Subtilisin Inhibitor Activity in Legume Seeds

Extracts of seeds of 12 legume species were tested for subtilisin inhibitor activity by a microelectrophoretic method. Activity was detected in garden beans, mung beans, jack beans, cow peas, broad beans, and chick peas. No inhibitor was detected in field peas, lentils, pigeon peas, soybeans, lupine, and wing beans.

Most legume seeds contain one or several inhibitors of trypsin and chymotrypsin which have been extensively studied (see Laskowski and Sealock, 1971). Inhibitors for other proteinases have been detected in some legume seeds but little is known about their distribution and chemical characteristics. A specific inhibitor for subtilisin was isolated recently from black garden beans and some of its chemical and physical properties have been described (Seidl et al., 1978a). It had been discovered by an electrophoretic screening method for protease inhibition. Inhibitory factors active on different proteolytic enzymes can be detected by this method (Seidl et al., 1978b), which has now been applied for screening different legume seed samples for the presence of subtilisin inhibitor activity.

EXPERIMENTAL SECTION

Seed extracts were prepared by stirring a 10% suspension of ground seeds in water for 24 h at 4 °C, followed by centrifugation. The protein concentration in the extracts was adjusted to about 2%. Microscopic slides were covered with a casein–agarose solution consisting of 83 mL of agarose, 6.7 mg/mL, 18 mL of 0.2% (w/v) casein and 9 mL of 0.5 M $CaCl_2$, each one dissolved in 0.025 M veronal–acetate–HCl buffer, pH 8.6. Samples of 5 μL of seed extracts were submitted to electrophoresis at 22 mAmp (200 V) for 2 h.

The slides were then covered with a filter paper strip soaked in an appropriate enzyme solution and incubated for 1 h at 37 °C. Coloring with amido black revealed blue spots where the casein was protected from digestion by inhibitors. Blanks were run on casein-free agar plates. The enzyme concentrations and the respective amounts of seed extract had to be previously standardized (Seidl et al., 1978b).

RESULTS

The screening revealed the presence of subtilisin inhibitor activity in garden beans, jack beans, mung beans, cow peas, broad beans, and chick peas. No such activity was detected in lentils, peas, soybeans, pegeon peas, lupine, and wing beans (Table I, Figure 1).

Samples of 63 different cultivars or strains of garden beans have been tested; each one showed subtilisin inhibitor activity.

The size and intensity of the inhibition zone of seed extracts from different samples varied considerably. All inhibition spots had similar electrophoretic mobility. In contrast, very different mobility patterns were observed for trypsin inhibitors (Seidl et al., 1978b). For jack beans and cow peas the subtilisin inhibition spot was very faint.

In order to demonstrate its presence in bean leaf extracts, it was necessary to fractionate the extract by chromatography on Bio-Gel P-10 and concentrate the active fraction. We were unable to obtain evidence for subtilisin inhibitor in the bean leaves by testing the extracts directly. No trypsin inhibitor activity could be found in any of the bean leaf protein fractions. In the extracts of the green and dry bean seed pods trypsin, but no subtilisin inhibition could be detected.

Table I. Detection of Subtilisin Inhibitor Activity in Extracts of Legume Seeds

name	no. of samples[a] tested	result
garden beans *(Phaseolus vulgaris)*	63	+
mung beans *(Vigna aureus)*	1	+
cow peas *(Vigna sinensis)*	3	+
Jack beans *(Canavalia ensiformis)*	2	+
chick peas *(Cicer arientinum)*	3	+
broad beans *(Vicia faba)*	1	+
field peas *(Pisum sativum)*	2	–
lentils *(Lens esculenta)*	2	–
pigeon peas *(Cajanus cajan)*	1	–
soybeans *(Glycine max)*	9	–
wing beans *(Psophocarpus tetragonolobus)*	1	–
lupine *(Lupinus sp.)*	5	–

[a] The samples were from different cultivars or genetical selections.

DISCUSSION

The microelectrophoretic method used was very convenient for testing the presence of different proteinase inhibitor in seed extracts. The test can easily be performed with the water extract of a single seed. It is noteworthy that a subtilisin inhibitor was detected only in about half of the legume species studied and only one well-defined spot was observed in each positive case. The spot revealing subtilisin inhibition was clearly distinguishable from those due to trypsin inhibition in all positive samples. Indeed, one specific subtilisin inhibitor is present in garden beans (Seidl et al., 1978a). No indication for the existence of isoinhibitors separable under the experimental conditions used was found. The presence of a subtilisin inhibitor in legumes has not been reported previously as far as we are aware with the exception of an inhibitory activity toward this enzyme in broad beans (Matsubara and Nishimura, 1958). Several protease inhibitors of broad specificity have been isolated from these beans. They were inactive toward subtilisin (Warsy et al., 1974).

The bean subtilisin inhibitor shows some reaction also with pronase and the protease from *Aspergillus oryzidae*, both fungal enzymes, but with none of the common proteases of animal or plant origin.

It is tempting to speculate on a possible protective action

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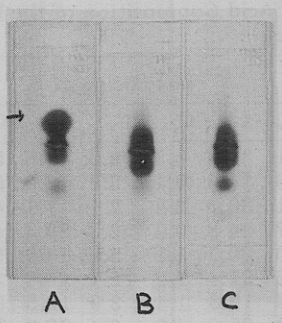

Figure 1. Electrophoretic pattern of (A) bean extract in casein-containing agar gel digested with subtilisin; the marked spot represents the area where the inhibitor protected the casein from digestion; (B) same with lentil extract, negative for subtilisin inhibitor; and (C) control, bean extract in casein-free agar gel; all slides stained with amino black.

of the subtilisin inhibitor against bacterial and fungal attacks in legume plants and seeds.

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Phospholipids of Barley Grain

Fractionation of the phospholipids of two barley (*Hordeum vulgare* L.) varieties, "Kearney" (winter type) and "Prilar" (spring type), by thin-layer chromatography produced ten lipid classes. Phosphorus analysis of each fraction showed that phosphatidylcholine, lysophosphatidylcholine, and phosphatidylethanolamine were present in highest amounts. Lesser amounts of phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol, and small amounts of phosphatidic acid and two unknown phospholipids were present. Fatty acids present in the fractions ranged from lauric (12:0) to arachidic (20:0). Linoleic acid (18:2) was the principal fatty acid in seven fractions and palmitic (16:0) was predominant in the other three fractions. This study provides a basis for monitoring of changes in phospholipids during cold acclimation of barley seedlings.

Phospholipids are essential components of the cytoplasmic membranes of vegetative and reproductive tissues, even though they only represent about 0.6% of the dry weight of barley grain (Price and Parsons, 1974). The presence of phospholipids in the structure and function of cell membranes relates to the movement of charged and uncharged molecules, the transport of triglycerides, and levels of enzyme activity.

The composition of phospholipids has been reported for most cereal grains. A few studies have been conducted on the phospholipids of barley (Aylward and Showler, 1962), barley malt (Silberusen and Anthon, 1967), and wort (Perkins, 1969). None of the studies on barley phospholipids have involved fractionation by thin-layer chromatography (TLC), quantification of inorganic phosphorus by colorimetry, or fatty acid analysis by gas–liquid chromatography (GLC).

This study was initiated to obtain detailed information on the phospholipids of the barley (*Hordeum vulgare* L.) varieties "Kearney" (winter type) and "Prilar" (spring type). The information will provide a basis for monitoring changes which occur in barley phospholipids during cold acclimation and the relationship of these changes to winterhardiness.

MATERIALS AND METHODS

Whole grain samples of the barley varieties Kearney and Prilar were ground in a Udy cyclone mill to pass a 0.6-mm screen. Total lipids including the phospholipids were extracted with chloroform–methanol–water (1.0:1.0:0.9) in the modification of the methods (Bligh and Dyer, 1959; Folch et al., 1957; Weber, 1970; Atkinson et al., 1972) and were purified as described previously (Price and Parsons, 1974). The lipids were separated into classes by silicic acid column chromatography (Hirsch and Ahrens, 1958). The phospholipid class was eluted with methanol, and the solvent was removed by a rotary vacuum evaporator at 40 °C. The phospholipids were then transferred to vials, flushed with nitrogen, and stored at −20 °C.

The phospholipids were separated by two-dimensional TLC (Parsons and Patton, 1967) on silica gel HR coated glass plates with the solvent systems of chloroform–methanol–water–28% aqueous ammonia (130:70:7:0.5, v/v) and chloroform–acetone–methanol–acetic acid–water (100:40:20:20:7, v/v). The individual phospholipids, separated by TLC, were identified by cochromatography with authentic reference lipids (Applied Science Laboratories, State College, PA; Supelco, Bellefonte, PA; and Analabs, North Haven, CT) and from published R_f values (Lepage, 1967; Nichols, 1964). Specific sprays were also used to identify phospholipids (Dittmer and Lester, 1964; Stahl, 1969). The phosphorus content of the individual lipids was determined by spraying the developed TLC plates with 50% sulfuric acid, heating the plates for 60 min at 180 °C, and scraping each charred spot into a test tube (Kahovcova and Odavic, 1969). Digestion and color de-

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