Use of Denatured Radioalbumin for Determination of Trypsin and Chymotrypsin Inhibitors in Different Plant Seeds

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The procedure for determination of trypsin and chymotrypsin inhibitors with urea-denatured albumin labeled by ^{125}I is described. The content of both types of inhibitory activity has been determined in crude extracts of soybean, bean, lentil, pea, horse bean, maize, and 20 pea cultivars. The method is sufficiently sensitive, reliable, and particularly suitable when estimations must be done in crude plant extract with low inhibitory activity.

Natural trypsin and chymotrypsin inhibitors are substances of protein or peptidic nature occurring at different levels in plant and animal cells. In contrast to other protein substrates they frequently have a million times higher affinity for the corresponding proteases, and the enzyme firmly bound in the complex usually loses either entirely or partly its ability to hydrolyze the natural or synthetic substrates (Means, 1974). This fact is generally considered to be one of the reasons for the antinutritive properties of the protein inhibitors, and other in vivo negative effects of these substances (Moregan et al., 1986; Roebuck, 1986) point to the need for their accurate, reliable, and sensitive determination.

Methods of antitryptic and antichymotryptic activity determination are based on measurement of enzyme activity reduction of a certain amount of the enzyme in the presence of an inhibitor. Methods for the enzymatic activity estimation that are most widely used are based on colorimetric determination using synthetic chromogenic substrates. Colorimetric methods are simple, relatively quick, and sufficiently reliable; when, however, they are used for measurements in solutions of nonpurified preparations with low inhibitory activity, their application is frequently limited by turbidity, color, or high values of control samples (Fritz et al., 1974). Moreover, the presence of synthetic substrates in the reaction may generally affect dissociation of the enzyme–inhibitor complexes as a consequence of the competitive nature of inhibition. A similar effect can also be induced by organic solvents, the presence of which is necessary in the reactions because of the low solubility of synthetic substrates in water. All the circumstances mentioned above may lead to incorrect results when synthetic chromogenic substrates are used.

These drawbacks are not present in the methods in which radioactive labeled natural proteins are used as substrates for inhibitory activity determination. In the present paper trypsin and chymotrypsin inhibitor activity determination is described in plant material by means of urea-denatured ^{125}I-labeled albumin. In comparison to native radioalbumin, this substrate is about 20 times more sensitive to enzymatic hydrolysis of both enzymes. Good sensitivity and reliability of the method make it possible to use it for determination of trypsin and chymotrypsin inhibitor activities in crude extracts of different plant seeds. It is particularly suitable when the estimation must be done in crops with low inhibitory activities.

MATERIALS AND METHODS

Material. Trypsin and chymotrypsin were obtained from Merck and Fluka AG, respectively. Stock solutions of both enzymes were prepared in 0.001 M HCl and stored at 4–6 °C for a maximum period of 10 days.

Labeling of bovine serum albumin was carried out by the chloramine oxidation method (Greenwood et al., 1963) using noncarrier radioactive sodium [^{125}I]iodide of specific activity of 385 MBq/mL. Radioalbumin was separated from the reaction mixture by gel filtration on Sephadex G-25. ^{125}I-Labeled bovine albumin denatured by alcalic urea solution was used as substrate for enzymatic trypsin and chymotrypsin activity measurements (Blahovec, 1980). The procedure of preparation, slightly modified, is as follows: 250 mg of bovine serum albumin was dissolved in 3 mL of redistilled H_2O, and then successively 2.25 g of urea, 0.5 mL of 1 M NaOH, and 1 mL of ^{125}I-labeled albumin (about 3 MBq) were added. The reaction mixture was kept at room temperature for 60 min and then neutralized by 1 M HCl to about pH 7.5. The stock solution of the substrate used as incubation mixture was prepared by dilution of denatured ^{125}I-labeled albumin with 0.1 M Tris-HCl buffer, pH 7.5, supplemented by 0.02 M $CaCl_2$ so that 0.9 mL of the substrate had an activity of approximately 3.7–5.5 kBq (0.10–0.15 μCi). The procedure used for radioalbumin denaturation does not increase the degree of spontaneous deiodination.

Preparation of inhibitor extracts was as follows: Commercial samples of peas, beans, horse beans, lentils, soybeans, and maize were obtained from the Central Agricultural Control and Testing Institute in Košice. The pea cultivars 701–720 were obtained from the Research Institute of Plant Production in Piešťany, Improving Station Horná Streda. The seeds were ground and sieved through a series of nets. The samples of 160 mesh were extracted by 0.2 M NaCl (0.5 g of sample into 50 mL of NaCl). Extraction was carried out for 1 h at room temperature, and then suspensions were centrifuged at 6000g for 30 min. The supernatant was kept at 4–6 °C until used. To determine inhibitor activity, the original inhibitor extracts were diluted so that 0.2 mL of diluted inhibitor contained 150, 100, 50, and 25 μL of the original extract. Dilution was made by 0.1 M Tris-HCl buffer of pH 7.5 with addition of $CaCl_2$.

The standard curves for trypsin and chymotrypsin activity determination were obtained as follows: 1.25–20.0 μg of enzyme at a volume of 0.1 mL (stock solution) was added into 0.9 mL of the incubation mixture (5.5 kBq of ^{125}I-labeled native or denatured albumin in 0.1 M Tris-HCl buffer, pH 7.5, with 0.02 M $CaCl_2$). The samples were incubated for 10 min in a water bath at 37 °C, and then 0.2 mL of 7% albumin was added. The reaction was stopped by adding 0.8 mL of 10% trichloroacetic acid. After 30 min of centrifugation at 5000g, the radioactivity of 1 mL of deproteinized supernatant was measured in a well-type scintillation crystal (Gama Automat, Tesla). The rate of spontaneous hydrolysis and deiodination was determined by means of control samples into which the corresponding volume of incubation buffer had been added instead of enzyme.

Determination of antitryptic and antichymotryptic activity was carried out by measuring the decreased digestion of denatured radioalbumin with corresponding enzyme in the presence of

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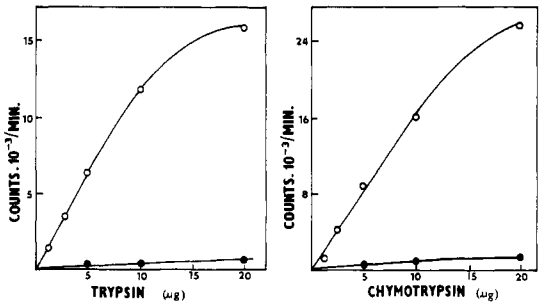

Figure 1. Enzymatic activity of trypsin and chymotrypsin as a function of enzyme concentration. (O) Denatured radioalbumin; (●) native radioalbumin. The details of conditions were described under Materials and Methods.

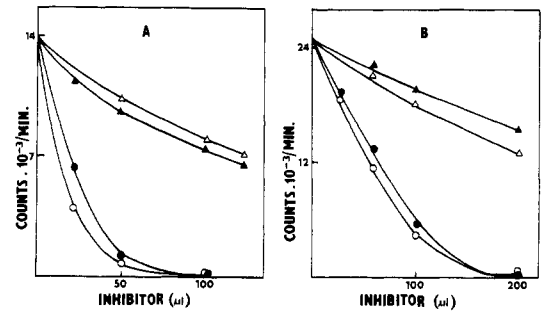

Figure 2. Inhibition of trypsin activity (A) and chymotrypsin activity (B) as a function of level of crude seed extract. (O) Soybean; (●) bean; (▲) lentil; (△) pea. The details of conditions were described under Materials and Methods.

various inhibitor extract concentrations. 0,2 mL of diluted or undiluted inhibitor solution were usually added into 0,2 mL of the enzyme solution (10 or 5 μg), and after 30 s 1 mL of the incubation mixture was added. The methods used for incubation, stopping of the reaction, measurement of radioactivity, and control samples were similar to those mentioned above. All determinations were carried out in triplicate and in most cases did not differ from the mean values by more than 5%. The amount of trypsin and chymotrypsin inhibitors is given in inhibitory units (IU). One inhibitory unit inhibits 1 unit of trypsin or chymotrypsin at the test conditions as defined in Merck and Fluka catalogs, respectively.

RESULTS AND DISCUSSION

By use of natural nonmodified radioiodine labeled substrate (Mešter et al., 1970) the optimal level of enzyme to be determined is relatively high (approximately 100 μg). Application of such enzyme concentrations for trypsin and chymotrypsin inhibitor determination in extracts with low inhibitory activities is not suitable. In such cases the decrease of enzyme activity is too small, which considerably affects the precision of determination.

In our procedure with urea-denatured radioalbumin it is possible to decrease both enzyme concentrations to 2.5 μg (Figure 1). From the data shown in this figure it is also evident that application of urea-denatured radioalbumin makes it possible to obtain a linear dependency of the enzymatic reaction on trypsin and chymotrypsin levels within a range of 1–10 μg. Specific activity within this range of concentration is constant for both enzymes so that enzymatic reaction in this case follows zero-order kinetics. To eliminate error that may be eventually caused by the hydrolytic effect of plant proteases on the substrate used, it is important to use the shortest possible incubation time, which in our case could be shortened to 10 min.

Figure 2 illustrates the inhibition of trypsin and chymotrypsin activity as a function of the various amounts of soybean, bean, pea, and lentil extracts. The inhibitory

Table I. Presence of Trypsin and Chymotrypsin Inhibitors in Some Plant Seeds[a]

seed	trypsin inhibition, IU/g	% of inhibition[b]	chymotrypsin inhibition, IU/g	% of inhibition[b]
soybean	94	100	638	100
bean	76	78	489	76
lentil	15	16	82	13
pea	13	14	132	20
horse bean	3	3	33	5
maize	3	3	27	4

[a] Values are given in inhibitory units (IU) per gram of sample. An IU represents the amount of inhibitor inhibiting 1 unit of the corresponding enzyme. [b] Inhibition of the corresponding enzyme by soybean inhibitors was taken for 100%. The other values have been calculated to this number.

curves in soybean and bean are linear to approximately 70% of both enzymes; when compared to the curves of pea and lentil extracts, their slope is steeper. Evident differences in slope of the inhibitory curves of the single-seed extracts prove the presence of different inhibitor types with various affinities for the corresponding enzymes. Deviations from linearity at higher inhibitor levels are probably due to endeavor to occupy the binding sites of the enzyme simultaneously by a greater number of inhibitor molecules. This has also been seen in many other well-known proteolytic enzyme inhibitors (Fritz et al., 1974).

The presence of trypsin and chymotrypsin inhibitors in soybean, bean, lentil, pea, horse bean, and maize is listed in Table I. The values given in inhibitory units (IU) per gram of sample were obtained from the inhibitory curves by reading the values for 50% inhibition of the given amount of enzyme and their calculation for 100% inhibition. Because of the variability of enzyme unit value (it depends on the substrate used and test conditions), the inhibitory values are often expressed in weight of inhibited enzyme per gram of sample (Liener and Tomlinson, 1981; Tan et al., 1984). This is more practical, and provided that the determinations are made with pure enzyme (or values are corrected on pure enzyme), it is possible to do direct comparison of the inhibitory values. When the inhibitory units listed in Table I were calculated in terms of milligrams of inhibited enzyme per gram of sample, inhibitory activity of all seed extracts to chymotrypsin—when compared to trypsin—was evidently lower. In the soybean and bean it forms only approximately 40% of trypsin inhibitory activity. Similarly, values of less than 50% of trypsin inhibitory activity were found in soybean for chymotrypsin inhibitory activity by Liener and Tomlinson (1981) and by Baintner (1981). As expected, the highest levels of both types of inhibitory activities are present in soybean and bean. As compared to soybean values only about 14% of trypsin inhibitors and about 20% of chymotrypsin inhibitors are present in peas, whereas both types of inhibitory activities do not surpass 5% in horse bean and maize.

The good sensitivity of denatured radioalbumin to trypsin and chymotrypsin hydrolysis enabled us to estimate the differences in the presence of both types of inhibitory activity in different pea cultivars. The results given in Table II were obtained from the inhibitory curves of three different amounts of pea extracts. Results reveal different trypsin and chymotrypsin inhibitor levels in the pea cultivars tested. The difference of antitryptic activity concentrations in cultivars 706 and 718 reached approximately double values, whereas the difference of antichymotryptic activity between them was even greater.

With regard to the fact that trypsin activity is strictly

Table II. Trypsin and Chymotrypsin Inhibitor Content of Different Pea Cultivars[a]

cultivar no.	trypsin inhibitor, IU/g	chymotrypsin inhibitor, IU/g
701	21	291
702	13	176
703	18	247
704	19	225
705	16	176
706	12	137
707	16	176
708	22	231
709	21	236
710	18	181
711	23	225
712	19	231
713	24	335
714	15	165
715	22	225
716	24	231
717	21	280
718	25	341
719	17	330
720	23	297

[a] Values are given in inhibitory units (IU) per gram of sample. An IU represents the amount of inhibtor inhibiting 1 unit of the corresponding enzyme.

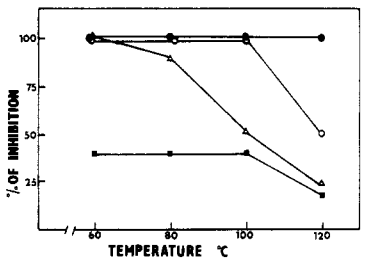

Figure 3. Effect of heat treatment on chymotrypsin inhibitor activity. Crude seed extracts were submitted to various temperatures for a period of 60 min. (○) Soybean; (●) bean; (△) pea; (■) horse bean.

specific to hydrolysis of peptide bonds formed by basic aliphatic amino acids, the prevailing part of dietary protein is better hydrolyzed by the effect of chymotrypsin than by that of trypsin. Total protein digestibility can, with regard to the above-mentioned, be markedly affected by the presence of chymotrypsin inhibitors. From the practical point of view the lowering of the undesirable antinutritive effects of these substances is very important. For this reason the effect of temperature on the inhibitory activity of trypsin inhibitors was often examined (Rackis, 1974; de Lumen and Salamat, 1980). In our experiments the resistance of chymotrypsin inhibitors present in soybean, bean, pea, and horse bean extracts to heat denaturation at 60, 80, 100, and 120 °C for 60 min was examined (Figure 3). Inhibitor concentrations were chosen so that in their presence (with exception of horse bean) enzymatic activity of chymotrypsin was entirely inhibited. From this figure it is evident that the chymotrypsin inhibitors of the above-mentioned leguminous seeds, except pea, resist heat denaturation up to a temperature of 100 °C for 60 min. Incubation at 120 °C resulted in evident destruction of inhibitory activities in soybean and horse bean. In beans even this temperature did not change chymotrypsin inhibitor conformation sufficiently to affect their inhibitory activity.

It is generally accepted that for evaluation of true tryptic or chymotryptic activity the proteins as substrates are most suitable, because they best simulate in vivo digestion. The values of antitryptic activity obtained with chromogenic substrates may be lower or higher than that obtained with protein substrates (Weder, 1986). The variable values of trypsin inhibitory activity of the same soybean extract were reported by Kakade et al. (1969) when determinations were carried out with N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPA) at various levels of inhibitory extract. When our method was compared with a standard method that uses BAPA, the values of antitryptic activity in crude soybean and bean extracts with BAPA substrate were about 70% of that obtained with urea-denatured radioalbumin. This difference may be due to the influence of various features on the inhibitory activity such as the competitive nature of inhibition and organic solvent. Both are present in the case of BAPA substrate. In addition, when synthetic chromogenic substrates are used, some of the hydrophobic interactions affecting catalytic enzyme activities with natural protein substrates do not take place. The above-mentioned reasons and some drawbacks of colorimetric methods already mentioned make it possible to conclude that the values for both types of inhibitors obtained by the use of modified radioalbumin are more correct and more approach the true inhibitory activity of plant seed extracts.

Summary. We have described a procedure for determination of trypsin and chymotrypsin inhibitors by use of urea-denatured radioalbumin. By this substrate we have determined the content of both types of inhibitors in crude extracts of soybean, bean, lentil, pea, horse bean, maize, and 20 pea cultivars. The levels of inhibitors expressed in inhibitory unit (IU) per gram were as follows: 94, 76, 15, 13, 3, and 3, for trypsin and 638, 489, 82, 132, 33, and 27 for chymotrypsin, respectively. There are smaller or bigger differences between the individual pea varieties in both types of inhibitory activity. The method is sufficiently sensitive, reliable, and particularly suitable when estimations must be done in crude plant extract with low inhibitory activity.

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