Occurrence and Purification of α-Amylase Isoinhibitors in Bean (*Phaseolus vulgaris* L.) Varieties

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One hundred fifty bean varieties (*Phaseolus vulgaris*) were screened to check for differences in α-amylase inhibitory activity by extraction at room temperature. Some evaluations were made in heated crude water extracts (in 41 varieties) to differentiate stabilities. The inhibitors from selected varieties were purified to homogeneity using hydrophobic chromatography on octyl-Sepharose. The results demonstrated the presence of two α-amylase inhibitors with different electrophoretical mobilities and heat stabilities. Some varieties have only one of the two inhibitors.

INTRODUCTION

Proteinaceous inhibitors of α-amylase from beans are glycoproteins first isolated and partially characterized by Marshall and Lauda in 1975 from a white variety (Great Northern). Since then, several of the properties of these inhibitors have been reported such as composition and structure (Powers and Whitaker, 1977; Wilcox and Whitaker, 1977; Frels and Rupnow, 1984; Lajolo and Finardi Filho, 1985), specificity (Powers and Whitaker, 1977; Pick and Wober, 1978), mechanisms of binding with α-amylase (Wilcox and Whitaker, 1984; Tanizaki and Lajolo, 1985; Whitaker et al., 1988), biological activity in animals (Jaffé and Lette, 1968; Mancini Filho and Lajolo, 1984; Menezes and Lajolo, 1987), and physiological characteristics in bean cotyledons (Moreno and Chrispeels, 1989; Altabella and Chrispeels, 1990; Moreno et al., 1990).

Not much is known yet about the inhibitor's physiological role in the seed and also about its occurrence and possible heterogeneity in different varieties. The occurrence seems to be restricted to the species *Phaseolus vulgaris* since amylase inhibitory activity was not found in other species of the genus such as *Phaseolus lunatus* (lima beans) (Powers and Whitaker, 1977) or *Phaseolus mungo* (Reddy and Salunkhe, 1980).

In a previous paper we reported the α-amylase inhibitor and also hemagglutinin activity of several Brazilian bean varieties, measured in a heated saline extract of the flour (Mancini Filho and Lajolo, 1981). More recently, Frels and Rupnow (1984) found in kidney bean (cultivar Ebony) two amylase inhibitors with different thermal stabilities.

In this paper we report studies on the occurrence of α-amylase inhibitors in 150 Brazilian (*P. vulgaris*) varieties of dried beans and evidence of the existence of at least two different inhibitors that can occur together or each one independently according to the variety. The difference in amount of α-amylase inhibitor activity according to the variety can be explained by the existence of either different amounts and/or different types of amylase isoinhibitors.

MATERIALS AND METHODS

Materials. The different cultivars of *P. vulgaris* were obtained from the Instituto Agronômico of Campinas, São Paulo, Brazil. Hog pancreatic α-amylase type I-A, bovine serum albumin, and sodium borohydrate were products of Sigma Chemical Co. Soluble starch and 3,5-dinitrosalicylic acid were obtained from Merck, and octyl-Sepharose CL-4B was from Pharmacia Fine Chemicals.

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Table I. α-Amylase Inhibitory Activity of 150 Varieties of Common Beans Extracted by Water Using Human Salivary α-Amylase

color	*n*	act., AIU/mL	sp act., AIU/mg of protein
beige (Be)	16	3.59[a] (2.17–4.54)[b]	0.26[a] (0.14–0.40)[b]
purple (Pu)	4	3.06 (2.38–3.44)	0.19 (0.17–0.22)
light brown (Lb)	11	2.91 (2.12–4.10)	0.20 (0.09–0.32)
yellowish brown (Yb)	27	2.91 (1.70–3.89)	0.29 (0.16–0.40)
white (W)	9	2.87 (2.88–3.15)	0.23 (0.14–0.33)
red (R)	14	2.85 (1.66–3.63)	0.25 (0.16–0.37)
brown (B)	20	2.82 (1.81–3.53)	0.26 (0.14–0.35)
pink (Pi)	14	2.81 (2.20–3.26)	0.21 (0.16–0.28)
black (Bl)	26	2.76 (2.01–3.57)	0.19 (0.11–0.30)
dark brown (Db)	9	2.71 (1.99–3.73)	0.25 (0.19–0.33)

[a] Average value. [b] Minimum and maximum values.

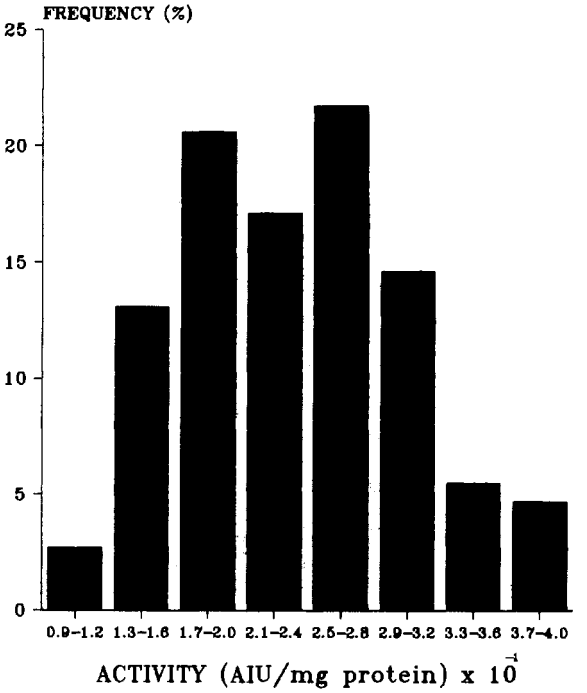

Figure 1. Frequency of distribution of specific inhibitory activity toward human salivary α-amylase.

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Table II. Thermal Stability of the Amylase Inhibitors in Water Extracts

variety	$A(15)^a$	$A(80)^a$	variety	$A(15)^a$	$A(80)^a$
Aeté 1/38 (Pi)	76	37	Olho de Pomba (B)	65	30
Aeté 1/40 (Yb)	75	40	Paquinho (Lb)	76	38
Bico Roxo Precoce (Pu)	75	25	Porto R. B. Grande (W)	72	38
Brilhante 819 (Be)	78	38	Rapé EEP 12/553 (Lb)	62	28
California Red (R)	70	40	Regente (B)	79	48
Cara Suja (Lb)	72	38	Rico Baio 1040 (B)	74	16
Carioca (Be)	76	36	Rico 23 (Bl)	76	47
Carioca 80 (Be)	76	44	Rosinha Cia 63 (Pi)	78	30
Chumbinho Lustroso (Lb)	70	32	Rosinha Precoce (Pi)	74	33
Copinha Enxofre Precoce (B)	76	46	Rosinha 93 (Pi)	66	20
Enxofre 4 (Yb)	65	22	Roxinho Opaco (B)	76	39
Gurgutuba (B)	68	14	Roxinho Ribeirão (Pu)	70	30
La Victore (Bl)	66	10	Royal Red (R)	60	10
Lambe Beiço (R)	64	36	Sutter Pink (B)	79	40
Manteiga 74 (Be)	57	0	Venezuela 77 (Lb)	73	40
Moruna 80 (Bl)	60	32	Wisconsin Hbr 72 (W)	74	15
Mulata Rosa (Pi)	72	26	31 III-58 A_2(200)696 (Yb)	70	16
Mulatinho (Be)	56	18	464-55-1-6 (B)	80	46
Mulatinho Gordo (Yb)	74	19	5965 Caetê-1 (Bl)	60	26
Mulatinho Precoce (Be)	70	30	6002-29PS-Seca 288-7/50 (Bl)	70	21
Navy B. Shemnfarm (B)	76	40	6007-4591-54-1 (Pi)	74	38
			6130 Rosinha 2 (B)	66	40

[a] Residual activity after 15 [$A(15)$] or 80 min [$A(80)$] of heating at 70 °C using hog pancreatic α-amylase.

Table III. Comparison between Specific Inhibitory Activity on Human Salivary and Hog Pancreatic α-Amylase (Water Extracts)

variety	α-HS[a]	α-HP[a]	variety	α-HS	α-HP
Aeté 1/38	0.26	0.16	Olho De Pomba	0.14	0.14
Aeté 1/40	0.32	0.21	Paquinho	0.50	0.18
Bico Roxo Precoce	0.22	0.19	Porto R. B. Grande	0.14	0.14
Brilhante 819	0.32	0.20	Rapé EEP 12/553	0.09	0.11
California Red	0.30	0.24	Regente	0.37	0.29
Cara Suja	0.16	0.14	Rico Baio 1040	0.35	0.20
Carioca	0.30	0.26	Rico 23	0.19	0.19
Carioca 80	0.20	0.18	Rosinha Cia 63	0.16	0.18
Chumbinho Lustroso	0.16	0.11	Rosinha Precoce	0.16	0.14
Copinha Enxofre Precoce	0.27	0.22	Rosinha 93	0.13	0.13
Enxofre 4	0.40	0.29	Roxinho Opaco	0.23	0.22
Gurgutuba	0.28	0.19	Roxinho Ribeirão	0.17	0.13
La Victore	0.26	0.19	Royal Red	0.25	0.22
Lambe Beiço	0.28	0.20	Sutter Pink	0.35	0.29
Manteiga 74	0.32	0.19	Venezuela 77	0.32	0.10
Moruna 80	0.14	0.10	Wisconsin Hbr 72	0.33	0.27
Mulata Rosa	0.19	0.15	31 III-58 A_2(200)696	0.37	0.28
Mulatinho	0.40	0.31	464-55-1-6	0.32	0.22
Mulatinho Gordo	0.32	0.26	5965 Caete-1	0.16	0.15
Mulatinho Precoce	0.14	0.11	6002-29-PS-Seca 288-7/50	0.16	0.14
Navy Bean Shemnfarm	0.16	0.15	6007-4691-54-1	0.16	0.17
			6130 Rosinha 2	0.32	0.21

[a] α-HS, human salivary α-amylase in AIU/mg of protein. α-HP, hog pancreatic α-amylase in AIU/mg of protein.

Methods. Protein concentration was determined according to the Lowry et al. (1951) procedure with bovine serum albumin as standard.

Human Salivary α-Amylase. Human saliva was collected in the presence of a few drops of octanol and toluene. The saliva was cleared by centrifugation for 1 h at 3000*g*. The supernatant was fractionated with acetone between 50 and 70% (v/v). The precipitate obtained was separated by centrifugation for 1 h at 3000*g* and dissolved in 0.07 M sodium acetate. It was fractionated again under the same conditions, and the precipitate obtained was dissolved in 0.02 M phosphate buffer, pH 6.9, containing 38 mM NaCl and 0.1 mM $CaCl_2$ and stored at −18 °C. α-Amylase activity was determined according to the Bernfeld (1955) technique using sodium borohydrate reduced starch according to the method of Strumeyer (1967). The activity was measured at 37 °C in 0.02 M phosphate buffer, pH 6.9, containing 38 mM NaCl and 0.1 mM $CaCl_2$ after addition of the reduced starch and incubation for 5 min. One unit of α-amylase activity (1 AU) is defined as the amount of enzyme that liberates 1 μmol of maltose/min under the above assay conditions.

Assays for Inhibitors. α-Amylase inhibitor activity was assayed by measuring the residual α-amylase activity after enzyme and inhibitor were preincubated for 45 min, at 37 °C, in 0.02 M phosphate buffer, pH 6.9, containing 38 mM NaCl and 0.1 mM $CaCl_2$. After addition of the reduced starch and incubation for 5 min at 37 °C, the residual activity was measured. A control without inhibitor was run to calculate the percentage of inhibition. One unit of inhibitor activity (1 AIU) is the amount that causes total inhibition of 10 units of α-amylase activity under the assay conditions.

Extraction and Purification of α-Amylase Inhibitors. Beans, with the seed coat removed, were ground to a 60-mesh flour. The flour was extracted with water (5% suspension) for 2 h at room temperature and centrifuged at 9220*g* for 1 h, and the supernatant was fractionated with ammonium sulfate. The precipitate obtained between 30 and 60% saturation was dissolved in phosphate buffer containing 25% saturation of ammonium sulfate and applied to the octyl-Sepharose column (0.5 × 10 cm) previously equilibrated with the same buffer. The column was then washed with the buffer until negligible absorbance at 280 nm. Afterward, a linear gradient was applied from 0 to 30% ethylene glycol (v/v) and at the same time from 25 to 0% saturation of ammonium sulfate as described by Frels and Rupnow (1984). Flow rate was kept at 0.3 mL/min, and 5-mL fractions were collected. The absorbance was monitored at 280 nm. The tube contents were assayed for amylase inhibitory

Table IV. Purification: Varieties with Two Inhibitors

variety	step	act.,[a] AIU	protein, mg	sp act., AIU/mg	yield, %	purifn, x-fold
Rico 23	water extract	62.50	312.30	0.20	100	1.0
	$(NH_4)_2SO_4$	43.00	122.45	0.35	69	1.8
	column (I-1)	8.74	1.43	6.12	14	30.6
	column (I-2)	14.26	4.80	2.97	23	14.9
Vermelhão	water extract	35.20	212.16	0.17	100	1.0
	$(NH_4)_2SO_4$	31.80	97.58	0.38	88	2.2
	column (I-1)	9.20	5.84	1.58	26	9.3
	column (I-2)	19.52	14.40	1.36	55	8.0
Enxofre 4	water extract	33.49	159.46	0.21	100	1.0
	$(NH_4)_2SO_4$	15.46	64.32	0.24	46	1.1
	column (I-1)	3.92	6.16	0.64	12	3.0
	column (I-2)	12.16	6.08	1.88	36	9.0
Olho de Pomba	water extract	23.04	212.04	0.11	100	1.0
	$(NH_4)_2SO_4$	20.86	99.28	0.24	91	2.2
	column (I-1)	6.88	2.48	2.77	30	21.2
	column (I-2)	11.97	7.42	1.61	52	14.6
Mulatinho	water extract	40.92	294.44	0.14	100	1.0
	$(NH_4)_2SO_4$	38.48	103.84	0.37	94	2.6
	column (I-1)	7.68	1.04	7.38	19	52.7
	column (I-2)	14.32	6.80	2.11	35	15.1
Brilhante 819	water extract	33.66	213.51	0.16	100	1.0
	$(NH_4)_2SO_4$	27.48	96.12	0.29	82	1.8
	column (I-1)	8.08	0.96	8.42	24	52.6
	column (I-2)	15.04	5.20	2.91	45	18.2

[a] Using hog pancreatic α-amylase.

Table V. Purification: Varieties with Only One Inhibitor

variety	step	act.,[a] AIU	protein, mg	sp act., AIU/mg	yield, %	purifn, x-fold
Manteiga 74	water extract	38.38	237.12	0.16	100	1.0
	$(NH_4)_2SO_4$	32.41	95.90	0.34	84	2.1
	column (I-1)[b]	14.40	11.12	1.29	38	8.1
Royal Red	water extract	72.01	266.70	0.27	100	1.0
	$(NH_4)_2SO_4$	37.80	107.76	0.35	52	1.3
	column (I-1)	30.12	14.04	2.15	42	8.0
Jalo	water extract	45.60	315.60	0.14	100	1.0
	$(NH_4)_2SO_4$	40.20	85.20	0.44	88	3.1
	column (I-1)[b]	25.68	9.24	2.78	56	19.9
Jalinho	water extract	36.12	358.68	0.10	100	1.0
	$(NH_4)_2SO_4$	26.88	95.92	0.30	80	3.0
	column (I-1)[b]	25.33	25.65	0.98	70	9.8
Regente	water extract	0.94	7.59	0.12	100	1.0
	$(NH_4)_2SO_4$	4.96	18.02	0.28	78	2.3
	column (I-2)	2.47	1.17	2.11	62	17.6
Rosinha Precoce	water extract	0.95	5.44	0.17	100	1.0
	$(NH_4)_2SO_4$	3.40	12.06	0.28	68	1.6
	column (I-2)	2.79	1.71	1.63	65	9.6

[a] Using hog pancreatic α-amylase. [b] Inhibitors with characteristics of both types.

Table VI. Electrophoretic Mobility (R_f), Thermal Inactivation Constants (K) at 70 °C, and Concentration of Ethylene Glycol Needed for Elution of I-1 and I-2

	ethylene glycol, %		K ($\times 10^{-2}$)		R_f	
variety	I-1	I-2	I-1	I-2	I-1	I-2
Vermelhão	0.5–3.0	3.0–8.0	1.32	0.575	0.62	0.68
Enxofre 4	1.5–4.5	4.5–8.0	1.16	0.192	0.62	0.69
Olho de Pomba	0.0–3.0	3.0–8.0	1.33	0.349	0.62	0.67
Mulatinho	2.0–4.0	4.0–7.0	1.50	0.499	0.61	0.68
Brilhante 819	0.0–3.0	3.0–8.0	1.52	0.458	0.62	0.66
Rico 23	1.0–3.5	3.5–7.0	1.05	0.100	0.60	0.65
Rosinha Precoce		4.0–8.0		0.308		0.66
Regente		3.0–7.0		0.563		0.68
Royal Red	0.0–4.5		1.45		0.63	
Jalo	3.5–7.5		0.83		0.62	
Manteiga 74	3.0–7.0		1.39		0.63	
Jalinho	3.0–7.5		0.99		0.65	

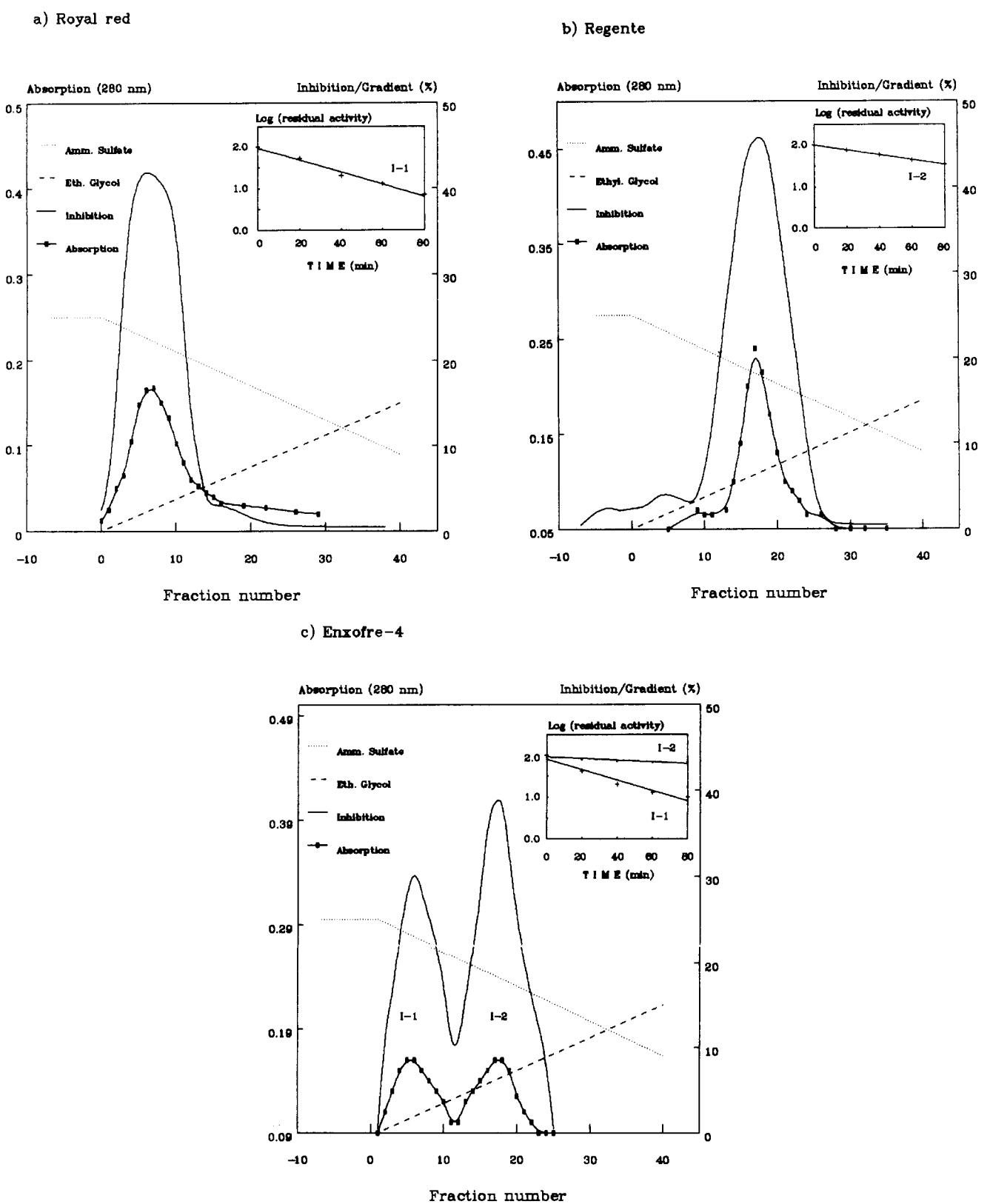

Figure 2. Elution profiles obtained with hog pancreatic α-amylase with varieties having (a) I-1, (b) I-2, and (c) I-1 and I-2 inhibitors.

activity, and after identification, the peaks were pooled and dialyzed against 0.05 M phosphate buffer, pH 6.9.

Electrophoresis. Polyacrylamide gel electrophoresis of purified inhibitors under nondissociating conditions (PAGE) was performed with 7.5% acrylamide gel in 0.025 M Tris–glycine buffer, pH 8.3, at a constant current of 3 mA/tube, according to the method of Davis (1964). The gels were stained for protein using 0.2% Coomassie brilliant blue G-250.

Thermal Stability. The inhibitors were tested for their stability to heat. They were heated at 70 °C in 0.05 M sodium phosphate buffer. Aliquots of 0.50 mL were removed at various times and cooled in an ice bath, and remaining inhibitory activity was determined. The results were plotted as log residual activity vs time (minutes). The heat stability is represented by K (slope of the curve).

RESULTS AND DISCUSSION

Occurrence of α-Amylase Inhibitor Activity in 150 Varieties. The α-amylase inhibitor activity (AIA) of un-

heated water extracts of 150 bean varieties is shown in Table I. Human salivary α-amylase was used in the screening. All of the varieties studied had inhibitory activity.

The results are arranged according to the color of seed coat of the variety to show the nonexistence of correlation between amylase inhibitor activity (AIA) and seed color. The inheritance of AI and seed pigments are not linked. This fact also indicates that it is not appropriate in the study of these compounds to refer to "black bean" or "white bean" inhibitor as done by several authors (Cinco et al., 1985; Whitaker et al., 1988). It is possible to have similar inhibitors in beans of different color and different inhibitors in beans with the same color.

Figure 1 illustrates the frequency of distribution of the specific inhibitory activity, indicating that the variation from the lowest to the highest value is from 3 to 4 times, the same order of magnitude as observed for variation of the lectin content (Mancini Filho and Lajolo, 1981). This statement should be understood considering the whole bean population since beans with the highest lectin activity do not have necessarily the highest amylase inhibitor activity.

Effect of Heating on α-Amylase Inhibitor Activity. A reduced number (41) of varieties were studied further. On the basis of the observation that the two isoinhibitors found by Frels and Rupnow (1984) and Cinco et al. (1985) had different thermal stabilities, we measured the AIA after a heat treatment of the crude water extract. As shown in Table II, depending on variety, the stabilities vary from high thermal resistance for Regente, Rico 23, 464-55-1-6, and Copinha Enxofre Precoce to very low stability for Manteiga 74. Fractionation with ammonium sulfate (results not shown) was done to partially purify the extract to reduce eventual interference by tannin and low molecular weight compounds during the thermal stability study. The results were parallel to those obtained in the crude water extract but with a higher sensitivity to heat because of the dilution of the system due to a reduced protein concentration. Those results were taken as indications of possible differences in types of α-amylase inhibitors in different bean varieties and helped to identify varieties for further studies.

In addition to the activity with salivary amylase the inhibitory activity with hog pancreatic α-amylase was also obtained for the 41 varieties (Table III). The ratio of activities was not constant, varying from 0.89 to 1.85, indicating different structural features and heterogeneous distribution. No relationship of specific inhibitor activities was found with the thermal stability.

Purification of α-Amylase Inhibitors. On the basis of their sensitivity to heat, 12 varieties with high or low thermal stability were selected for purification of the inhibitors present by hydrophobic chromatography. Figure 2 shows typical profiles obtained with varieties having both I-1 and I-2 or only I-1 or I-2 inhibitors. The inserts show the thermal stability curve for each situation. I-2 was more thermally stable than I-1. Tables IV and V present the yield, specific activity, and level of purification obtained.

These elution profiles, heat sensitivities, and R_f values demonstrated the existence of a heterogeneous distribution of amylase inhibitors in the *P. vulgaris* species. These results are in agreement with those obtained by Frels and Rupnow (1984) and Cinco et al. (1985). At least two inhibitors with different hydrophobic properties and heat stabilities were present in some beans, and one or the other inhibitor was present in other beans. In varieties having both inhibitors the total amount of I-2 was always higher than the amount of I-1. The specific activity of I-1 was usually higher than that of I-2, but it was equal (Vermelhão) or even lower (Enxofre 4) in some beans. When the inhibitors occurred independently, total I-1 activity was higher than that of I-2 and the specific activity was of the same order of magnitude. The total activity in the bean varied independently of the presence of one or both inhibitors. In other words, the existence of only one inhibitor did not necessarily reduce the α-amylase activity of the bean.

The electrophoretic mobilities (R_f) varied from 0.60 to 0.63 for I-1 and from 0.65 to 0.69 for I-2 for the purified inhibitors, and the ethylene glycol concentrations needed for elution were 0.0–4.5% for I-1 and 3.0–8.0% for I-2 (Table VI). As already shown by Frels and Rupnow (1984), I-2 is more thermally stable and has higher R_f than I-1. Cinco et al. (1985) isolated α-amylase inhibitory activity from red kidney bean eluted as a single peak from a phenyl-Sepharose column, but they did not show its thermal stability.

The thermal inactivation constants (Table VI) were similar among I-1 and I-2 inhibitors with some exceptions (Enxofre 4 and Rico 23) and allow a distinction between both inhibitors. The same can be said for the concentration of ethylene glycol needed for elution. Jalo and Manteiga 74 have R_f and K values compatible with those of I-1 (0.62/0.63 and 0.83/1.39) but an elution profile similar to that of I-2 (3.0–7.5%). The results indicate there are at least two isoinhibitors with different surface hydrophobicities, electrophoretic mobilities, and thermal stabilities corresponding to different molecular structures. Other inhibitors with minor alterations may also occur. The results also suggest the possibility of two genes coding for these compounds. More studies on the subject are underway.

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