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An Endogenous α-Amylase Inhibitor in Barley Kernels[1]

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RANDALL J. WESELAKE[2], ALEXANDER W. MACGREGOR, AND ROBERT D. HILL
Grain Research Laboratory, Canadian Grain Commission, Winnipeg, Manitoba R3C 3G8 (R. J. W., A. W. M.) and Department of Plant Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada (R. J. W., R. D. H.)

ABSTRACT

Barley (*Hordeum distichum* cv Klages) kernels were shown to contain a factor that converted malted barley α-amylase II to the α-amylase III form. After purification by ammonium sulfate fractionation, ion exchange chromatography on DEAE-Sephacel, and gel-filtration on Bio Gel P60, the factor gave a single band of protein on isoelectric focusing. The purified factor inhibited hydrolysis of soluble starch by α-amylase II from malted barley and germinated wheat (*Triticum aestivum* cv Neepawa). However, α-amylase I from these cereals was not affected. The inhibitor was not dialyzable and was retained by a PM 10 ultrafiltration membrane suggesting a molecular weight greater than 10,000 daltons. Heat treatment of the inhibitor at 70°C for 15 minutes at pH 5.5 and 8.0 resulted in considerable loss of inhibitory activity.

Isoelectric focusing studies on crude extracts of germinated barley kernels have shown previously that the total α-amylase activity was distributed among three groups of enzyme bands (8). The groups were designated α-amylases I, II, and III, in order of increasing isoelectric point.

Quantitative extraction of enzymes from focused gels showed that a high proportion of total activity was found in the α-amylase III group (8). However, when the applied sample was pretreated for 15 min at 70°C, there was a significant decrease in α-amylase III activity and a corresponding increase in α-amylase II activity. This suggests that the heat treatment converted α-amylase III to α-amylase II. Furthermore, α-amylases II and III were shown to share immunochemical identity (9).

Preliminary investigation revealed that kernels of mature barley contained a factor that appeared to convert α-amylase II to α-amylase III and also to inhibit α-amylase II. Protein-like inhibitors of native α-amylases have been reported previously in winter wheat (15, 16) and in one cultivar of maize (1). Activation, after affinity chromatography, of α-amylase in extracts from germinated triticale kernels suggested that triticale also may contain enzyme inhibitors (17).

The present investigation was undertaken to purify and characterize the factor in mature barley responsible for interconversion of malted barley α-amylases II and III and for inhibition of α-amylase II.

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MATERIALS AND METHODS

Inhibitor Purification. Barley (*Hordeum distichum* cv Klages) kernels were dehusked for 20 s in a pearling machine. Eighty-five g of pearled kernels were ground to flour in a Udy Mill. The flour was extracted with 420 ml of 20 mM sodium acetate (1 mM $CaCl_2$, pH 5.5) at 4°C for 60 min. After centrifugation for 30 min at 13,000*g*, the supernatant solution was subjected to $(NH_4)_2SO_4$ fractionation. Material precipitating between 40 and 70% $(NH_4)_2SO_4$ was resuspended in 15 ml of 5 mM Tris-HCl (1 mM $CaCl_2$, pH 8.0). The suspension was dialyzed at pH 8.0 and then centrifuged at 18,000*g* for 20 min to remove insoluble material. The supernatant solution was put on a DEAE-Sephacel (Pharmacia) column (2 × 44 cm) equilibrated with dialysis buffer. The column was eluted with a linear gradient consisting of 300 ml equilibration buffer and 300 ml of buffer containing 150 mM NaCl. Inhibitory fractions were concentrated by pressure ultrafiltration (Amicon UM2) and subjected to gel-filtration on a column (2.6 × 77 cm) of Bio-Gel P60 (100–200 mesh) at pH 8.0. Active fractions from the sieving gel were then concentrated (Amicon PM10) and frozen (−15°C) for subsequent analysis. Protein content was determined by the method of Lowry *et al.* (5).

Enzyme Purification. Malted barley α-amylase II was separated from other amylase groups as described by MacGregor *et al.* (10) and was purified further by affinity chromatography on cycloheptaamylose-epoxy Sepharose 6B (17, 18). Malted barley α-amylase I was purified as described by MacGregor (7). Alpha-amylases I and II were purified from germinated wheat (*Triticum aestivum* cv Neepawa) kernels as described by Weselake and Hill (18).

Inhibitor Assay. Inhibition assays were conducted at pH 5.5 (200 mM sodium acetate, 1 mM $CaCl_2$) and at pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$) with an incubation and reaction temperature of 35°C. One hundred μl of appropriately diluted inhibitor solution was preincubated with an equal volume of appropriately diluted α-amylase. The diluted enzyme contained 1 mg/ml BSA as a stabilizing agent (8). A control system was set up without inhibitor. The reaction was initiated by addition of 200 μl of 1% soluble starch and activity was determined after 7 min by the appearance of reducing groups (11, 13). The extent of inhibition was determined by the difference in reducing power of the two

Table I. *Purification of α-Amylase Inhibitor*

Fraction	Total Protein	Total Inhibitor Activity
	mg	*anti IDC[a] units*
Crude extract	1272	561,100
40–70% $(NH_4)_2SO_4$	240	235,500
DEAE-Sephacel	7.8	167,000
Bio-Gel P60	1.9	105,000

[a] Iodine Dextrin Color.

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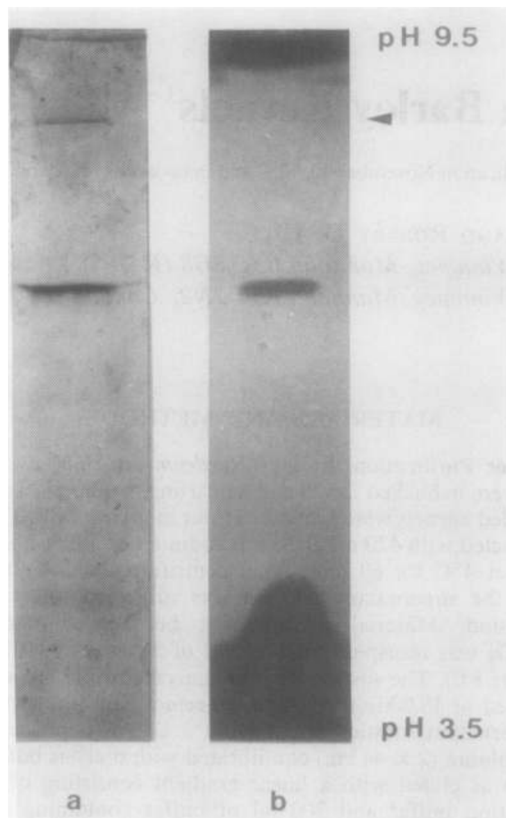

FIG. 1. Isoelectric focusing of purified α-amylase inhibitor. a, Gel section stained for protein (20 μg protein applied). b, Gel section showing inhibitory activity (7 μg protein applied). The isoelectric focusing gel was flooded with α-amylase II, incubated against a starch gel plate, and then developed with I_2-KI. Arrow indicates sample application point.

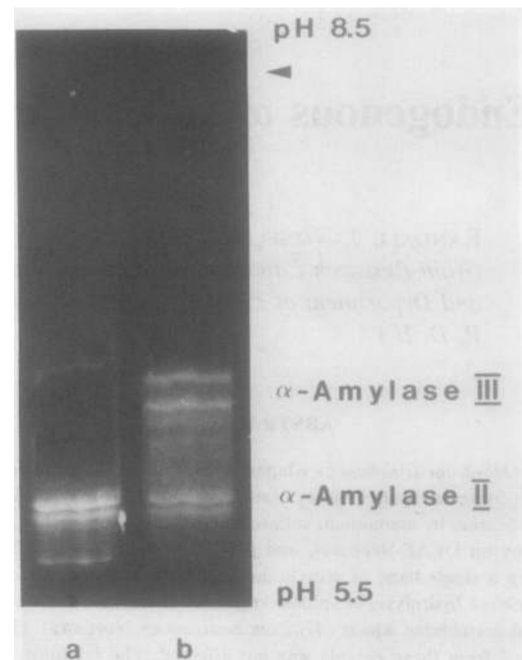

FIG. 2. Isoelectric focusing zymograms demonstrating the effect of inhibitor on α-amylase II. a, α-Amylase II. b, α-Amylase II + inhibitor. Arrow indicates sample application point.

digests.

Under the conditions of the assay: Inhibitor Activity = (Amylase Activity without Inhibitor) − (Amylase Activity with Inhibitor). A specific α-amylase assay (2) was used during inhibitor purification because the presence of reducing compounds and β-amylase in the crude extract would have interfered with determinations based on appearance of reducing groups.

Heat treatment of purified inhibitor, in the presence of 500 μg/ml BSA and 10 mM $CaCl_2$ was conducted at pH 5.5 and 8.0 at 70°C for 15 min. Inhibitory activity remaining was then assayed at pH 8.0.

Isoelectric Focusing. Analytical isoelectric focusing in flat-bed polyacrylamide gels was carried out using precast gels (LKB-Produkter AB, Box 305, S-161 26 Bromma, Sweden) according to manufacturer specifications. The effect of inhibitor on α-amylase isoelectric focusing patterns was analyzed in a pH 5.5 to 8.5 ampholine gradient. Two μl of malted barley α-amylase II (1.8 μg) was incubated with 40 μl of inhibitor solution, containing 10 μg of BSA, for at least 15 min at room temperature. Twenty μl of incubation mixture was applied to an adsorptive pad on the gel surface and then was focused. Zymograms were prepared with β-limit dextrin substrate as described elsewhere (6). In a separate experiment, the following samples were applied to a pH 3.5 to 9.5 focusing gel: (a) 6 μg inhibitor; (b) 8 μg inhibitor, 10 μg α-amylase II, and 10 μg BSA; and (c) 10 μg α-amylase II and 10 μg BSA. Protein bands were visualized using a silver stain procedure (3).

Two adjacent applications of purified inhibitor were focused in a pH 3.5 to 9.5 ampholine gradient. One lane of focused inhibitor was visualized for protein by the silver stain procedure. The other lane of focused inhibitor was removed in a 2× 11-cm section of gel and the surface was flooded with 200 μl of barley α-amylase II (18 μg) in 5 mM Tris-HCl (1 mM $CaCl_2$, pH 8.0) containing 1 mg/ml BSA. The gel was incubated at room temperature for 30 min, sandwiched with a starch substrate plate for 15 min at 35°C (pH 8.0) and then stained with acidified I_2-KI solution.

RESULTS AND DISCUSSION

Preliminary results showed that endosperm extracts of mature Klages barley contained a factor that partially converted malted barley α-amylase II to α-amylase III. This factor was isolated and purified using $(NH_4)_2SO_4$ precipitation followed by ion exchange and gel permeation chromatography. Initially, the purification of the factor was monitored, qualitatively, by its ability to convert α-amylase II to α-amylase III. However, the purified factor was found to inhibit α-amylase II and subsequent purifications were monitored using the ability of the factor to inhibit α-amylase II. The inhibitor peak was eluted from the ion exchange column at a salt concentration of 70 mM NaCl and from the gel permeation column at one-half bed volume. The inhibitor was purified 125-fold from the crude extract and recovered in approximately 20% yield (Table I).

The purified inhibitor gave a single protein band after isoelectric focusing (Fig. 1a) indicating that it was essentially homogeneous. A corresponding band on the zymogram (Fig. 1b) was the area where α-amylase II was inhibited. Undegraded starch in this region gave a blue iodine color on an otherwise clear background. Positions of the two bands on the gel were identical, giving direct evidence that the inhibitor was proteinaceous.

The ability of the inhibitor to convert α-amylase II to α-amylase III is shown in Figures 2 and 3. Addition of the inhibitor to α-amylase II resulted in a mixture of α-amylases II and III as assessed by isoelectric focusing. This combined pattern was similar to the α-amylase II and III pattern reported previously for malted barley α-amylase (8). The zymogram (Fig. 2b) still indicated the presence of both α-amylase II and III, even under conditions in which inhibitor was in excess over α-amylase II. Evidently, there was some degree of dissociation of the complex (α-amylase III) during isoelectric focusing. Visualization of α-amylase III activity

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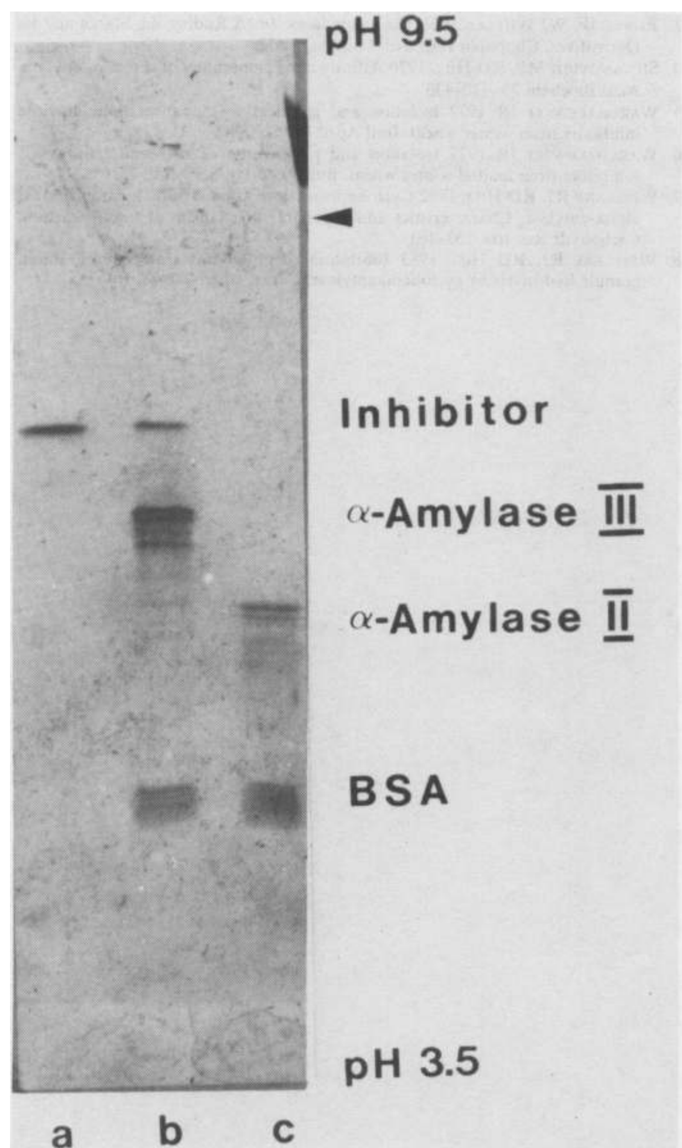

FIG. 3. Protein stain after isoelectric focusing demonstrating the effect of inhibitor on α-amylase II. a, 6 μg inhibitor. b, 8 μg inhibitor, 10 μg α-amylase II, and 10 μg BSA. c, 10 μg α-amylase II and 10 μg BSA.

Table II. *Inhibition of α-Amylase Activity at pH 8.0 and 35°C*

Approximately equal amounts (protein) of inhibitor and α-amylase were used.

	Enzyme Activity		
	No inhibitor	Inhibitor	Inhibition
	μmol glucose/min·ml		%
Malted barley α-amylase I	0.067	0.069	0
Malted barley α-amylase II	0.109	0.013	88
Germinated wheat α-amylase I	0.100	0.104	0
Germinated wheat α-amylase II	0.094	0.018	81

in the zymogram suggested that further dissociation of the complex may have occurred during activity staining. In addition, the appearance of α-amylase III was demonstrated directly by protein staining (Fig. 3). After isoelectric focusing of a mixture of inhibitor and α-amylase II, the stained gel (Fig. 3b) had new bands (α-amylase III) which were intermediate in charge to the contributing species.

Table III. *Inhibition of Barley α-Amylase II at pH 8.0 before and after Heat Treatment of the Inhibitor at 70°C for 15 Minutes*

pH during Heating	Inhibition	
	Before heating	After heating
	%	
5.5	93	22
8.0	97	14

The purified inhibitor was not dialyzable and it was retained by a PM 10 ultrafiltration membrane. Both observations suggest that the inhibitor has a mol wt greater than 10,000 D.

Enzyme inhibition was greater at pH 8.0 than at pH 5.5 and, consequently, inhibition was monitored at pH 8.0 to attain greater sensitivity of detection. Enzyme and inhibitor had to be preincubated for at least 5 min to attain maximum inhibition, indicating that the α-amylase II-inhibitor interaction was time dependent. A similar phenomenon has been observed previously for the interaction of animal α-amylase with inhibitor proteins from wheat kernels (12). Addition of starch solution prior to addition of enzyme reduced inhibition considerably. Therefore, it was possible that interaction of starch with α-amylase II reduced the affinity of the enzyme for the inhibitor.

α-Amylases I and II, purified from malted barley and germinated wheat, were tested against a fixed concentration of inhibitor at pH 8.0 (Table II). Both α-amylase II enzymes were inhibited extensively but α-amylase I enzymes were not. This suggests that the inhibitor was not a protease because one might expect that a proteolytic enzyme effective against α-amylase II would digest α-amylase I, at least to some extent. Isoelectric focusing of a preincubated mixture of inhibitor and malted barley α-amylase I failed to reveal the presence of additional enzyme bands. This lack of interaction supports the inhibition data. These results demonstrate the biospecificity of the inhibitor for α-amylase II from malted barley and germinated wheat.

Heat treatment (70°C for 15 min) of the inhibitor at both pH 5.5 and 8.0 resulted in a large diminution of inhibitory activity (Table III). This agrees with previous findings, which showed that heating extracts of germinated barley tended to convert α-amylase III to α-amylase II (8). Presumably, the conversion factor in germinated barley was similar to, if not the same as, the purified inhibitor. Heat treatments have been used routinely during purification of α-amylases (4, 10) and this could be the reason that the inhibitor has not been detected in the past.

The findings in this communication were the result of preliminary investigations. A detailed account of the purification, characteristics, and interaction of the inhibitor with α-amylases is in preparation.

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