

Endogenous Alpha-Amylase Inhibitor in Various Cereals¹

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ABSTRACT

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An endogenous α -amylase inhibitor was detected in various cereal grains by an immunochemical method and by inhibition of α -amylase activity. Immunochemical studies showed that barley cultivars contained a protein that had complete immunochemical identity with the purified inhibitor. Wheat cultivars, rye, and triticale contained protein that showed only partial immunochemical identity with the purified inhibitor, but sorghum,

oats, millet, rice, and maize did not elicit an immunochemical response. Inhibitor activity was found in all barley cultivars, wheat, rye, and triticale but not in sorghum, oats, millet, rice, or maize. These findings indicate that endogenous α -amylase inhibitors are not restricted to barley but are also present in other cereals. Examination of pearling fractions of barley indicated that the inhibitor was distributed throughout the endosperm.

Cereals contain proteins with inhibitor activity against animal α -amylases (Kneen and Sandstedt 1943, 1946; Shainkin and Birk 1970; Silano et al 1973), but endogenous inhibitors of cereal α -amylases have been reported only recently. Warchalewski (1977a,b) showed that endogenous α -amylase inhibitors were in both durum and winter wheat. Jones and Meredith (1982) suggested that purothionins might inhibit wheat α -amylase by controlling calcium availability. A protein, isolated from a maize cultivar, was found to inhibit maize α -amylase but was not effective against other cereal α -amylases tested (Blanco-Labra and Iturbe-Chinas 1981). Endogenous α -amylase inhibitors with similar properties have been isolated from both barley (Hejgaard et al 1983, Mundy et al 1983, Weselake et al 1983a,b) and wheat (Mundy et al 1984). These inhibitors were also found to have inhibitor activity against subtilisin, a microbial protease (Mundy et al 1983, 1984), and many properties in common with a subtilisin inhibitor previously isolated from barley (Yoshikawa et al 1976). The barley α -amylase inhibitor was biospecific for α -amylase II (Weselake et al 1983a), the major α -amylase component of malted barley (MacGregor and Ballance 1980), and germinated wheat (Sargeant and Walker 1978, Weselake and Hill 1983). When this inhibitor protein is incubated with barley α -amylase II an enzyme-inhibitor complex (Weselake et al 1983a,b), previously referred to as α -amylase III (MacGregor and Daussant 1979), is formed.

This study was done to determine whether the α -amylase inhibitor from barley was present in cereal grains other than barley and wheat. Also, an attempt was made to determine the distribution of the inhibitor in the barley kernel.

MATERIALS AND METHODS

Enzyme and Inhibitor Purification

α -Amylase II was purified from a green malt of barley (*Hordeum vulgare* cv. Bonanza), and α -amylase II inhibitor was purified from barley kernels (*Hordeum distichum* cv. Klages), as described previously (Weselake et al 1983a,b). Protein was determined by the method of Lowry et al (1951), using bovine serum albumin (BSA) as a standard.

Antibody Production

A young female rabbit, weighing approximately 2.5 kg, was bled at the ear for control serum, which was kept frozen at -15°C until

required. A stock solution of purified inhibitor (the antigen) containing 1.74 mg/ml of protein was prepared in a 5 mM Tris-HCl buffer, pH 8, that contained 0.9% NaCl and 1 mM CaCl_2 . Freund's complete adjuvant ([0.40 ml] Difco Laboratories, Detroit, MI; cat. no. 0638/59; batch no. 5864/82) was mixed thoroughly with an equal volume of stock inhibitor solution. The mixture was administered to the rabbit intradermally in a shaved area on the lower back. The rabbit received 300 μg of protein in the primary injection. Subsequent booster shots (100 μg each) were administered at 14 and 31 days after the primary injection. Bleedings of 5-10 ml were taken between injections and the serum retained at -15°C for future analysis.

Antiserum (2.5 ml) from a bleeding taken at day 54 was thawed, and the immunoglobulin G (IgG) fraction was isolated. The antiserum was diluted to 7.5 ml with distilled water and treated with 1.84 g of ammonium sulphate. The mixture was equilibrated overnight at room temperature and then centrifuged at $10,000 \times g$ for 20 min. The pellet was resuspended in 5 ml of 10 mM sodium phosphate buffer (pH 8), and the IgG component was isolated by anion-exchange chromatography, essentially as described by Fahney (1967) except that diethylaminoethyl (DEAE)-Sephacel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was substituted for DEAE-cellulose. The unretained peak, containing the IgG fraction, was concentrated to 2.5 ml (Amicon PM10), dialyzed overnight at 4°C against 0.9% NaCl, and kept frozen at -15°C .

Analysis of Cereal Extracts by Ouchterlony Double Diffusion

Barley cultivars, (Klages, Bonanza, and Himalaya), wheat cultivars (Neepawa, Columbus, and Northstar), durum wheat, rye, triticale, sorghum, oats, millet, rice, and maize were surveyed. Approximately 10-g portions of cereal kernels were finely ground in a coffee mill (Braun AG, type KSM1, Frankfurt, W. Germany). Samples of 500 mg were extracted with 2.5 ml of 0.9% NaCl at room temperature for 15 min on a Labquake shaker (Lab Industries, Berkeley, CA). Supernatant solutions were retained after being centrifuged at $13,000 \times g$ for 20 min. One milliliter of each extract was concentrated approximately sevenfold in a B-15 Minicon (Amicon) concentrator unit. The remainder of each extract was frozen (-15°C) and retained for determination of inhibitor activity. The concentrated extracts were qualitatively analyzed for inhibitor, using the Ouchterlony (1967) double diffusion system and apparatus purchased from the Gelman Instrument Co. (Ann Arbor, MI).

The diffusion matrix, consisting of 1% agarose A (Pharmacia), 0.9% NaCl, 1% polyethylene glycol 8000, and 0.02% sodium azide, was poured on microscope slides in an immunodiffusion frame. Control serum or purified IgG (6 μl of 11.6 mg/ml protein) were placed in center wells. Outer wells received 6- μl aliquots of purified inhibitor (1 μg) or concentrated extracts, and diffusion was allowed to proceed for 24 hr at room temperature. Diffusion plates were then washed over a period of two days, with three changes of 0.9% NaCl. After a final wash with distilled water for 1 hr, the agarose was dried with a hair dryer to a thin film on the microscope slides. Slides were removed from the immunodiffusion frame and stained

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individually for protein in plastic trays, using the silver stain procedure of Willoughby and Lambert (1983).

Analysis of Inhibitor Activity in Cereal Extracts

Frozen extracts were thawed, and a modified Briggs (1961) assay was adapted to determine inhibitor activity at pH 8 (40 mM Tris-HCl, 1 mM CaCl₂), essentially as described by Weselake et al (1983b). Extracts of Klages and Bonanza barley were diluted 500-fold with assay buffer. Extracts of Himalaya barley, all wheat cultivars, durum wheat, rye, and triticale were diluted 200-fold, and the remaining cereal extracts were diluted 100-fold. One milliliter of diluted extract was preincubated (15 min at 35°C) with 1 ml of α -amylase II (0.043 μ g, 30 IDC units at pH 5.5) in a buffer

containing 100 μ g of BSA. Reactions were allowed to proceed for 15 min after β -limit dextrin solution (2 ml) was added. The coefficient of variation for the determination of inhibitor activity in extracts was found by extracting six portions (500 mg) of ground Klages barley and assaying each for inhibitor activity.

Preparation and Analysis of Pearling Fractions

Pearling fractions were prepared, using a barley pearler, from 25 g of barley (*Hordeum distichum* cv. Klages) kernels. Fractions were removed at 20-sec intervals up to 120 sec, and the remaining core was ground to meal in a Udy mill. Samples of 500 mg from each fraction, including the core, were extracted with 2.5 ml of 20 mM sodium acetate buffer (1 mM CaCl₂, pH 5.5) at room temperature

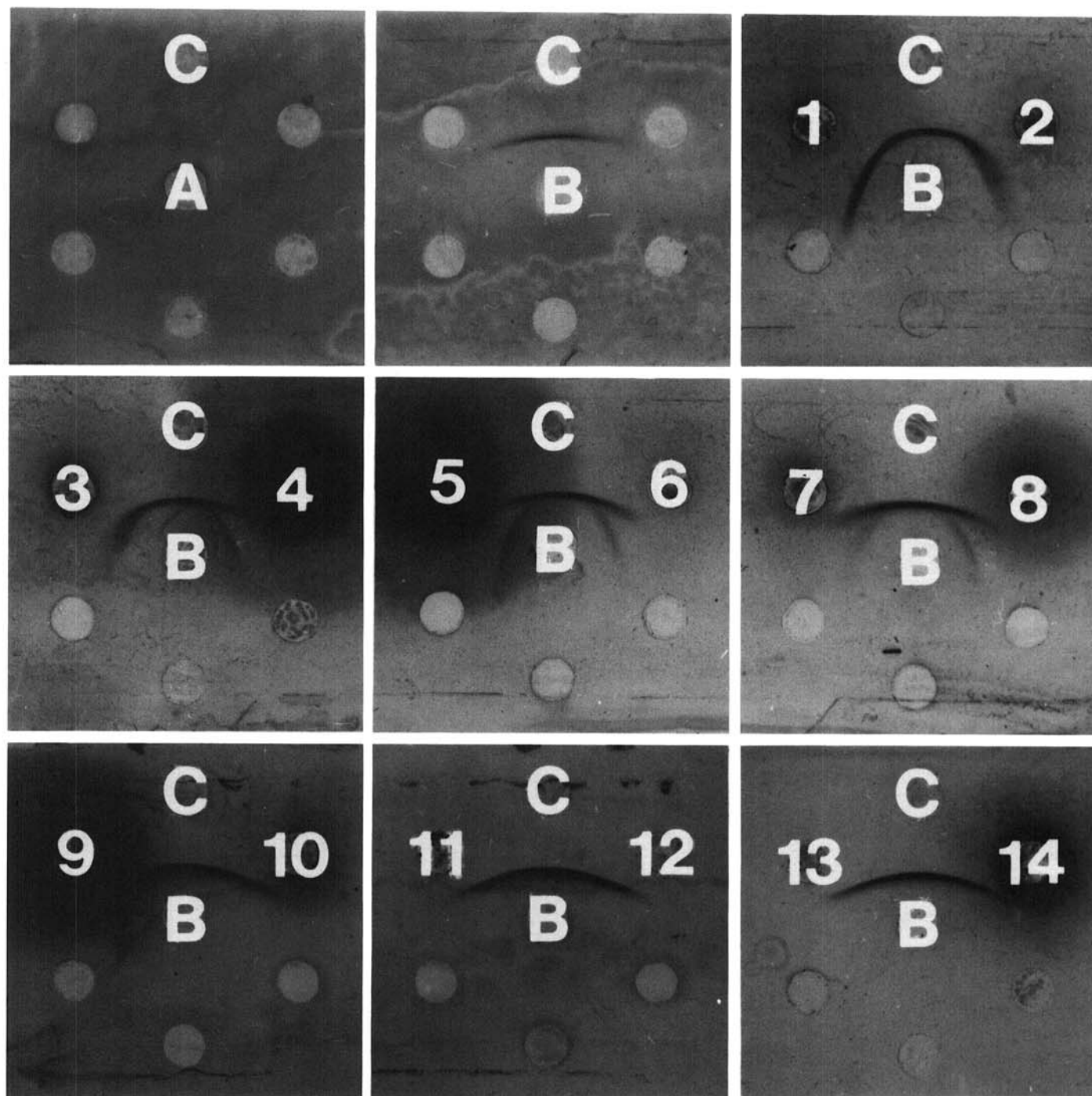


Fig. 1. Ouchterlony double-diffusion analysis of extracts of cereal kernels: (A) preimmune serum, (B) anti-inhibitor IgG, (C) purified inhibitor, (1) *Hordeum distichum* cv. Klages (barley), (2) *H. vulgare* cv. Bonanza (barley), (3) *H. vulgare* cv. Himalaya (barley), (4) *Triticum aestivum* cv. Neepawa (wheat), (5) *T. aestivum* cv. Columbus (wheat), (6) *T. aestivum* cv. Northstar (wheat), (7) *T. turgidum* cv. Wakooma (durum wheat), (8) *Secale cereale* cv. Puma (rye); (9) *Triticosecale wittmack* (secondary hexaploid triticale), (10) *Sorghum bicolor* (white sorghum), (11) *Avena sativa* (oats), (12) *Pennisetum americanum* (millet), (13) *Oryza* spp. (rice), and (14) *Zea mays* (maize).

for 60 min on a Labquake shaker. Supernatant solutions were retained after being centrifuged at $13,00 \times g$ for 20 min. The extracts were analyzed for protein content and inhibitor activity at pH 8.

Removal of the Husk

Barley kernels were soaked in 70% sulphuric acid for 90 min, filtered over glass wool, and washed thoroughly with distilled water. The remaining portions of the husk were removed by hand, and the naked kernels were allowed to dry at room temperature overnight. Both intact and dehusked kernels were finely ground in a coffee mill and assayed for inhibitor activity.

RESULTS AND DISCUSSION

Cereal extracts were assayed by two techniques for the presence of α -amylase II inhibitor. In one series of experiments, antibodies raised against purified inhibitor from barley were tested for their ability to form an insoluble antigen-antibody complex when allowed to diffuse against cereal extracts in an agarose matrix. A second approach involved the direct determination of inhibitor activity in cereal extracts. Assays were done with appropriately diluted extracts at pH 8 under conditions in which the affinity of the inhibitor for α -amylase II was high (Weselake et al 1983b).

Specific cultivars of barley, wheat, durum wheat, and rye were used, but complete identifying information was not available for the other cereals examined. Before immunological assays were done, extracts were concentrated to intensify precipitin reactions. Results of immunodiffusion experiments are shown in Figure 1. Purified antibody, applied to a central well in the agarose matrix, was allowed to diffuse against the extract in question, which was applied to a peripheral well. Another well, adjacent to the test extract, received inhibitor purified from Klages barley. The precipitin line produced by the antibody-inhibitor interaction served as a criterion of identity when characterizing the precipitin reactions that occurred when related antigens in the test extracts reacted with the same antibodies. Following diffusion and washing and drying of immunodiffusion plates, the precipitin lines were silver stained for protein. Preimmune serum (Fig. 1, well A) failed to show a precipitin reaction with purified inhibitor (C), thereby serving as a control to eliminate the possibility of artifacts. Antibody was used in the form of an isolated IgG fraction because the absence of other serum proteins reduced the washing time required to remove nonreacted proteins from the system. This was important, especially when using the highly sensitive silver stain. In all cases, the antibody fraction (B) and the purified inhibitor (C) formed a precipitin line in the matrix between the two wells.

Extracts of Klages (1), Bonanza (2), and Himalaya (3) barleys formed precipitin lines with purified antibody (B). These lines fused with the lines formed from the precipitin reaction of purified inhibitor and antibody fraction, which indicated that all of these barley cultivars contained an antigen that was immunochemically identical to the purified inhibitor—thus providing evidence that the same inhibitor was present in each cultivar of barley examined. Extracts of Neepawa (4), Columbus (5), and Northstar (6) hexaploid wheats also reacted with the antibody. Precipitin lines generated by the wheat extracts fused with the precipitin line formed by the purified inhibitor (C), but part of the precipitin line extended further as a spur, due to the purified inhibitor. Therefore, the wheat cultivars contained a component that shared only partial immunochemical identity with the purified inhibitor. These results suggest that an inhibitor protein in wheat shares a high degree of amino acid sequence homology with the α -amylase II inhibitor from barley, but the sequences of the two proteins are not identical. Recently, an endogenous α -amylase inhibitor was isolated from wheat (*Triticum aestivum* cv. Solid) kernels (Mundy et al 1984). N-terminal sequencing studies indicated that this inhibitor shared 96% homology with barley α -amylase II inhibitor in the 45 N-terminal amino acid residues examined. Partial immunochemical identity was also found for extracts of durum wheat (7), rye (8), and triticale (9), which indicates that these cereals probably contain inhibitor also. Extracts of sorghum (10), oats (11), millet (12), rice (13), and maize (14) failed to show immunochemical precipitin lines, strongly indicating that these cereals did not contain a similar inhibitor or even a closely related protein.

Results of the immunochemical analysis and determinations of extractable inhibitor activity are summarized in Table I. The coefficient of variation of inhibitor activity was 4.5% for six extractions of Klages barley, which indicates that the overall procedure of extraction and subsequent determination of inhibitor activity was highly reproducible. Inhibitor activity was found only in extracts that showed an immunochemical reaction with the antibody. Therefore, the two analyses were correlated in this respect. There was considerable variation in extractable inhibitor activity among barley cultivars examined, but the values were higher than those obtained for the other grains. Bonanza barley had the highest inhibitor activity per gram of ground seed, and this activity was more than twice the activity of Himalaya barley. Inhibitor activity in extracts of wheat cultivars, rye, and triticale ranged from 3,330 to 4,330 anti-IDC units per gram of ground seed, indicating that the levels of inhibitor in these seeds were comparable. The wheat cultivar, Columbus, is known to be highly resistant to sprouting (Campbell and Czarnecki 1981, Noll 1983), but inhibitor levels in extracts of this cultivar were not abnormally high when compared to Neepawa and Northstar wheats. Therefore, it is unlikely that sprouting resistance of Columbus wheat can be attributed to this type of endogenous inhibitor. However, inhibitors from the wheat cultivars, rye, and triticale may be more

TABLE I
Immunochemical Reactivity and Inhibitor Activity
of Cereal Kernel Extracts

Cereal	Immunochemical Identity	Inhibitor Activity ^a (anti-IDC units/g of seed)
<i>Hordeum distichum</i> cv. Klages (barley)	Complete	8,325
<i>H. vulgare</i> cv. Bonanza (barley)	Complete	13,325
<i>H. vulgare</i> cv. Himalaya (barley)	Complete	5,330
<i>Triticum aestivum</i> cv. Neepawa (wheat)	Partial	3,795
<i>T. aestivum</i> cv. Columbus (wheat)	Partial	3,330
<i>T. aestivum</i> cv. Northstar (wheat)	Partial	4,330
<i>T. turgidum</i> cv. Wakooma (durum wheat)	Partial	3,665
<i>Secale cereale</i> cv. Puma (rye)	Partial	3,330
<i>Triticosecale wittmack</i> (secondary hexaploid triticale)	Partial	4,000
<i>Sorghum bicolor</i> (white sorghum)	np ^b	... ^c
<i>Avena sativa</i> (oats)	np	...
<i>Pennisetum americanum</i> (millet)	np	...
<i>Oryza</i> spp. (rice)	np	...
<i>Zea mays</i> (maize)	np	...

^a Coefficient of variation = 4.5%.

^b No precipitin reaction.

^c Not detectable.

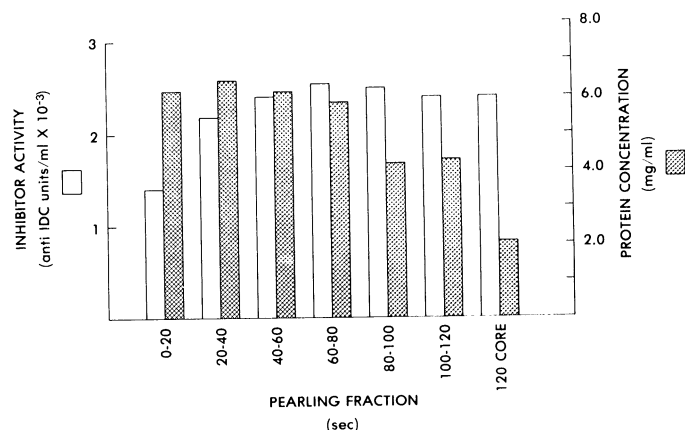


Fig. 2. Inhibitor activity and protein content of extracts of barley pearling fractions.

or less effective against their own endogenous α -amylases. Therefore, the study could be somewhat misleading because barley α -amylase II provided the reference enzyme activity upon which all inhibitor activity determinations were based. Results of both the immunochemical study and inhibitor activity survey do suggest, however, that endogenous α -amylase inhibitor proteins are not restricted to barley.

Pearling studies were done to determine the distribution of inhibitor in the barley kernel. Pearling fractions of barley kernels were sequentially collected after different degrees of abrasion and after 120 sec of pearling, the remaining core fraction constituted approximately 40% of the starting material. Equal portions of each pearling fraction were extracted with a buffer, and the extracts were analyzed for inhibitor activity and protein content. Inhibitor activity of the extracts remained relatively constant at 2,000–2,500 anti-IDC units/ml of extract from the 20- to 40-sec pearling fraction to the remaining core fraction (Fig. 2). Inhibitor activity of the first pearling fraction (0–20 sec) was considerably lower (1,400 anti-IDC units/ml) than in other fractions. This reduction in activity was probably attributable to the presence of a high proportion of abraded husk in the first pearling fraction. Removal of the husk by sulphuric acid treatment did not affect the total inhibitor activity per barley kernel, indicating that husks did not contain detectable inhibitor activity.

The protein content of extracts of pearling fractions decreased as the core of the seed was approached (Fig. 2). This would be expected because the highest concentration of protein in cereal kernels is in the outer regions of the kernel (Morris et al 1946, Novacek et al 1966). Extracts of the 20- to 40-sec pearling fraction and the remaining core fraction contained 6 and 2 mg/ml of protein, respectively. Based on this extractable protein, the specific activity of the inhibitor was 340 and 1,250 anti-IDC units/mg of protein in these two fractions, respectively, which indicated that the inhibitor was not localized where soluble protein concentration was highest in the kernel. A more precise localization of inhibitor in the endosperm would require analysis by more elaborate techniques, such as immunohistochemistry.

It is not known whether the total inhibitor content per kernel changes during germination, but the presence of a high proportion of α -amylase III relative to α -amylase II in green malt (MacGregor and Ballance 1980) indicates that the inhibitor remains active during germination. Therefore, the inhibitor does not appear to be degraded by proteolytic enzymes during germination, indicating that it probably does not serve as a reserve protein.

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LITERATURE CITED

- BLANCO-LABRA, A., and ITURBE-CHINAS, F. A. 1981. Purification and characterization of an α -amylase inhibitor from maize (*Zea mays*). *J. Food Biochem.* 5:1.
- BRIGGS, D. E. 1961. A modification of the Sandstedt, Kneen, and Blish assay of α -amylase. *J. Inst. Brew.* 67:427.
- CAMPBELL, A. B., and CZARNECKI, E. M. 1981. Columbus hard red spring wheat. *Can. J. Plant Sci.* 61:147.
- FAHNEY, J. L. 1967. Chromatographic separation of immunoglobulins.

- Page 321 in: *Methods in Immunology and Immunochemistry*. C. A. Williams and M. W. Chase, eds. Academic Press, New York, NY.
- HEJGAARD, J., SVENDSEN, I., and MUNDY, J. 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.
- JONES, B. L., and MEREDITH, P. 1982. Inactivation of alpha-amylase activity by purothionins. *Cereal Chem.* 59:321.
- KNEEN, E., and SANDSTEDT, R. M. 1943. An amylase inhibitor from certain cereals. *J. Am. Chem. Soc.* 65:1,247.
- KNEEN, E., and SANDSTEDT, R. M. 1946. Distribution and general properties of an amylase inhibitor in cereals. *Arch. Biochem.* 9:235.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265.
- MacGREGOR, A. W., and BALLANCE, D. 1980. Quantitative determination of α -amylase enzymes in germinated barley after separation by isoelectric focusing. *J. Inst. Brew.* 86:131.
- MacGREGOR, A. W., and DAUSSANT, J. 1979. Evolution of α -amylase components during initial stages of barley germination with and without prior steeping. *Cereal Chem.* 56:541.
- MORRIS, V. H., ALEXANDER, T. L., and PASCOE, E. D. 1946. Studies of the composition of the wheat kernel. III. Distribution of ash and protein in central and peripheral zones of whole kernels. *Cereal Chem.* 23:540.
- MUNDY, J., HEJGAARD, J., and SVENDSEN, I. 1984. Characterization of a bifunctional wheat inhibitor of endogenous α -amylase and subtilisin. *FEBS Lett.* 167:210.
- MUNDY, J., SVENDSEN, I., and HEJGAARD, J. 1983. Barley α -amylase/subtilisin inhibitor. I. Isolation and characterization. *Carlsberg Res. Commun.* 48:81.
- NOLL, J. S. 1983. Peroxidases and their relationship to dormancy and germination in the wheat kernel. Page 132 in: *Third International Symposium on Pre-Harvest Sprouting in Cereals*. J. E. Kruger and D. E. LaBerge, eds. Westview Press: Boulder, CO.
- NOVACEK, E. J., PETERSON, C. F., and SLINKARD, A. E. 1966. A separation of anatomical parts of barley from the by-products and barley pearling. *Cereal Chem.* 43:384.
- OUCHTERLONY, O. 1967. Immunodiffusion and immunoelectrophoresis. Page 655 in: *Handbook of Experimental Immunology*. D. M. Weir, ed. Blackwell Scientific Publications, London, England.
- SARGEANT, J. G., and WALKER, T. S. 1978. Adsorption of wheat α -amylase isoenzymes to wheat starch. *Starke* 30:160.
- SHAIKIN, R., and BIRK, Y. D. 1970. α -Amylase inhibitors from wheat: isolation and characterization. *Biochem. Biophys. Acta* 221:502.
- SILANO, V., POCCHJARI, F., and KASARDA, D. D. 1973. Physical characterization of α -amylase inhibitors from wheat. *Biochem. Biophys. Acta* 317:139.
- WARCHALEWSKI, J. R. 1977a. Preliminary investigation of purification of alpha-amylase inhibitors from durum wheat. *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* 24:559.
- WARCHALEWSKI, J. R. 1977b. Isolation and purification of native alpha-amylase inhibitors from winter wheat. *Bull. Acad. Pol. Sci. Ser. Sci. Biol.* 25:725.
- WESELAKE, R. J., and HILL, R. D. 1983. Inhibition of alpha-amylase catalyzed starch granule hydrolysis by cycloheptaamylose. *Cereal Chem.* 60:98.
- WESELAKE, R. J., MacGREGOR, A. W., and HILL, R. D. 1983a. An endogenous α -amylase inhibitor in barley kernels. *Plant Physiol.* 72:809.
- WESELAKE, R. J., MacGREGOR, A. W., HILL, R. D., and DUCKWORTH, H. W. 1983b. Purification and characteristics of an endogenous α -amylase inhibitor from barley kernels. *Plant Physiol.* 73:1,008.
- WILLOUGHBY, E. W., and LAMBERT, A. 1983. A sensitive silver stain for proteins in agarose gels. *Anal. Biochem.* 130:353.
- YOSHIKAWA, M., IWASAKI, T., FUJII, M., and OOGAKI, M. 1976. Isolation and some properties of a subtilisin inhibitor from barley. *J. Biochem.* 79:765.

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