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Extraction, purification and some partial characterization of α-amylase inhibitors from wheat Iapar 28-Igapó

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SUMMARY. α-Amylase inhibitors from wheat (*Triticum aestivum*) cultivar Iapar 28-Igapó were extracted with water in a 1:10 (w:v) ratio and precipitated with ammonium sulfate between 20-50% saturation, followed by DEAE and CM-cellulose chromatography. One inhibitor was purified and designated as CMC-IB, and had electrophoretic mobilities of 0.23 and 0.54 in alkaline and acidic conditions, respectively. This inhibitor was 750 times more active on human salivary α-amylase (HSSA) than porcine pancreatic σ-amylase (PPA). The preincubation time required for maximum complexation with HSA was 20 minutes and optimum pH of inhibition was 7.5. The inhibitor CMC-IB was stable at 0°C and maintained 50% of inhibitory activity against HSA, when incubated at 98°C for one hour.

RESUMEN. Extracción, purificación y algunas características parciales de los inhibidores de α-amilasa del trigo 28-Igapó. Los inhibidores de α-amilasa del trigo (*Triticum aestivum*) Iapar 28-Igapó fueron extraídos con agua en la proporción de 1:10 (p/v) precipitado como sulfato de amonio entre 20-50% de saturación, en seguida fue realizada una cromatografía en DEAE y CM-celulosa. Un inhibidor fue purificado e identificado como CMC-IB y su mobilidad eletroforética fue de 0,23 y 0,54 en medio alcalino y ácido, respectivamente. Este inhibidor fue 750 veces más activo en α-amilasa humana que la α-amilasa pancreática de puerco (APP). El tiempo de preincubación para la complexación máxima con ASH fue de 20 minutos y el pH óptimo de inhibición fue de 7,5. El inhibidor CMC-IB fue estable a 0°C y mantuvo 50 % de su actividad inhibidora contra ASH, cuando fue incubado a 98°C durante una hora.

INTRODUCTION

Proteinaceous inhibitors of α-amylase (1,4-alpha D-glucan glucanohydrolase, EC 3.2.1.1) have been the subject of extensive investigation, based on reasons such as: (a) the possible interference of these compounds with starch digestion in live organisms (1,2); (b) lack of knowledge about their physiological function (3,4); (c) possible undesirable effects related to tooth decay in children (5); (d) potential for utilization as therapeutic agents (6); (2) analytical application in purification of α-amylase from different origin (7) and (f) in selection and genetic improvement of varieties of wheat more resistent to insect attack during storage (8).

These inhibitors are widely distributed in plants and most inhibit the human saliva (HSA) and porcine pancreatic α-amylases (PPA). The literature describes also the inhibition of α-amylases obtained from fungi and bacteria (9-14); insects (15, 16); marine animals and plants other than those from which the inhibitors were isolated (17). On the other hand, there are descriptions of inhibitors that inhibit their own α-amylases. These are designated as endogenous inhibitors and these have been isolated from wheat (4), barley, triticale, rye (8) and corn (18).

Purification to homogerety of these protein inhibitors from wheat was done by conventional techniques such as ion exchange and/or gel filtration chromatography (12, 14, 19, 20, 21, 22).

Silano et al (9) confirmed the presence of six albumin inhibitors of α-amylase from wheat, codified as '0.19;; 0.28; 0.32; 0.35, 0.39 and 0.48', according to relative electrophoretic mobilities. The 0.19 inhibitor inhibited only HSA, while the inhibitors of the '0.28 family' (0.28-0.48) did not inhibit HSA, but inhibited the α-amylases from insects. O'Donnel and McGeeney (19) separated one homogeneous fraction from wheat inhibitor (designated as '0.20'), that was 600 times

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more active for HSA inhibition than for PPA. Another albumin inhibitor from wheat (codified as '0.53') was purified and described as belonging to '0.19 family' with inhibition specificity against HSA.

α-amylase inhibitor of gliadinic nature also have been described in wheat, which was extracted with 70%, ethanol and purified to homogeneity (12). Three wheat α-amylase inhibitors, designated as 0.19; 0.28 and 0.55 (electrophoresis mobility), exhibited different inhibition characteristics against HSA.

The levels and especific activities inhibition of aqueous extract of 28 wheat cultivars recommended in Paraná State (Brazil) against HSA and PPA were determined and clustered. The wheat Iapar 28-Igapó showed higher specific activity inhibition against HSA (23) and was selected for this study.

The α-amylase inhibitor from wheat in Brazil has never been studied, and in this paper we describe the extraction, purification and partial characterization of α-amylases inhibitors from wheat Iapar 28-Igapó.

MATERIAL AND METHODS

Materials: The wheat (*Triticum aestivum)* cultivar Iapar 28-Igapó was furnished by Instituto Agronómico do Parana (IAPAR) - Londrina-PR. Human saliva α-amylase (HSA) crude extract was prepared from saliva collected from individuals in the laboratory. The pooled material was centrifuged at 4°C/ 7000 g for 30 minutes and the supernatant partially fractionated with 0-50% saturated acetone. After centrifugation, the supernatant was fractionated by saturation with 70% (25). The precipitate obtained was suspended with 10 mM sodium phosphate buffer pH 6.9, added to 38 mM NaCl, 0.1 mM $CaCl_2$, and 0.02% sodium azide. This enzyme crude extract was frozen and stocked for further use. Porcine pancreatic α-amylase (PPA) of IA type, two times crystalized was acquired from Sigma. The ion exchange resins were DEAE-cellulose and CM-cellulose from Sigma. Other materials and reagents were of analytical grade.

Methods: Grinding of a grain. The wheat flour was obtained by grinding in a mill of hammer type, without heating the flour and equipment.

Substrate preparation: Five percent maize starch solution was reduced with sodium borohydride and stocked at 4°C, according to Strumeyer (24). Aliquots from this solution were neutralizated daily and diluted to 1% with the same buffer used for suspension of the HSA.

Determination of the enzyme activity of α-amylases: HSA and PPA activities were determined following the method of Bernfeld (25). Suitable amounts of the adequately diluted enzyme were preincubated in the buffer for 20 minutes at 37°C and added to 1.0 ml of 1% solution of reduced starch. After incubation of 5 minutes, the reaction was terminated by the addition of 2.0 ml of 3,5 dinitrosalisilic acid (DNS) reagent in the alkaline medium. Simultaneously, a blank without enzyme was prepared. The tubes were heated at 100 °C for 5 minutes and immediately cooled in tap water. Each tube was distilled with 5 ml of the destilated water and the amylase activity was determined by absorbance read at 540 nm. One unit of α-amylase activity (1 UA) corresponded to production of one micromol of maltose per minute at 37 °C, in 10 mM sodium phosphate buffer pH 6.9 containing 38 mM NaCl and 0.1 mM $CaCl_2$.

Determination of the activity of the α-amylase inhibitor: The inhibitory activity on HSA and PPA was determined by incubating 100 µl of the adequately diluted inhibitor with 100 µl of enzyme solution and the volume completed to 1.0 ml with the same buffer used for amylase activity determination. The procedure was followed as described previously. Simultaneously, a blank without inhibitor and enzyme, and a control without inhibitor were prepared. The control served as a parameter for inhibition percentage calculation. It was considered that the control had 100% α-amylase activity. The results were expressed in α-amylase inhibitor units (AIU), considering that 1 AIU was equivalent to 50% α-amylase inhibition under assay condition. The inhibitory specific activity was defined as the ratio between the total inhibitory activity and the total protein. The relation between HSA and PPA inhibitory activity was referred to as HSAIU/PPAIU.

Protein determination: The amount of soluble protein, in all the steps of this study, was quantified by the method of Lowry et al. (26). Bovine serum albumin was used as standard. The protein concentration in the eluted fractions during chromatography was estimated by absorbance at 280 nm.

Extraction and fractionation of α-amylase inhibitors: The extraction of the α-amylase inhibitors from lapar 28-Igapó wheat was accomplished with water and 200 g of the meal in a 1:10 (w/v) ratio, with constant stirring for one hour at room temperature. During fractionation of the inhibitors, the precipitate obtained from 0-20% saturation of ammonium sulfate was discarded of and the supernatant submitted to fractionation with 50% saturation of ammonium sulfate. This solution was left overnight at 4 °C. Afterwards, it was centrifuged and the precipitate P_{20-50} suspended with 5 mM sodium phosphate buffer pH 6.9 and dialysed against the same buffer for 48 hours at 4 °C.

Purification of α-amylase inhibitors: Six hundred mg of the P_{20-50} protein extract was applied in the DEASE-cellulose anion exchange column which had been previously treated and equilibrated with 5 mM sodium phosphate buffer pH 6.9. In the next step, the same buffer with 20 mM Na_2HPO_4 was used, followed by 20 mM Na_2HPO_4 with 0.5 M NaCl,

maintaining the same pH. Four ml fractions were collected in the tubes using an automatic collector (LKB 2070). The protein concentration in each fraction was monitored at 280 nm and the inhibitory activity against HSA and PPA was also determined. The fractions presenting the higher inhibitory activity against HSA were pooled and concentrated. The pooled fractions DEASE-I and DEAE-II were concentrated with a molecular filter of immersion type with 10,000 exclusion limit. The pooled and concentrated fractions from DEAE-I and DEAE-II were applied to the CM-cellulose cation exchange columns equilibrated with 0.1 M sodium acetate buffer pH 4.8. Initially, the fractions were eluted with equilibrium buffer and followed by linear gradient concentration (0.1-0.5 M) of acetate buffer pH 4.8. The protein concentration in the tubes was read at 280 nm and the activity against HSA and PPA was determined. The fractions with inhibitory activity against HSA and PPA were combined and frozen for characterisation studies.

Electrophoretical studies of inhibitory fractions: Gel electrophoresis of amylase inhibitors under nondissociating conditions were performed using 7% acrylamide gels with 0,025 M tris-glycine, pH 8.3, as the running buffer. The gels were loaded with 50-100 µg protein and stained with comassie brilliant blue R-250 according to the method of Davis (27). The electrophoresis in acidic medium (pH 4.3) was conducted as described by Reisfeld et al (28).

Effect of preincubation time on HSA inhibition by CMC-IB: Inhibitor CMC-IB was diluted in water and preincubated with 100 µl of HSA at 37 °C in a volume of 1.0 ml with 10 mM phosphate buffer containing 38 mM of NaCl and 0.1 mM of $CaCl_2$. After intervals of time, varying from zero to 80 minutes the residual activity toward HSA was determined according to the procedure previously described.

Effect of pH on CMC-IB interaction with HSA: The inhibitor CMC-IB was diluted in destiled water and equilibrated in different pHs using the following buffers solution: 20 mM sodium acetate (pH 4.0 to 5.5), 20 mM sodium phosphate (pH 6.1 to 7.9), 20 mM tris-HCL (pH 7.4 to 8.8) and a 0.04 M tris solution (pH 10.0). Following, the inhibitory activity was determined throughout the incubation of 100 µl of each solution containing the inhibitor in different pHs with 100 µl of enzyme solution. After 20 minutes of preincubation, 1.8 ml of reduced starch solution (24) was added in tubes. The further steps were done according with process previously described.

Thermal stability of inhibitor CMC-IB: The thermal stability of the purified inhibitor CMC-IB was conducted at temperatures of 0°, 23°, 37° and 98 °C over a range time. The residual inhibitory activity was then tested against HSA as previously described.

RESULTS AND DISCUSSION

Extration, fractionation and purification of α-amylase inhibitors from Wheat Iapar 28-Igapó.

Results from different phases of separation and purification of the α-amylase inhibitors against HSA and PPA are presented in Table 1. Fractions P_{20-50} resulted in a yield of 74.35% and 31.12% in the inhibitory activity against HSA and PPA, respectively. In this step, the purification increased by 2.83 times for HSA and 1.18 times for PPA in relation to crude extract. The relation between HSAIU and PPAIU demonstrated that the inhibitory activities in the crude extract and P_{20-50} were respectively of about 4.0 and 10.0 times more active against HSA than the PPA.

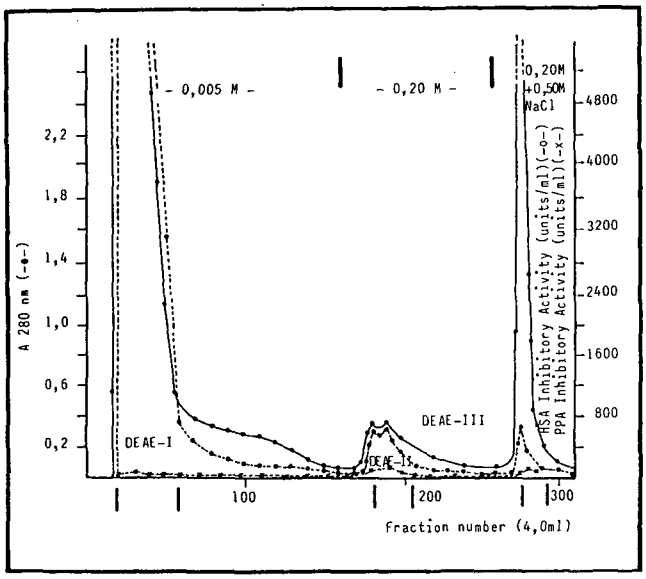
TABLE 1
SEPARATION AND PURIFICATION OF AMYLASE INHIBITORS FROM WHEAT IAPAR-28 IGAPO

	Crude extract	P_{20-50}	DEAE-I	DEAE-II	DEAE-III	CMC-IB	CMC-II
Vol. (ml)	1,565	34	120	80	40	80	17,5
Total units (HSA IUx103)	3,461.78	2,573,83	1,952.46	57,83	25,86	1,094.74	16.10
Total protein (mg)	3,317.80	872.10	272.09	24.88	48.84	76.74	5.57
Specific activity (HSA IU x 10^3/mg)	1,043.40	2,951.30	7,175.79	2,324.36	429.48	14,265.57	2,890.48
Purification times	1	2.8	6.9	2.2	0.51	13.7	2.8
Yield[1] (%)	100	74.35	56.40	1.67	0.75	31.62	0.47
Total units (PPA IU x 10^3)	839.07	61.15	2.23	11.76	5.68	1.46	0.57
Specific activity (PPA IU x 10^3/mg)	252.90	299.45	8.20	472.67	116.29	19.02	102.33
Ratio HSA IU/PPA IU	4.13	9.85	875.54	4.92	4.55	749.82	28.25
Purification times	1	1.2	0	1.9	0.5	0.1	0.4
Yield[2] (%)	100	31.12	0.27	1.40	0.68	0.17	0.07

1 For total inhibitory activity against HSA
2 For total inhibitory activity against PPA

The chromatography in DEAE-cellulose of the fraction P20-50 separated three protein peaks and was designated DEAE-I, DEAE-II and DEAE-III, according to the elution order (Figure 1). The elution system of this chromatography was similar to that used by Granum & Whitaker (12) for the purification of α-amylase inhibitors from wheat.

FIGURE 1
Chromatography on DEAE-cellulose from wheat flour extract P20-50 of wheat flour. Elution with phosphate buffer pH 6,9 in different concentration: 5 mM, 20 mM and 20 mM with 0.5 M NaCl. The pool fraction are indicated with bars.



Date in Figure 1 and Table 1, show that the DEAE-1 fraction had higher protein concentration than other fractions and elevated inhibition activity against HSA, but low inhibition against PPA.

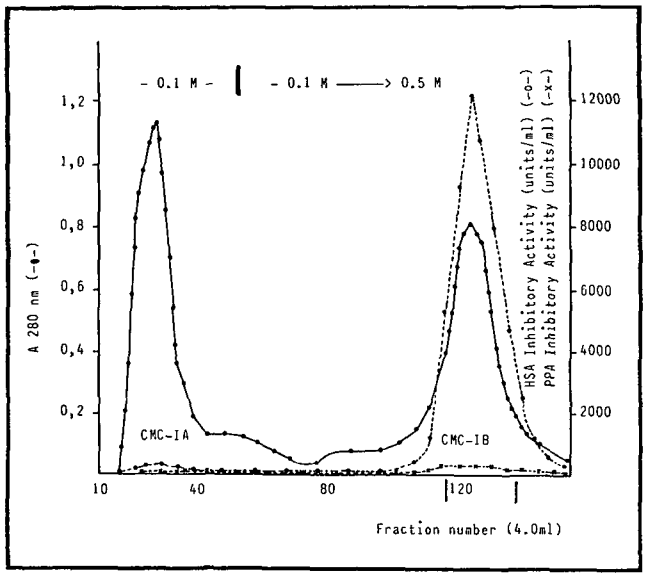
Related to the HSA inhibition (Table 1), greater yield was observed in the DEAE-1 fraction containing 56.40% in relation to initial crude extract. A low yield was obtained for the DEAE-II and DEAE-III fractions, with values of 1.67% and 0.75%, respectively. Granum and Whitaker (12) obtained two major peaks (I and II) with inhibitory activities against HSA, but they did not mention the quantitative inhibition against HSA for these peaks.

The DEAE-I fraction presented low total inhibitory activity against PPA (2.23x103 PPAIU), while the DEAE-II and DEAE-III fraction presented higher inhibition values $11.76x10^3$ and $5.68x10^3$ PPAIU, respectively.

The relation ship between HSAIU and PPAIU (Table 1) for the DEAE-I, DEAE-II and DEAE-III fractions was 875.5, 4.9 and 4.6 respectively.

The DEAE-I fraction with high inhibitory activity against HSA was chromatographed by CM-cellulose as described by Granum and Whitaker (12). In this chromatography system (Figure 2), two protein fractions were separated and designed CMC-IA and CMC-IB. The CMC-IA fraction presented low activity against HSA and PPA, while the CMC-IB fractions presented high inhibitory activity against HSA. The total protein of the CMC-IB fraction was 76.74%, the total inhibitory activity against HSA yield was 31.62% and the purification was 13.67 times that of initial crude extract. The specific inhibitory activity against HSA from this fraction was twice that of the DEAE-I fraction. This CMC-IB fraction was characterized by its high inhibition coefficient against HSA (Table 1 and Figure 2). The fraction I from DEAE-cellulose, described by Granum and Whitaker (12), after chromatography in CM-cellulose, resolved in three protein groups; the first and second (designated 0.28) did not present activity against HSA and the third (designated 0.19) presented inhibitory activity against HSA and PPA. Additional investigation is necessary for the confirmation of these possible differences.

FIGURE 2
Chromatography on CM-cellulose from pooled fractions of DEAE-I. Elution with sodium acetate buffer pH 4.8 - 0.1 M and linear gradient concentration (0.1 - 0.5M). The pooled fractions are indicated with bars.

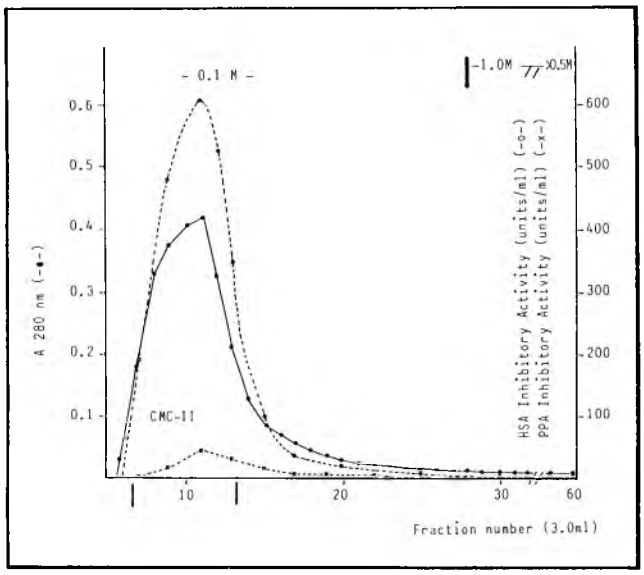


The ratio between HSAIU and PPAIU showed that the inhibitory activity in the CMC-IB was 749.82 times more active against HSA than PPA, confirming that the inhibition activity of CMC-IB was more specific against HSA. O'Donnell and McGeeney (19) purified until homogeneity one albumin inhibitor from wheat, codified 0.20, that was 600 times more active against HSA than PPA. On the basis of the high specificity of inhibition of α-amylase inhibitors from wheat against HSA, O'Donnell et al (29) suggested the utilization of these inhibitors for analytical purpose, in the differentiation of

human salivary and pancreatic amylases.

The DEAE-II fraction with low inhibition yield against HSA and PPA was applied in another CM-cellulose column using the same system of elution. In this system, only one inhibitory fraction (designated as CMC-II) against HSA and PPA was observed after elution with equilibrium buffer (Figure 3). The linear gradient concentration which followed did not produce further protein fractions. Granum and Whitaker (12) also observed that the fraction II of DEAE-cellulose which had separated initially in a fraction designated 0.55, did not produce any further inhibitory fractions.

FIGURE 3
Chromatography on CM-cellulose from pooled fractions of DEAE-II. Elution with sodium acetate buffer pH 4.8 - 0.1 M and linear gradient concentration (0.1 - 0.5 M). The pooled fractions are indicated with bars.



Electrophoretical studies of the inhibitory fractions: Next, the purification steps were accompanied by electrophoresis study in PPA gel (pH 8.3) of crude extract, P_{20-50}, DEAE-a, DEAE-II, DEAE-III, the result of which are presented in the Figure 4. In this electrophoresis study, the DEAE-I fraction showed to have a strong inhibitory against HSA. For this reason this fraction CM-cellulose chromatography.

The fraction, of the CM-cellulose chromatography (designated CMC-IB) presented one unique band in the alkaline (pH 8.3) and acidic (pH 4.3) electrophoresis, with electrophoretical mobility of 0.23 and 0.54 respectively (Figure 5). This mobility was similar to α-amylase inhibitors from wheat with mobility of 0.20 (In 1) and 0.21 (In 2) (14). Bedetti et al. (30) separated α-amylase inhibitors from wheat with different electrophoretical mobilities, and one them presented mobility of 0.23 and was active against HSA and *Tenebrio molitor* amylase.

The electrophoresis of the CMC-II fraction (Figure 5) indicated the presence of a protein band with eleectrophoretical mobility of 0.62 (pH 8.3) and 0.41 (pH 4.3). In this electrophoresis it was possible to observe the presense of other protein bands of low colouration intensity. The fraction CMC-II, although having a low inhibitory activity against HSA and PPA, was studied in a attempt to compare wich other α-amylase inhibitor from wheat described in the literature. The electrophoretical mobility of 0.62 in the alkaline medium for the wheat α-amylase inhibitors was equivalent to 0.64 as described by Bedetti et al (30).

FIGURE 4
Eletrophoresis in PAA gel (pH 8.3) of crude extract (100 µg); P_{20-50} (50µg); DEAE-I (50 µg); DEAE-II (100 µg) and DEAE-III (100 µg)

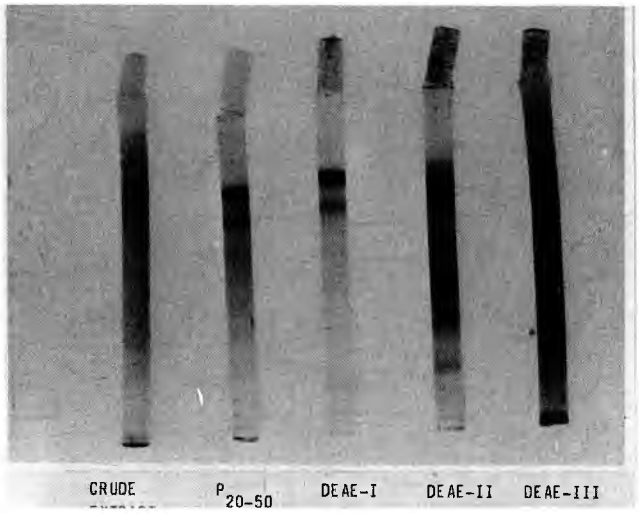
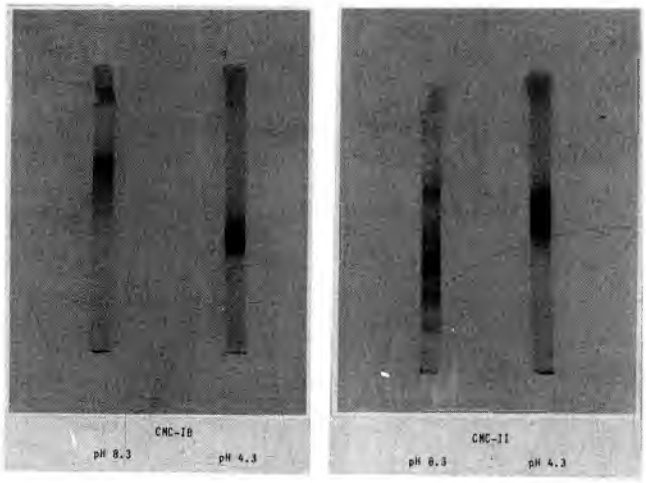
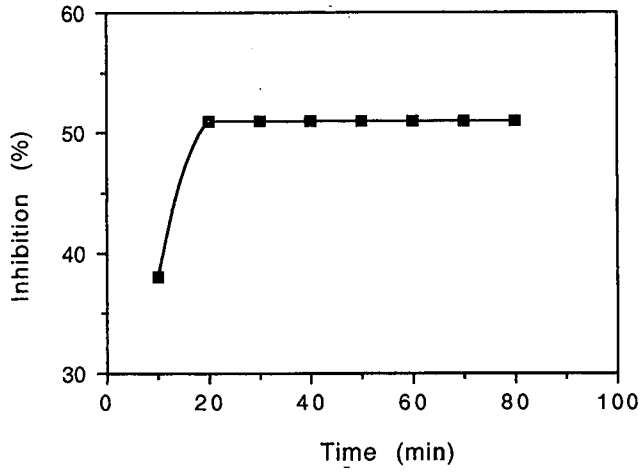


FIGURE 5
Electrophoresis in PAA gel in pH 8.3 and pH 4.3 of fractions CMC-IB and CMC-II



Effect of preincubation time of inhibitor CMC-IB with HSA: The preincubation time of inhibitor CMC-IB with HSA necessary for maximum inhibition was 20 minutes or more at 37 °C (Figure 6). The literature indicates that the preincubation time for maximum inhibition against HSA depends on the inhibitors from differents plants. The preincubation time necessary for the α-amylase inhibitors from wheat was 30 minutes (12, 14, 19, 21, 30, 31). However, for the triticale, the preincubation time was 45 minutes (32), and for bean, there are reports with 20 (11), 30 (10) and 45 minutes (33).

FIGURE 6
Effect of preincubation time of inhibitor CMC-IB and HSA at 37°C.



Effect of pH in the interaction of inhibitor CMC-IB with HSA: According to Figure 7, the optimal pH for HSA inhibition of inhibitor CMC-IB was pH 7.5. O'Donnell & MGeeney (19) observed a broad pH optimum from 6.4 to 8.0 for the interaction of wheat α-amylase inhibitors with HSA. Shainkin & Birk (21) related pH 7.0 as optimal inhibition pH to the wheat inhibitors Am1 and Am2 HSA and PPA, while for the inhibitor 0.19, the optimal pH observed was 8.0 (12).

Thermal stability of inhibitor CMC-IB: Figure 8, shows that at 0 °C, the inhibitor CMC-IB maintained its activity completely. At 23 °C there was gradual lost of inhibition for two hours and, after this period, the loss was less drastic. At 37 °C, about 80% of its inhibitory activity was maintained after one hour inhibition. Under the same conditions, O'Connor & McGeeney (14) previously showed that the inhibitors In1, In2 and In3 were slightly inactivated. At 98 °C for five minutes the inhibitor CMC-IB lost about 18% of activity and, under the same conditions, the inhibitor 0,19 lost about 80% of its activity (31), while inhibitor AmI_2 described by Shainkin & Birk (21) was thermoresistent. After one hour incubation at 98 °C, the inhibitor CMC-IB lost about 50% of its activity, while inhibitors In2, In3 and In4 were completely inactive at the same conditions (14).

FIGURE 7
Effect of pH in the interaction of inhibitor CMC-IB with HSA. Buffer 20 mM sodium acetate pH 4.0 - 5.5; 20 mM sodium phosphate pH 6.1 - 7.9; 20 mM tris - HCl pH 7.4 - 8.8 and 0.04 M tris pH 10.0

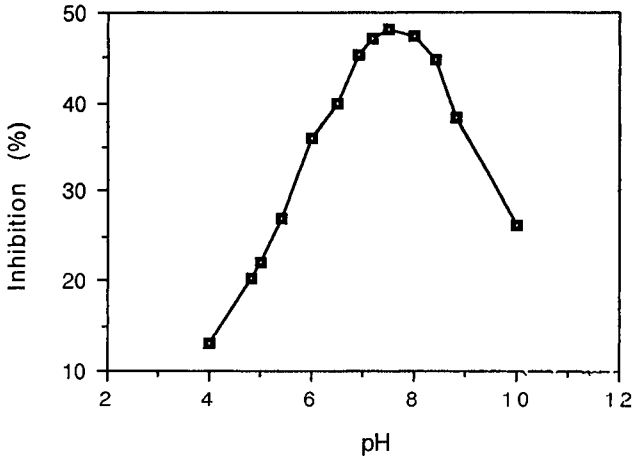
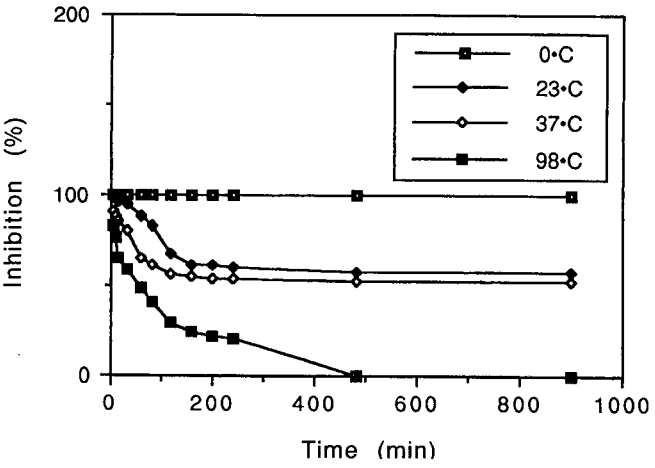


FIGURE 8
Thermal stability of alpha-amylase inhibitors CMC-IB at 0°, 23° and 98° C



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