

## 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<sub>2</sub>, 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, pigeon 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

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

name	no. of samples <sup>a</sup> tested	re-sult
garden beans ( <i>Phaseolus vulgaris</i> )	63	+
mung beans ( <i>Vigna aureus</i> )	1	+
cow peas ( <i>Vigna sinensis</i> )	3	+
Jack beans ( <i>Canavalia ensiformis</i> )	2	+
chick peas ( <i>Cicer arietinum</i> )	3	+
broad beans ( <i>Vicia faba</i> )	1	+
field peas ( <i>Pisum sativum</i> )	2	-
lentils ( <i>Lens esculenta</i> )	2	-
pigeon peas ( <i>Cajanus cajan</i> )	1	-
soybeans ( <i>Glycine max</i> )	9	-
wing beans ( <i>Psophocarpus tetragonolobus</i> )	1	-
lupine ( <i>Lupinus sp.</i> )	5	-

<sup>a</sup> The samples were from different cultivars or genetic selections.

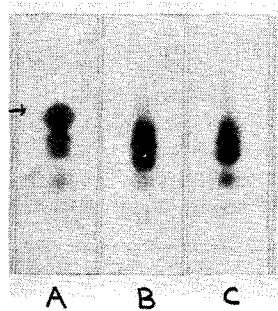
of the green and dry bean seed pods trypsin, but no subtilisin inhibition could be detected.

### 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 oryzae*, 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



**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.

#### LITERATURE CITED

Laskowski, M., Jr., Sealock, R. W., in "The Enzymes", Vol. 3,

- Bayer, G. D., Ed., Academic Press, New York, 1971, p 375.  
 Seidl, D. S., Abreu, H., Jaffé, W. G., *FEBS Lett.* **92**, 245 (1978a).  
 Seidl, D. S., Jaffé, W. G., González, E., Callejas, A., *Anal. Biochem.* **88**, 417 (1978b).  
 Matsubara, H., Nishimura, S. J., *Seikagaku* **45**, 503 (1958).  
 Warsy, A. S., Norton, G., Stein, M., *Phytochemistry* **13**, 2481 (1974).

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Received for review August 21, 1978. Accepted January 12, 1979.  
 Supported by Grant No. S1-0273 from the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT).