Localization and Physical Properties of Endogenous Germination Inhibitors in White Wheat Grain[1]

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ABSTRACT

Preharvest sprouting in wheat (*Triticum aestivum* L.) seriously reduces end-use quality of flour and is associated with lack of dormancy in mature seed. Knowledge of the location of endogenous germination inhibitor(s) within the wheat kernel and their solubility and chemical stability would greatly aid in the isolation, identification, and determination of the mode of action of these compound(s). Six mill fractions were produced from white wheat grain using laboratory break rolls and sieves. Fractions were break 2 large, medium, and small and break 3 bran, medium, and small. Break 2 large and break 3 bran and medium fractions were greatly enriched in bran, and the other three fractions were mostly endosperm. Endogenous germination inhibitory activity of water-soluble extracts of mill-fractions and whole-grain meal was measured by a wheat embryo bioassay. Inhibitory activity was mostly localized in the bran and little occurred in the endosperm fractions. Most inhibitory activity was soluble in water and up to 90% ethanol, insoluble in petroleum ether, and heat stable. The endogenous inhibitory activity was similar to 1 to 2.5 μM abscisic acid in its effect on germination of embryos from dormant wheat seeds. The results support the hypothesis that control of germination and preharvest sprouting resides in the embryo and is mediated by inhibitors in the bran.

Preharvest sprouting seriously reduces the end-use quality and market value of wheat grain (Perten 1964, Greenaway 1969, USDA 1978). The primary means of increasing cultivar resistance to preharvest sprouting conditions is through genetic manipulation of seed dormancy. Control of seed dormancy is poorly understood, however, making breeding for this trait difficult. Endogenous inhibitors in wheat grain may play a major role in the control of seed dormancy and germination (Mosheov 1938, Miyamoto and Everson 1958, Ching and Foote 1961, Miyamoto et al 1961, McCrate et al 1982, Paulsen and Heyne 1983). Mosheov (1938) reported that wheat grain contained extractable, heat-labile germination inhibitors, but nondormant wheat seeds were used to bioassay extracts.

Miyamoto and Everson (1958) found catechin and catechin tannin (CT) in the testa (seed coat) but not in the pericarp of dough-stage wheat seeds. No other seed tissues were analyzed. A water extract of tea leaves inhibited germination of nondormant wheat seeds and contained CT. A later study (Miyamoto et al 1961) utilized embryos excised from nondormant wheat seeds to identify and quantify endogenous inhibitors. Water-soluble extracts from the endosperm and bran of hard dough-stage wheat seeds were noninhibitory and variably inhibitory, respectively. Inhibitory activity of bran extracts was correlated with seed dormancy, and four different types of inhibitors were characterized on the basis of solubility and physical properties.

Stoy and Sundin (1976) used excised wheat embryos to study the inhibitory effects of CT and abscisic acid (ABA) on germination. Embryo germination response paralleled the level of seed dormancy, and ABA was relatively more inhibitory than CT.

McCrate et al (1982) demonstrated that the germination of embryos excised from dormant seeds was inhibited by a water-soluble extract from ground grain. Inhibitory activity of extracts was not related to seed dormancy or pericarp color and did not diminish during afterripening of the grain. Extracts from deembryonated distal portions of seeds and whole-grain meal gave similar results. Paulsen and Heyne (1983) confirmed and extended the results of McCrate et al (1982) by using bran from dormant and afterripened red and white grain as the inhibitor source and embryos from dormant and afterripened wheats for bioassays.

A thorough understanding of the role of endogenous germination inhibitor(s) is needed for improving preharvest sprouting resistance of wheat by breeding. This requires that endogenous inhibitory compound(s) be isolated and characterized. Objectives of this study were to localize the endogenous inhibitory activity in mill-fractioned wheat seed and to characterize some of the physical properties of the compound(s). A bioassay utilizing embryonic axes from dormant wheat seed was used to identify inhibitory activity.

MATERIALS AND METHODS

Sources of Inhibitors

Grain. Clark's Cream hard white winter wheat (*Triticum aestivum* L.) grain was harvested from unreplicated field plots at the North Agronomy Farm, Manhattan, KS, in 1985. The grain was cleaned by sieving, air-fractionated using a South Dakota seed blower (Seedburo Equip. Co., Chicago, IL), and stored about one month at ambient temperature. The resulting grain weighed 16.5 kg and had a mean kernel weight of 33.4 mg. Approximately 300 g of grain was ground in a Udy cyclone mill to pass a 1-mm screen and stored at −20° C (whole-grain meal).

Mill fractionations. Grain for milling was tempered from approximately 11 to 14% moisture in a rotary drum for 1 hr and passed through three laboratory break rolls (Ross, Oklahoma City, OK) (Fig. 1). Break rolls were 22.9 (diam) × 15.2 cm and rotated with a 2.5:1 differential. Corrugations per centimeter (roll 1/roll 2, respectively) and gap were break 1, 47.2/39.4 and 0.58 mm; break 2, 47.2/55.1 and 0.30 mm; and break 3, 63.0/63.0 and 0.15 mm. Following each break roll, the material was sifted on a no. 20 light wire screen (0.86-mm opening) at 180 rpm (10.2-cm-diameter throw) for 2 min. The mill fractions were separated further by briefly sieving on hand-shaken U.S. Standard testing sieves, numbers 18 and 30 (1.00 and 0.59-mm opening, respectively). Fractions were dried in a forced-air oven at 35° C for 48 hr and sealed in plastic bags. The break 3 bran fraction was ground in a Udy cyclone mill and stored at −20° C, and other fractions were stored at 5° C. A composite of the mill fractions was prepared according to the proportional yield of each fraction and stored at −20° C. Ash concentration of mill fractions and whole-grain meal was determined in duplicate 10-g samples following AACC method 08-01 (AACC 1983).

Localization and Stability of Inhibitors

Localization in mill-fractionated grain. Mill fractions and whole-grain meal were extracted with excess water on a wrist arm shaker (Burrell Corp., Pittsburgh, PA) at about 22° C for 2 hr. The resulting slurry was quantitatively transferred to 260-ml centrifuge jars and centrifuged at 16,300 × g for 30 min. The supernatant was vacuum filtered through prerinsed Whatman 42 filter paper, immediately frozen, and lyophilized. Dried extracts were redissolved in water so that 1 ml of solution contained the water-soluble material from 0.5 g of the mill fractions or whole grain

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meal. Solutions were stored at −20° C. Extracts were thawed, diluted where appropriate, and bioassayed on embryos from dormant Clark's Cream grain.

Heat stability. Thawed extracts (2.5 ml) were heated in a boiling water bath for 2 min and centrifuged 12,100 × *g* for 1 min (excluding acceleration and deceleration time). The supernatant and pellet were separated, lyophilized, redissolved in water, and bioassayed.

Solubility of Inhibitors

Thawed extracts (5 ml each) of the break 3 medium and bran and composite mill fractions were heated in a boiling water bath for 2 min (Fig. 2). The heat-precipitated pellets were extracted three times more, and the combined supernatants and pellets were lyophilized and resuspended in 5 ml of water. Supernatants were brought to 50% ethanol (v/v) and centrifuged at 12,100 × *g* for 15 min. The supernatants were decanted, and the ethanol-precipitated pellets were reextracted three times more with 50% ethanol and lyophilized. The combined supernatants were taken to near dryness under a stream of air at 45° C and lyophilized. Both the water-soluble and ethanol-precipitated fractions were redissolved in 5 ml of water. The water-soluble fraction was defatted three times with petroleum ether without adjustment of the pH. The aqueous phase was lyophilized and redissolved in 5 ml of water, and the ether fraction was evaporated under a stream of air and dissolved in 1 ml of water. All fractions were bioassayed for inhibitory activity using embryos from dormant Clark's Cream grain.

Quantitative Extraction of Whole-Grain Meal

Whole-grain meal (50 g) was extracted with 500 ml water with constant stirring at about 22° C for 2 hr (Fig. 3), and the slurry was centrifuged at 4,100 × *g* for 30 min. The supernatant and pellet were separated, lyophilized, and weighed, and the water-insoluble fraction was discarded. A subsample of the dried, water-soluble supernatant was redissolved in three volumes of water (w/v), brought to 90% ethanol, and centrifuged at 12,100 × *g* for 10 min. The supernatant was decanted, and the ethanol-precipitated pellet was lyophilized, redissolved in three volumes of water, and extracted again with 90% ethanol. The extraction procedure was repeated twice more, and the combined supernatants were taken to near dryness under a stream of air at 45° C and lyophilized. All fractions were bioassayed for inhibitor activity relative to ABA using embryos from dormant Clark's Cream and nondormant, afterripened Parker 76 wheats.

Wheat Embryo Bioassay

Embryo sources. Clark's Cream and Parker 76, hard white and red winter wheat cultivars, respectively, were harvested from unreplicated field plots at the North Agronomy Farm, Manhattan, KS, in 1985. Harvest-ripe grain was immediately cleaned, sealed in plastic bags, and stored at −20° C to arrest afterripening (Mares 1983). A portion of the Parker 76 grain was afterripened at about 22° C for four weeks and then returned to −20° C.

Embryo isolation. Wheat embryonic axes were isolated following a modified procedure of Johnston and Stern (1957) (cf. Marcus et al 1974). A 50–100-ml volume of seeds was placed in a Waring Blendor, and the motor was quickly turned on and off, producing a pulse of blade rotation lasting < 1 sec. After each pulse, the seeds were transferred to a set of U.S. Standard testing sieves, numbers 10, 18, and 30 (2.00, 1.00, and 0.59-mm openings,

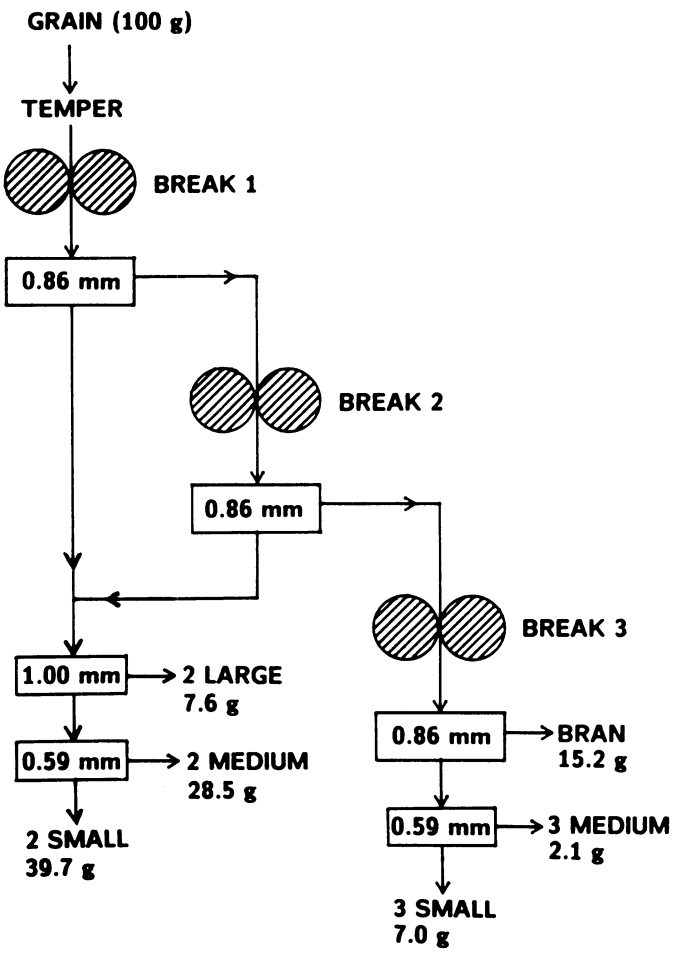

Fig. 1. Schematic representation of the mill fraction of 16.5 kg of Clark's Cream grain. Fraction designations are break 2 large, medium, and small and break 3 bran, medium and small. Striated circles and rectangles represent break rolls and sieves, respectively.

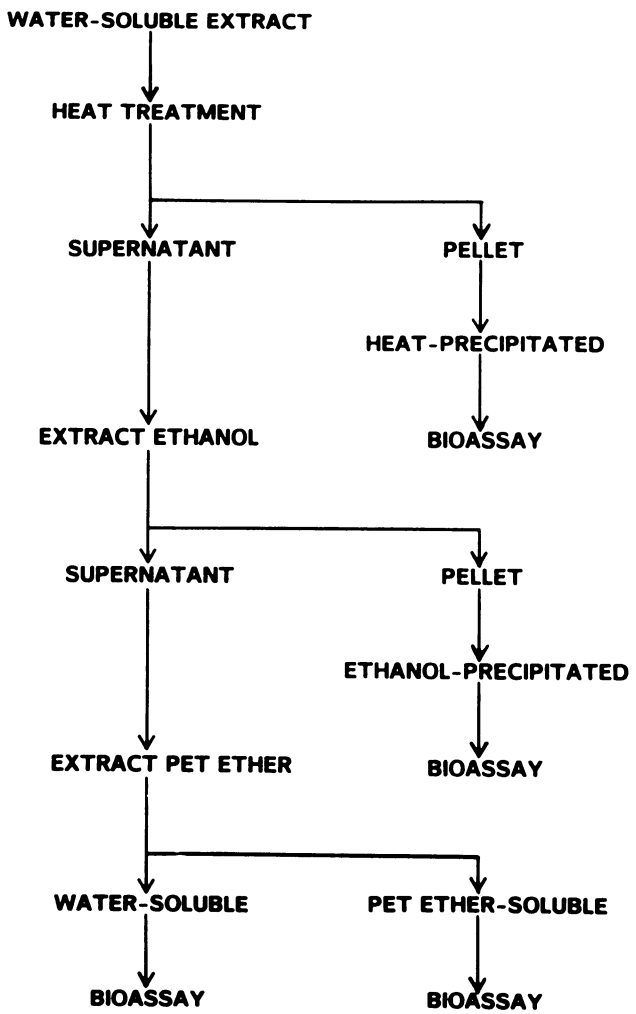

Fig. 2. Solubility of endogenous germination inhibitors of the water-soluble extract from break 3 medium and bran and composite mill fractions of Clark's Cream wheat.

respectively), and briefly shaken. Material remaining on the top sieve (no. 10) was returned to the blender, and the cycle was repeated. The blender pulse and sieving cycle was repeated 20–50 times or more as needed. Visibly undamaged whole embryonic axes (hereafter termed embryos) that were free of adhering pericarp and endosperm were selected under magnification and were stored at −20° C.

Embryo germination. Excised wheat embryos were germinated in 96-well tissue culture plates (no. 3596, Costar, Cambridge, MA). Double-strength sucrose agar (Difco Laboratories, Detroit, MI) was autoclaved and mixed 1:1 (v/v) with test solutions or, for the control, with water to give a final concentration of 20 g/L sucrose and 6.8 g/L agar. Each plate well received 0.2 ml of agar-test solution and, after cooling, one embryo that was placed scutellum side down. Twelve embryos were used for all treatments, which were replicated twice. Operations were conducted in a laminar flow hood. Plates were taped shut and incubated at 22° C in darkness. Embryos were periodically examined under magnification for germination over a 72-hr period; they were considered germinated if radicle, lateral root, or coleoptile growth exceeded 1 mm.

Analyses. A promptness index (PI) was calculated as the summation of (n_i/h_i), where n_i equals the percentage of embryos germinated on the ith hour of the bioassay, and h_i equals the ith hour of the bioassay. Error degrees of freedom were reduced by the number of treatments that produced a PI = 0 for both replications. Corrected mean square errors are reported in Tables I and III as a measure of experimental precision and were used to calculate the least significant differences. Data in Table II could not be analyzed by conventional procedures, but the standard deviation of the control treatment is provided as a measure of experimental precision.

Distilled, resin-deionized water was used throughout. Unless noted, all chemicals were of reagent-grade or better.

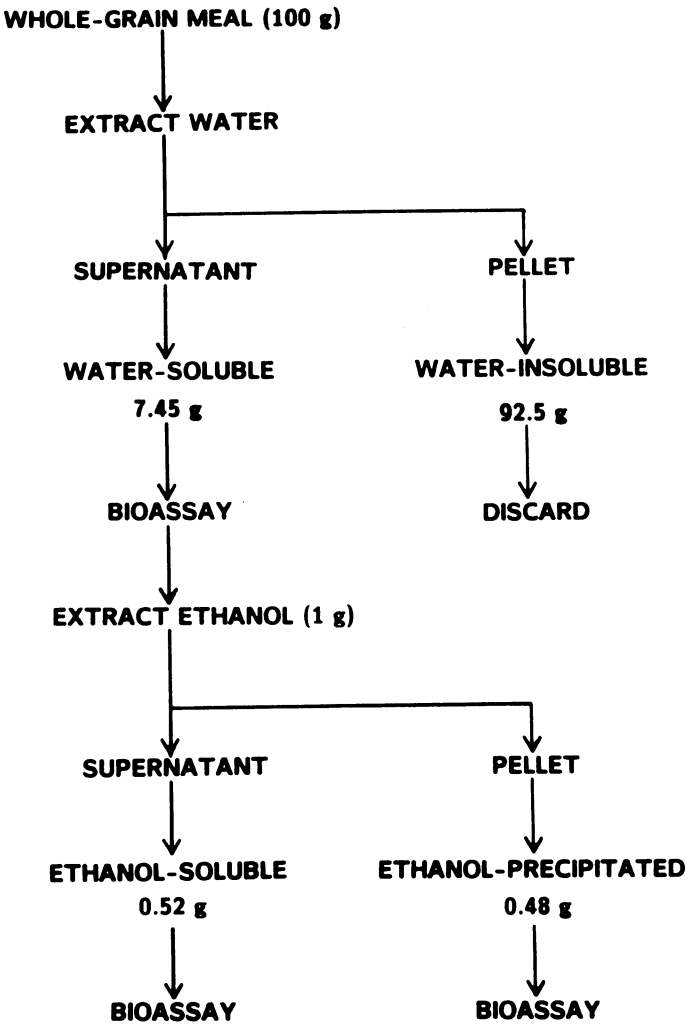

Fig. 3. Quantitative extraction of endogenous germination inhibitors from whole-grain meal of Clark's Cream wheat.

RESULTS

Ash concentrations in the six mill fractions in Figure 1 were break 2 large, 20; medium, 10; small, 6; break 3 bran, 57; medium, 44; small, 9; and whole grain meal, 20 mg/g. The average standard deviation was 0.5 mg/g. Most of the milled material (68.2%) passed easily through a 1.00-mm-opening sieve after the first two break rolls (break 2 medium and small). An additional 7% was represented by material less than 0.59 mm after the third break roll (break 3 small). These three mill fractions were high in endosperm and low in bran as indicated by visual appearance and ash concentration. The three remaining fractions (break 2 large and break 3 bran and medium) represented about 25% of the grain and contained most of the bran portion.

Localization and Stability of Inhibitors

Crude water-soluble extracts from all the mill fractions and composite and whole-grain meal were highly inhibitory to wheat embryo germination (Table I). Mill fractions enriched in bran tended to be more inhibitory than other fractions after dilution. The lowest concentration of crude extract assayed (0.02 g/ml) represented 4 mg of original fraction per embryo.

No loss of inhibitory activity was apparent after the crude water-soluble extract was briefly heated in boiling water (Table I). In some cases (break 3 bran, composite, and whole-grain meal), a significant amount of inhibitory activity was present in the heat-precipitated material at the higher concentration. The heat precipitation of some activity was probably from incomplete extraction of the precipitated material or the reversible precipitation of one or more inhibitors.

Solubility of Inhibitors

Break 3 medium and bran and composite mill fractions had high specific inhibitory activity on excised embryos (Table I). After the

TABLE I
Germination Promptness Index (PI) Response of Embryos Treated with Crude and Heat-Treated Extracts of Individual and Composite Mill Fractions and Whole-Grain Meal of Clark's Cream Wheat[a]

Treatment Description	Mill Fraction Extracted (g/ml)	Control	Break 2			Break 3			Composite	Whole-Grain Meal
			Large	Medium	Small	Bran	Medium	Small		
Crude extract	1.0	3.28	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.00
Crude extract	0.2	3.17	0.86	1.68	1.50	0.40	0.43	1.32	0.64	0.43
Crude extract	0.02	2.50	2.49	2.31	2.33	2.07	2.29	2.52	2.07	2.56
Heated, supernatant	1.0	3.17	0.00	0.00	0.14	0.00	0.00	0.14	0.00	0.00
Heated, supernatant	0.2	3.06	0.81	2.47	1.90	0.52	0.17	1.53	1.42	0.98
Heated, precipitate	1.0	2.84	1.25	1.28	1.72	0.00	1.14	1.38	0.21	0.75
Heated, precipitate	0.2	2.49	2.14	2.10	2.79	1.34	2.89	2.52	1.89	2.01

[a] Least significant difference (0.05) = 0.95; mean square error (50 df) = 0.224.

TABLE II
Germination Promptness Index (PI)[a] of Embryos Treated with Fractions from Break 3 Medium and Bran and Composite Mill Fractions of Clark's Cream Wheat

Fraction	Break 3		Composite
	Medium	Bran	
Heat-precipitated	0.80	1.98	1.40
Ethanol-precipitated	1.14	1.11	1.27
Water-soluble	0.00	0.00	0.23
Petroleum ether-soluble	1.60	1.44	1.19

[a] Control PI (no inhibitor) = 2.07±0.28.

TABLE III
Germination Promptness Index (PI) of Embryos Treated with Whole-Grain Meal Fractions of Clark's Cream Wheat and Abscisic Acid (ABA)[a]

Treatment Fraction	Embryo Genotype	
	Clark's Cream	Parker 76
Water-soluble	0.23	1.56
Ethanol-soluble	0.27	1.82
Ethanol-precipitated	2.95	3.13
ABA 1.0 μM	0.78	2.81
ABA 2.5 μM	0.00	2.01
Control	2.69	2.99

[a] Least significant difference (0.05) = 1.74; mean square error (11 df) = 0.63.

three fractions were separated into heat- and ethanol-precipitated components and water- and petroleum ether-soluble components (Fig. 2), most of the inhibitory activity was in the water-soluble fraction (Table II). Thus, the inhibitor(s) is clearly soluble in water, insoluble in organic extractant, and stable to brief heating.

Quantitative Extraction of Whole-Grain Meal

Whole-grain meal fractions of Clark's Cream wheat that were quantitatively extracted with water and ethanol following the procedure outlined in Figure 3 caused differential inhibition when they were bioassayed with embryos from dormant Clark's Cream and nondormant Parker 76 wheats (Table III). Treatments containing 1.0 and 2.5 μM ABA were included for comparison. A 2-hr extraction of the whole-grain meal recovered 7.45% of the total meal; germination of Clark's Cream but not Parker 76 embryos was significantly inhibited by this water-soluble extract. Nearly all of the inhibitory activity was in 90% ethanol-soluble extracts, and negligible amounts were in 90% ethanol-precipitated material. The endogenous inhibitory activity of the extracts was similar to that of 1.0–2.5 μM ABA.

DISCUSSION

Endogenous inhibitors in wheat grain have been implicated in the control of seed dormancy and germination (Mosheov 1938, Miyamoto and Everson 1958, Ching and Foote 1961, Miyamoto et al 1961, Stoy and Sundin 1976, McCrate et al 1982, Paulsen and Heyne 1983). Our results support this general hypothesis and show that wheat seed dormancy and germination are controlled by action of water-soluble inhibitors localized in the bran on the embryo (McCrate et al 1982, Paulsen and Heyne 1983).

Before the report of Stoy and Sundin (1976), lack of dormancy at maturity or loss of dormancy during afterripening was attributed to a decrease in the concentration or activity of inhibitor(s) (Miyamoto and Everson 1958, Ching and Foote 1961, Miyamoto et al 1961). Since control was believed to lie outside the embryo, nondormant wheat seed and embryos were used for detecting inhibitory activity. Stoy and Sundin (1976) found that embryos from dormant and nondormant wheat seeds were sensitive and insensitive, respectively, to exogenous catechin tannin and ABA. McCrate et al (1982) and Paulsen and Heyne (1983) demonstrated that embryo sensitivity to endogenous inhibitors explained genetic differences in seed dormancy at harvest ripeness and loss of dormancy during afterripening.

Our results indicate that most inhibitory activity resides in tissues outside the endosperm—probably in one or more of the layers comprising the bran. Miyamoto et al (1961) and Paulsen and Heyne (1983) also used bran milled from wheat grain as an inhibitor source but did not compare other seed tissues. Since embryo excision releases the wheat embryo from imposed dormancy (Stoy and Sundin 1976, McCrate et al 1982), the inhibitor(s) probably lie outside the embryo itself. The high solubility of endogenous inhibitors in water would facilitate their movement to the embryo during imbibition (Woodbury and Wiebe 1983).

Most of the inhibitory activity of water-soluble extracts is neither destroyed nor precipitated by heat. This stability is an indication, but certainly not firm evidence, that one major inhibitor or class of inhibitors is involved. Additional classes of inhibitors that are less active or less plentiful may also be present, however, because some activity was not completely extracted by water and some was precipitated by brief heating.

The data indicate that the inhibitors are hydrophilic but are freely soluble in solvents considerably less polar than water. Precipitating a water-soluble extract with 50 or 90% ethanol removed little, if any, inhibitory activity. A petroleum ether extract also exhibited little or no activity.

The endogenous inhibitory activity was similar to low micromolar concentrations of ABA, and embryo germination response (i.e., sensitivity) to ABA parallels the level of seed dormancy in the two wheat genotypes (Paulsen and Heyne 1983). Endogenous ABA may contribute to the inhibitory activity of the extracts described above; however, the involvement of ABA in the control of dormancy in mature wheat seed has not been demonstrated (cf. Walker-Simmons 1987).

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