Phytochemistry, 1980, Vol. 19, pp. 2517–2519. © Pergamon Press Ltd. Printed in England. 0031-9422/80/1201-2517 $02.00/0

THE RELEASE OF PROTEINASE INHIBITORS FROM LEGUME SEEDS DURING GERMINATION*

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(*Revised received* 15 *March* 1980)

Key Word Index – *Glycine*; *Pisum*; *Vicia*; Leguminosae; trypsin inhibitors; chymotrypsin inhibitors; germination; inhibitor release.

Abstract The seeds of twelve common species of legumes were examined for the release of proteinase inhibitor activity during germination. All species released inhibitory activity against bovine trypsin (EC 3.4.21.4), ranging from 1.0 unit per g dry wt. of seed in 24 hr for soybean (*Glycine max*), to 0.07 unit per g for broad beans (*Vicia faba*) and sugar pod peas (*Pisum sativum*). This release corresponds to approximately 1–13% of the total trypsin inhibitory activity of the seed, with lentils (*Lens culinaris*) releasing the greatest percentage, and the scarlet runner bean (*Phaseolus coccineus*) the least. In most species the amount of inhibitor released increases until 24–48 hr of germination, and then remains roughly the same or decreases slightly by 72 hr of germination. Five species of legumes were also examined for the release of inhibitory activity against bovine chymotrypsin (EC 3.4.21.1). In each case chymotryptic inhibitory activity was released in a manner similar to the trypsin inhibitor.

INTRODUCTION

Plant seeds have long been known to contain high levels of protein proteinase inhibitors [1]. While much progress has been made in elucidating the physical and chemical properties of these proteins *in vitro* [2, 3], their physiological function *in vivo* remains poorly understood. A number of functions for these proteins have been proposed, including the regulation of endogenous proteinases during germination, the storage of sulfur amino acids during dormancy, and the protection of the seed from insects and soil microorganisms [3]. The recent observations of Hwang *et al.* [4] are particularly interesting in regard to this last possible role. These workers observed the release of five Bowman–Birk type proteinase inhibitors from soybean seeds during germination. The release of such inhibitors might inhibit the activity of extracellular microbial proteinases, inhibiting the growth of the microbes and retarding or preventing their invasion of the germinating seed. In this publication we demonstrate that the release of proteinase inhibitors is a general phenomenon occurring in many common legumes.

RESULTS AND DISCUSSION

The germination of all twelve species of legumes tested resulted in the release of trypsin inhibitory activity into the medium (Table 1). However, the amount of inhibitor released per g dry wt. of seed varies greatly among different species, ranging from a low of 0.07 units/g with broad beans and peas to 1.01 units/g with the soybean. To a large extent these differences reflect the different levels of inhibitor in the ungerminated seeds of these species. While nine of the species differ greatly in the level of inhibitor activity in the seed, all release approximately 1–3% of their total inhibitor to the medium in 24 hr. However, three species, lentil, alfalfa, and adzuki bean, show a larger fractional release of their inhibitors. Differences in seed viability do not explain these exceptions. The lima bean and scarlet runner seeds used have comparable germination rates, as do the lentil, alfalfa and pea seeds.

The different levels of inhibitor release in different species could also result from different kinetics of release. This is to some extent true, as evidenced by the release curves shown in Fig. 1 for ten of the species studied. For example, while both lima and garden beans reach the same maximal value of inhibitor released per g dry wt. of seed, the release from the garden bean is somewhat more rapid. In general, the maximal level of released inhibitor is reached by 24 hr, or, in several instances 48 hr, and remains constant or declines slightly thereafter. The one exception to this is the soybean, which continues to release inhibitor throughout the period of the experiment (72 hr). The early part of this release curve is similar to that observed by Hwang *et al.* [4] with the Tracy cultivar of the soybean.

Many of the legume species studied are known to contain inhibitors active against chymotrypsin as well as trypsin. Indeed, in many cases, reactive sites for both types of proteinase have been found in the same inhibitor molecule, e.g. in the double-headed Bowman–Birk type inhibitors [5–9]. The release of chymotrypsin inhibitor was therefore examined in the leachates of several species known to possess such inhibitors. The chymotrypsin inhibitor content of dry seeds, and the dependence of inhibitor release upon germination time is shown in Table 2. As with the trypsin inhibitors, chymotryptic inhibitory activity is released from the germinating seeds in a roughly

* Supported by grant-in-aid No. 240-7412A from the Research Foundation of the State University of New York at Binghamton.

Table 1. The release of trypsin inhibitor from legume seeds during germination*

Legume	Source†	Germination (%)	Extractable inhibitor U/g	Inhibitor released U/g‡	% inhibitor released
Chick pea (*Cicer arietinum*)	L	65	6.20	0.08	1.3
Soybean (*Glycine max*) cv Fiskeby V	TM	50	41.82	1.01	2.4
Lentil (*Lens culinaris*)	L	95	2.57	0.33	12.9
Alfalfa (*Medicago sativa*)	JG	90	3.53	0.13	3.6
Adzuki bean (*Phaseolus angularis*)	JSS	82	27.74	0.13	4.5
Scarlet runner (*P. coccineus*)	NK	95	14.07	0.13	0.9
Lima bean (*P. lunatus*) cv Fordhook 242	JSS	90	15.69	0.26	1.7
Garden bean (*P. vulgaris*) cv Great Northern	L	95	19.03	0.54	2.8
Pea (*Pisum sativum*) cv Oregon Sugar Pod	JSS	95	2.85	0.07	2.3
Broad bean (*Vicia faba*) cv Windsor	JSS	76	3.29	0.07	2.2
Mung bean (*Vigna radiata*)	JSS	99	5.24	0.12	2.3
Black-eyed pea (*V. sinensis*)	L	85	6.57	0.08	1.2

* Seeds were germinated for 24 hr as described in the text.

† Abbreviations of sources: JG, Jonathan Green & Sons, Farmingdale, N.J.: JSS, Johnny's Selected Seeds, Albion, ME: L, Local grocery store: NK, Northrup King Seeds, Minneapolis, Minnesota: TM, Thompson & Morgan Inc., Somerdale, N.J., U.S.A.

‡ Average of at least two experiments.

similar manner. By 48 hr the soy, adzuki, lima and garden beans and the black-eyed pea released 14.2, 1.0, 4.8, 4.9 and 2.0%, respectively of the total extractable chymotryptic inhibitor of the seeds. This is comparable to a corresponding release of 3.1, 1.0, 3.1, 2.3 and 1.6%, respectively of the total extractable tryptic inhibitory activity of the seed.

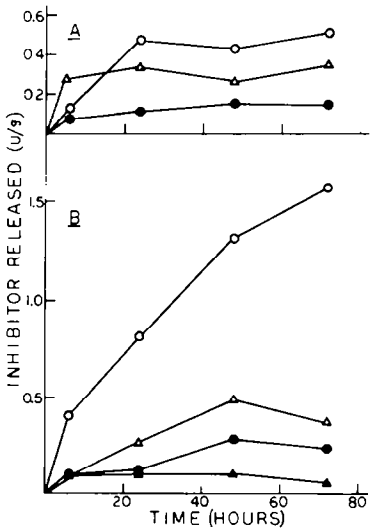

Fig. 1. Time course of the release of trypsin inhibitory activity during legume seed germination. Conditions were as given in the text. (A) ○--○, garden bean: △--△, lentil: ●--●, mung bean. (B) ○ ○, soy bean; △-△, lima bean; ●-●, adzuki bean; ▲-▲, black-eyed pea. The release curves for the snow pea, chick and alfalfa closely resemble that for the black-eyed pea.

A number of the trypsin and chymotrypsin inhibitor release curves indicate a decline in inhibitor activity after 48 hr. This decline in inhibitor activity may result from proteolytic degradation of the inhibitor in the leachate. Traces of activity capable of hydrolysing α-*N*-benzoyl-L-arginine ethyl ester at pH 8.0 were found in the 72 hr leachates.

As first hypothesized by Hwang *et al.* [4], the release of proteinase inhibitors may play an important role in the establishment of a 'normal' microbial complement in the rhizosphere of legumes and possibly other plant families. Such an effect may be due both to the inhibition of pathogenic microorganisms and the stimulation of

Table 2. The release of chymotrypsin inhibitor from germinating legume seeds

Legume	Extractable inhibitor U/g*	Inhibitor released U/g†		
		24 hr	48 hr	72 hr
Soybean	2.25	0.26	0.32	0.34
Adzuki bean	2.86	0.01	0.03	0.05
Lima bean	1.66	0.03	0.08	0.04
Garden bean	2.04	0.01	0.10	0.05
Black-eyed pea	1.00	0.00	0.02	0.01

* Total extractable inhibitor was determined as described in the text.

† Seeds were germinated as described in the text for the indicated lengths of time.

beneficial symbionts. The finding that all of the common legumes tested here display such a release lends additional evidence to this idea.

EXPERIMENTAL

Materials. Seeds were obtained from the sources indicated in Table 1. Per cent germination was determined by imbibing overnight in distilled water and incubating at 22° in the dark between moist sheets of filter paper. Seeds were examined for germination every day for up to 7 days after imbibition.

Methods. Trypsin inhibitory activity was assayed for by a modification of the method of Schwert and Takenaka [10] as described by Kassell *et al.* [11], using α-*N*-benzoyl-L-arginine ethyl ester as substrate. Chymotrypsin inhibitor assays using α-*N*-benzoyl-L-tyrosine ethyl ester were performed as described by Kress *et al.* [12]. In each case 1 unit of inhibitor was defined as that amount of inhibitor required to inhibit 1 mg of active proteinase. The concentrations of trypsin (bovine) and α-chymotrypsin (bovine) in stock solutions was determined spectrophotometrically using $E^{1\%}_{280}$ values of 14.9 and 20.4, respectively. The values reported have been corrected for the fraction of active enzyme in the trypsin and chymotrypsin preparations used. The trypsin used in this study was found to be 57% active by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate [13]. To determine the fraction of active chymotrypsin, the fluorometric titrant 4-methylumbelliferyl-4'-trimethylammonium cinnamate HCl was used [14]. To calibrate the spectrofluorometer, known quantities of bovine trypsin were reacted with 4-methyl-umbelliferyl-4'-guanidinobenzoate [14].

Extractable inhibitor was determined using seeds ground to a fine meal in a Waring blendor. The meal was extracted for 1 hr at 22° with 5 ml 0.05 M Tris Cl + 0.01 M NaCl, pH 8.0, per g of meal. The extract was clarified by centrifugation at 28 000 *g* for 30 min. The recovered pellet was then re-extracted in the same manner for 30 min. Both extracts were combined, and assayed for trypsin and chymotrypsin inhibitors as described above. We have found this procedure to be efficient in extracting the bean inhibitors (K. A Wilson, unpublished results).

In germination studies, weighed samples of seeds for each time point were surface-sterilized with 70% EtOH for 2 min. After rinsing thoroughly with sterile distilled water, the seeds were transferred to petri dishes containing 6 ml of sterile distilled water per g dry wt of the seeds. Germination was at 23° in constant darkness. After the desired length of time, the leachate was removed from the seeds. The seeds and dish were washed with two 1-ml aliquots of water, with the washes being added to the leachate. The leachate was centrifuged at 28 000 *g* for 30 min to clarify if necessary, and assayed for proteinase inhibitors as described above.

Acknowledgement The technical assistance of Michele Bogaards during the initial stages of this study is gratefully acknowledged.

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