Isolation and Characterization of an Amylase Inhibitor from Sorghum Seeds, Specific for Human Enzymes

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An α-amylase inhibitor was purified 53-fold from an acid extract (0.1 N HCl) of sorghum grains by neutralization, ammonium sulfate fractionation (0–40% saturation), and affinity chromatography on Red-Sepharose CL-6B. The homogeneity of the final product was confirmed by polyacrylamide gel electrophoresis and gel chromatography on BioGel P-30. The molecular weight was found to be 21K. The inhibitor was made up of two polypeptide chains of equal size linked by disulfide bridges. It acted on human salivary amylase and human pancreatic amylase equally efficiently but had no effect on porcine pancreatic α-amylase, *Bacillus subtilis* amylase, and *Aspergillus oryzae* amylase. It was labile to heat and to alkaline conditions. Pepsin, pronase, α-chymotrypsin, and trypsin inactivated the inhibitor. Chemical modification of amino groups and guanido groups and reduction of disulfide bridges of the inhibitor resulted in loss of biological activity.

Proteinaceous inhibitors of α-amylase are widespread in plants, notably in cereals (Buonocore et al., 1977; Warchalewski, 1983). Apart from wheat inhibitors which have been studied extensively (Petrucci et al., 1978; Mundy et al., 1984), several of these factors have been purified and characterized from grains like finger millet (Shivaraj and Pattabiraman, 1980), maize (Blanco-Labra and Iturbe-Chinas, 1981), and barley (Weselake et al., 1983). The presence of condensed tannins capable of inhibiting enzymes nonspecifically are known, and they have been reported in sorghum (Davis and Hoseney, 1979). Studies in this laboratory, on the other hand, revealed the presence in sorghum of an amylase inhibitor acting on human pancreatic and salivary amylase but not on porcine pancreatic amylase (Chandrasekher et al., 1981). It was considered worthwhile to study this amylase inhibitor in sorghum and characterize its nature. In this paper we report the purification and characterization of a proteinaceous α-amylase inhibitor from sorghum seeds.

MATERIALS AND METHODS

Materials. *Sorghum bicolor* (Var. Co-24) grains were obtained from Tamil Nadu Agricultural University, Coimbatore, India. 1,2-Cyclohexanedione (CHD) was from Aldrich Chemical Co., Milwaukee, WI. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Pierce Chemical Co., Rockford, IL. Red-Sepharose CL-6B (Lot No. HA 23183) and Sephadex G-100(fine) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium dodecyl sulfate (SDS) and BioGel P-30 were obtained from Bio-Rad Laboratories, Richmond, CA. Molecular weight marker (bovine hemoglobin, cross-linked, containing monomer Mr 16 K, dimer, trimer, and tetramer), crystalline porcine pepsin, ninhydrin, sodium trinitrobenzenesulfonate (TNBS), pepstatin, diazoacetyl-DL-norleucine methyl ester, *Aspergillus oryzae* α-amylase, *Bacillus subtilis* α-amylase, and twice-crystallized procine pancreatic α-amylase (PPA) were purchased from Sigma Chemical Co., St. Louis, MO. Crystalline bovine trypsin and crystalline bovine α-chymotrypsin were the products of Worthington Biochemical Corp., Freehold, NJ. Human salivary amylase (HSA) and human pancreatic amylase (HPA) were partially purified up to the acetone fractionation stage as described by Bernfeld (1955). Human pancreatic amylase used in complex formation studies was further purified by chromatography on Sephadex G-100. The details were the same as described in Complex Formation Studies. Other reagents used were analytical grade commercial chemicals.

Amylase activity was measured according to the method of Bernfeld (1955) as described earlier (Shivaraj and Pattabiraman, 1980). One enzyme unit is the amount that liberated 1 μmol of reducing equivalent (as maltose) under the assay conditions (pH 6.9, 5 min, 37 °C) from starch. A 1.2-μg portion of HSA protein 0.2 μg of PPA protein, 18 μg of HPA protein, 15 μg of *A. oryzae* amylase protein, and 8.2 μg of *B. subtilis* amylase protein were used for routine assays to provide 4 units of enzyme activity. Under the same conditions 1.0 μg of HPA protein purified by gel chromatography gave 4 units of enzyme activity.

To determine the inhibitory activity, suitable aliquots of the sorghum seed extracts were included in the assay medium. For routine assays, 20-min preincubation of the target enzyme with the inhibitor was performed. One unit of inhibitory activity is defined as the amount that suppressed amylase activity by 1 unit. The purified inhibitor reduced the amylase activity of HPA preparation purified upto acetone fractionation stage and the preparation purified by gel chromatography on Sephadex G-100 to equal extents.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Isolation of the Inhibitor. All operations were carried out at 4 °C unless otherwise mentioned. Finely powdered sorghum grains (200 g) was homogenized with 1000 mL of 0.1 N HCl containing 0.15 M NaCl in a blender. After 60 min of stirring, the homogenate was centrifuged at 10000*g* for 20 min. The supernatant (acid extract, volume 880 mL, pH 1.5) was adjusted to pH 7.0 by adding 1 N NaOH. The precipitate formed during this step was removed by centrifugation at 10000*g* for 20 min. The clear supernatant (neutral extract, volume 960 mL) was subjected to 40% saturation with ammonium sulfate (234 g). After the mixture was allowed to stand for 24 h, the precipitate formed was collected by centrifugation at 10000*g* for 30 min and dissolved in 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl. The solution was then dialyzed against the same buffer for 24 h. The cloudy solution was centrifuged, and the clear supernatant was collected.

The above supernatant (ammonium sulfate fraction, volume 30 mL) was allowed to flow through a column of Red-Sepharose (0.9 × 35 cm^2, bed volume 20 mL, flow rate 30 mL h^{-1}) equilibrated with 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl. The column was washed with

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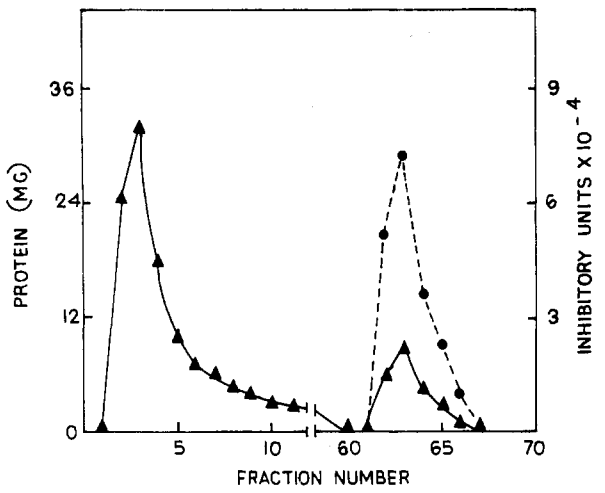

Figure 1. Affinity chromatography of the ammonium sulfate fraction on Red-Sepharose CL-6B during purification of the inhibitor: ▲ protein; ● inhbitory units. Experimental details are described under Materials and Methods.

60 bed volumes of the buffer. The bound inhibitor protein was then eluted with 0.05 M Tris-HCl buffer, pH 7.0, containing 1 M NaCl. Twenty-milliliter fractions were collected. The active fractions (tubes 62–66, Figure 1) were pooled and concentrated to 5 mL by ultrafiltration. The solution was then dialyzed against 0.02 phosphate buffer, pH 6.9, containing 0.1 M NaCl and was used for further studies.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed with 5% gel in 0.05 Tris–glycine buffer, pH 8.3, at a current of 3 mA/tube for 3 h. Electrophoresis in the presence of 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol was done for 10 h at 7 mA/tube at pH 7.2 using 10% gel. Protein bands were stained with Coomassie Brilliant Blue R-250. For molecular weight determination, cross-linked bovine hemoglobin was used as standard.

Gel Chromatography. The purified inhibitor (1.5 mg of protein) in 0.02 M sodium phosphate buffer, pH 6.9, containing 0.1 M NaCl was allowed to flow through a column of BioGel P-30 (0.9 × 60 cm^2, bed volume 38 mL) previously equilibrated with 0.02 M phosphate buffer, pH 6.9, containing 0.1 M NaCl. The column was eluted with the equilibration buffer. One-milliliter fractions were collected at a flow rate of 6 mL h^{-1}. The protein content and inhibitory activity were estimated in all the fractions. For molecular weight determination, lysozyme (14.3K), cytochrome *c* (13K), myoglobin (17K), and pepsin (34K) were used as standards.

Effect of Temperature and pH on the Stability of the Inhibitor. The inhibitor (0.3 μg of protein in 0.1 mL of 0.1 M phosphate buffer, pH 6.9) was subjected to heat treatment. After 10-min exposure to different temperatures, the samples were cooled and assayed for residual inhibitory activity against HPA. To study the pH effect, the inhibitor, (0.3 μg of protein) was preincubated with 10 μmol each of HCl (pH 1.0), HCl–KCl buffer (pH 2 and 3), acetate buffer (pH 4 and 5), phosphate buffer (pH 6 and 7), Tris-HCl buffer (pH 8, 9, and 10), and 0.1 N NaOH (pH 12.0) in a volume of 0.1 mL for 18 h at 4 °C. After 18-h incubation, 200 μmol of phosphate buffer, pH 6.9, was added to the system. The residual activity against HPA was assayed. Controls were run simultaneously.

Effect of pH on the Preincubation System. The inhibitor protein (0.3 μg) and human pancreatic amylase (18 μg of protein) together were preincubated with 10 μmol of different buffer (pH 1–12) as mentioned above at 37 °C for 20 min. After preincubation, 200 μmol of phosphate buffer, pH 6.9, was added and the residual enzyme activity was measured. Enzyme and inhibitor controls were run simultaneously.

Effect of Proteolytic Enzymes on the Inhibitor. The inhibitor protein (50 μg) was treated with pronase (40 μg of protein), trypsin (50 μg of protein), and α-chymotrypsin (50 μg) for 2 h at 37 °C in a total volume of 1.5 mL in the presence of 100 μmol of phosphate buffer, pH 7.6. Similarly, the inhibitor was treated with pepsin (25 μg of protein) in HCl–KCl buffer (100 μmol), pH 2.0, in 1 mL for 2 h. At intervals, aliquots were withdrawn and assayed for residual HPA inhibitory activity. Controls without inhibitor were run simultaneously. Under the assay condition the proteases had no effect on HPA.

Chemical Modification of the Inhibitor. Amino groups were modified by treatment with TNBS (Haynes et al., 1967). The inhibitor (100 μg of protein) was treated with 500 μg of TNBS in the presence of 150 μmol of phosphate buffer, pH 7.6, in a volume of 2 mL at 30 °C. After definite intervals of time, aliquots were withdrawn and dialyzed against 0.05 M NaCl solution for 8 h at 4 °C and residual inhibitory activity against HPA was determined. Guanido groups were modified with CHD (Abe et al., 1978). The inhibitor (100 μg of protein) was treated with 600 μg of CHD in 2 mL in the presence of 150 μmol of borate buffer, pH 9.0, and processed as described above. Guanido groups were also modified with ninhydrin as described by Chaplin (1976). The inhibitor (50 μg of protein) was treated with 200 μg of the modifier in the presence of 100 μmol of Na_2HPO_4 in 1 mL and processed as above. The sulfhydryl groups were modified by treating with DTNB (Ellman, 1959). The reagent (500 μg) was treated with 50 μg of protein in presence of 50 μmol of phosphate buffer, pH 8.0, in 1 mL. Aliquots were withdrawn at intervals and processed as above. The disulfide bridges were reduced by treating the inhibitor (50 μg of protein) with 1% mercaptoethanol in a final volume of 1 mL in the presence of 50 μmol of phosphate buffer, pH 7.2. Aliquots were assayed for residual HPA inhibitory activity. Controls for inhibitor were run simultaneously in all cases without modifiers. To rule out the presence of contaminating proteases in the inhibitor preparation that could inactivate amylase, the following experiments were done: The inhibitor protein (10 μg) was treated with 500 μg of diazoacetyl-DL-norleucine methyl ester known to be a thiol protease inhibitor (Takahashi et al. 1972) or 200 μg of *p*-chloromercuribenzoate in the presence of 40 μmol of acetate buffer, pH 5.0, in a volume of 1.0 mL for 12 h. Similarly, the inhibitor was treated with 300 μg of pepstatin, a specific carboxyl protease inhibitor (Takahashi et al. 1974). The samples were dialyzed, and the amylase inhibitory activity of the treated protein was assayed.

Complex Formation Studies. The purified inhibitor (200 μg of protein) was preincubated with 800 μg of protein of human pancreatic amylase at 37 °C for 30 min in the presence of 20 μmol of phosphate buffer, pH 6.9, containing 100 μmol of NaCl in a volume of 1 mL. The solution was then applied onto a column of Sephadex G-100 (0.9 × 60 cm^2, bed volume 38 mL) equilibrated with 0.02 M phosphate buffer, pH 6.9, containing 0.1 M NaCl at a flow rate of 8 mL h^{-1}. The same buffer system was used as eluant. Two-milliliter fractions were collected, and fractions were analyzed for protein, amylase activity, and amylase inhibitory activity.

The enzyme and inhibitor mixture was also subjected to affinity chromatography on Red-Sepharose. The inhibitor protein (200 μg) was incubated with 800 μg of hu-

Table I. Purification of the Amylase Inhibitor from Sorghum Grains

fraction	total protein, mg	HPA inhib, units × 10^{-3}	sp act., units/mg	yield, %
acid extr	2610	415	159	100
neutral extr	1340	390	291	94
ammonium sulfate	181	285	1575	69
red-sepharose	24.2	204	8430	49

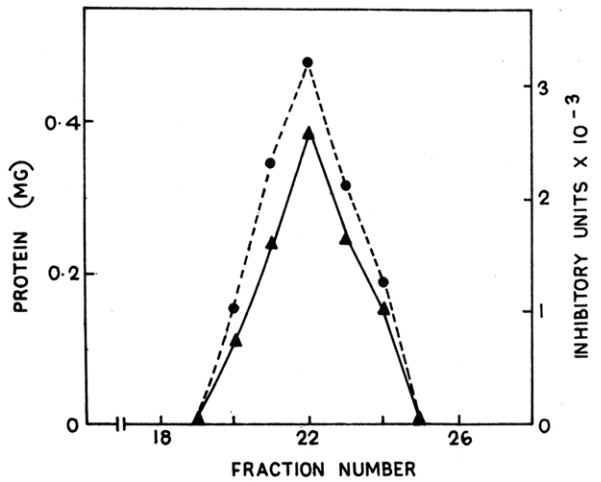

Figure 2. Gel chromatography of the purified inhibitor on BioGel P-30: protein; inhibitory units. Experimental details are given under Materials and Methods.

man pancreatic amylase at 37 °C for 10 min in the presence of 50 μmol of Tris-HCl buffer, pH 7.0, containing 100 μmol of NaCl in a total volume of 1 mL. The solution was applied onto a column of Red-Sepharose previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl (0.9 × 3.14 cm^2, bed volume 2 mL). The column was then extensively washed (flow rate 30 mL h^{-1} with equilibration buffer until washings did not contain any protein. The column was then eluted with 0.05 M Tris-HCl buffer, pH 7.0, containing 1.0 M NaCl. Four-milliliter fractions were collected, and all the fractions were analyzed for protein, enzyme activity, and amylase inhibitory activity.

RESULTS

The amylase inhibitor from sorghum grains was purified 53-fold with 49% recovery of the activity. The data on the purification process are shown in Table I. The major purification was achieved by chromatography on Red-Sepharose. The inhibitor obtained by this step was homogeneous as assessed by PAGE and gel chromatography on BioGel P-30. During gel chromatography the inhibitor was eluted as a single peak with constant specific activity across the peak, and the M_r was calculated to be 18K. The elution pattern is shown in Figure 2. On polyacrylamide gel electrophoresis at pH 8.3 the inhibitor moved as a single protein band (Figure 3A). Inhibitory activity when estimated by extraction of sections of gel with 0.1 M phosphate buffer, pH 6.9, showed that the inhibitory activity coincided with the protein band. The molecular weight of the purified inhibitor was found to be 21K by SDS-PAGE.

SDS-PAGE in the presence of mercaptoethanol also showed a single protein band (Figure 3B). The molecular weight of the reduced protein was found to be 10K. This suggests that the native inhibitor protein is probably made of two similar polypeptide fragments linked by disulfide bridges.

The purified inhibitor affected the activity of both HSA and HPA. The relative ratio of inhibition was 1.25:1 based

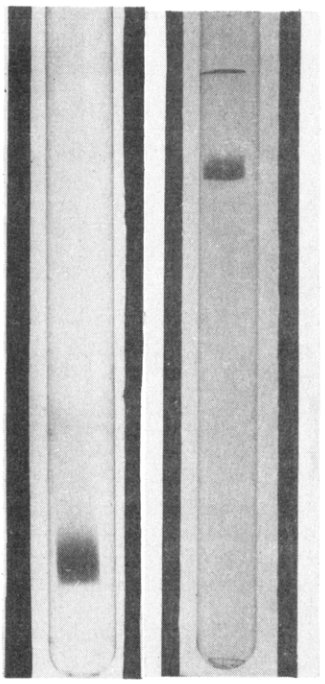

Figure 3. (A) Polyacrylamide gel electrophoresis of the purified inhibitor. (B) SDS-polyacrylamide gel electrophoresis of the inhibitor in the presence of mercaptoethanol. Experimental details are described under Materials and Methods.

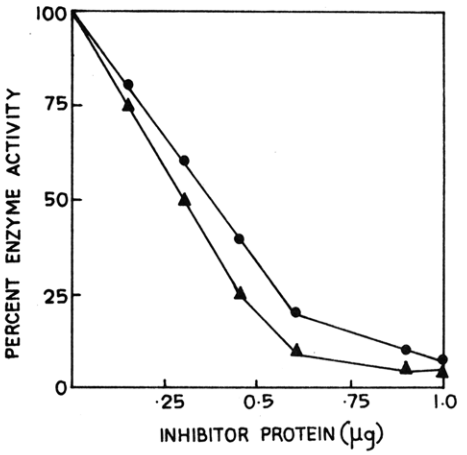

Figure 4. Effect of varying concentrations of the purified amylase inhibitor on the activity of HSA (▲) and HPA (●). Experimental details are given under Materials and Methods.

on the linear range of action. The decrease in enzyme activity was linear with respect to inhibitor concentration up to 80%. Beyond this level, the enzyme activity gradually decreased to 3–5% of the initial activity (Figure 4). The purified inhibitor had no effect on crystalline porcine pancreatic α-amylase, *B. subtilis* α-amylase, and *A. oryzae* α-amylase at concentrations up to 1 μg of inhibitor protein.

The inhibition was dependent on the time of preincubation of the inhibitor with the target enzyme. A minimum of 20 min of preincubation was necessary to elicit maximal response. The inhibitory activity observed against HPA without preincubation was only 20% of the activity observed after 20 min of preincubation. Further increase in time up to 1 h did not increase the magnitude of inhibition. Pretreatment of the inhibitor with starch solution for 10 min at 37 °C before assay resulted in complete loss of inhibitory activity.

The inhibitor was found to be heat labile. Heat treatment at 50 °C for 10 min and at 80 °C for 10 min resulted in 50% and 100% loss of HPA inhibitory activity, re-

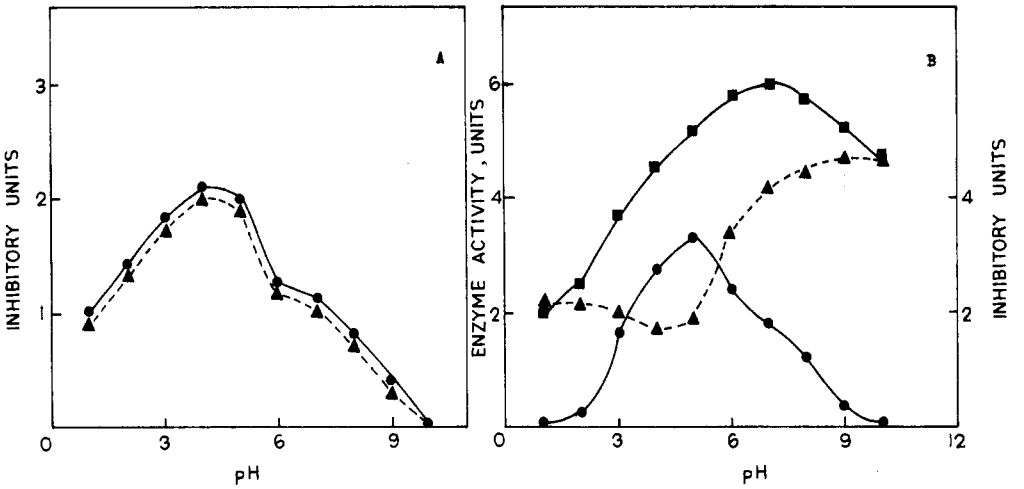

Figure 5. (A) Effect of pH on the stability of the amylase inhibitor in terms of amylase inhibitory activity: ▲, 18-h exposure at 4 °C; ●, 20-min exposure at 37 °C. (B) Effect of varying hydrogen ion concentration on the interaction of human pancreatic amylase and sorghum inhibitor: ■, HPA activity; ●, inhibition by sorghum inhibitor; ▲, residual enzyme activity of the enzyme–inhibitor mixture. Experimental details are given under Materials and Methods.

spectively. When the inhibitor was exposed to different pH values in the range pH 2–7 either for 18 h at 4 °C or for 20 min at 37 °C, the activity did not decrease compared to the activity measured directly at pH 6.9. When the inhibitor solutions was rendered alkaline, there was rapid loss of activity. Data shown in Figure 5A on exposure of the inhibitor to different pH values indicate that the inhibitory activity was more on exposure to mild acidic conditions (pH 4–5) compared to values obtained at pH 2 or 6. This increase in activity was found to be a time-dependent activation process. To rule out the possibility of enhancement in inhibitory activity due to the effect of acetate ions (used at pH 4–5), enzyme controls with 10 μmol of sodium acetate were run simultaneously at pH 6.9. Acetate alone did not cause any increase in inhibitory activity.

The effect of varying hydrogen ion concentration on the interaction of enzyme and inhibitor was studied. The results are shown in Figure 5B. The data suggest that enzyme inhibitor interaction is also maximal at pH 4 and 5.

Treatment of inhibitor protein with diazoacetylnorleucine methyl ester, pepstatin, or *p*-chloromercuribenzoate over a period of 12 h did not diminish the inhibitory activity, suggesting that the increased inhibition of α-amylase by the inhibitor under mild acidic condition could not be due to inactivation of the amylase by traces of proteases present in inhibitor preparation. The inhibitor preparation also did not display any trypsin-like activity.

The effect of proteolytic enzymes on HPA inhibitory activity of the purified factor is shown in Figure 6. Pepsin and pronase inactivated the inhibitor rapidly. The action of chymotrypsin or trypsin on the inhibitor was relatively slow.

The data on chemical modification of the inhibitor with various reagents and the effect of the modified inhibitor on HPA inhibitory activity are summarized in Table II. Treatment with TNBS, an amino group modifier, resulted in rapid loss of inhibitory activity. Treatment with CHD, a guanido group modifier, caused a slow, time-dependent loss of inhibitory activity. However, ninhydrin, another reagent for guanido group modification under the conditions employed, abolished the action of the inhibitor rapidly. Treatment with DTNB did not alter the inhibitory activity, ruling out the role of sulfhydryl groups for the action of the inhibitor. Treatment with 2-mercaptoethanol abolished the activity of the inhibitor.

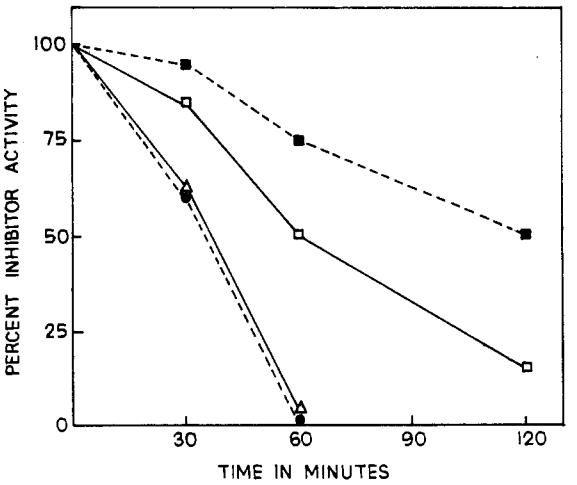

Figure 6. Effect of treatment with proteolytic enzymes on the amylase inhibitory activity of sorghum inhibitor: ●, pepsin; △, pronase; ■, trypsin; □ chymotrypsin. Experimental details are given under Materials and Methods.

Table II. Effect of Chemical Modification on the Action of the Inhibitor

modifier	time of treatment, h	% resid act. against human pancreatic amylase
TNBS	0.5	40
	1.0	0
CHD	3	90
	6	90
	6	72
	12	25
ninhydrin	0.5	25
	1.0	0
DTNB	6	100
	12	100
mercaptoethanol	0.5	50
	1.0	0

The mode of inhibition of HPA by the purified inhibitor was found to be noncompetitive (Figure 7). The K_i value calculated based on a molecular weight of 21K was 2.02 × 10^{-8} M.

The chromatographic profiles of a mixture of purified inhibitor and human pancreatic amylase on Red-Sepharose was studied. Unlike the native inhibitor, which was bound to the immobilized dye, the mixture was eluted from the column during washing. Native amylase was eluted from the column (12 bed volumes) during the washing (in terms

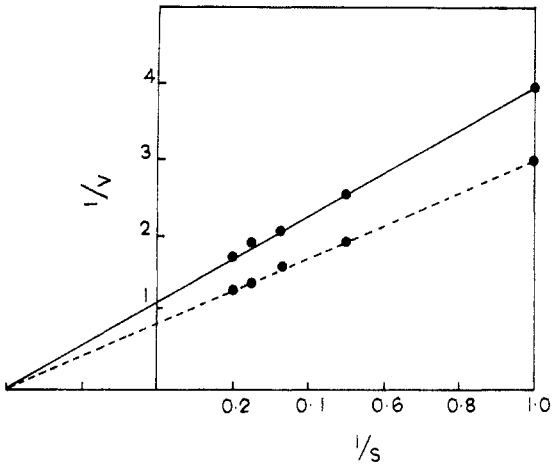

Figure 7. Mode of inhibition of HPA by the purified sorghum inhibitor: ●, enzyme activity without inhibitor; ●, enzyme activity in the presence of the inhibitor. $1/S$ represents the reciprocal of starch concentration (mg). $1/V$ represents the reciprocal of enzyme activity (o.d. units).

of amylase activity). The inhibitor enzyme mixture also showed a similar elution behavior (in terms of protein). The eluant did not exhibit significant amylase activity but had measurable amylase inhibitory activity. These data suggest formation of a complex between human amylase and the sorghum inhibitor.

Complex formation was also analyzed by gel chromatography. The elution profile of the mixture of human pancreatic amylase and the sorghum inhibitor on Sephadex G-100 is shown in Figure 8A. Two protein peaks could be identified. The first peak had both measurable amylase activity (15% of the total enzyme applied) and inhibitory activity (50% of the total inhibitor applied). The second peak displayed neither of these activities. However, this protein fraction on treatment with 2-mercaptoethanol (0.5%) for 1 h at 37 °C exhibited amylase activity (20% of the enzyme applied). Mercaptoethanol did not have any effect on native amylase under these conditions. Free amylase was eluted in the same region as the second peak, and the native inhibitor, in the region of the first peak (Figure 8B). Amylases are known to show an anomalous elution pattern on Sephadex G-100 (Minamiura et al., 1974). During gel chromatography, HPA was retained on the column and was eluted with a Ve value higher than that of the inhibitor.

DISCUSSION

The sorghum inhibitor reported here is similar in its size to 0.19 inhibitor from wheat (Petrucci et al., 1978) and pearl millet inhibitor (Chandrasekher and Pattabiraman, 1983). Like most other millet inhibitors, the sorghum inhibitor was stable under acid conditions and required preincubation with target enzymes to elicit maximal inhibitory activity (Chandrasekher and Pattabiraman, 1983). Loss in inhibitory activity observed on modification of amino and guanido groups of the sorghum inhibitor is similar to the observations made with AmI_1 and AmI_2 wheat inhibitors (Shainkin and Birk, 1970), finger millet inhibitor (Shivaraj and Pattabiraman, 1980), and yam amylase inhibitor (Sharma and Pattabiraman, 1982). However, the chemical modification studies reported here are preliminary in nature and cannot be construed as evidence for the involvement of amino or guanido groups as part of the inhibitory site.

SDS-PAGE with and without mercaptoethanol showed that the sorghum inhibitor is made of two polypeptide chains similar in size if not identical, linked by disulfide bridges. Dismutation of the native inhibitor by reduction results in the loss of inhibitory activity. A similar observation has been made with pearl millet inhibitor (Chandrasekher and Pattabiraman, 1983).

Even though Kneen and Sandstedt (1946) reported amylase inhibitory activity in aqueous extracts of sorghum a long time before, Strumeyer and Malin (1969) concluded that the inhibition was due to oligomeric condensed tannins that acted as general protein denaturants. Davis and Hoseney (1979) confirmed this view by demonstrating inhibitory activity against porcine pancreatic amylase by condensed tannins of bird-resistant sorghum varieties. The inhibitor reported in this paper is distinctly different from those described by earlier workers. The α-amylase inhibitor isolated by us was found to be highly specific in its action unlike the condensed tannins. While it inhibited human amylases, it had no action on crystalline porcine pancreatic amylase and bacterial amylases. The tannin inhibitor in sorghum (Kneen and Sandstedt, 1946) was highly thermostable and alkali stable unlike the present inhibitor. The heat and alkali lability, inactivation by proteases and inactivation by amino and guanido group

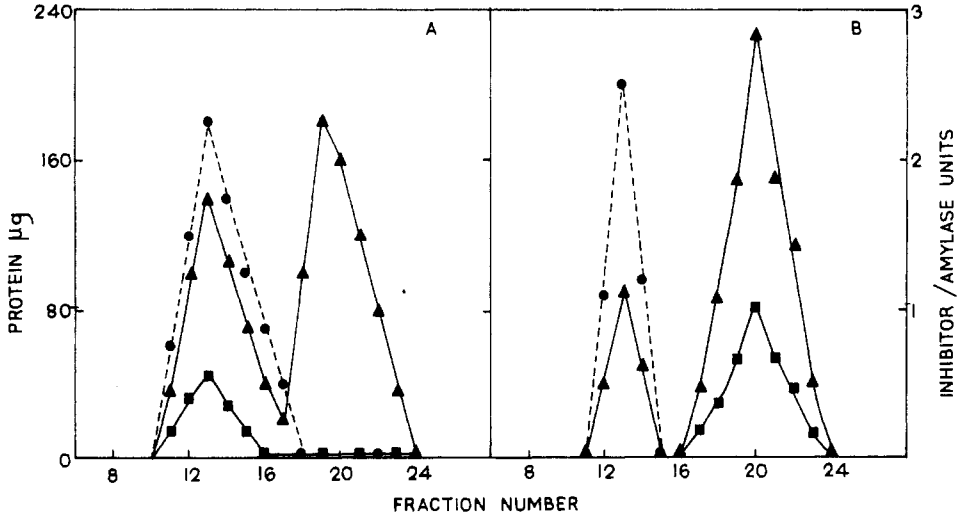

Figure 8. (A) Studies on sorghum inhibitor–HPA complex formation by chromatography on Sephadex G-100: ▲, protein of inhibitor–HPA mixtures; ■, residual amylase activity in the mixture; ●, residual inhibitory activity. (B) Chromatography of purified sorghum inhibitor and purified HPA individually on Sephadex G-100 columns: ▲, protein; ●, amylase inhibitory activity of sorghum inhibitor; ■, amylase activity of HPA. 1 Division = 300 inhibitory/amylase units.

reagents, loss in activity on reduction by mercaptoethanol, and electrophoretic studies confirm that the new inhibitor is proteinaceous in nature.

We could not unequivocally demonstrate the formation of an amylase–inhibitor complex during our studies. When inhibitor and human pancreatic amylase were chromatographed on Red-Sepharose, all the protein was eluted from the column whereas the native inhibitor was bound tightly to the immobilized ligand. While it should be expected that the inhibitor–enzyme complex would be eluted from Sephadex G-100 column earlier than the free enzyme and inhibitor, in our studies such a protein peak could not be identified. Instead, the inhibitor enzyme mixture was eluted in two protein peaks. The first peak with a V_e value corresponding to the native inhibitor had measurable amylase activity. The second peak corresponding to native human pancreatic amylase had neither amylase activity nor inhibitory activity. However, treatment of this fraction with mercaptoethanol resulted in the appearance of amylase activity. These data suggest that the two protein peaks obtained during gel chromatography of amylase–inhibitor mixture represent two types of complexes. The fraction with amylase activity eluted first during gel chromatography could represent a complex formed due to loose interaction of inhibitor and enzyme dissociating under assay conditions exhibiting amylase activity. The second protein fraction may represent a complex formed by strong noncovalent interaction between the inhibitor and amylase, which exhibited amylase activity on inactivation of the inhibitor by treatment with mercaptoethanol. Further studies are needed to substantiate this view.

Registry No. α-Amylase, 9000-90-2.

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Received for review April 22, 1985. Revised manuscript received November 18, 1985. Accepted February 27, 1986. This work was supported by a Grant-in-Aid from the Department of Science and Technology, Government of India.

Racemization Kinetics of Free and Protein-Bound Amino Acids under Moderate Alkaline Treatment

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Amino acid racemization was measured in α-lactalbumin, β-lactoglobulin, α-casein, lysozyme, and BSA and in a free amino acid mixture exposed to pH 9 at 83 °C for times ranging from 0.5 to 24 or 96 h. Inversion rate constants were determined for 14 to 15 amino acids in the six models. Conclusions derived from these data were as follows: (1) Free amino acids racemize about 10 times more slowly than bound residues. (2) The main driving force in free amino acid racemization is the electron-withdrawing ability of the side chain (σ^* constant), except for Asp, the inversion of which seems to involve an intramolecular assistance effect. (3) The racemization of bound residues is governed by both amino side chain effects and protein-related factors. (4) Bound amino acid racemization is also affected by alkali-induced denaturation of the proteins (e.g., partial hydrolysis).

INTRODUCTION

Structural and chemical changes occurring in food proteins on processing may produce undesirable nutritional effects. These include cross-linking (Provansal et al., 1975; Friedman, 1977; Finot, 1983), degradation (Asquith and Otterburn, 1977; Sen et al., 1977), and reactions with sugars (Finot, 1982) or with other food constituents such as polyphenols (Mauron, 1983; Nielsen et al., 1985). Recent investigations have focused on the racemization of amino acid residues, a process that may also be nutritionally detrimental (Hayase et al., 1973, 1975, 1979; Masters and Friedman, 1979; Friedman et al., 1981; Friedman and Masters, 1982; Liardon and Hurrell, 1983; Liardon and Ledermann, 1984; Jenkins et al., 1984). The conversion of L amino acids in food proteins into D isomers generates nonutilizable forms of amino acids, creates peptide bonds

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0021-8561/86/1434-0557$01.50/0