

Inhibition of Rye α -Amylase Activity by Barley α -Amylase Inhibitor

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ABSTRACT

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Endogenous α -amylase inhibitors from barley, wheat, soy, and rye were found to inhibit rye α -amylase. The inhibitor from barley was studied in detail. This proteinaceous molecule was stable at alkaline pH range and most active when the pH was over 7.0. The rye α -amylase-inhibitor

complex was not heat stable, but bovine serum albumin stabilized the complex. The falling number and amylograph tests showed that the inhibitor could be considered a potential α -amylase inhibitor in breadmaking when flour made of sprout-damaged rye is used.

The endogenous α -amylase inhibitor of barley was first studied by Mundy et al (1983) and Weselake et al (1983a,b). They found that the inhibitor is a low-molecular-weight protein (21,000) with an isoelectric point of 7.1-7.3. The inhibitor inhibits endogenous α -amylase II (high-pI amylases) of barley and wheat. The inhibitor activity is also detectable in wheat and rye (Weselake et al 1985). The inhibitor content of barley seeds was found to be highest at early seed development (Robertson and Hill 1989).

In Scandinavia, especially in Finland, consumption of rye bread is high. Depending on the harvest year, a significant amount of the rye produced can be damaged by preharvest sprouting. α -Amylase inhibitors have been previously studied in breadmaking by Zawistowska et al (1988). They found that the baking result from wheat flour containing malted barley flour could be improved by adding the inhibitor.

The aim of this work was to study the endogenous α -amylase inhibitor of barley as a potential improver of the baking quality of a flour made of sprout-damaged rye. The stability of the barley inhibitor and the heat stability of the inhibitor-enzyme complex

were studied. Falling number and amylograph tests were used to evaluate the potential value of the inhibitor in rye bread-baking.

MATERIALS AND METHODS

Extraction of Ground Kernels

The inhibitor activity of seeds of two-rowed barley, oat, rye, spring wheat (varieties commonly used in Finland), and soy were studied. Rye α -amylase was purified from germinated rye kernels (falling number 60).

The kernels were ground in a Buhler's laboratory mill. Meals were extracted with acetate buffer (pH 5.5, 0.05M) at a ratio of 1 kg of kernels to 3 L of buffer (1 hr at room temperature). The extracts were centrifuged (9,000 rpm, 30 min, 5°C) and the supernatants clarified by filtration.

The α -amylase inhibitor activities were determined in the supernatant aliquots that were acid-treated to eliminate endogenous α -amylase activities according to Henson and Stone (1988). The acid inactivation of α -amylase was done by lowering the pH of supernatants to 3.5-3.8 with acetic acid. After 1 hr of incubation at room temperature, the pH of the acidified aliquots was adjusted to 7.5.

Purification Procedures

Rye α -amylase II was purified from the supernatant of germinated rye kernels (falling number 60). The α -amylase II (α -amylase

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isoenzymes with high pI; MacGregor et al 1988) was prepared by ion-exchange. Extract from 500 g of rye was bound to 50 ml of CM-Sepharose cation exchanger (Pharmacia) in 0.05M Na-acetate buffer (pH 5.0) containing 1 mM CaCl₂. The gel was packed into a Pharmacia K26/40 column and was eluted first with 0.05M Na-acetate (pH 5.0) and then with 1.0M NaCl (pH 5.0) at a flow rate of 2 ml/min. The total volume of each solvent was 100 ml. The α -amylase fractions were determined by isoelectric focusing followed by enzymatic activity staining (zymogram). Fractions containing the high-pI (pI > 5.0) enzyme were pooled. Proteins from this solution were precipitated by ammonium sulfate (40% w/v).

Inhibitor was purified from barley extract with Pharmacia Cuminodaoacetic acid-epoxy (IDA)-Sepharose 6B gel according to the method of Zawistowska (1988). The IDA-Sepharose 6B was washed with deionized water, and the copper was bound to the gel from a solution containing CuSO₄·5H₂O (6 mg/ml). The extract from 1 kg of barley was bound to 50 ml of gel equilibrated with 0.05M Tris/HCl-buffer, pH 7.5, containing 0.15M NaCl (equilibrium buffer). The gel was packed into the Pharmacia K26/40 column and eluted with 0.05M and 0.2M glycine in equilibrium buffer at a flow rate of 0.5 ml/min. The fractions containing inhibitor were combined. The copper that was eluted from the column was removed from this solution by gel filtration on Sephadex G25 (Pharmacia). From 1 kg of barley, 100 mg of inhibitor protein was obtained.

α -Amylase and Inhibitor Assays

α -Amylase activities were assayed by an α -amylase test kit (Food Application) from Pharmacia. The assay is based on the use of dyed amylase substrate. Amylase solution was added to a test tube containing 5 ml of buffer (maleic acid-NaOH, pH 6.0, with 1 mM CaCl₂) and one substrate tablet. After 15 min of incubation at 45°C, the reaction was stopped with 1 ml of 1M NaOH. Samples were filtered, and the absorbance of supernatants was measured at 620 nm. Activities were calculated in Phadebas Units, which were obtained from the standard curve based on the European Brewery Convention's α -amylase units per milligram of malt (1 U = 0.0167 μ mol/sec).

In inhibitor assays, the appropriately diluted inhibitor solution and the α -amylase II solution were preincubated for 15 min at room temperature. From this preincubated solution, 1 ml was taken for the α -amylase assay, which was performed as described above. Inhibitor activity was calculated in anti- α -amylase units.

Half-Life Studies

Half-life studies were done at different pH values (6.5–10.5) at 60°C. The inactivation was followed by taking samples every 5 min for 30 min. To stop thermal inactivation, the samples were placed in an ice bath. Samples were analyzed according to the inhibitor assay described above.

Half-lives of inhibitor at different pH levels at 60°C were calculated with the aid of an RS/1-computer program (Bolt Beranek and Newman Inc. 1984). It is based on the Arrhenius equation:

$$T_{1/2} = \ln 2/k,$$

where $T_{1/2}$ = the half-life of the protein and k = the rate constant of inactivation.

Heat Stability Studies

The heat stability of inhibitor-amylase complexes was studied by using starch tablets from Pharmacia as substrate. Samples were preincubated at different temperatures (25–70°C) for 15 min and then assayed at a fixed temperature as usual. Activities were calculated as described for α -amylase and inhibitor assays.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (gradient 8–25% total polyacrylamide, 2% cross-linker) was performed with the Pharmacia Phast System. Proteins

were detected on the gels by the Pharmacia silver staining procedure optimized for native gels. For the isoelectric focusing gels, staining methods for amylase and inhibitor activity were also used. In amylase activity staining, gels were first incubated in 1% starch solution in distilled water for 15 min at 45°C and then stained with a solution of 4.0% KI in 0.01% I₂. The bands showing amylase activity were white on the blue stained gel. In the inhibitor activity staining, the gel was first covered with solution containing α -amylase II (1 hr at room temperature) and was then treated with starch and iodine solutions as described above. The bands showing inhibitor activity were blue on the colorless gel.

Falling Number and Amylograph Tests

Falling number was determined according to the standard method of the AACC (1983), and the amylograph test was performed according to the ICC method (1984).

RESULTS AND DISCUSSION

Purified rye α -amylase II represented about 30% of the total amylase activity of this rye sample, according to the CM-Sepharose eluate. The amount was quite low considering that MacGregor et al (1988) found that high-pI amylase made up 80% of total α -amylase activity in sprout-damaged rye. Isoelectric focusing showed some α -amylase II still present in the supernatant after CM-Sepharose treatment, which is one reason for the low yield of α -amylase II in the eluate. On the other hand, in this study the total amylase activity may also include β -amylase activity because no heat treatment was done on the crude extract to eliminate the β -amylase activity. The pIs of β -amylases are below 5.0, so in CM-Sepharose treatment at pH 5.0 they did not bind to the gel. The eluted proteins that showed amylase activity were high-pI α -amylases (α -amylase II).

The α -amylase II of rye was inhibited by the acid-treated extracts of barley, rye, wheat, and soy. The oat extract did not show any inhibition activity. According to Weselake et al (1985), oat extract does not inhibit wheat and barley α -amylases. The specific inhibitor activity of the tested kernels was highest in barley (Table I). We estimate that as much as 25 kg of barley would be needed to inhibit a significant proportion of the α -amylase activity of 1 kg of sprout-damaged rye. Thus, the purification of the inhibitor from barley seeds for industrial applications is not economically feasible.

The inhibitor of barley was studied further. From 1 kg of barley, 100 mg of enriched inhibitor protein was obtained. The yield was 67%, which was somewhat lower than the 80.9% obtained by Zawistowska et al (1988). SDS-PAGE (Fig. 1) showed that the major part of this protein fraction consisted of a single protein with a molecular weight of 21,000 and an isoelectric point of 7.3. Thus the molecular properties of the inhibitor were the same as described earlier by Mundy et al (1983) and Weselake et al (1983a).

The inhibitor was most stable at the alkaline pH range. The half-lives of the inhibitor at different pH levels at 60°C are shown in Figure 2.

The effect of dosage of the purified inhibitor on the constant

TABLE I
Inhibitor Activity in Different Grains and in Soy Extracts^a

Source of Inhibitor	Protein (mg/ml)	Inhibitor Activity		Equivalent Amount ^b (g)
		(anti-U/ml)	(anti-U/mg protein)	
Barley	3.0	0.36	0.120	26
Rye	10.2	0.30	0.030	32
Wheat	5.6	0.08	0.010	120
Oat	6.4	Negligible
Soy	27.0	0.17	0.006	56

^a Amount of protein and activities correspond to 0.5 g of starting material.

^b Amount of raw material needed for 80% inhibition of an α -amylase II preparation.

amount of α -amylase II at pH 7.5 can be seen in Figure 3. Maximally, about 80% inhibition could be obtained. Similar results were obtained by Mundy et al (1983).

The degree of inhibition was dependent on the pH of the reaction solution (Fig. 4). α -Amylase was inhibited completely by the inhibitor only in alkaline conditions above pH 8.0. The α -amylase activity is pH-dependent also, but at pH 5.0, 8.0, and 9.0 the

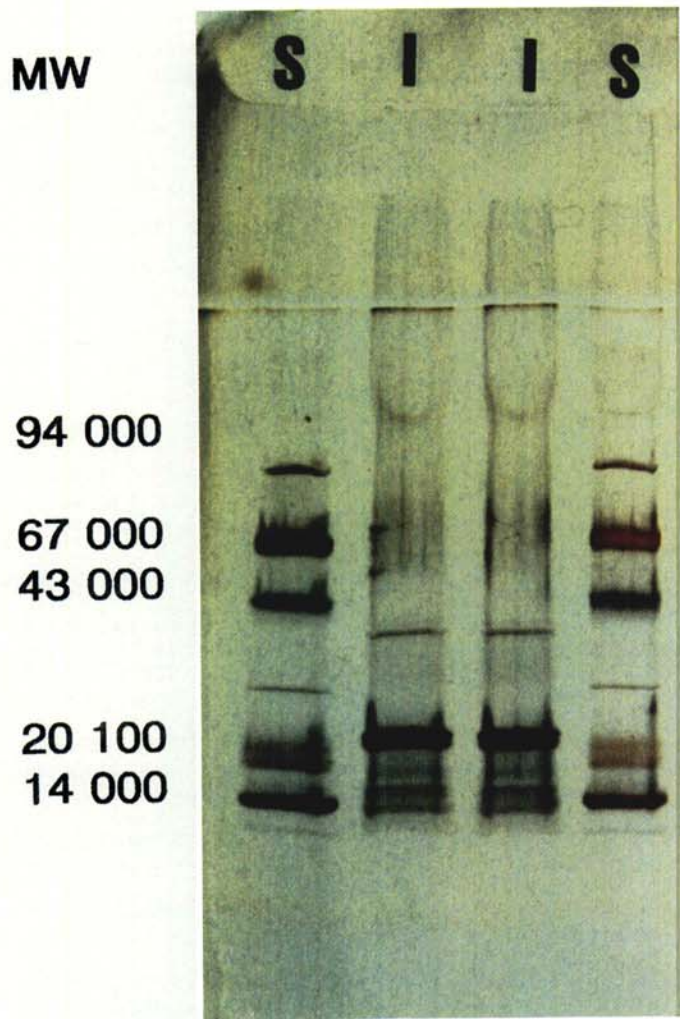


Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; silver staining. S = standard proteins (Pharmacia), I = inhibitor preparation (10 μ g of protein per gel), MW = molecular weight.

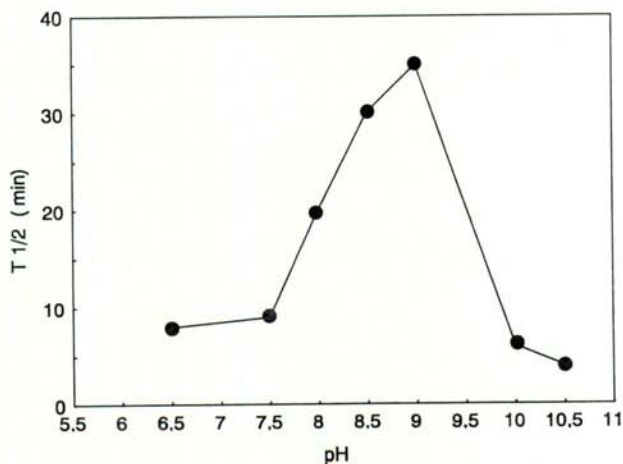


Fig. 2. Half-lives ($T_{1/2}$) of the purified inhibitor at different pH levels. The protein concentration in the inhibitor preparation was 150 μ g/ml and the α -amylase II content 30 μ g/ml in the inhibitor assays.

enzyme is still active. This result implies that the inhibitor cannot be fully active (<40%) in Scandinavian sour bread processes, where the pH of the dough can be as low as 4.5. On the other hand, the pH of dough used in crisp bread production is around 6. In such doughs, about 50% of the α -amylase II activity could be inhibited. In real doughs, however, the inhibition reaction may function differently than in the ideal conditions used in this study.

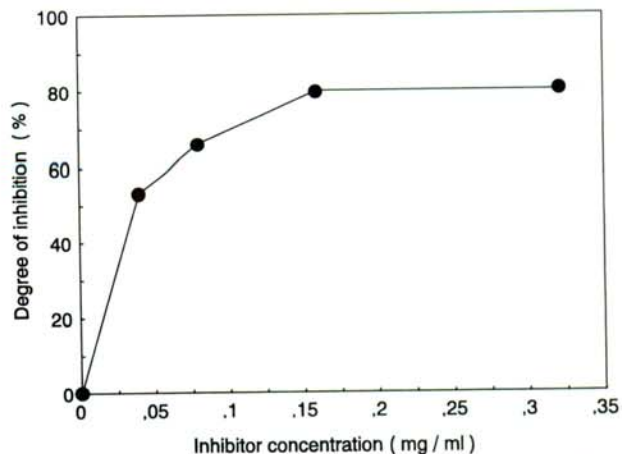


Fig. 3. Effect of inhibitor dosage on the activity of α -amylase II of rye at pH 7.5. The concentration of α -amylase in the test was 30 μ g/ml.

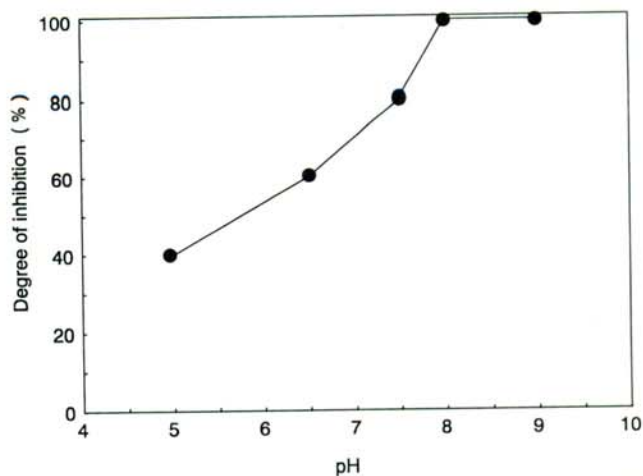


Fig. 4. pH dependence of the inhibition of α -amylase II activity. The protein concentration in the enriched inhibitor preparation was 150 μ g/ml and the α -amylase II concentration 30 μ g/ml in the inhibitor assays.

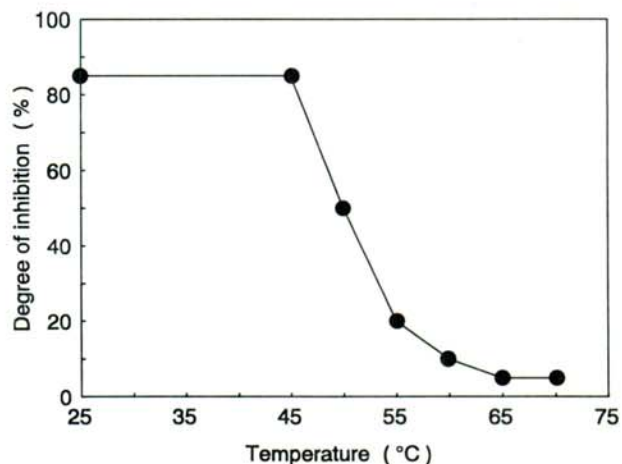


Fig. 5. Heat stability of the inhibitor- α -amylase II complex. The protein concentration in the enriched inhibitor preparation was 150 μ g/ml, and α -amylase concentration was 30 μ g/ml; pH 7.5.

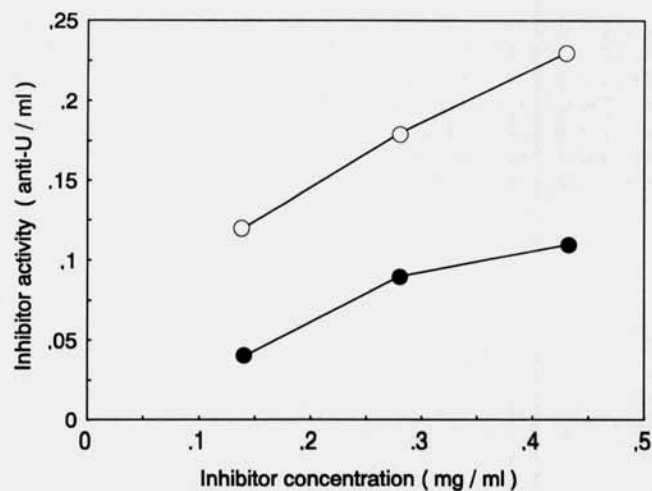


Fig. 6. Stabilizing effect of bovine serum albumin (BSA) on the inhibitor-amylose complex at pH 6.0. ● = reaction in buffer at pH 6.0, ○ = reaction in 0.2% BSA solution at pH 6.0.

The heat stability of α -amylase inhibition is shown in Figure 5. When the temperature reached 55°C, decomposition of the complex was rapid, showing that the inhibition is temperature dependent. The pH and temperature dependence of the inhibitor reaction was similar to that described previously by Weselake et al (1983a,b). Although the inhibitor-amylose complex is not very heat stable, this is not crucial since at temperatures over 55°C heat denaturation of α -amylase is rapid. In real baking conditions, the complex may be stabilized by various components of the dough. For example, bovine serum albumin stabilized the complex at room temperature (Fig. 6), indicating that other proteins in dough might have a stabilizing effect on the inhibition.

Falling number and amylograph tests were used to study the effects of the α -amylase II inhibitor from barley. The effect of dosage of the inhibitor on the falling number of the rye flour can be seen in Figure 7. The falling number was raised from 120 to 170 sec by the addition of the inhibitor. The maximum increase could be obtained when the amount of inhibitor preparation in the flour was 0.37%. In an amylograph test of a low-quality rye flour, a peak increase from 260 to 320 BU was found with 0.07% (w/w) inhibitor in the flour.

According to these results, to significantly improve the baking quality of the sprout-damaged rye, the inhibitor content should be around 0.4% (w/w) in the flour. This means that for 300-g loaves, 1.1 g of the purified inhibitor is needed. If the yield of the inhibitor is approximately 0.01 (w/w) in barley, about 11 kg of barley is needed to obtain 1.1 g of the purified inhibitor. The use of purified inhibitor to increase the applicability of sprout-damaged rye seems to be economically infeasible. A sponge and dough method, where bacteria and/or yeasts of the sponge are able to excrete α -amylase inhibitor in necessary quantities, might be economical. Such bacteria or yeasts are not known, but such organisms could be constructed with gene engineering techniques. Alternatively, the properties of the inhibitor might be modified by protein engineering techniques.

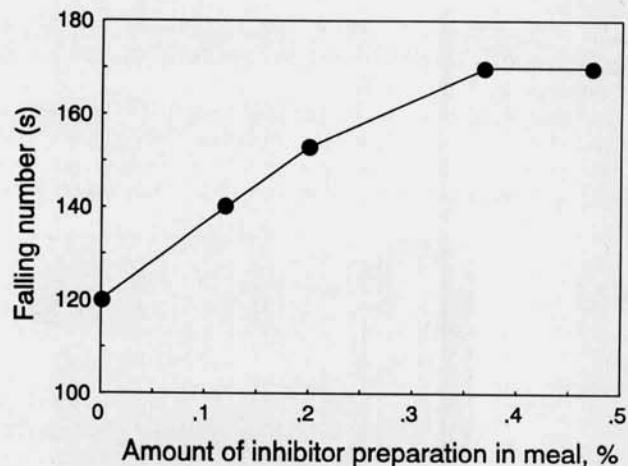


Fig. 7. Falling number tests of rye flour containing different amounts of the inhibitor preparation. The amount of inhibitor protein (0.37%) needed for maximal effect was 0.29 mg.

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