-HCl (I = 0 01, pH 9.2). 2 col vol buffer were eluted before starting a NaCl gradient (0 0—0 5 M) at fraction No 65 (indicated by arrow) Fraction volume = 6 ml The four inhibitor fractions eluted were collected, lyophilised and used as starting material for further purification.

TABLE I

YIELD AND POTENCY OF PURIFIED INHIBITOR PREPARATIONS

s/p Ratio, the ratio of inhibitory activity towards human salivary α-amylase relative to human pancreatic α-amylase

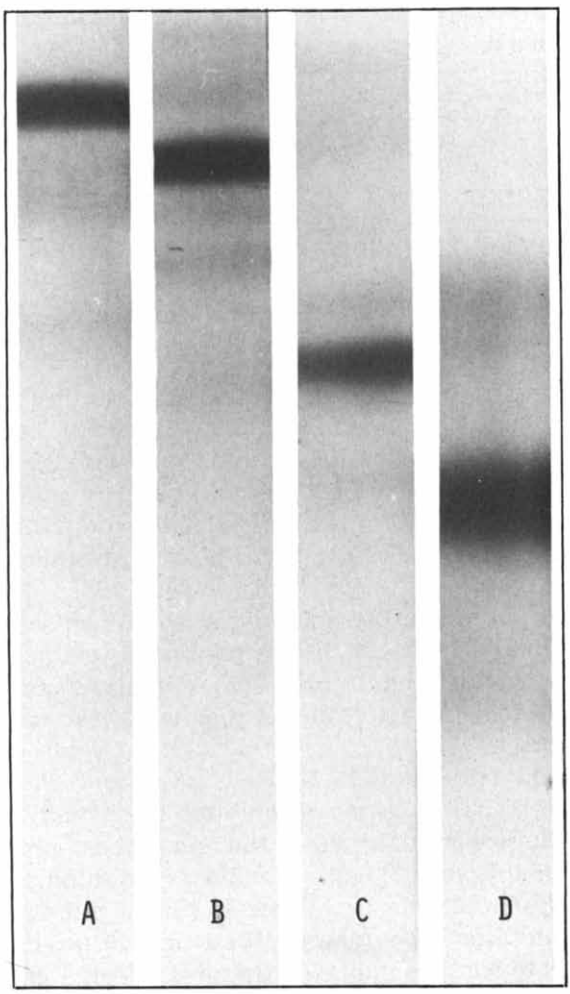
Inhibitor	Total i u * vs salivary amylase	Total protein (mg)	Specific activity	s/p ratio	% Recovery (from lyophilised fraction)
Inhibitor 1	28 800	26 3	1094	140	10
Inhibitor 2	7 154	8 36	856	25	3
Inhibitor 3	5 605	6 56	854	2 3	5
Inhibitor 4	42 048	47 2	891	1 4	7

* i u , inhibitory units defined as the quantity of inhibitor required to reduce the activity of 2 I U of amylase by 50%

0.00—0.5 M NaCl gradient. All peaks inhibited salivary and pancreatic α-amylases to different degrees. The salivary/pancreatic ratios were 10.6, 2 2, 1.13 and 2.7 for inhibitors 1, 2, 3 and 4, respectively. Further separation of inhibitor 1 from inhibitor 2 was achieved primarily by rechromatography on DEAE-Sephacel using shallow NaCl gradients. Preliminary electrophoretic studies indicated that inhibitors 3 and 4 were basic proteins with strong negative charges at pH 7.0, hence a cation-exchange chromatography step was included in the purification of these two proteins. Table I summarises the yield and potency of the final inhibitor preparations. The electrophoretic profiles of the four preparations are shown in Fig. 3. The apparent molecular weights of the inhibitors, determined from their elution volumes on Sephadex G-100, were 22 500 for inhibitor 1; 21 400 for inhibitor 2; 25 000 for inhibitor 3 and 63 000 for inhibitor 4. Electrophoresis in a dissociating system (SDS) gave a molecular weight estimation of 14 000 for inhibitors 1, 2 and 3. Only one protein band was observed in the gels (Fig. 4). In the case of inhibitor 4, however, two bands of almost equal intensity, corresponding to molecular weights of 14 000 and 15 000 were noted. Similar profiles were observed with reduced and non-reduced samples of the inhibitor preparations These results indicate the presence of two subunits of similar size in each of the inhibitor 1, 2 and 3 proteins. Inhibitor 4 would seem to consist of four subunits, two of molecular weight 14 000 and two of 15 000.

Reversibility of dissociation. To evaluate the reversibility of the dissociation of inhibitors into subunits, samples were incubated with 1% SDS or 6 M urea Upon removal of the dissociating agent only partial loss of inhibitory activity (20%) was observed and the salivary/pancreatic ratio of the inhibitors remained unchanged When examined by electrophoresis, the mobilities of the major bands in the treated samples corresponded to the major bands in control samples, indicating a re-association of subunits to form the 'parent' protein upon removal of the dissociating agent. Inclusion of 2-mercaptoethanol in the dissociating system caused a total loss of inhibitory activity and no distinct electrophoretic pattern could be obtained from the treated samples.

Deamidation. Electrophoresis of deamidated inhibitor samples showed the presence of several protein bands with mobilities greater than the untreated



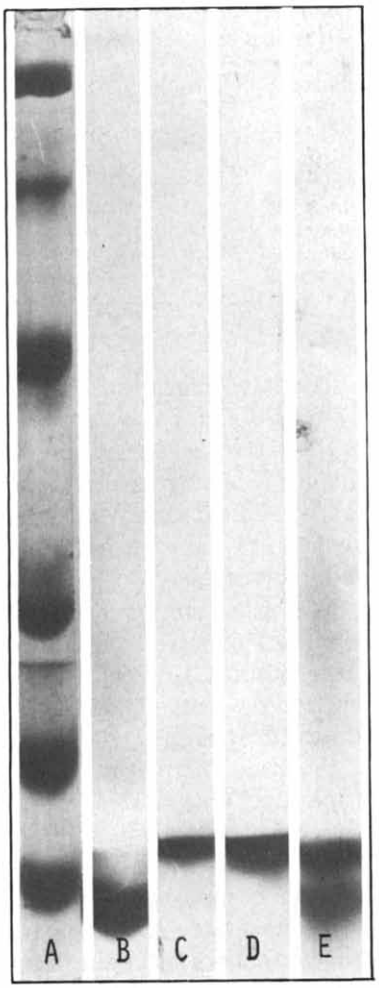


Fig 3 Polyacrylamide gel electrophoretic patterns of the purified inhibitors (A) Inhibitor 1, (B) Inhibitor 2, (C) Inhibitor 3 and (D) Inhibitor 4.

Fig 4 SDS-polyacrylamide gel electrophoretic profiles of (A) Reference proteins, (B) Inhibitor 1, (C) Inhibitor 2, (D) Inhibitor 3 and (E) Inhibitor 4. Reference proteins are (from top) phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme

inhibitors. However, when assayed for inhibitory activity, the deamidated preparations showed no change in their relative specificities towards human salivary and pancreatic α-amylases. This indicated that the isolated inhibitor proteins were not simply deamidated forms of one another or of a 'parent' inhibitor.

Proteolysis and heat inactivation. Incubation of inhibitor samples with pepsin resulted in the total inactivation of the four inhibitors within 2 h. This inactivation was due to proteolytic digestion, as control samples in 0.02 M HCl sustained only minor losses of activity. Similar destruction of inhibitor 4 was caused by trypsin and α-chymotrypsin, whereas the other three inhibitors were

TABLE II

INHIBITION OF α-AMYLASES FROM VARIOUS SOURCES BY WHEAT INHIBITORS

Inhibitor	Quantity (μg)	Percentage reduction in amylase activity				
		Human salivary	Human pancreatic	*Aspergillus oryzae*	*Bacillus subtilis*	Hog pancreatic
Inhibitor 1	12 0	92	50	8	15	0
Inhibitor 2	2 0	95	65	0	19	20
Inhibitor 3	1 0	95	75	8	0	26
Inhibitor 4	0 25	95	95	0	0	28

resistant to digestion by these proteases. When maintained at temperatures of 37 or 60°C for 1 h, inhibitors 1, 2 and 3 sustained little loss of potency, but 1 h at 100°C resulted in complete inactivation of these inhibitors. Inhibitor 4 was also rapidly inactivated at 100°C yet, unlike the other inhibitors, this protein sustained significant loss of activity at 60°C. The action of the inhibitors on both human α-amylases was affected in the same manner by heat treatment and proteolysis.

Inhibition of α-amylases from different sources. In addition to human salivary and pancreatic α-amylases, several other α-amylases were tested for their susceptibility to inhibition by the purified inhibitors. The results are summarised in Table II No inhibition of α-amylase from germinated wheat was observed by any of the four inhibitors.

Chemical analyses. On disulphide reduction 14.5, 13 7, 16.7 and 65.4 sulphydryl groups were titrated with DTNB/molecule of inhibitor 1, 2, 3 and 4, respectively. No free sulphydryl groups were detected in the non-reduced proteins. Thus, each molecule of the inhibitors 1, 2 and 3 contain 7—8 disulphide linkages compared to 32 S-S linkages/molecule of inhibitor 4. Since reduction is not necessary to dissociate the inhibitors into subunits, these linkages would appear to be intrachain. Carbohydrate analysis indicated the presence of 1.46, 2 3 and 2.0 mol reducing sugar/mol protein for inhibitors 1, 2 and 3, respectively. No carbohydrate was detected in inhibitor 4. Table III summarizes the observed properties of the four inhibitors.

TABLE III

A SUMMARY OF THE OBSERVED CHARACTERISTICS OF THE ISOLATED INHIBITORS

Carbohydrate content, mol carbohydrate/mol protein Sulphydryl content, number of free -SH groups/ molecule of reduced protein

Inhibitor	Molecular weight	Electrophoretic mobility (pH 8 5)	Subunit composition	Carbohydrate content	Sulphydryl content
Inhibitor 1	22 500	0 20	2	1 46	14 5
Inhibitor 2	21 400	0 21	2	2 30	13 7
Inhibitor 3	25 000	0 34	2	2 00	16 7
Inhibitor 4	63 000	0 45	4	—	65 4

Discussion

The separation and purification of four α-amylase inhibitors from wheat was achieved primarily by ion-exchange chromatography The methods employed proved eminently suitable for separating individual inhibitors from the mixture present in wheat albumin. Three inhibitors with similar physical properties but with different specificities for human salivary and pancreatic α-amylases were separated and purified. Retention of the salivary/pancreatic ratios of these three inhibitors on deamidation indicated that these proteins differ from one another in ways other than the substitution of glutamate or aspartate for glutamine or asparagine in their primary structures

The presence, in wheat extracts, of at least three inhibitors with molecular weights in the 20 000—25 000 region, which exhibit pronounced differences in specificities for human salivary and pancreatic α-amylases may explain why Silano et al. [9] did not observe a sizeable difference in the inhibition of these amylases by an inhibitor fraction (molecular weight 24 000) separated from wheat albumin by gel filtration. This fraction has been shown to be heterogeneous [2], consisting of at least ten proteins, all capable of inhibiting human salivary α-amylase. It is possible, that the proteins of this group also inhibit human pancreatic α-amylase, to varying degrees. By direct comparison, inhibitor 1 was found to be identical to the inhibitor isolated by O'Donnell and McGeeney [7]. The properties of inhibitors 1, 2 and 3 strongly suggest that these inhibitors are members of the '0.19 isoinhibitor family'. It may be that inhibitor 1 is identical to the 0.19 inhibitor [5] since it is the major protein in this group isolated from wheat flour. The molecular weight of inhibitor 2 closely resembles that reported for the inhibitor II protein of Saunders and Lang [4]. AmI_2 (molecular weight 26 200) was purified by Shainkin and Birk [3] by CM-cellulose chromatography. These authors indicate that this protein is quite basic. This property, and the similarity in molecular weights, would suggest a close homology between inhibitor 3 and AmI_2.

The fourth isolated inhibitor, inhibitor 4, differs significantly from the other three in physical characteristics (Table III) This inhibitor has the highest specificity for human pancreatic α-amylase and may be similar to the proteins described by Frerichs et al. [28] as being 'potent inhibitors of pancreatic α-amylase' It is likely that inhibitor 4 is one of the components of the 60 000 molecular weight fraction separated from wheat albumin by Deponte et al. [2].

All four isolated inhibitors consist of subunits of similar molecular size. Silano et al. [5] found that the inhibitors of the '0 28 isoinhibitor family' were single-protomer proteins of molecular weight 13 000. Thus, the three inhibitor groups in wheat all consist of proteins which dissociate into subunits of similar size. This is suggestive of different association forms of polypeptides from a single 'pool' However, experiments in which the inhibitors were dissociated into subunits and allowed to reassociate at random show that the major protein formed on re-association corresponds both in electrophoretic mobility and salivary/pancreatic ratio to the original inhibitor. This indicates highly specific subunit-subunit interactions within the individual proteins and suggests that the inhibitors are products of distinct, though possibly phylogenetically related genes.

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