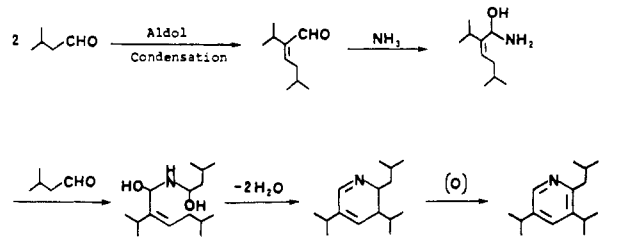

Figure 3. Possible mechanism of the formation of 2-isobutyl-3,5-diisopropylpyridine.

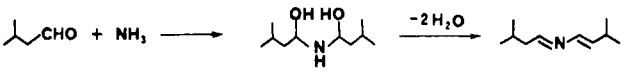

Figure 4. Possible mechanism of the formation of 3-methyl-*N*-(3-methylbutylidene)butenylamine.

should yield 1,2-dihydro-2-isobutyl-3,5-diisopropylpyridine instead of the 2,3-dihydro corresponding pyridine. On the contrary, from the current study, the 2,3-dihydro corresponding pyridine has been found; therefore, an alternate formation mechanism (Figure 3) has been suggested.

Formation of 3-methyl-*n*-(3-methylbutylidene)butenylamine isomers may be via the mechanism shown in Figure 4.

Peaks 7 and 9 were 2,4,6-triisobutyl-1,3,5-trioxanes, which were the isovaleraldehyde trimers formed by oxidation. None of the components from peak 1 to peak 11 showed bacon aroma.

Registry No. Isovaleraldehyde, 590-86-3; 5,6-dihydro-2,4,6-triisobutyl-4*H*-1,3,5-dithiazine, 74595-94-1; 3,5-diisobutyl-1,2,4-trithiolane, 92900-67-9; 2-isobutyl-3,5-diisopropylpyridine, 7033-68-3; 2-methyl-2-propanol, 75-65-0; trimethylacetaldehyde, 630-19-3; isoamyl alcohol, 123-51-3; 3-methyl-*N*-(3-methylbutylidene)butenylamine, 92900-68-0; 2,4,6-triisobutyl-1,3,5-trioxane, 68165-40-2; diisobutyl disulfide, 1518-72-5; 2,3-dihydro-2-isobutyl-3,5-diisopropylpyridine, 92900-69-1; ammonium sulfide, 12135-76-1.

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Partial Characterization of the Amylase Inhibitor of Black Beans (*Phaseolus vulgaris*), Variety Rico 23

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The amylase inhibitor of black (kidney) beans (*Phaseolus vulgaris*) has an apparent molecular weight of 53 000 by gel filtration, an isoelectric point of 4.35, and a sedimentation coefficient ($s^0_{20,w}$) of 4.4 S. It contains mannose, xylose, galactose (5.4%), and glucosamine (3%) and is highly resistant to proteolysis. The N-terminal amino acids are alanine, glutamic acid, and threonine; the C terminals identified were leucine and tyrosine. The inhibitor can be dissociated into three different subunits that can reassociate with complete restoration of activity but it's molecular structure is modified as shown by the fluorescence and circular dichroism spectra. The inhibitor is active against mammalian α-amylases and amyloglucosidase from *Rhizopus genus* and *Aspergillus niger*; it is not active against *Bacillus subtilis* or *Aspergillus orizae* α-amylases. The optimum pH for inhibitor is 4.5 for salivary and 5.5 for pancreatic α-amylase. Several anions, nitrate > chloride > bromide > iodide > thiocyanate, increase the rate of salivary amylase inhibition.

Proteinaceous inhibitors of amylases have been detected in different organs of several vegetable species: in cereal seeds such as wheat (Buonocuore et al., 1977), rye (Granum, 1978), triticale (Finardi Filho and Lajolo, 1982), corn (Blanco-Labra and Iturbe-Chiñas, 1981), and ragi (Shivaraj and Pattabiraman, 1980) and in legumes such as kidney beans (Mancini Filho and Lajolo, 1981), peanuts (Irshad and Sharma, 1981), black grams (Reddy and Salunkhe, 1980), and chickpeas (Singh et al., 1982). They were also found in tubers (Shivaraj et al., 1979) and fruits (Mattoo and Modi, 1970). Their proteinaceous nature differentiates them from the low molecular weight oligosaccharide and peptide amylase inhibitors produced by microorganisms (Frommer et al., 1979), but very little is still known of its chemical properties and physiological functions.

The presence of amylase inhibitors in kidney (navy) beans was reported as early as 1945 (Bowman, 1945) and rediscovered by Jaffé and Lette (1968). Only recently they started to receive a more systematic investigation, contrary to what happened to the inhibitors found in wheat that have been more studied and already have their primary structure established (Kashland and Richardson, 1981). More recently Marshall and Lauda (1975), Pick and Wöber (1978), and Powers and Whitaker (1977a) established techniques for isolation and partially characterized the

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inhibitors of white and red kidney beans: they are glycoproteins with a carbohydrate content ranging from 8.6% to 15% and molecular weights varying from 43000 to 49000 and form 1:1 stoichiometric complex with hog pancreatic α-amylase. The red bean inhibitor can be dissociated into four subunits of three different types (Powers and Whitaker, 1977a), while the inhibitor of white beans seems to have three or four identical subunits (Pick and Wöber, 1978).

Several kinetic parameters of the combination of the kidney bean inhibitor with porcine pancreatic α-amylase were studied by Powers and Whitaker (1977b), and aspects on its combination with insect amylases have also been studied by Powers and Culbertson (1982).

In previous papers we reported studies related to the isolation (Lajolo and Finardi Filho, 1984), kinetics of interaction with pancreatic α-amylase (Tanizaki and Lajolo, 1984; Tanizaki et al., 1984), and nutritional properties in rats (Lajolo et al., 1984) of the inhibitor found in a Brazilian variety of black beans (Rico 23); in this paper we report data on several of its physicochemical properties.

EXPERIMENTAL SECTION

Materials. Black kidney beans (*Phaseolus vulgaris*), variety Rico 23, were obtained from the Agricultural Experimental Station of EMBRAPA (Minas Gerais).

Salivary α-amylase was obtained from human saliva by precipitation with acetone according to Bernfeld (1955). *Acanthoscelides* amylase was a crude extract prepared by homogenization of adult insects collected from spoiled beans with 0.9% NaCl solution followed by centrifugation at 10000 rpm for 20 min at 4 °C. α-Amylase from the fruit fly *Rhyncosciara americana* was a gift from A. G. Bianchi of the Biochemistry Department. Porcine pancreatic α-amylase (type 1A, 2× crystallized), trypsin (type III), chymotrypsin (type II), fungal protease (type XIII), pepsin (crystallized and lyophilized), *Aspergillus orizae* α-amylase (type IVa), *Bacillus subtilis* α-amylase (type III-A), and *Rhizopus genus*) (type II) amylase were from Sigma Chemical Co. Amyloglucosidase of *Aspergillus niger* was from Böehringer.

Sephadex and blue dextran were from Pharmacia. DEAE-cellulose, CMC-cellulose, and chromatography paper 1 MM and 3 MM were from Whatman. Ion-exchange resins AG1-X2 and AG50W-X2 were from Bio-Rad.

Acrylamide and bis(acrylamide) were purchased from BDH and recrystallized before using. Ampholytes used for isoelectric focusing were from LKB. Soluble starch was from Baker and 3,5-dinitrosalicylic acid from Carlo Erba; dimethyl suberimidate, sodium dodecyl sulfate (SDS), dansyl chloride, Tris, triethanolamine, Coomassie brilliant blue, β-mercaptoethanol, bovine serum albumin (2× crystallized) were also from Sigma.

The molecular weight standards used in column chromatography were bovine serum albumin (M_r 67000), ovalbumin (40000), chymotrypsinogen A (24000), and cytochrome c (12500). For flat bed chromatography we used the same standards plus aldolase (M_r 158000) and ferritin (M_r 540000) as a front marker. In the electrophoresis under dissociation conditions were used lysozyme (M_r 14300), β-lactoglobulin (18400), trypsinogen (24000), pepsin (34700), ovalbumin (45000), and serum albumin (66000). All the standards were from Pierce. All other reagents were analytical grade and deionized water was used.

Analytical Techniques. The amylase inhibitor was purified by water extraction and ethanol precipitation followed by DEAE-cellulose and Sephadex G-100 chromatography as previously described (Lajolo and Finardi Filho, 1984). Protein concentration was determined by the Lowry et al. (1951) procedure, using bovine serum albumin as a standard.

The carbohydrate content of the amylase inhibitor was evaluated either in the whole inhibitor by using the phenol–sulfuric acid reaction of DuBois et al. (1956) or after separation of the carbohydrate moiety. In the second case we proceeded as follows: 2 mg of the inhibitor was hydrolyzed with 2 N HCl for 10 h at 100 °C in a closed Pyrex tube under nitrogen; the solution was then passed through a 2-mL Bio-Rad AG1-X-2 column, and the free sugars were eluted with 70% ethanol. To separate neutral sugars from amino sugars, we used a Bio-Rad AG50W-X2 column according to Krystal and Graham (1976). The determination of the amount of sugars obtained was done according to Krystal and Graham (1976). The identification of individual carbohydrates was accomplished on Whatman 1 MM paper chromatography (Spiro, 1966).

Amino acids analysis was performed in a Beckman 120 C analyzer according to Spackman et al. (1958) after hydrolysis with 6 N HCl in closed tubes, under nitrogen, at 110 °C, for 22, 48, and 72 h. Cysteine and methionine were determined respectively as cysteic acid and methionine sulfone by oxidation with performic acid (Moore, 1963). Tryptophan was evaluated according to Beaven and Holiday (1952). For amino sugar determination the inhibitor was hydrolyzed with 4 N HCl at 110 °C for 4 and 6 h (Spiro, 1973).

Terminal amino acids were identified basically as described by Gray (1972).

Electrophoresis in polyacrylamide gel rods were run either in alkaline pH according to Davis (1964) or in acid media as proposed by Reisfield et al. (1963). In neutral pH and in the presence of SDS we used the Weber and Osborn (1969) technique. Protein bands were visualized by staining with Coomassie brilliant blue or by the reaction with fucsine to demonstrate glycoproteins (Segrest and Jackson, 1972).

The cross-linking reaction with dimethyl suberimidate was performed as described by Davis and Stark (1970). Dissociation of the inhibitor was studied by using different media: SDS (1%), urea (6 M), guanidine (6 M), and β-mercaptoethanol (1%) in 0.1 M phosphate buffer, pH 6.9, according to Tanford (1968). The elimination of the reagents after the dissociation was done by dialysis against deionized water for 24 h followed by dialysis against 0.1 M phosphate buffer, pH 6.9; SDS was removed from the media by using an ion-exchange (AG1-X8) resin (Lenard, 1971).

The isoelectric point of the purified inhibitor was evaluated by electrophoresis in polyacrylamide gel rods containing 2% ampholytes (pH 4–6) according to Wrigley (1971).

The molecular weight was obtained by three techniques: (a) electrophoresis in SDS–polyacrylamide gel rods (Weber and Osborn, 1969); (b) chromatography on a 1.2 × 90 cm calibrated Sephadex G-100 column (Andrews, 1964); (c) flat bed chromatography on a 20 × 20 cm plate covered with a 1-mm Sephadex G-150 superfine gel bed. After the run the spots were visualized by contact of the plate with a Whatman 3 MM paper followed by drying the paper and staining with 0.25% Coomassie blue solution in acetic acid–methanol (1:9).

Sedimentation coefficient was measured in a Beckman Model E analytical ultracentrifuge with schlieren optics by using solutions of the inhibitors in 0.05 M acetate buffer, pH 5.0, containing from 0.6 to 2.8 mg of protein/mL; the experiments were carried out at room temperature

(19–22 °C). The sedimentation coefficient was calculated from the movement of the maximum ordinate of the schlieren curve and were corrected to standard conditions ($s^0_{20,w}$). Near-UV–visible spectra of inhibitors were determined either under alkaline (0.1 N NaOH solution) or under acid (0.05 M sodium acetate buffer at pH 5.4) conditions in an Acta III spectrophotometer from Beckman Instruments. Fluorescence spectra of the native or dissociated–reassociated inhibitor were obtained in a Perkin-Elmer Mod-204 spectrofluorometer at 280 nm as the excitation wavelength; the inhibitor was dissolved in 0.05 M phosphate buffer (pH 6.9), and the dissociating agents were removed as described above before recording the spectra. Circular dichroism (CD) measurements were made with a Cary Model 60-705 by using the same solvents as above; results are reported as mean residue elipticity Θ with units of deg cm^2 mol^{-1}. In the digestion trials pepsin, trypsin, chymotrypsin, or Pronase were incubated with the inhibitor in appropriate media (0.05 N HCl solution for pepsin and 0.2 M phosphate buffer, pH 7.6, for the other enzymes) at 37 °C, for different times up to 72 h. The enzyme to substrate ratio was the 1:40 w/w.

Amylase inhibitor activity was evaluated as previously described (Finardi Filho and Lajolo, 1982).

RESULTS AND DISCUSSION

The amylase inhibitor used was an homogeneous preparation showing only one band by electrophoresis either in acid or in alkaline media. The homogeneity was checked also by ultracentrifugation, which showed only a single peak, not separated into other, even after prolonged centrifugation. The sedimentation coefficient ($s^0_{20,w}$) was 4.45 S, corresponding roughly to a M_r of 40 000. The specific activity of the inhibitor was 7.1 inhibitor units/mg of protein (1 inhibitor unit is the amount of inhibitor that inhibits 10 international units of salivary α-amylase at pH 6.9 and 37 °C after 30 min [see Finardi Filho and Lajolo (1982)].

Analysis of Carbohydrates. The glycoprotein nature of the purified inhibitor was shown by staining the polyacrylamide (PAA) gel after electrophoresis with the fucsine–sulfite reagent. The total carbohydrate content estimated by the phenol–sulfuric acid reagent was 14.5%; using the same techniques Marshall and Lauda (1975) and Powers and Whitaker (1977b) obtained lower values, 10% and 8.6%, respectively. By hydrolysis followed by paper chromatography we identified in the inhibitor only three monosaccharides, mannose, galactose, and xylose, and one amino sugar, glucosamine. No sialic acid was found either by paper chromatography or by the specific reaction with thiobarbituric acid (Warren, 1958). After controlled hydrolysis with 0.1 N H_2SO_4 for a time varying from 0.5 to 3 h, only mannose and xylose in constant ratios (1:2.34) were liberated, indicating both are terminal carbohydrates.

The total amount of carbohydrates obtained after acid hydrolysis of the inhibitor was 8.4%, corresponding to 5.4% neutral sugars and 3% amino sugars.

None of the carbohydrates could be released by β-elimination in alkaline conditions, but after reacting with anhydrous hydrazine (Yoshima et al., 1980), several oligosaccharides were detected by paper chromatography. These facts suggest the carbohydrate moiety in the inhibitor is not linked to a serine or threonine residue but probably to an asparagine group through a linkage with glucosamine.

Amino Acid Analysis: Terminal Amino Acids. Amino acid composition of the black kidney bean inhibitor is shown in Table I, which also shows, for comparison, data obtained for the red kidney bean inhibitor by Powers and Whitaker (1977a). Both are similarly rich in aspartic acid, threonine, serine, and glutamic acid and poor in sulfur amino acids. Significant differences were detected in the cysteine, lysine, histidine, proline, and tryptophan contents; we observed, for instance, only one cysteine, two tryptophan, and thirteen proline groups while they detected respectively four cysteines, thirteen tryptophans, and no proline.

Table I. Amino Acid Composition[a] of Black and Red Kidney Bean Amylase Inhibitors

amino acids	black beans[b]	red beans[c]	amino acids	black beans[b]	red beans[c]
Asp	77	66.9	Ile	18	18.4
Thr	32	37.8	Leu	20	21.6
Ser	52	77.3	Tyr	16	24.2
Glu	36	35.0	Phe	23	22.9
Pro	13	0	His	15	5.3
Gly	20	19.3	Lys	2	17.5
Ala	23	21.4	Arg	13	11.4
Cys	1	3.7	Trp	2	13.5
Val	36	38.2	glucosamine[d]	2	n.d.
Met	3	2.4			

[a] Numbers of residues per mole. [b] Based on a M_r of 53 000. [c] From Powers and Whitaker (1977a) and based on a M_r of 49 000. [d] After hydrolysis with 4 N HCl for 4 h.

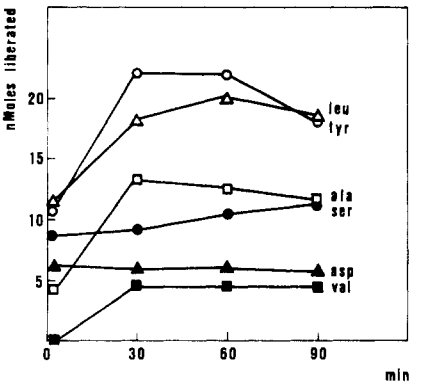

Figure 1. Amino acids produced by hydrolysis of the inhibitor with carboxypeptidase A, as a function of time. A molar ratio enzyme:inhibitor of 1:20 was used. The amino acids liberated were identified by using the amino acid analyzer.

Terminal amino acids were identified to help establish the real number of subunits presented by the amylase inhibitor.

After the reaction with dansyl chloride followed by hydrolysis and paper or polyamide chromatography in different solvents, we identified alanine, glutamic acid, and threonine as N-terminal amino acids.

Using controlled hydrolysis with carboxypeptidase A and following the amount of amino acids liberated as a function of time (Figure 1), we could identify leucine and tyrosine as C terminals. The third C-terminal amino acid probably is serine, alanine, or aspartic acid, but the data obtained did not allow a conclusion and this should be confirmed.

Preliminary studies were also undertaken to establish which were the N-terminal amino acids of each one of individual subunits. By preparative PAA–SDS gel electrophoresis (Weber and Osborn, 1969) using the UNIPHOR LKB system, we could separate fractions a + b from fraction c (see Figure 2). Fraction a + b presented as N terminal only threonine and alanine; in consequence, it follows that band c corresponds to a subunit having glutamine as the N terminal, which confirms again the existence under dissociating conditions of three types of subunits in the inhibitor molecule.

Molecular Weight and Isoelectric Point. The apparent molecular weight (M_r) of the black bean inhibitor

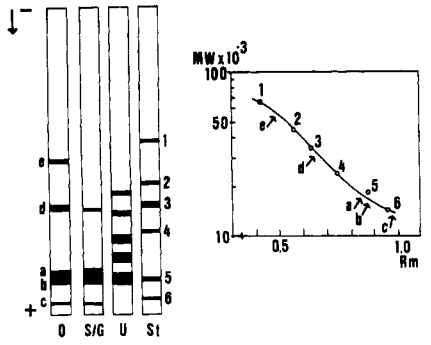

Figure 2. Disc gel electrophoresis of the inhibitor under dissociating conditions and molecular weights of the subunits. The treatments were as follows: D = dimethyl suberimidate plus SDS; S/G = either SDS or guanidine (produced the same profile); U = urea; St = M_r standards (1 = serum albumin, 2 = ovalbumin, 3 = pepsin, 4 = trypsinogen, 5 = β-lactoglobulin, and 6 = lysozyme). The M_r of bands were as follows: e = 49 000–56 000; d = 32 000; a = 17 500; b = 16 000; c = 13 500.

obtained by gel permeation chromatography on a calibrated Sephadex G-100 column was 56 000 ± 2500 (average of three determinations). Using flat bed chromatography on Sephadex G-150, we obtained the value 53 000 ± 1500; this value was used for calculation of molar concentrations because of the lower standard deviation of the data.

By chromatography and PAA–SDS and the addition of the M_rs of each of the three subunits (bands a, b, and c), the value obtained was lower: 47 000 (Figure 2). This was probably due to the glycoprotein nature of the inhibitor, which has a larger volume in solution resulting in higher M_r in Sephadex (Andrews, 1964). The M_r of the cross-linked inhibitor (band c) varied from 49 000 to 56 000 (Figure 2).

Our data are well in agreement with that of Marshall and Lauda (1975) and Powers and Whitaker (1977a) obtained respectively for white and red kidney beans the values 45 000–50 000 and 49 000. Pick and Wöber (1978) obtained also for a white bean inhibitor a lower value: 42 600.

The isoelectric point was obtained in PAA gels by using ampholytes from pH 4 to pH 6. The value 4.35 was similar to what had been obtained by Powers and Whitaker (1977a) (4.50–4.65) and lower than that observed by Pick and Wöber for white beans (5.2). The inhibitors isolated from wheat have higher isoelectric points (around 6.7) and curiously also have higher pH optima, reaching values in the alkaline range (Saunders and Lang, 1973).

Optical Properties. The UV–visible spectra in acidic media showed the protein typical peak at 280 nm (specific absorbance 55.0 × 10^{-2} cm^{-1} L^{-1} g) and another at 237 nm (Figure 3).

In alkaline solution, besides the peak at 280 nm (specific absorbance 52.8 × 10^{-2} cm^{-1} L^{-1} g), a new peak appeared at 294 nm (specific absorbance 51.7 × 10^{-2} cm^{-1} L^{-1} g) due to dissociation of aromatic hydroxyl groups and was used to calculate the tryptophan content. The peaks at 234–243 nm probably have contributions from aromatic and cysteine residues (Beaven and Holiday, 1952).

The fluorescence spectra of the native inhibitor (Figure 3) showed a maximum emission at 323 nm. After dissociation followed by reassociation a new peak appeared at 276–280 nm, the area of aromatic groups. The same effect was shown by the circular dichroism spectra (Figure 4), indicating a molecular alteration.

Subunit Structure. The subunit structure of the black bean amylase inhibitor was studied under several dissociating conditions. Disc gel electrophoresis in the presence of either SDS or guanidine (associated or not) produced

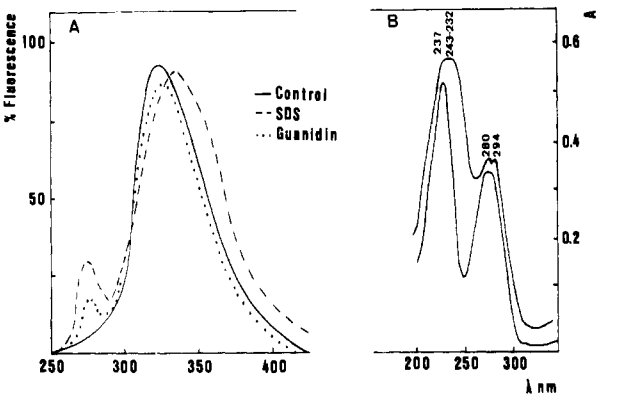

Figure 3. Fluorescence and near-ultraviolet spectra of amylase inhibitor. A = fluorescence spectra of the inhibitor in 0.05 phosphate buffer, pH 6.9, before (control) or after dissociation–reassociation using either SDS or guanidine. B = spectra in acid media, 0.05 M sodium acetate buffer, pH 5.4 (lower curve), or in alkaline media (0.1 NaOH solution) (upper curve).

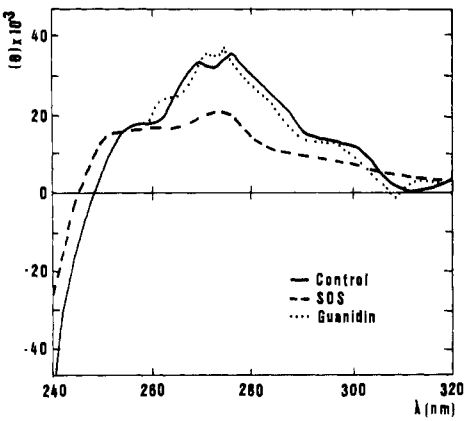

Figure 4. Circular dichroism spectra in the near-UV. The spectra was obtained in 0.1 M phosphate buffer, pH 6.9, either before (control) or after dissociation with SDS or guanidine followed by reassociation of the inhibitor. θ = mean ellipticity.

the same results: the appearance of four bands, a (R_m = 0.86), b (R_m = 0.88), c (R_m = 0.96), and d (R_m = 0.65), all stained as proteins with Coomassie blue and as glycoproteins by the fucsine reagent (Figure 2).

Bands a, b, and c correspond to apparent molecular weights of 17 500, 16 000, and 13 500, respectively, and can be considered the three different subunits present, resulting in a molecular weight of 47 000 daltons for the undissociated inhibitor, which is in agreement with the data reported with the other techniques used (see Molecular Weight and Isoelectric Point). Band d, because of its high M_r (32 000), is probably an undissociated part of the inhibitor molecule.

The addition of β-mercaptoethanol to SDS or guanidine did not alter the electrophoresis profile obtained before, showing disulfide linkages are not important for the bean inhibitor molecular structure as also suggested by Powers and Whitaker (1977a) and contrary to the wheat inhibitors (Buonocore et al., 1977). This was also indicated by the low cysteine (one residue per molecule) content of our inhibitor (see Table I).

When the inhibitor was cross-linked by dimethyl suberimidate and subjected to electrophoresis in SDS, besides bands a–d already shown, a new band e (R_m = 0.49–0.55) having a M_r of 49 000–56 000 appeared (see Figure 2); this band corresponds to the undissociated cross-linked inhibitor, confirming its subunit structure.

The use of urea (4–6 M) as the dissociating agent produced different and variable patterns with the appearance

of four new bands between bands d and e; these results were interpreted as artifacts due to the formation of cyanate from urea and its reaction with the inhibitor.

The data we obtained are similar to what was observed by Powers and Whitaker (1977a). Our bands a (M_r 17 500), b (M_r 16 000), and c (M_r 13 500) probably correspond to their bands a (M_r 15 000–17 000), b (M_r 12 000–15 000), and c (M_r 11 000–12 000). On the basis of the even subunit structure presented by most oligomeric proteins, Powers and Whitaker (1977a) interpreted their data as indicating the existence of four subunits, two of them being equal. From our data on electrophoresis and on the basis of the N-terminal amino acids identified, it is possible to conclude the existence of only three different subunits.

Contrarily, Pick and Wöber (1978) after electrophoresis under dissociating conditions with SDS observed only a single band having a M_r around 11 000 and suggested the inhibitor they isolated from white kidney beans (M_r 42 600) is composed of three or four identical subunits. Probably we are facing different inhibitors.

The dissociation of the black bean inhibitor seems to be reversible with partial or total restoration of the original activity after removal of the dissociating agent by dialysis and ion exchange. The restoration of activity after denaturation with guanidine was total, only partial for urea (55%), and almost none (5%) when SDS was used. Since SDS and guanidine as shown by electrophoresis produced the same pattern of bands, one would expect a similar reactivation. This did not happen, probably because of the strong binding of SDS that can remain linked in small amounts even after removal by ion exchange (Lenard, 1971). By cross-linking with dimethyl suberimidate, the inhibitor retained 87% of its original activity.

After reassociation of its subunits, the inhibitor suffered alterations of the molecular structure as demonstrated by its fluorescence spectra (Figure 3); the undissociated inhibitor showed only one maximum emission peak at 323 nm while the dissociated–reassociated form showed a new peak at 276 nm typical of exposition of internal aromatic groups. The same effect was also shown by the circular dichroism spectra in the near-UV (Figure 4), which showed a marked difference in the region corresponding to aromatic amino acids.

It is also important to report that the undenaturated inhibitor was totally resistant to acid (pH 2–3) and to hydrolysis by pepsin, trypsin, chymotrypsin, and Pronase, even after prolonged periods of time (72 h, 35 °C). The hydrolysis was followed by measurement of the activity of the inhibitor, the amino groups liberated with the ninhydrin reaction, and also by electrophoresis in polyacrylamide to check for a possible limited proteolysis, but in all cases the results were negative. This resistance to digestive enzymes explains the smoothing effect on the glycemic curve that the inhibitor we isolated can produce "in vivo" (Lajolo et al., 1984).

Andriolo et al. (1984) showed that a semipurified inhibitor obtained from white "lingot blanc" beans could be digested by chymotrypsin at pH 8.1 and was not resistant to pHs below 3. This difference from our results is probably due to the difference source of inhibitors studied and indicates structural differences.

Specificity of Black Bean Amylase Inhibitor. The purified inhibitor was tested for its ability to inhibit amylases from several sources at different pHs: 4.0, 5.5, and 7.4. The results shown in Table II suggest the black bean inhibitor, besides mammalian α-amylases, is also able to inhibit other amylases such as amyloglucosidases from fungi. Confirming data from Marshall and Lauda (1975) but contrary to what had been observed by Jaffé and Lette (1968), our inhibitor was not effective against *B. subtilis* α-amylase. It was also not active against *A. orizae* α-amylase nor against amyloglucosidases obtained from the jejunal wall of rats.

Table II. Specifity of Black Bean Amylase Inhibitor at Different pHs[a]

source	activity, inhibitor units, at pH 4.4	5.5	7.0
α-amylases			
salivary (human)	157	28	6
pancreatic (hog)	44	66	0
Aspergillus orizae	0	0	0
Bacillus subtilis	0	0	0
amyloglucosidase			
Rhyzopus genus	0	56	n.d.
Aspergillus niger	0	15	0
Rhyncosciara americana	2	4	4
Acanthoscelids sp.	4.4	n.d.	0

[a] Enzyme and inhibitor were incubated for 1 h and 37 °C at the indicated pH obtained with 0.05 M acetate or phosphate buffers containing NaCl, 0.02 M. After incubation the pH was adjusted to 6.9 with 0.2 M phosphate buffer and residual amylase activity was measured. Proper controls containing only the enzyme were run under the same conditions.

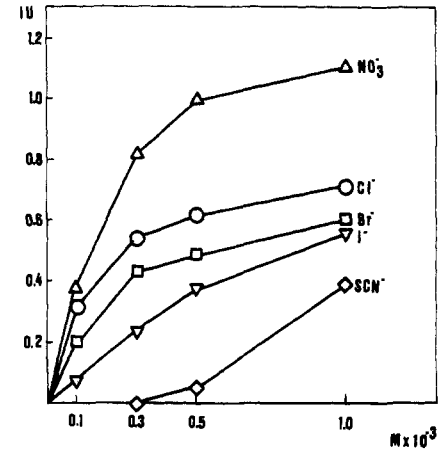

Figure 5. Effect of several anions on the amylase inhibitor activity over salivary α-amylase. Salivary α-amylase (0.3 inhibitor unit) plus inhibitor (2.5 μg) were incubated for 30 min at 37 °C in 0.025 M phosphate buffer, pH 6.9 (2-mL final volume), containing the stated concentration of sodium salts of different anions.

The observed action on *Acanthoscelides* amylases, even if low, allows speculation about its physiological role being related to some defense mechanism against insect and fungi as suggested for the wheat inhibitors (Buonocore et al., 1977).

Effect of Several Anions on the Action of the Inhibitors. During the early purification steps of the amylase inhibitor we observed that after a 48-h dialysis against deionized water, extracts containing the inhibitor seemed to completely lose their activity on human salivary α-amylase, even after prolonged incubation, but the activity could be restored by adding small amounts of NaCl to the media.

Searching the literature, we learned this fact had been reported by Militzer et al. (1946). Also, more recently O'Donnel and McGeeney (1976) verified the activity of dialyzed wheat inhibitor could be restored by addition of NaCl (5×10^{-3} M) to the phosphate buffers.

Our results (Figure 5) showed chloride ions have a strong influence on the reaction of the bean inhibitor with salivary α-amylase; plotting the data from Figure 5 according to

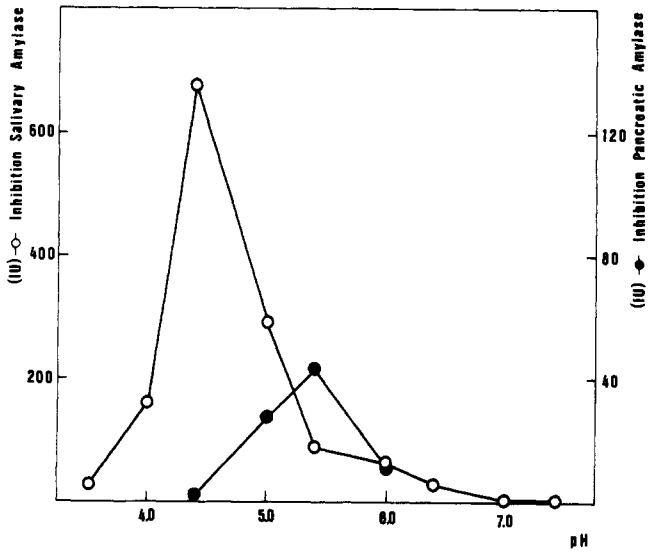

Figure 6. Effect of pH on the activity of the black bean inhibitor. Amylases and inhibitor were incubated at 37 °C for 30 min at the stated pH obtained with 0.02 M citrate–phosphate buffers containing 0.02 M NaCl. After incubation the pH was adjusted to 6.9 with 0.2 M phosphate buffer and the residual amylase activity evaluated to calculate the inhibitor activity. Proper controls containing only the amylase were run at the same pH. After the incubation time, residual amylase activity was measured and the inhibitor units were calculated.

Lineweaver–Burk as 1/inhibition units × $1/Cl^-$, it was possible to calculate a $K_M^{Cl^-}$ of 10^{-4} M.

Chloride ions are known to function as activators of α-amylase by modulating its action through a subtle conformational change that results in a 340 times increase in calcium binding ability. It is tempting to speculate that this allosteric effect could be involved in complex formation with the inhibitor.

Several other anions, known to affect α-amylase action on starch, similar to chloride (Fisher and Stein, 1960), were also tested, and as chloride they also showed a defined pattern of influence on the extent of inhibition (Figure 5). Particularly effective was the nitrate ion, which enhanced the rate of inhibition by several times for only a small (10^{-5} M) increment in its concentration. The effect we reported seems different from the crude effect observed at high ion strengths (up to 0.9 M) by Powers and Whitaker (1977a) with pancreatic α-amylase and from the effect observed by Powers and Culbertson (1982) with *Tenebrio molitor* amylases and deserves more investigation.

Effect of pH and Temperature on the Inhibitory Activity. The effect of pH on the inhibition of either salivary or pancreatic α-amylase was studied on a broad range of pHs, from 3.0 to 7.4, obtained with citrate or phosphate buffers as described under Experimental Section.

The optimum pH for inhibition of porcine pancreatic α-amylase was about 5.4 (Figure 6); differently, the optimum pH for the inhibition of human salivary α-amylase was lower, around 4.5, and decreased sharply either for lower or for higher pHs. The efficiency of the inhibitor, compared at the respective optimum pHs, was 15 times higher over salivary than pancreatic amylase.

The effect of temperature was studied by using only pancreatic α-amylase and at the optimum pH (pH 5.4) for inhibition. Contrary to Marshall and Lauda (1975) and Powers and Whitaker (1977b), who observed a 10-fold increase in activity of the inhibitor when the temperature of the reaction was raised from 25 to 35 °C, in our case the effect of temperature was not so marked (Table III). One

Table III. Effect of Temperature on the Inhibition of Porcine Pancreatic Amylase[a]

	activity, inhibitor units (×10^3) at			
time, min	15 °C	25 °C	35 °C	45 °C
15	30	73	72	143
30	70	120	147	172
45		126	161	206
60	106	136	192	241

[a] Measured at pH 5.4 in a 0.05 M sodium acetate–0.02 M NaCl–0.1 mM $CaCl_2$ buffer.

explanation might be related to the different pHs of the experiments since they worked at pH 6.9 (optimum for amylase action) while our experiments were conducted at pH 5.4, which is the pH optimum for inhibition (see Figure 6), a situation in which the energy barrier is lower. The pH and temperature effect on the activity can possibly be related to pH-induced conformational changes of the inhibitor as suggested by UV–visible spectral changes observed at different pHs (Figure 3).

A rough estimate of the Arrhenius activation energy of the reaction at pH 5.4, based on a first-order plot of the data, gave a value around 5 kcal/mol. The first-order kinetics for complex formation at this pH was demonstrated previously in our laboratory (Tanizaki and Lajolo, 1984). The data on composition and in vitro action of the black bean inhibitor indicated there are significant structural differences among amylase inhibitors of different bean varieties. The dissociation–reassociation of the inhibitor and the anion effect on its action we identified had not been described before and deserve more detailed studies. The resistance of our inhibitor to proteolysis, as opposed to other inhibitors, is also another factor to be explored and is probably important to explain its antinutritional actions in vivo.

Registry No. NO_3^-, 14797-55-8; Cl^-, 16887-00-6; Br^-, 24959-67-9; I^-, 20461-54-5; SCN^-, 302-04-5; amylase, 9000-92-4.

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Volatile Constituents from Guava (*Psidium guajava*, L.) Fruit

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Concentrates of fresh guava (*Psidium guajava*, L.) fruit pulp were obtained by standard controlled high-vacuum distillation with subsequent solvent extraction (pentane–dichloromethane, 2:1) and silica gel LC preseparation using a pentane–diethyl ether gradient. The concentrates were analyzed by capillary gas chromatography and coupled capillary gas chromatography techniques, i.e., on-line mass spectrometry and FTIR spectroscopy. A total of 154 substances was identified, from which 116 compounds could be described for the first time as guava fruit constituents. Quantitatively, lipid peroxidation products such as C_6 aldehydes and alcohols were predominant.

Guava is the fruit of *Psidium guajava*, L., a tree native to the American tropics. The round–oval fruit is green-yellow and shows a light yellow or pink pulp. The aroma impression of the fruit is often described as "quince banana" like (Herrmann, 1983).

Several publications about guava fruit volatiles have already been provided; the literature until 1978 has been reviewed by Shiota (1978). Recent studies were carried out by McLeod and Gonzales de Troconis (1982) and Shiota et al. (1980), the latter comparing the volatiles of strawberry guava (*Psidium cattelianum*, Sabine), yellow guava (*Psidium cattelianum* Sabine var. lucidum Hort.), and feijoa (*Feijoa sellowiana* Berg). Additional work has also been done exclusively on the volatile constituents of feijoa (Hardy and Michael, 1970; Shaw et al., 1983).

EXPERIMENTAL SECTION

Fruits. Fresh ripe guava fruits (*P. guajava*, L.) were transported by air freight from Brazil and were analyzed the day after arrival.

Sample Preparation. A total of 3.3 kg of fruit pulp was obtained from 3.9 kg of total fruit after removal of the skin, homogenization by a Waring blender, and separation by a hydraulic press (Hafico). After addition of internal standards (butylbenzene, 0.98 mg; dimethyl fumarate, 1.27 mg; 1-decanol, 1.0 mg), the pulp was subjected to high-vacuum distillation.

High-Vacuum Distillation. In a 4-L three-neck flask an aliquot (1.1 kg) of the pulp was diluted with water (1 L) and high-vacuum distilled (40–50 °C/0.1 bar). Approximately 1 L of distillate was collected in two dry ice–methanol (−25 °C) and in two liquid nitrogen cooled traps. The four traps were thawed and the contents were combined for the following solvent extraction. In total, 3.3 kg of fruit pulp was distilled in three batches. The distillation residues were set aside for subsequent analysis of non-distillable aroma compounds by direct solvent extraction.

Liquid–Liquid Extraction. The aqueous distillates and the distillation residues were each extracted with pentane–dichloromethane (2:1) in several portions over 24 h (Drawert and Rapp, 1968). Each of the two extracts were dried over anhydrous sodium sulfate and carefully concentrated to approximately 0.2 mL by using a Vigreux column (45 °C).

Column Chromatography on Silica Gel. The concentrated extracts obtained from distillates were fractionated on silica gel 60 (Merck), activity grade II, by using a pentane–diethyl ether gradient (Idstein et al., 1984). Cooled (11–13 °C) glass columns, 2.0 cm i.d. × 30 cm, were used. The elution rate was 60 mL/h; three fractions were separated. Fraction I was eluted with 250 mL of pentane, fraction II was obtained by eluting with 250 mL of diethyl

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