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PURIFICATION AND PROPERTIES OF AN α-AMYLASE PROTEIN-INHIBITOR FROM *ARACHIS HYPOGAEA* SEEDS

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Summary

A protein showing highly specific inhibitory activity towards hog pancreatic and human salivary α-amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1), but not towards plant and bacterial α-amylases, has been purified 197-fold from an aqueous extract of peanut cotyledons using heat treatment, $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography on DEAE- and CM-cellulose. The purified inhibitor was homogeneous by polyacrylamide gel electrophoresis. Its molecular weight, as determined by Sephadex G-100 gel-filtration, and its electrophoretic mobility at pH 8 relative to bromophenol blue, were 25 000 and 0.14, respectively. The inhibitory activity was relatively resistant to thermal treatment and markedly increased when the inhibitor was preincubated with the enzyme before the addition of starch. Further, the inhibition was found to be pH-dependent and non-competitive in nature.

Introduction

α-Amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) protein-inhibitors have been reported from a few plant sources such as wheat [1—5], kidney bean [6—8] and *Colocasia esculenta* [9]. Recently some molecular species of albumin showing varied degrees of specificity towards α-amylases of different origins have been isolated from wheat kernel [10,11]. However, the presence of these proteinaceous inhibitors in other seeds and their exact physiological significance are still unknown. During our preliminary survey of other sources for the presence of such an inhibitor of α-amylase we found that peanut

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cotyledons also contain a water-soluble protein capable of inhibiting pancreatic and human salivary α-amylases. Thus, in the present report we have described the purification procedure and properties of an α-amylase protein-inhibitor from peanut cotyledons.

Materials and Methods

Materials. Unless specified all chemicals used in the present study were of analytical reagent grade. Soluble starch was obtained from B.D.H. Chemicals, Poole (U.K.). Bacterial α-amylase (spec. act. 200 units/mg protein) was purchased from E. Merck AG, Darmstadt (F.R.G.). Hog pancreatic α-amylase, bovine serum albumin, Sephadex G-100 and G-50, DEAE-cellulose and CM-cellulose were obtained from Sigma. Peanut and other seeds were purchased from the local seed store. α-Amylases from maize and barley were prepared as described elsewhere [12].

Inhibition of α-amylases. Unless otherwise stated, the reaction mixture contained the following in a final volume of 2 ml: 100 μmol phosphate buffer, pH 7; 12 μmol NaCl; and 0.1—0.2 unit α-amylase. For inhibition measurements, the enzyme was first preincubated with and without 50—200 μg inhibitor for 30 min (time required for complete equilibrium between enzyme and the inhibitor) at 30°C. The enzyme action was then started by adding 0.5 ml buffered starch solution (6 mg starch) to the reaction mixture. After 5 min incubation at 30°C, the enzyme activity was measured with I_2/KI reagent [12]. One unit of α-amylase was defined as the amount of enzyme that digests 1 mg starch per min under the above assay conditions. Similarly one unit of inhibitor was defined as the amount of inhibitor (in mg) required to inhibit 1 unit of α-amylase activity by 50%. Inhibition of α-amylase was measured by the same procedure and under the same experimental conditions as were used for α-amylase assay.

Protein determination. Protein was determined by the method of Lowry et al. [13] using bovine serum albumin as the standard.

Purification of α-amylase inhibitor

Extraction and preliminary treatments. All operations were conducted at 0—5°C, unless otherwise indicated. In a typical purification, peanut meal (250 g) was defatted with chilled acetone, dried under vacuum and extracted by constant stirring for 18 h with 1 l distilled water. The homogenate was filtered through cheesecloth and centrifuged for 1 h at 12 000 × *g*. The clear extract was then heated for 30 min at 65°C in a water bath to deactivate completely all the α- and β-amylases present. The denatured protein was removed by centrifugation at 16 000 × *g* for 1 h. The supernatant fluid containing α-amylase inhibitor was subjected to $(NH_4)_2SO_4$ fractionation. Protein fractions precipitating at different $(NH_4)_2SO_4$ saturations were collected by centrifugation. The protein fraction containing α-amylase inhibitor was dissolved in 0.05 M phosphate buffer, pH 7.2, and dialysed for 18 h against the same buffer.

Column chromatography on DEAE-cellulose. A 25 ml aliquot of the dialysed protein fraction (inhibitor) was applied to a DEAE-cellulose column (2.5 × 25 cm), which had been previously equilibrated with 0.05 M phosphate buffer

(pH 7.2). The column was first washed with 200 ml of the same buffer to remove unabsorbed protein. The absorbed proteins were then eluted by a 600 ml linear salt gradient of 0.05 M phosphate buffer containing 0—0.8 M NaCl. Flow rate was 1 ml/min and 10-ml fractions were collected. Aliquots (0.2 ml) from each fraction were analysed for protein and the fractions in individual protein peaks were pooled and assayed for α-amylase inhibitory activity. The inhibitor protein was precipitated from the solution by the addition of $(NH_4)_2SO_4$ to 100% saturation and centrifuged.

Column chromatography on CM-cellulose. The inhibitor protein fraction from the DEAE-cellulose column was dissolved in a minimal volume of 0.05 M acetate buffer, pH 4.8, and dialysed for 18 h against the same buffer. The dialysed fraction (10 ml, containing 24 mg protein) was applied to a CM-cellulose column (1.25 × 35 cm) which had been previously equilibrated with 0.05 M acetate buffer, pH 4.8. The column was first eluted with 200 ml of the same buffer to wash off the unbound protein. The absorbed protein was then eluted by a 600 ml salt gradient of 0.05 M acetate buffer, pH 4.8, containing 0—0.8 M NaCl. 10 ml fractions were collected at a flow rate of 1 ml/min. Each fraction was analysed for protein and the effluent in tubes in individual protein peaks was pooled and tested for the α-amylase inhibitory activity. The protein fraction showing α-amylase inhibitory activity was precipitated by $(NH_4)_2SO_4$ and centrifuged as before.

Gel electrophoresis. The electrophoresis was carried out on 5% acrylamide gel in 0.05 M phosphate or acetate buffers (pH 5—8) as described by Davis [14]. About 40—60 μg protein was layered through the electrode buffer in 40% sucrose solution and bromophenol blue was used as tracker dye. The gels were run at a constant current of 5 mA per tube for 2.5 h and protein bands were located by staining the gels with Coomassie blue [15]. The electrophoretic mobility of the protein was determined with respect to bromophenol blue at pH 8.

Molecular weight. Molecular weight was measured by gel filtration on Sephadex G-100 as described by Andrews [16].

Results and Discussion

Purification of α-amylase protein-inhibitor

The results of the purification of a water-soluble protein from peanut seeds showing inhibitory activity towards human salivary and hog pancreatic α-amylases are summarized in Table I. The purification scheme involved four steps in the following sequence: (i) acetone and water extraction: (ii) $(NH_4)_2SO_4$ fractionation: (iii) DEAE-cellulose and (iv) CM-cellulose chromatography. The acetone treatment of the peanut meal and heating of the aqueous extract at 65°C for 30 min were the essential preliminary treatments. The former removed the lipids, pigments and other such materials which usually interfere in the isolation of enzymes and proteins, and the latter completely deactivated the amylases present in the aqueous extract without any apparent adverse effect on the inhibitory power of the protein-inhibitor. The $(NH_4)_2SO_4$ fraction precipitating between 60 and 80% saturation contained most of the inhibitor. Other fractions showed little or no inhibitory activity

TABLE I

PURIFICATION OF AN α-AMYLASE INHIBITOR FROM *ARACHIS HYPOGAEA* SEEDS

Purification (Fraction)	Protein (mg)	Inhibitory activity * (munits)	Specific activity (munits/mg)	Yield (%)	Purification (fold)
Crude extract **	12 500	20 000	1.6	100	—
$(NH_4)_2SO_4$ frac.					
60—80%	980	12 300	12.5	61.5	7.5
80—100%	780	700	0.9	3.5	—
DEAE-cellulose chromatography ***					
Peak III	26	8 000	308	40	187.5
CM-cellulose chromatography					
Peak II	16.5	5 200	315	26	196.9

* One unit of protein-inhibitor was defined as the amount of protein (mg) capable of inhibiting one unit of hog pancreatic α-amylase by 50% at pH 7.0.

** The crude extract data represent the aqueous extract prepared from the acetone defatted peanut meal followed by heating for 30 min at 65°C.

*** Total of four runs on the same column.

towards α-amylase. The purification and the yield of inhibitor were 7.5-fold and 61.5%, respectively.

The protein-inhibitor thus obtained was further purified by chromatography on DEAE-cellulose and CM-cellulose columns. The elution profile of proteins from the DEAE-cellulose column is shown in Fig. 1. It was observed that the bulk of the protein remained unabsorbed on the anion exchanger column at pH 7.2 (peak I) and that it did not show any inhibitory activity towards α-amylases. The absorbed protein upon elution with the NaCl linear gradient (0—0.8 M) yielded two protein peaks (II and III) at 0.12 M and 0.44 M salt gradients, respectively. The latter peak contained the highly active α-amylase protein-inhibitor with 187.5-fold purification and a yield of 40%. The elution profile of this fraction from the CM-cellulose column is shown in Fig. 2. Again, a large amount of protein remained unabsorbed on the CM-cellulose at pH 4.8 and emerged out of the column with washing (peak I). This fraction did not exhibit any inhibitory activity towards α-amylases. The absorbed protein eluted in a single symmetrical sharp peak (II) at 0.41 M salt gradient and showed strong inhibitory activity towards α-amylase. The purification and yield were 197-fold and 26%, respectively.

The α-amylase protein-inhibitor so obtained was homogeneous by disc electrophoresis carried out on 5% polyacrylamide gel at various pH values between 5 and 8. The electrophoretic mobility of the inhibitor relative to bromophenol blue at pH 8 was 0.14.

Properties of inhibitor

The inhibitory activity of 200 μg/ml purified protein-inhibitor from peanut seeds towards equivalent amounts of α-amylases of different origin and towards the sweet potato β-amylase was examined. The results clearly showed that the activity of the inhibitor was highly specific towards the human salivary and hog

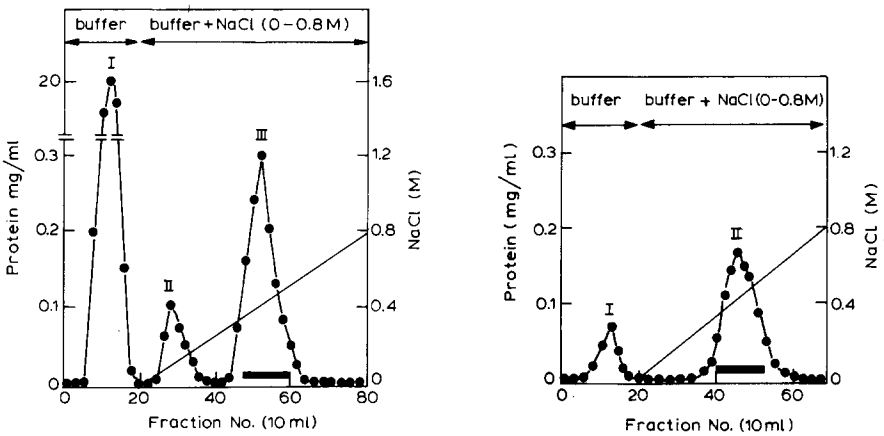


Fig. 1. Chromatography of $(NH_4)_2SO_4$ fraction (60—80% saturation) on DEAE-cellulose column (2.5 X 25 cm). The elution was first performed with 0.05 M phosphate buffer (pH 7.2) and then by a linear NaCl gradient (0—0.8 M). (●———●), protein; and (■) fractions showing inhibitory activity towards α-amylase.

Fig. 2. Chromatography of peak III from DEAE-cellulose column (fraction Nos. 26—38, Fig. 1) on CM-cellulose column (1.25 X 35 cm). Elution was performed first with 0.05 M acetate buffer (pH 4.8) and then by a linear NaCl gradient (0—0.8 M). (●———●), protein; and (■) fractions with inhibitory activity towards the hog pancreatic α-amylase.

pancreatic α-amylases (58 and 43% inhibition, respectively). Enzymes from plant sources, namely, maize, barley and wheat, from bacteria (*Bacillus subtilis*), and β-amylase from sweet potato were not at all inhibited, indicating that these enzymes differ from those of pancreatic and salivary α-amylases. Whether the same is true in the case of other plant and bacterial α-amylases is uncertain at the moment. However, α-amylase protein-inhibitors showing varied inhibitory activity and inhibiting a very large number of amylases of very different origins have been reported in wheat [4,5,10,11,18,19].

The α-amylase protein-inhibitor from peanut was found to be extremely thermostable. After exposing it for 30 min at 65, 70, 75 and 80°C, the inhibitory activity was reduced by 0, 5, 12 and 20%, respectively. In contrast, however, the amylolytic activity completely disappeared after 30 min exposure at only 65°C. These results are in general agreement with those reported by Petrucci et al. [18] with 0.19 α-amylase protein-inhibitor from wheat. The thermal stability is, thus, an important property which has been of advantage in removing many thermolabile proteins, including amylases, during purification without damaging the inhibitory activity of protein-inhibitor.

Fig. 3 shows the effect of pH on the activity and percentage inhibition of hog pancreatic α-amylase by the inhibitor. The optimum inhibition was obtained in the pH range between pH 6 and 8. On either side of this pH the inhibition decreased appreciably. In contrast, the pH optimum activity curve was sharply peaked at pH 7. Preincubation of the inhibitor with enzyme at optimum pH before the addition of substrate markedly increased the enzyme inhibition and a 30 min incubation was found essential for the maximum inhibition of hog pancreatic α-amylase (Fig. 4). Preincubation of the inhibitor with

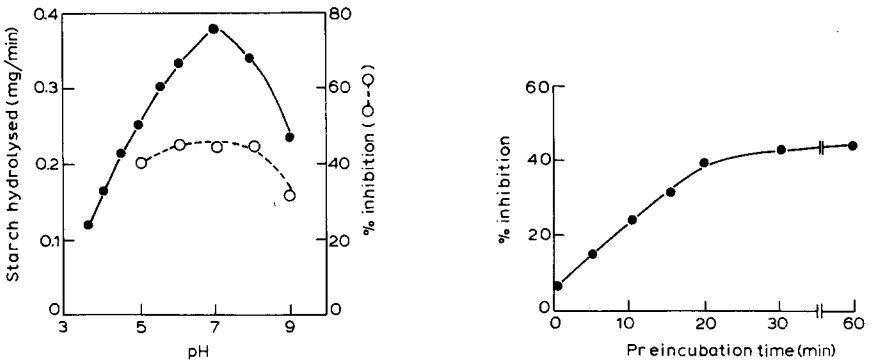


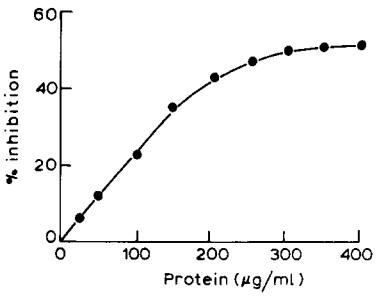
Fig. 3. The effect of pH on hog pancreatic α-amylase inhibition by the peanut protein. Enzyme activity without inhibitor (●———●); and % inhibition (○ - - - - - ○). Experiments with and without the inhibitor were carried out simultaneously. The enzyme activity at each pH in the absence of inhibitor was taken as 100%. Buffers were 0.05 M acetate (pH 3.6—5.6) and 0.05 M phosphate (pH 6—9) containing 50 mM NaCl.

Fig. 4. Dependence of inhibition on preincubation period of hog pancreatic α-amylase with the inhibitor. In a typical experiment 0.5 ml of the enzyme (0.5 units) were incubated with 100 μg inhibitor or without the inhibitor at 30°C in a total volume of 2 ml containing 100 μmol phosphate buffer (pH 7). At timed intervals, aliquots (0.2 ml) were assayed for α-amylase. The enzyme activity without inhibitor was taken as 100%.

starch (substrate) before the addition of enzyme did not significantly affect the extent of α-amylase inhibition (the effect was less than 5%), indicating that there was practically no interaction between the inhibitor and the substrate. The results are in agreement with those reported in the case of α-amylase inhibition by phytate [12] and also in the case of α-amylase protein-inhibitor, 0.19, from wheat [17]. The preincubation data were interpreted to mean that the formation of enzyme-inhibitor-starch complex was much slower than the formation of enzyme-substrate complex. As regards the interaction with starch, the α-amylase protein-inhibitor from peanut appears to be different from the inhibitor isolated by O'Donnell and McGeeney [11] from the alcohol extract of wheat, as the latter showed a strong binding with starch.

Fig. 5 shows the increase in the percentage inhibition as a function of inhibitor concentration. It was noticed that even in the most favourable experimental conditions, it was not possible to obtain 100% inhibition. The maximum inhibition in the case of hog pancreatic α-amylase was about 50%. These results indicate that the enzyme was perhaps partially saturated relative to the inhibitor. The mechanism for this effect is unknown. However, it seems likely that the relatively large size of the inhibitor molecule prevents complete saturation of the enzyme due to the steric effect.

The nature of inhibition was determined by the method of Lineweaver and Burk [21]. The results obtained (Fig. 6) indicate that the inhibition of hog pancreatic α-amylase by peanut protein-inhibitor was of non-competitive type i.e., inhibitor had no effect on K_m, whereas V was appreciably decreased with increasing inhibitor concentration. These results are identical to those of Marshall and Lauda [6] who have reported a non-competitive inhibition of hog



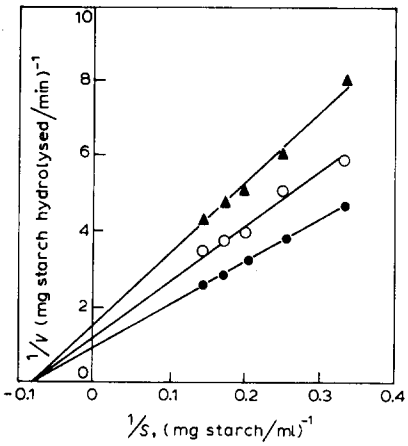


Fig. 5. The effect of inhibitor concentration on the inhibition of hog pancreatic α-amylase. A fixed amount of the enzyme (0.2 units) was first equilibrated with various amounts of inhibitor for 30 min at pH 7.0 and 30°C and the residual enzyme activity was assayed.

Fig. 6. Lineweaver-Burk plots showing non-competitive type of inhibition of hog pancreatic α-amylase by peanut protein-inhibitor. The enzyme was preincubated without (●———●) and with 100 μg (○———○) and 200 μg (▲———▲) inhibitory protein for 30 min at pH 7.0 and the reaction was started by the addition of buffered starch (varying from 3 to 7 mg starch per ml of the reaction mixtures). The velocity was expressed as mg starch hydrolysed per min per ml of the reaction mixture.

pancreatic α-amylase by phaseolamine. The apparent molecular weight of the inhibitor, as determined by the Sephadex gel filtration method [16], was 25 000. This value was in close agreement with the molecular weights of other α-amylase protein-inhibitors, namely, inhibitor I [5], inhibitor III [18], 0.19 [4] and A_m I_2 [10].

Though, in view of the general properties and inhibition data noted above, it may be assumed that the peanut inhibitor is identical to the α-amylase protein-inhibitor from wheat [4,19,20] and kidney bean [6—8], it had still to be ascertained whether this inhibition was due to a specific enzyme-inhibitor interaction or simply due to some proteolytic inactivation of α-amylase. This was finally explained with the help of some experiments similar to those of Marshall and Lauda [6]. In these experiments, α-amylase, protein-inhibitor and enzyme-inhibitor complex (the complex was prepared by incubating α-amylase with protein-inhibitor in the ratio of 1 : 2 (w/w) for 1 h at 30°C and pH 7.0; this ratio of 1 : 2 was used in response to our finding that the stoichiometric relation of enzyme and inhibitor in the complex, as calculated by the method in Ref. 6, was 1 : 2) were separately chromatographed on the same column of Sephadex G-100 under identical conditions and the values of V_e/V_0 (where V_e and V_0 represent the elution volume and void volume [16], respectively) were determined for each one of them. The value of V_e/V_0 for α-amylase, protein-inhibitor and complex were found to be 2.05, 2.28 and 1.82, respectively. These values clearly indicate that the complex is eluted first, followed by enzyme and inhibitor. This accorded with our expectations of higher values of molecular weight and Stokes' radius for the complex as compared to those for

enzyme and inhibitor. In this manner, gel filtration data demonstrate that inhibitor binds directly to the enzyme molecule to form an inactive complex and, therefore, that inhibition of α-amylase was due to the formation of this inactive complex rather than proteolytic inactivation. These experiments also indicate that peanut inhibitor does not act by chelating calcium or removal of chloride, species which are needed for the stability and activity of α-amylases.

Thus, in addition to various other sources, peanut represents yet another plant source which contains an α-amylase protein-inhibitor showing a similar inhibition pattern.

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