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Purification and Characteristics of an Endogenous α-Amylase Inhibitor from Barley Kernels[1]

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ABSTRACT

An inhibitor of malted barley (*Hordeum vulgare* cv Conquest) α-amylase II was purified 125-fold from a crude extract of barley kernels by $(NH_4)_2SO_4$ fractionation, ion exchange chromatography on DEAE-Sephacel, and gel filtration on Bio-Gel P 60. The inhibitor was a protein with an approximate molecular weight of 20,000 daltons and an isoelectric point of 7.3. The protein was homogeneous, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid analysis indicated the presence of about 9 half-cystine residues per mole. The neutral isoelectric point of the inhibitor suggested that some of the apparently acidic residues (glutamic and aspartic) existed in the amide form. The first twenty N-terminal amino acids were sequenced. Some homology appeared to exist between the α-amylase II inhibitor and trypsin inhibitor from barley. Complex formation between α-amylase II and the inhibitor was detected by the appearance of a new molecular weight species after gel filtration on Bio-Gel P 100. Enzyme and inhibitor had to be preincubated for 5 min, prior to assaying for enzyme activity before maximum inhibition was attained. Inhibition increased at higher pH values. At pH 5.5, an approximately 1100 molar excess of inhibitor over α-amylase II produced 40% inhibition, whereas, at pH 8.0, a 1:1 molar ratio of inhibitor to enzyme produced the same degree of inhibition.

Frydenberg and Nielsen (7) made an in-depth study of the polymorphism of germinated barley α-amylase using zone electrophoresis on agar gel. They showed that heating of malted barley extracts at 70°C for 15 min resulted in disappearance of some α-amylase bands and enrichment of others. The heat treatment appeared to cause the conversion of a heat labile band of α-amylase to a more stable form having a different electrophoretic mobility. A similar phenomenon was described later by MacGregor and Ballance (14). Using isoelectric focusing, these authors reported the presence of three major polymorphic groups of α-amylase, designated α-amylases I, II, and III, in extracts of malted barley. Quantitative determination of these groups before and after a 15-min heat treatment at 70°C showed almost complete conversion of α-amylase III to α-amylase II. More recent results (24) have indicated that α-amylase III is a complex formed by α-amylase II and a proteinaceous factor. Furthermore, this factor inhibited α-amylase II of both germinated barley and wheat but did not inhibit the α-amylase I group. Because the inhibitor is heat labile, a 70°C heat treatment disrupts the α-amylase II-inhibitor complex and liberates α-amylase II thus explaining the conversion of α-amylase III to α-amylase II during heating.

Heat stable inhibitors of cereal α-amylases have been reported in winter wheat (22, 23) and maize (1) kernels. Purothionins have been implicated also as inhibitors of wheat α-amylase (9). However, most research on α-amylase inhibitors has been restricted to a family of albumins isolated from wheat kernels (3). These albumins do not have inhibitory activity against cereal α-amylases.

The current investigation describes the purification, amino acid composition, and some physicochemical properties of the factor that binds specifically to, and inhibits α-amylase II, converting it to α-amylase III. Some parameters affecting the enzyme-inhibitor interaction will be discussed.

MATERIALS AND METHODS

Purification of Inhibitor. The inhibitor was purified from barley (*Hordeum distichum* cv Klages) kernels, essentially as described by Weselake *et al.* (24). Flour prepared from dehusked barley kernels (85 g) was extracted for 1 h (4°C) with 420 ml of 20 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5). Subsequent purification procedures were performed at 4°C. After centrifugation, the material precipitating from the supernatant between 40% and 70% $(NH_4)_2SO_4$ was resuspended and dialyzed against 5 mM Tris-HCl buffer (1 mM $CaCl_2$, pH 8.0). After dialysis, the material (15 ml) was applied to a DEAE-Sephacel (Pharmacia) ion-exchange column (2 × 44 cm) equilibrated with the same buffer. After sample application, the column was washed with approximately one bed volume of equilibration buffer at a flow rate of 40 ml/h. A linear gradient consisting of 300 ml equilibration buffer and 300 ml of buffer containing 150 mM NaCl then was applied. The same flow rate was maintained and 5.3-ml fractions were collected. Conductivity measurements were made at room temperature on every tenth fraction using a YSI model Conductivity Bridge (Yellow Springs Instrument Co.) equipped with a type CDC 314 conductivity probe (Radiometer). Inhibitor activity and A at 280 nm were determined. The inhibitor solution, eluting in a volume from 405 to 450 ml, was pooled and concentrated by pressure ultrafiltration (Amicon UM2). The concentrate (10 ml) was applied to the bottom of a Bio-Gel P 60 (100–200 mesh) gel filtration column (2.6 × 77 cm), equilibrated

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with 5 mM Tris-HCl buffer (1 mM $CaCl_2$, pH 8.0). The column was eluted using an upward flow rate of 15 ml/h and 5.6-ml fractions were collected. Active fractions, eluting in the range of 220 to 270 ml were pooled, concentrated (Amicon, PM 10), and frozen at −15°C for subsequent analysis.

Preparation of α-Amylase II. One kg of green malt from Conquest barley was extracted with 2 L of 200 mM acetate buffer (1 mM $CaCl_2$, pH 5.5). After centrifugation (10,000*g*, 10 min), the extract was heated at 70°C for 15 min to remove β-amylase, was cooled, centrifuged, and dialyzed extensively against 20 mM acetate buffer (1 mM $CaCl_2$, pH 4.75). The extract was added to a column (4.3 × 36 cm) of carboxymethyl cellulose equilibrated with the same buffer and, after thorough washing with starting buffer, the column was eluted sequentially with 80 mM acetate buffer (pH 4.75) to remove α-amylase I (13) and 200 mM acetate buffer (pH 4.75) to remove α-amylase II. This enzyme was dialyzed against 100 mM acetate buffer (1 mM $CaCl_2$, pH 4.75) and purified further on a column (2 × 90 cm) of carboxymethyl cellulose using a linear gradient of 1 L of 100 mM acetate buffer and 1 L of 200 mM acetate buffer (both at pH 4.75 and containing 1 mM $CaCl_2$). Affinity chromatography (21) was used as a final purification step to yield an enzyme of high purity as assessed by isoelectric focusing followed by protein and enzyme activity staining.

Protein Determination. The protein content of inhibitor and α-amylase solutions was determined by the method of Lowry *et al.* (12). Absorbance measurements at 280 nm were used to detect calibration proteins after gel filtration on Bio-Gel P 100.

Assay of α-Amylase and Inhibitor Activity. All enzyme and inhibition assays were performed at 35°C. The Briggs (2) assay for α-amylase was adapted to measure inhibitor activity at pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$) during purification steps. One ml of inhibitor solution was preincubated for 15 min with 1 ml of appropriately diluted barley α-amylase II, containing 100 μg of BSA. Control digests without inhibitor were prepared. The reaction was started by adding 2 ml of β-limit dextrin (0.65 mg/ml) and, after 15 min, stopped by adding 10 ml of acidified I_2-KI (0.05 N HCl, 0.5 mg KI/ml, 0.05 mg I_2/ml). Loss of iodine-binding-capacity was determined at 540 nm.

In other experiments, with purified inhibitor, a reducing sugar assay (20) was used to determine α-amylase and inhibitor activity. One hundred μl of inhibitor solution was preincubated with 100 μl of α-amylase II, containing 1 mg/ml of BSA. The reaction was started by adding 200 μl of 1% soluble starch (Baker) and terminated by adding alkaline copper reagent. One unit of α-amylase activity liberated 1 μmol glucose equivalent/min.

Under the conditions of the assay:

Inhibitor Activity = (Amylase Activity Without Inhibitor) − (Amylase Activity With Inhibitor)

One unit of inhibitor activity nullified 1 unit of α-amylase activity.

Preincubation time required for maximum inhibition to occur was studied at pH 5.5 (200 mM sodium acetate, 1 mM $CaCl_2$) and at pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$). Solutions of enzyme and inhibitor were preincubated for different time periods prior to addition of soluble starch. Enzyme solution was used to initiate the reaction where the effect of zero preincubation time was studied. Enzyme reactions in these time dependent studies were allowed to proceed for 7 min.

The effect of different concentrations of inhibitor on a fixed amount of enzyme (0.10 μg) was studied at pH 5.5 (200 mM sodium acetate, 1 mM $CaCl_2$) and 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$). Enzyme and inhibitor were preincubated for 15 min prior to the addition of soluble starch. Enzyme reactions were allowed to proceed for 8 min at pH 5.5 and 22 min at pH 8.0.

Mol Wt Estimation. The mol wt of the inhibitor was estimated by SDS-PAGE using the Laemmli (11) system. Marker proteins were from Pharmacia and 10 μg of purified inhibitor was used.

Mol wt of inhibitor, α-amylase, and enzyme-inhibitor complex were estimated by gel filtration on a column (1.6 × 54 cm) of Bio-Gel P 100 (100–200 mesh) at pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$, 4°C). Samples were applied to the bottom of the column in 0.50 ml of equilibration buffer. The column was eluted with an upward flow rate of 6 ml/h and 1.93-ml fractions were collected. Calibration proteins were from the Sigma Chemical Co.

Twenty μg of inhibitor were applied to the column and fractions were assayed for inhibitor activity at pH 8.0. In a separate run α-amylase II (9 μg) was applied to the column and fractions were assayed for α-amylase at pH 5.5 (200 mM sodium acetate buffer, 1 mM $CaCl_2$). A mixture of inhibitor (76 μg) and α-amylase II (9 μg) was preincubated for at least 15 min prior to column applications. Elution of α-amylase II-inhibitor complex was detected by assaying fractions for α-amylase activity at pH 5.5 and elution of excess inhibitor was detected by assaying fractions at pH 8.0 for inhibitor activity.

Isoelectric Point Determination. Analytical polyacrylamide gel isoelectric focusing (1°C) of the inhibitor and subsequent visualization was done as described previously (24). The gradient of pH in the gel was determined using a surface electrode.

Amino Acid Analysis and Sequencing. The method for amino acid determination has been described by Duckworth and Bell (5). Tryptophan was determined as described by Edelhoch (6) and half-cystine was determined after oxidation to cysteic acid by the method of Moore (17). Amino-terminal sequencing was performed using a Beckman 890C Sequencer, with 2 mg Polybrene in the spinning cup. A 0.1 M Quadrol program was used, Beckman catalogue no. 030176. Phenylthiohydantoins of amino

Table I. *Purification of α-Amylase Inhibitor*

Fraction	Volume	Total Protein	Total Inhibitor Activity[a]	Specific Activity	Purification	Recovery
	ml	*mg*	*anti units*	*anti units/mg*	*-fold*	*%*
Crude extract	353	1272	561,100	440	1	100
40–70% $(NH_4)_2SO_4$ (after dialysis)	15	240	235,500	980	2	42
DEAE-Sephacel (after concn.)	10	7.8	167,000	21,410	49	30
Bio-Gel P 60 (after concn.)	5	1.9	105,000	55,260	125	19

[a] Based on Briggs (1961) assay.

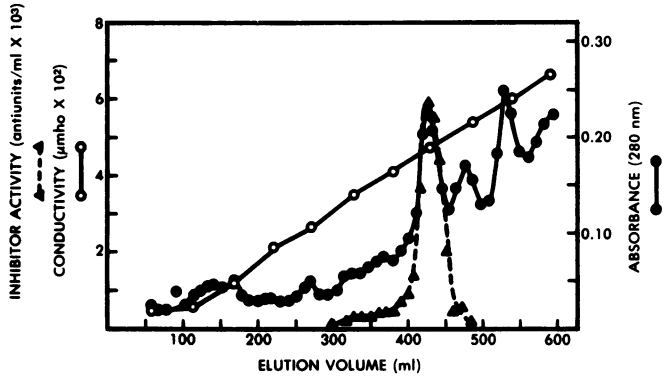

FIG. 1. Ion-exchange chromatography on DEAE-Sephacel of the 40% to 70% $(NH_4)_2SO_4$ fraction of the barley kernel extract. The inhibitor was eluted using a gradient of 300 ml of 5 mM Tris-HCl (1 mM $CaCl_2$, pH 8.0) and 300 ml of the same buffer containing 150 mM NaCl.

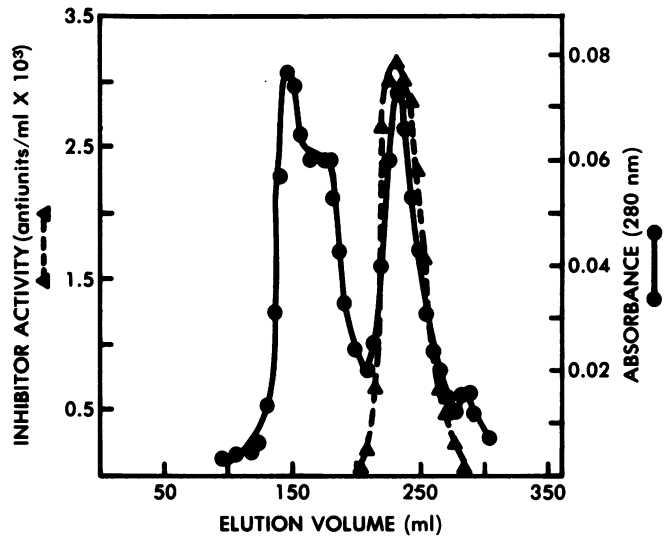

FIG. 2. Gel filtration on Bio-Gel P 60 (100–200 mesh) of the inhibitor peak eluted from the DEAE-Sephacel column. The gel filtration column was eluted with 5 mM Tris-HCl (1 mM $CaCl_2$, pH 8.0).

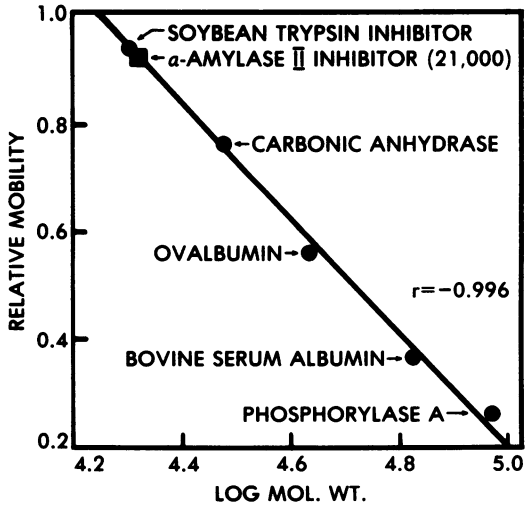

FIG. 3. Mol wt of α-amylase inhibitor estimated by SDS-PAGE.

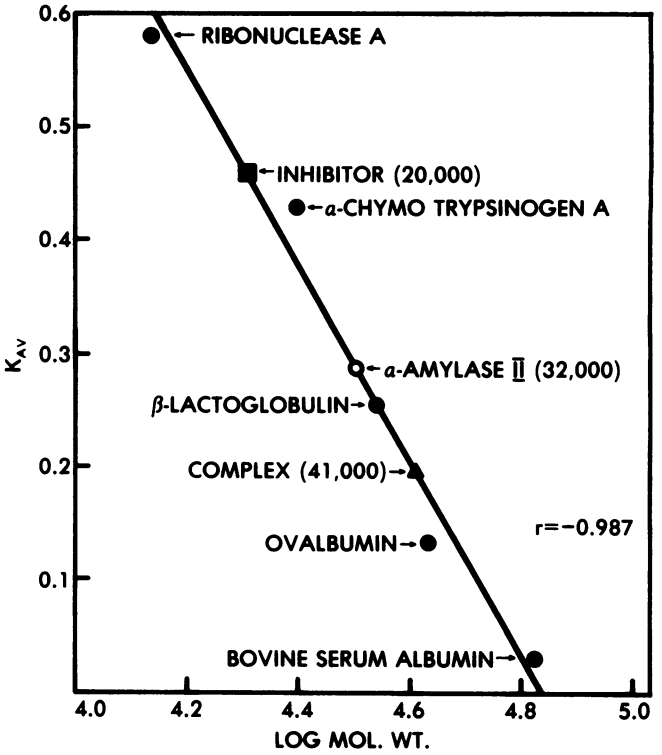

FIG. 4. Mol wt of inhibitor, α-amylase, and the α-amylase-inhibitor complex estimated by gel filtration on Bio-Gel P100 (100–200 mesh). The column was eluted with 50 mM Tris-HCl (1 mM $CaCl_2$, pH 8.0).

Table II. *Amino Acid Composition of α-Amylase Inhibitor*

Amino Acid	Residues/20,000 g
Asp	19.0
Thr	10.0
Ser	8.4
Glu	14.7
Pro	13.4
Gly	17.5
Ala	20.8
Val	14.0
Met	nil
Ile	8.2
Leu	9.9
Tyr	6.0
Phe	5.3
His	4.6
Lys	7.0
Arg	13.1
Trp	2.6
Half-cystine	8.8

acids were identified by HPLC on a Perkin-Elmer HS-3 reversed phase column, using a Perkin-Elmer System 4 Liquid Chromatograph and an elution system recommended by the manufacturer.

RESULTS AND DISCUSSION

The inhibitor was purified 125-fold from the crude extract with an overall recovery of approximately 20% (Table I). Almost 60% of the total inhibitor activity present in the crude extract was lost after $(NH_4)_2SO_4$ fractionation and dialysis. It is possible that part of the α-amylase inhibitory activity present in the crude extract was due to components other than the protein inhibitor. For example, polyphenolics have been implicated as cereal α-

Table III. *N-Terminal Sequence of α-Amylase Inhibitor Compared to Barley Trypsin Inhibitor*

A single sequenator run on 0.46 mg of α-amylase inhibitor (23 nmol assuming 20,000 D) gave 112% coupling (26 nmol alanine in cycle 1) and a repetitive yield of 91.4%.

α-Amylase Inhibitor	Barley Trypsin Inhibitor
	1 Phe[b]
	Gly
1 Ala[b]	Asp
Asp	Ser
Pro	5 Cys
Pro	Ala
5 Pro	Pro
Val	Gly
His	Asp
Asp	10 Ala
Thr	Leu
10 Asp	Pro
Gly	His
His	Asp
Glu	15 Pro
Leu	Leu
15 Arg	Arg
Ala	Ala
Asp	Cys
Ala	20 Arg
Asn	Thr
20 Tyr	Tyr

[a] From Odani *et al.* (18).
[b] N-terminal amino acid.

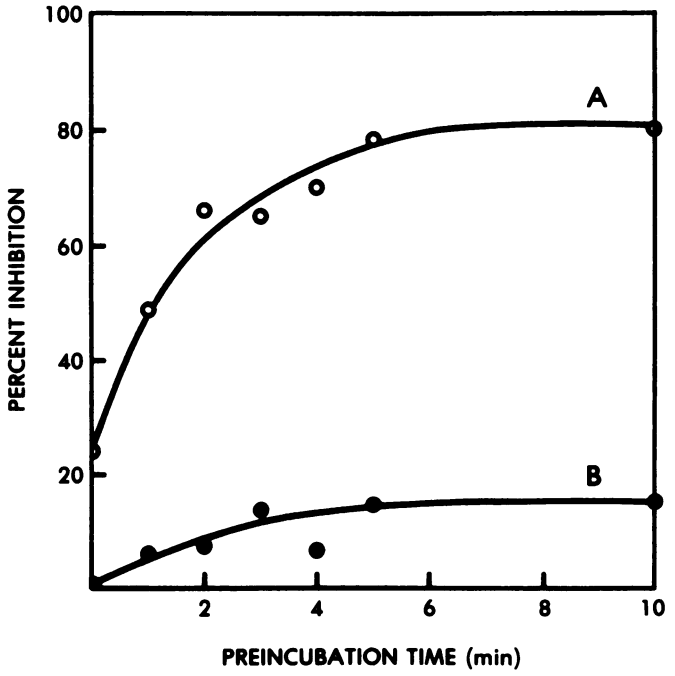

FIG. 5. Effect of preincubation time on per cent inhibition of α-amylase activity at pH 8.0 (A) and pH 5.5 (B). Protein used at pH 8.0 was 0.30 μg α-amylase and 0.20 μg inhibitor. Protein used at pH 5.5 was 0.15 μg α-amylase and 1.90 μg inhibitor. Enzyme reactions were allowed to proceed for 7 min.

amylase inhibitors (4). The development of a specific assay for the inhibitor would resolve this question.

During DEAE-Sephacel chromatography (Fig. 1), the inhibitor activity emerged in one peak at a conductivity between 400 and

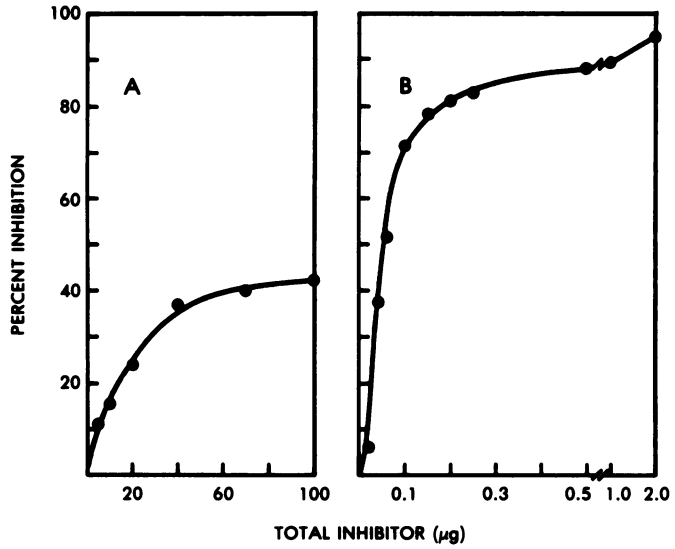

FIG. 6. Effect of inhibitor concentration on α-amylase activity at pH 5.5 (A) and pH 8.0 (B). A fixed amount of α-amylase (0.10 μg) was used. Enzyme and inhibitor were preincubated for 15 min prior to the addition of starch solution. Enzyme reactions were allowed to proceed for 8 min at pH 5.5 and 22 min at pH 8.0.

500 μMho in the salt gradient. The peak fraction had a salt concentration of approximately 70 mM NaCl and the peak of inhibitor activity coincided with a peak of *A* at 280 nm. Some starch hydrolyzing activity, as detected by the reducing sugar assay, was eluted later in the salt gradient and was due probably to β-amylase. Overall, ion-exchange chromatography was the most effective purification step, giving a purification of about 25-fold (Table I).

The inhibitor peak from DEAE-Sephacel chromatography was separated into two major protein peaks by gel filtration on a column of Bio-Gel P 60 (Fig. 2), but only one of these had inhibitory activity against α-amylase II. Total inhibitor activity from both chromatographic steps was determined after concentration of the active fractions. In each series of chromatography and concentration, a 10% loss was incurred (Table I). Some of this loss may have been due to adsorption of the inhibitor to the ultrafiltration membrane.

The homogeneity of the isolated inhibitor was demonstrated by the appearance of only one protein band after SDS-gel electrophoresis. High purity of the preparation also was supported by the sequencing of the first 20 N-terminal amino acids which readily identified a unique amino acid with each cycle of the sequenator. Previous analysis by polyacrylamide gel isoelectric focusing concomitant with silver staining for protein similarly revealed the high purity of the isolated inhibitor (24).

Electrophoresis of the inhibitor on SDS gels indicated a mol wt of 21,000 D (Fig. 3). Gel filtration on Bio-Gel P 100 (Fig. 4) indicated a mol wt of approximately 20,000 D for the inhibitor which is in good agreement with the electrophoretic data and suggests that the inhibitor is not a dimer. The mol wt of α-amylase II was estimated to be 32,000 D (Fig. 4) which is anomalously low compared to the generally accepted value of about 45,000 D (8). Similar low mol wt have been reported for wheat α-amylases using Bio-Gel as a sieving matrix (15).

Gel filtration of inhibitor plus α-amylase II at pH 8.0 yielded a new peak having a mol wt of 41,000 D (Fig. 4). This peak, presumably, was due to formation of an enzyme-inhibitor complex and would correspond to α-amylase III. Weselake *et al.* (24) have demonstrated the formation of an enzyme-inhibitor complex by isoelectric focusing of a solution of α-amylase and inhibitor. The complex appeared to correspond to α-amylase III.

A mol wt of about 52,000 D would be expected for the complex but the lower value obtained suggests that the complex may not behave like a globular protein during gel filtration.

Amino acid analysis (Table II) showed that the inhibitor contained about 9 half-cystine residues/mol but no detectable methionine. At this stage, the degree of disulfide bonding has not been determined. The presence of a relatively high proportion of aromatic amino acids accounts for the absorbance of the inhibitor at 280 nm. The ratio of aspartic and glutamic acid residues to basic residues was 1.4. This would suggest that some of the apparently acidic residues exist as amides since the isoelectric point of the inhibitor was found to be 7.3. The amino acid composition indicates clearly that the inhibitor is not a purothionin analog (19).

The relatively high content of half-cystine is a characteristic shared by barley trypsin inhibitor (16) and α-amylase inhibitor albumins from wheat kernels (3). However, α-amylase II inhibitor has a greater proportion of basic amino acid residues than does inhibitor albumins from wheat.

Results obtained from twenty cycles of the sequenator are shown in Table III. Despite the high half-cystine content of the inhibitor (Table II), no half-cystine was found in the first 20 N-terminal residues. Some homology appears to exist between the α-amylase inhibitor and trypsin inhibitor from barley (Table III). The amino acid residues were numbered sequentially from the N-terminal amino acid. Residues 14 to 16 (Leu-Arg-Ala) of the inhibitor coincided with residues 16 to 18 of the barley trypsin inhibitor. Once this degree of homology was established, proline 5 and tyrosine 20 of the inhibitor coincided then with proline 7 and tyrosine 22 of the barley trypsin inhibitor. However, there was no strong homology in the N-terminal region between inhibitor albumins from wheat (10, 18) and α-amylase II inhibitor.

Inhibition of enzyme activity did not occur instantaneously upon addition of inhibitor. Maximum inhibition was attained after 5 min of preincubation of enzyme and inhibitor (Fig. 5). The preincubation time required for maximum inhibition was similar at both pH 5.5 and 8.0. No further changes were observed after extended preincubations of up to 60 min. Similar preincubation requirements have been reported for the inhibition of animal α-amylase by inhibitor albumin from the wheat kernel (3).

Inhibition studies carried out between pH 5.5 and 8.0 showed that the inhibitor became increasingly more powerful as pH was increased. A detailed investigation of the effect of inhibitor concentration on α-amylase activity was made at pH values of 5.5 and 8.0 (Fig. 6). Under the experimental conditions used, an approximately 1100 M excess of inhibitor over α-amylase II was required to attain 40% inhibition at pH 5.5 (Fig. 6a), assuming mol wt of 20,000 and 45,000 for inhibitor and α-amylase, respectively. At pH 8.0, however, much higher levels of inhibition were achieved at significantly lower inhibitor concentrations (Fig. 6b). Only 1 mol of inhibitor/mol of enzyme was required to achieve a 40% inhibition of α-amylase II and 90% inhibition was achieved with a 23 M excess of inhibitor. These results offer an explanation for the α-amylase activity exhibited by α-amylase III (α-amylase II-inhibitor complex) after isoelectric focusing (24) or after gel filtration experiments carried out at pH 8.0 in this study. Although a strong complex between α-amylase II and inhibitor can be formed at pH 8.0 (forming α-amylase III) when the pH is lowered to 5.5 for α-amylase activity analysis, the complex is weakened. Thus, some α-amylase II is liberated and its activity then may be detected.

At present, the effect of pH on inhibitor activity is not clearly understood and more detailed studies must be made to clarify this effect as well as to determine the precise mechanism of inhibition. In the germinating seed, compartmented pH effects and location of the inhibitor could have an impact on physiological regulation of α-amylase. Immunochemical methods are being developed for the localization and specific determination of inhibitor in cereal kernels.

Note Added in Proof. Since this manuscript was submitted, Mundy *et al.* (1983 [*Carlsxberg Res. Commun.* 48: 81–90] and Hejgaard *et al.* (1983 [*Carlsberg Res Commun.* 48: 91–94] have published a similar study.

Mundy J, IB Svendsen, J Hejgaard 1983. Barley α-amylase/subtilisin inhibitor. I. Isolation and characterization. *Carlsberg Res. Commun.* 48: 81–90.

Hejgaard J, IB Svendsen, J Mundy 1983. Barley α-amylase/subtilisin inhibitor. II. N-terminal amino acid sequence and homology with inhibitors of the soybean trypsin inhibitor (Kunitz) family. *Carlsberg Res. Commun.* 48: 91–94.

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