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α-AMYLASE INHIBITORS IN *TRITICUM AESTIVUM*: PURIFICATION AND PHYSICAL–CHEMICAL PROPERTIES*

ROBIN M. SAUNDERS and JOHN A. LANG

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California, CA 94710, U.S.A.

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Abstract—Two α-amylase inhibitors in aqueous extracts of wheat flour have been resolved by DEAE-Sephadex chromatography. α-Amylase inhibitor I, the major inhibitor, was homogeneous by disc gel electrophoresis. It had a MW of 20 000 daltons and an isoelectric point of 6·7. α-Amylase inhibitor II had two minor contaminants when analysed by electrophoresis. These inhibitors were classified as typical wheat albumin proteins. A third α-amylase inhibitor was discovered when it was observed that an albumin protein which is found only in *Triticum aestivum* varieties of wheat could also inhibit pancreatic α-amylase. All three of these inhibitors could be distinguished by their characteristic electrophoretic mobilities.

INTRODUCTION

IN THE early 1940s Kneen *et al.* described the isolation and general properties of an α-amylase inhibitor in wheat.[1–4] At that time, their conclusion was that the inhibitor was a water soluble protein. Sandstedt and Beckord also published work indicating that the wheat inhibitor was located in the endosperm and not the pericarp, and appeared when the kernel reached full size.[5] In the next 20 years or so, to our knowledge nothing further appeared in the literature on these inhibitors. In 1967, Japanese workers described results on the occurrence of an α-amylase inhibitor in baked wheat flour goods.[6] In 1970, Israeli workers reinvestigating the inhibitor isolated from wheat by Militzer, Ikeda and Kneen described the isolation and properties of two α-amylase inhibitors from wheat.[7] Both these inhibitors were water soluble proteins, but they differed in MW, electrophoretic mobility, and in specificity towards α-amylases from different origin. Recently, Strumeyer[8] reported the isolation of one α-amylase inhibitor from wheat. The physiological role of these inhibitors in the seed is not known. In view of a possible role in seed germination or interference with starch digestion by living organisms, our laboratory has carried out the investigations reported here. Three proteins which inhibit pancreatic α-amylase have been isolated from wheat flour.

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RESULTS AND DISCUSSION

A water-soluble protein extract was isolated from wheat flour, similar to that described by Militzer *et al.*[3,4] The extract was chromatographed on DEAE-Sephadex. Figure 1 shows the isolation by chromatography of two albumin proteins which have the characteristics of α-amylase inhibitors. Fractions 150–190 were combined, concentrated and desalted by ultrafiltration and are designated Inhibitor I. Inhibitor II is derived from fractions 212–227 by an analogous procedure. The material in Peaks I and II represents 31% (I, 24·4%; II, 6·6%) of the protein applied to the column, and contains 88% (I, 78%; II, 10%) of the inhibitory activity. Specific activity measurements indicate 3·2-fold purification for I, and 1·5-fold purification for II, over the extract. The isolation and purification of a third wheat albumin protein with α-amylase inhibitory activity, Inhibitor 0·19, has been described previously.[9] It must be emphasized however that prior to the present report the 0·19 inhibitor has been the subject of investigation only as a wheat albumin. None of the other purified wheat albumins prepared by Sodini *et al.*[9] inhibited mammalian α-amylase.

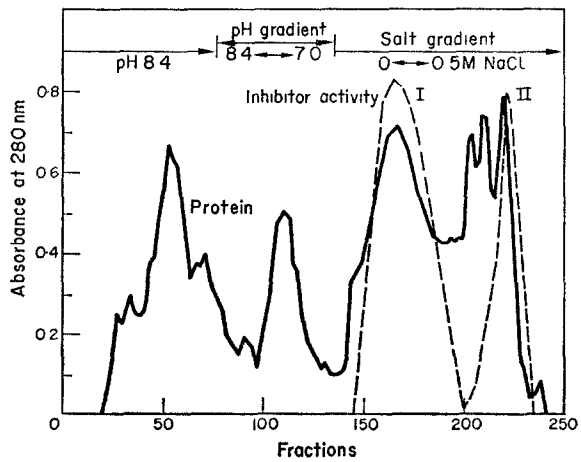

FIG. 1. CHROMATOGRAPHY ON DEAE-SEPHADEX OF α-AMYLASE INHIBITORS EXTRACTED FROM WHEAT FLOUR.

Disc gel electrophoresis with three different buffer systems at a running pH of 9·5,[10,11] pH 8·5[12] and pH 4·3[13] has established the degree of purity of the inhibitors. Inhibitors I and 0·19 showed essentially a single band with only a trace of impurity. Inhibitor II showed a major protein component with two minor protein components, but no I or 0·19 was present.

The behavior of these inhibitor proteins in electrofocusing, DEAE-Sephadex chromatography and disc gel electrophoresis consistently demonstrate that inhibitor II is more anionic than inhibitor I. For inhibitor I the MW was 20 000 by disc gel electrophoresis according to the method of Hedrick and Smith[14] (pI = 6·7). The literature value for the MW of 0·19 is

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23 800 (pI = 7·3).[9] For inhibitor II, disc gel electrophoresis established MWs of 21 000 and 30 000 for two of the components. Isolation of the protein bands from the gel after electrophoresis and measurement of activity established that the component with the highest mobility was the active inhibitor, which had a MW of 21 000 (pI = 6·5). No further heterogeneity was observed for I, II or 0·19 during these electrophoresis studies at higher acrylamide concentrations. For Inhibitor I, ultracentrifugation confirmed the MW (20 300 ± 400). The amino acid composition of Inhibitor I is listed in Table 1. Inhibitor II has been of insufficient quantity and purity to warrant an amino acid analysis. None of the inhibitors contained carbohydrate, as measured by the phenol–sulfuric acid method.[15]

TABLE 1. AMINO ACID PATTERN COMPOSITION OF α-AMYLASE INHIBITOR I IN *Triticum aestivum*

Amino acid	g AA/16 g N	Amino acid	g AA/16 g N	Amino acid	g AA/16g N
Lys	2·82	Glu	11·85	Met	1·82
His	1·57	Pro	5·91	Ile	2·29
Arg	7·19	Gly	4·69	Leu	7·50
Asp	5·87	Ala	8·40	Tyr	4·67
Thr	2·24	Cys/2	2·69	Phe	1·85
Ser	4·64	Val	6·57		

% Nitrogen recovered 90·6.

An inhibition curve for Inhibitor I, where 0·5, 1, 2, 4 and 8 μg of inhibitor were equilibrated with a fixed amount of chick α-amylase before addition of a fixed amount of starch gave 25, 65, 85, 87 and 90% inhibitors respectively. The time allowed for interaction of inhibitor with amylase was 30 min in all experiments. In an experiment where the equilibration times were 1, 10, 30 and 60 min, inhibition of chick α-amylase was 25, 64, 90 and 90% respectively. The amount of reducing sugar liberated by starch hydrolysis decreased (i.e. inhibition increased) as the concentration of inhibitor is increased. Inhibitors I, II and 0·19 showed this same type of curve. The incremental degree of inhibition becomes successively smaller at higher increments of inhibitor. The maximum is about 95% inhibition.

Pepsin digestion of the inhibitors caused a loss of inhibitory activity, presumably by proteolytic destruction of the inhibitors. The acidic condition of the pepsin digest was not responsible since in the control experiment where pepsin was omitted but the inhibitor was subjected to the acid treatment, full inhibitory activity was retained. Pepsin inactivation is evidence that inhibition of α-amylase is due to a functional property of the protein and not due to a non-protein contaminant.

Each of the α-amylase inhibitors exhibited reversible linear uncompetitive inhibition kinetics against chick pancreatic α-amylase, and had k_i values of 5×10^{-8} M.

In the original reports on α-amylase inhibitors from wheat[1–4] only one inhibitor was described. Likewise, Strumeyer has also isolated only one inhibitor from wheat.[8] Shainkin and Birk[7] however, have isolated two inhibitors from wheat. One of these inhibits only *Tenebrio molitar* larval midgut α-amylase, and has no activity towards α-amylase from mammalian sources. Their other inhibitor is active against mammalian α-amylase, and as judged from its physical characteristics and amino acid composition is similar to I and 0·19 described in this paper.

[15] DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. and SMITH, F. (1956) *Anal. Chem.* **28**, 350.

This report is the first evidence for the existence of multiple forms of wheat proteins which are capable of inhibiting mammalian α-amylase.

EXPERIMENTAL

Crystalline pepsin (1:60 000) (Calbiochem, Los Angeles, California) ovalbumin and deoxyribonuclease (Worthington Biochemical Corp., Freehold, New Jersey), and soybean trypsin inhibitor and cytochrome-*c* (Sigma Chemical Co , St. Louis, Missouri) were used. Chick pancreas α-amylase was prepared as described previously.[16] Starch used throughout was Merck soluble starch.

Extraction and separation of α-amylase Inhibitors I *and* II. Flour (long patent, hard red spring wheat) (1 kg) was suspended in 2 l. H_2O, stirred for 5 min, and centrifuged. The clear supernatant was lyophilized. The product was suspended in 100 ml H_2O heated to 70° to inactivate wheat β-amylase, cooled, then purified by fractional precipitation between 70 and 90% EtOH.[3] The entire product (3 g) was dissolved in 0·05 M Tris buffer pH 8·4 and dialyzed exhaustively against the same buffer. Extract (75 mg) in 25 ml of the buffer were chromatographed on a column of DEAE-Sephadex A50, (85 × 2·5 cm). A flow rate of 0·5 ml/min was maintained by a pump, and 6 ml fractions were collected. The column was first eluted with 0·05 M Tris buffer pH 8·4, then a linear pH gradient produced by a mixture of Tris pH 8·4 and Tris pH 7·0 (each at 0·05 M), and then by a linear NaCl gradient (0–1·0 M) in the pH 7·0 Tris buffer. Protein was assayed in the column fractions by measurement of absorbance at 280 nm, with a 4 cm light path cell. Concentration and desalting was achieved by ultrafiltration using an Amicon PM 10 membrane. The fractions were assayed for α-amylase inhibitory activity as described below.

Protein characterization. Analytical disc gel electrophoresis was carried out according to the methods of Davies[10] and Ornstein.[11] Protein (100 μg) was polymerized in a sample gel and electrophoresed in a discontinuous buffer system with a running gel pH of 9·5, 8·5 and 4·3. The separating gel was routinely run at both 7·5 and 10% acrylamide. Determination of MWs by mobilities on disc gel electrophoresis was carried out according to the method of Hedrick and Smith.[14] Separating gels, 7 cm in length, containing 8, 10, 12 and 14% acrylamide were used. Ovalbumin, deoxyribonuclease, soybean trypsin inhibitor, and cytochrome *c* were used as standards. Isoelectric points were determined by isoelectric focusing in 5% polyacrylamide gels containing 2% ampholine pH 3–10 according to the method of Catsimpoolas [17] The concentration of riboflavine was reduced from 10 to 2·6 μg. Protein (50–100 μg per gel) was exposed to 300 V for 7 hr. The proteins were fixed with 15% TCA, washed with 7% HOAc and stained with Coomassie Blue. The pH gradient was measured by cutting the gel into 12 segments, 1 cm in length, adding 1 ml H_2O then measuring pH of the soln 12 hr later.

MW measurement by sedimentation equilibrium was made with a Beckman-Spinco Model E ultracentrifuge equipped with absorption optics and scanner. The buffer was 0·2 M sodium phosphate pH 7 0 and the temp. 19 8°, with a partial specific vol. of 0·72. The centrifuge was run at 20 000 rpm for 26 hr, then 26 000 rpm for 17 hr. The MW was calculated from the slope of ln absorption vs. x^2 plot, where x is the distance of any point in the cell from the axis of rotation. Amino acid analysis was carried out on an acid hydrolysate (6 N HCl for 24 hr) using a modified Phoenix Amino Acid Analyzer, with computer programming for correction of amino acid destruction.[18]

Determination of α-amylase inhibition. The inhibitor protein (normally 0·1–5 μg) in 100 μl H_2O or buffer soln was added to buffer solution containing the α-amylase (normally 1–10 μg of protein). The mixture was made up to 1·4 ml with buffer and incubated at room temp. for 30 min. Starch soln (0·6 ml of a 10 mg/ml H_2O) was added, and the mixture was incubated at room temp. for 10 min. Then 2 ml of dinitrosalicylate reagent were added and the mixture was heated to 100° for 10 min. Water (15 ml) was added, mixed and absorbance at 530 nm was measured [19] The change in absorbance represents production of reducing sugars liberated by starch digestion, and indirectly, the extent of amylase inhibition. The buffer was 0 02 M sodium diethylbarbiturate solution containing 0·15 M NaCl, pH 7·0. A control experiment contained no inhibitor. Zero time controls were used to correct for any reducing sugar contamination. In order to compare the potency of different inhibitor solutions, a Dixon plot was employed. The reciprocal of the velocity of the hydrolysis of starch was plotted against the amount of inhibitor protein present (determined by a modification of the Lowry method[20]) The amount of inhibitor protein required to obtain the same amount of inhibition (1/V) was interpolated from the linear Dixon plots and represents the reciprocal of the specific activity of the inhibitor. For calculating the extent of purification the original extract was used as the reference

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inhibitor solution. Because the absorbance at 530 nm was directly proportional to the velocity of the reaction, and the velocity of the reaction was constant with respect to time during the incubation period, the velocity of the reaction was represented by the absorbance at 530 nm.

Treatment of inhibitors with pepsin. Under similar conditions to those described for determination of inhibition above, the inhibitor in 100 μl H_2O was pre-incubated with 1·1 mg of pepsin in 0·3 ml of 0·02 N HCl for 10 min. A control experiment contained no pepsin. The soln was neutralized with 0·3 ml $NaHCO_3$ soln, then subjected to the routine assay for inhibitory activity.

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