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A microassay for the analysis of trypsin inhibitor activity in peas

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A microassay has been developed, for determining trypsin inhibitor activities in peas, that requires only small amounts of sample (5 mg pea flour) and reagents. Acid extracts of peas were tested for their ability to inhibit the activity of bovine trypsin using the synthetic substrate, Nα-benzoyl-DL-arginine *p*-nitroanilide, in a microtitre plate. The extent of colour reactions were then rapidly determined using a plate reader. This new procedure allows a large number of assays to be performed in 1 day (up to 80), without significant loss of accuracy compared to the most commonly used larger scale Kakade assay. The method can be applied by plant breeders to screen the trypsin inhibitor activity of large numbers of pea samples and to the analysis of samples in genetics programmes where the quantity of material can be limited to a single seed.

INTRODUCTION

Pea seeds can be significant sources of protein for both human and animal diets (Augustin & Klein, 1989; Gatel & Grosjean, 1990). However, peas and most other legumes also contain small quantities of low molecular weight proteins, known as trypsin inhibitors (TI), that have the ability to form complexes with and inhibit the proteolytic activity of the digestive enzyme, trypsin (Leiner, 1989). This can lead to reduced availability of amino acids, reduced growth and, in extreme cases, to pancreatic enlargement.

Griffifths (1984) reported that the activity of proteinaceous TIs in pea were destroyed by moist heating and thus they are of little antinutritional consequence in properly prepared human diets, but when peas are compounded for animal consumption, the level of energy input during processing, e.g. pelleting, is insufficient to significantly reduce TI activity in animal feeds (Grosjean *et al.*, 1989). The amounts of these proteins in legumes are consequently of major interest to animal nutritionists, feed compounders, plant breeders and geneticists alike. Pea seeds contain typically 1–15 TI Units (TIU)/mg, an amount several times less than that found in soybean (Valdebouze *et al.*, 1980) where their antinutritional effects are well documented (Leiner, 1989), but this level is still believed by many animal nutritionists to be important (Jondreville *et al.*, 1992). Evaluation of the true nutritional effect of individual pea cultivars, high and low in TI activity is, however complex as there are many other factors, such as protein, starch and fibre levels and composition, that can have a significant influence on protein digestibility (Leterme *et al.*, 1990).

There has been a recent upsurge of interest in gaining data on TI activities in peas, particularly as high levels can incur penalty points in some countries within the European agricultural community, e.g. France (Anon, 1992). The most commonly used procedure for the assay of trypsin inhibitor activity in peas is based on the method of Kakade *et al.* (1974). This assay requires a relatively large amount of sample (at least 200 mg pea flour) and is time consuming and thus has disadvantages for plant breeders who would like to screen experimental lines at a much earlier stage in their programmes but are prevented from doing so by the constraints of the assay. The usefulness of the assay is also limited for plant geneticists who would like to obtain analytical information from a small part of a single seed and then germinate the remainder of that seed (Jones *et al.*, 1994). The object of this study was therefore to develop a rapid and robust assay using technology available in a routine analytical laboratory that would be applicable to very small quantities of sample, and the data obtained could be expressed in the same units and hence directly comparable with results from the established larger scale Kakade assay.

The analytical aspects of legume trypsin inhibitors were reviewed in detail by Belitz and Weder (1990). Most methods rely on the measurement of the inhibition of the enzymic reaction on a chosen substrate by the addition of sample extracts. Immunological techniques have also been used for the rapid assay of specific inhibitors in other legumes, i.e. soyabean

(Brandon *et al.*, 1988), but such methods first require highly pure fractions of an antigen, i.e. the inhibitor. Only a few of the family of proteins responsible for the TI activity of peas have been isolated and characterised (Domoney *et al.*, 1993) but as their relative inhibitory activities are not known, it is uncertain whether an immunoassay would reflect true TI activity and enzyme activity assays must therefore continue to be the preferred option. A number of choices of substrates for the enzymic assay of trypsin activity exist but whilst more sensitive, but more complex, substrates are available (Geiger & Fritz, 1984), BAPNA ($N\alpha$-benzoyl-DL-arginine *p*-nitroanilide), as used by Kakade *et al.* (1974), still remains the best option in terms of commercial availability and enzyme specificity. Additionally, amongst the available synthetic substrates the effect observed using amide substrates, which include BAPNA, is reported as being closest to that measured using natural substrates, i.e. proteins (Belitz & Weder, 1990). BAPNA was therefore used here together with bovine trypsin, but it should be noted that trypsins from different species of animal may have different relative activities with TIs from different plant species (Arentoft *et al.*, 1991) and therefore trypsin from a species appropriate to any study should be used to be relevant to *in-vivo* digestion.

MATERIALS AND METHODS

Materials

Four pea cultivars, Madria, Frijaune, Ballet and Frilene, were selected for analysis on the basis of widely differing trypsin inhibitor activities. Pea seed was supplied as part of the ECLAIR programme (AGRE CT90 0048). Subsamples of each cultivar were milled to a fine flour (less than 200 mesh) in a Spex shatterbox (Metuchen, NJ, USA). All chemicals, unless otherwise stated, were obtained from BDH Ltd., Poole, UK, and were of Analytical Reagent grade.

Methods

Enzyme solution
Ten mg of bovine trypsin (Sigma, Poole, UK, Cat. No. T8253) was dissolved in 500 ml of 0·001M HCl, stored at 0°C and discarded after 7 days.

Substrate solution
Four hundred mg (Sigma cat no. B4875) was dissolved in 20 ml dimethyl sulphoxide and allowed to stand for at least 30 min. One ml aliquots of the resulting solution were then stored at -20°C for future use. As required, 1 ml of BAPNA solution was diluted to 50 ml with freshly prepared 20 mM $CaCl_2$, 50 mM Tris–HCl pH 8·2, preheated to 37°C. The final substrate solution was then stored at 37°C and discarded after 4 h. Larger quantities (500 ml) were prepared for the large scale assay.

Extraction procedure

Extracts were prepared fresh daily as described previously by An *et al.* (1993). For the microassay, 30 mg of pea flour was extracted on an orbital shaker with 3 ml of 0·009M HCl for 1 h at 20°C and for the larger scale, 1 g of flour was similarly extracted with 100 ml of 0·009M HCl. The extract was then centrifuged at 10,000 *g* for 20 min at 20°C. The supernatants were diluted as required with water.

Large scale Kakade assay

Five aliquots of between 0 and 2·0 ml of supernatant solution were pipetted into test tubes. Sufficient distilled water was added to give a total volume of 2·0 ml. Two ml trypsin solution was added to each tube. To two other tubes, 2 ml of sample supernatant (Sample Blank) or 2 ml of water (Substrate Blank) was added. All the tubes were placed in a water bath at 37°C to equilibrate. After 10 min, 5·0 ml of substrate solution was added to every tube. After a further 10 min, the reaction was stopped by the addition of 1·0 ml of 30% v/v acetic acid to each tube. Two ml of trypsin solution was added to both the Substrate and Sample Blanks. The A_{410} of the tube contents were measured in a spectrophotometer using the Substrate Blank as reference. Several sets of tubes were analysed at once, starting each, in sequence, at timed intervals with the addition of the enzyme solution.

Microassay

Preliminary experiments were performed to establish appropriate time and oven temperature parameters, for the microassay, that closely mimicked the incubation conditions used in the Kakade assay. Thermocouples were inserted in the wells of microtitre plates and the time taken for them to equilibrate in an oven at various temperatures recorded. An oven temperature of 41°C was required to heat and maintain the microtitre plates at 37°C throughout the assay procedure described below.

For the purposes of assay development, sample extracts were analysed in quadruplicate. The diluted supernatants of two samples were each transferred into four consecutive channels of a multi-channel reservoir and using an octapipette, aliquots of 0, 20, 30, 40 and 60 μl were added to consecutive rows on a standard 96 well polystyrene microtitre plate. The contents of those wells were then made up to a total volume of 60 μl with water. A further 60 μl extract supernatant was added to the next empty row of wells for the Sample Blank. Sixty μl of water was added to all wells in an empty column of the plate for the Substrate Blank. Sixty μl enzyme solution was then added to all the sample wells, but not to the Sample Blank or Substrate Blank wells. The plate was covered and placed in the oven for 15 min. One hundred and fifty μl of substrate solution was then added to all wells and the plate returned to the oven. After exactly 10 min, the reaction was stopped by the addition of 30 μl 30% acetic acid

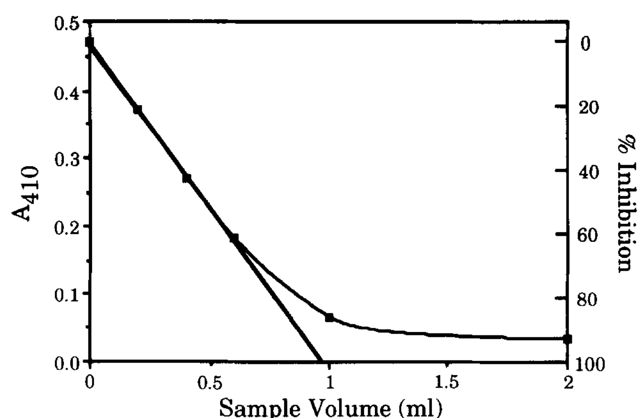

Fig. 1. Plot of trypsin activity (A_{410}) vs volume of added sample extract for Kakade assay.

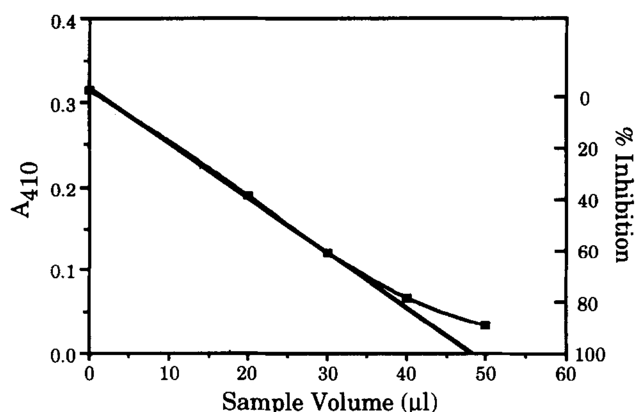

Fig. 2. Plot of trypsin activity (A_{410}) vs volume of added sample extract for microassay.

per well. Sixty μl enzyme solution was then added to the Sample and Substrate Blank wells. The plate was read at 410 nm on a Dynatech MR5000 plate reader zeroed with the Substrate Blanks. Several plates were analysed at once, starting each, in sequence, at timed intervals with the addition of the enzyme solution.

CALCULATIONS

All sample data values were corrected for sample blank as follows:

Sample (A_{410}) — (Sample blank (A_{410}) × Sample volume/ Blank volume*)

The corrected Sample (A_{410}) values were plotted vs Sample volume (Figs 1 and 2). TI activity was calculated from the slope of the linear part of the plot as follows:

TI activity (TIU)/mg sample = SLOPE × dilution factor/ (0·01 × (total assay volume(ml)/10) × sample concentration (mg ml^{-1})). One TIU is defined as a decrease in A_{410} by 0·01 in 10 min using the large scale assay. Data from the microassay were standardised using the average enzyme activity control value (i.e. the large scale assay performed with no sample) which was determined for each new batch of trypsin.

RESULTS AND DISCUSSION

Experiments were carried out to determine the relative precision of the microassay and the large scale assay. Least squares fits of the plot of absorbance vs volume of sample extracts were used to check the linearity of response. Correlation coefficients of 0·990 and 0·998 were obtained for the microassay and large scale method, respectively. For both methods a linear fitting of experimental data was observed over the 0–65% inhibition range.

Both assays were applied to the analysis of four pea flours. Each extract was assayed in quadruplicate and to include variations in extraction procedure, both methods were performed independently by two different analysts on several different days. For each sample both assays were performed at least 30 times. Table 1 shows the results obtained and as expected, Frijaune and Frilene, both winter cultivars, had high TI activities and all results were of a similar order to the results obtained by other workers (Leterme *et al.*, 1992) although variation due to agronomic and environmental influences must be expected (Bacon *et al.*, 1992). Both methods gave comparable overall levels with no significant difference between data sets being detected. Neither method could be shown to give higher or lower results than the other. The large scale method gave slightly better reproducibility with variance levels in the range 2–5%, with the exception of cv. Frilene, compared with 4–7% for the microassay. This degree of precision, however, is well within acceptable limits and in many applications a simple indication of high or low trypsin inhibitor activity is sufficient.

Using the microassay, an analyst could maintain a throughput of eight plates per day; allowing space for blank determinations this allows for a maximum of 10 assays per plate and hence a throughput of 80 assays per day. This allows time to complete sample and reagent preparation and calculation of results. In contrast, throughput using the large scale method was limited to 16 assays per day. The time advantage is principally gained by the use of rapid multichannel pipetting of samples and reagents and ease of reading and

*2·0 ml or 60 μl for large scale or microassay respectively.

Table 1. A comparison of data from large scale and microassays for trypsin inhibitors activity in pea cultivars

Sample	Trypsin inhibitor activity (TIU ml^{-1} dry matter)				
	Large scale Kakade assay		Microassay		
	TIA[a]	%cv	TIA[a]	%cv	a/b[b]
Madria	2·41 ± 0·12	5·0	2·53 ± 0·18	7·1	1·05
Frijaune	12·25 ± 0·23	2·0	11·75 ± 0·48	4·1	0·96
Ballet	5·63 ± 0·19	3·4	5·50 ± 0·27	4·9	1·02
Frilene	14·40 ± 1·15	8·0	15·25 ± 0·90	5·9	1·06

[a]Mean value ± SD of > 30 determinations.
[b]a/b = microassay mean/large scale assay mean.

recording data from microtitre plates relative to the lengthy procedure required to pipette out test tubes singly and the inefficient transfer of test solutions to cuvettes for measurement. Other lesser advantages may also accrue from a reduced scale, particularly from being able to handle many more sample extractions at once through procedures such as centrifugation.

For duplicate assays, the minimum volume of extract required is 500 μl for the microassay and 20 ml for the large scale assay. This is equivalent to sample masses of 5 and 200 mg respectively, a 40-fold difference in scale. Pea seeds weigh typically 150–350 mg. The microassay method can readily be applied to the analysis of parts of single seeds where only 30–40 mg of flour can be yielded from a single seed and still leave it viable for germination (Jones *et al.*, 1994). A microtitre based method for TI activity in small samples of flour from pea embryos has previously been developed, using similar reagents by Domoney and Welham (1992). However, the extraction and measurement protocol they used did not allow the direct comparison of their data with that obtained from the Kakade assay and because the authors wished to assay total protein and TI activity on the same extract they used NaOH solution to ensure complete dissolution of protein in their samples. Among the disadvantages of using an alkaline extract is that coextracted proteins are liable to precipitate during the course of the TI assay and require an extra time consuming step of centrifuging each assay mixture and returning to a clean plate before colour measurement, thus prohibiting its use for the analysis of a large numbers of samples.

The new procedure described here achieves the goal of this study and allows a large number of analyses to be performed in one day (up to 80), without significant loss of accuracy, and only requires small amounts of sample and analytical reagents. It can also be used either to screen the trypsin inhibitor activity of large numbers of samples or applied to the analysis of samples where the amount of material is limited, such as parts of single seeds.

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