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Notes & Tips

Detection of α-amylase inhibitors by a zymography method, performed in isoelectric focusing electrophoretic PhastGels

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Different electrophoretic methods for the analysis of α-amylase inhibitor isoforms have been tested and some of these methods include immunostaining after electrophoresis requiring specific antibodies which, in some cases, give false signals due to cross reactivity with other closely related proteins. However, zymography techniques have been shown to be effective tools for visualizing, isolating, and characterizing natural enzyme inhibitors [1–3]. Many of these zymography methods are based on the separation of proteinaceous inhibitors on an SDS–polyacrylamide gel containing the protein substrate copolymerized into the gel. Usually, the amylase inhibitors are tested on unique-percent native-polyacrylamide gels copolymerized with starch. After electrophoresis, the gel is washed and incubated with the target amylase solution to allow the substrate to be digested by the target amylase. The undigested amylase substrate remains where the inhibitor molecules are located and can be stained by using a KI/I_2 solution, which produces dark blue bands on a light background [4,5].

A similar zymography method was useful in searching for identification of mutants in pigeonpea (*Cajanus cajan* L. Millisp) containing diverse isoforms of α-amylase inhibitors; however the method was not useful to separate similar molecular-weight inhibitors with small differences in their isoelectric points [5]. We developed a simple reverse zymography method, performed in IEF[1] gels which were incubated in a soluble starch solution and then stained with a KI/I_2 solution, allowing a better resolution and separation of amylase inhibitors than the traditional native or SDS-based zymograms. By using this methodology, a wide range of quantities of plant amylase inhibitors could be selectively detected, allowing detection of new inhibitors with small differences in their isoelectric point.

Finely ground bean seeds of *Phaseolus vulgaris* L and *P. coccineus* were 3× defatted by shaking them with acetone (1:1 w/v) for 10 min and then decanting. The defatted flour (10 g) was mixed with 50 ml of 0.1 M NaCl and continuously stirred for 180 min at 4 °C. The soluble proteins in the slurry were obtained by centrifugation at 20,000*g* for 60 min and the supernatant was dialyzed extensively against water to allow protein precipitation. After centrifugation (20,000*g* for 20 min), the supernatant was freeze-dried. The resulting powder was used as initial source of inhibitor for zymography. Zymography technique was developed by using a PhastSystem instrument (GE Healthcare Life Sciences) operating with PhastGel Media IEF 3–9, which allows separation at a pH range from 3 to 9. Mixtures of p*I* markers between pH 3.5 and 9.3 were used. Electrophoresis was performed on a thermostatic plate maintaining the temperature at 15 °C. Samples were run as described by the manufacturer (Separation Technique File No. 100). In the IEF inhibitor zymogram, after electrophoresis the gel was incubated in 1.0% soluble starch in 10 mM Na-citrate, pH 5.0, containing 20 mM NaCl and 20 mM $CaCl_2$ for 1 h at 4 °C. The gel was rinsed quickly with the same buffer and incubated at 30 °C for 20 min with human salivary amylase dissolved with the activity buffer. After this, the gel was rinsed with water and then the starch–gel was stained with an iodine/iodide (0.5% I_2 and 5% KI) solution for 10 min. This treatment produced dark blue bands on a light background. To detect protein bands, a part of the gel was stained in a Coomassie brilliant blue solution prepared by adding one tablet of PhastGel Blue R (GE Healthcare) to 400 ml of bleaching solution (30% methanol and 10% acetic acid) for 30 min and then washing with the bleaching solution until the

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[1] *Abbreviation used:* IEF, isoelectric focusing.

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background of the gel was completely decolored. The isoelectric points were calculated using standard proteins with known isoelectric points and with software (Image Master VDS) provided by Pharmacia Biotech.

To determine whether this zymography technique could be used as a powerful tool to find new α-amylase inhibitors from plants, different accessions of *P. vulgaris* seeds were screened for the presence of these kinds of enzymatic inhibitors. In this experiment, two adjacent applications of protein samples from bean seeds containing the inhibitor were separated by using IEF-PhastGel focused in pH 3.0 to 9.0 ampholine gradients. One part of the PhastGel was visualized for protein and pI markers and the other part was used to locate the α-AI activity bands. Fig. 1 shows that this optimal zymography technique gives clear patterns containing a sharp blue α-AI band on the starch–gel background, because the amylase digests the starch everywhere in the gel, except where the inhibitor is located (Fig. 1, lanes 2a and 2b). It is very important to point out that, after longer incubation times the α-AI band could gradually disappear. We found two protein bands with isoelectric points (p*I*) of 4.7 and 5.0 (Fig. 1, lanes 1b and 1d). Similar results were previously reported by Valencia Jimenez et al. [6] and Veronique et al. [7]. In those experiments, the isoelectric point for α-AI isoform 1 was found to be 4.7 and 4.6, respectively. In addition, positions of the protein and inhibitory activity bands on the IEF PhastGel were identical, giving direct evidence that the α-amylase inhibitor was proteinaceous. α-AI-1, which inhibits mammalian and some insect α-amylases, is frequently found in many cultivated common bean varieties [8].

P. coccineus has been reported to contain α-amylase inhibitors [9–11], and we used our zymography methodology to detect the presence of this kind of α-amylase inhibitor in two different accessions of scarlet runner bean. By using this new technique, it was possible to find not only one but different α-AI-1 isoforms in some of the *Phaseolus* varieties that were tested. The results (Fig. 2) show that our methodology produces a very clear zymogram image (dark blue band of α-amylase inhibitor bands on a white background) and probes the high capacity of this technique to separate and to elucidate, with a very good resolution, the different α-AI-1 isoforms that could be present in the plant sample. The size and intensity of the dark blue band depend upon the protein concentration and activity of α-amylase inhibitor in the starch–PhastGel. Of particular interest is the different patterns found in the two seed *P. coccineus* accessions. The *P. coccineus* accession that was run in lane 1 shows a α-AI isoform (α-AI-1) with a superior level of expression compared to those of the others. In our opinion, any effort to purify α-amylase inhibitors from this scarlet runner bean accession must be addressed to this particular activity band because of the demonstrated abundance of this inhibitory activity band. Other zymography methods tested to detect α-amylase inhibitors in plant seed sources, based on starch–gels, were always run on homogeneous starch–gels needing, in some cases, additional experimental steps which require that the initial polyacrylamide gel must be sandwiched with other starch–gels [12], generating a probable diminution of inhibitory activity. The results show that, under the conditions of our assay, the zymography technique using IEF PhastGels is a promising tool to screen plant extracts for inhibitory activity.

In summary, we have developed a simple starch–PhastGel method very useful for detecting α-amylase inhibitors from natural sources, differing not only in their molecular weights, but also in their isoelectric points. Our methodology shows that it is possible to separate these proteinaceous inhibitors while keeping their biological activity under very good resolution conditions. By using this methodology

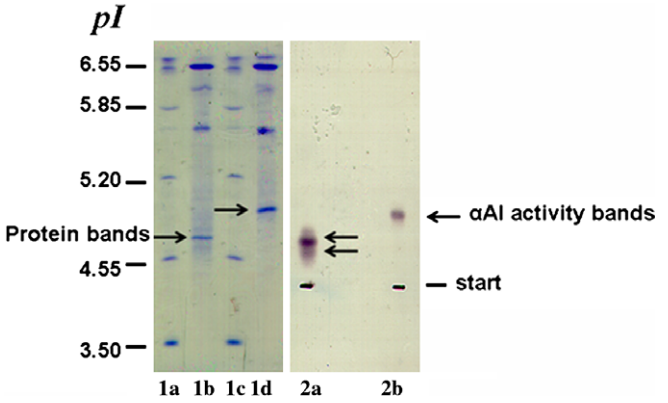

Fig. 1. Isoelectric focusing of α-amylase inhibitor from two accessions of *P. vulgaris*. Lanes 1a and 1c, mixture of p*I* standard proteins, human carbonic anhydrase B (p*I* 6.55), bovine carbonic anhydrase B (p*I* 5.85), ß-lactoglobulin A (p*I* 5.20), soybean trypsin inhibitor (p*I* 4.55), amyloglucosidase (p*I* 3.5). Lanes 1b and 1d, proteins from *P. vulgaris* Coomassie blue stained, showing the α-AI protein band. Lanes 2a and 2b, inhibitor zymogram. Isoelectric focusing was performed on PhastGel IEF 3-9. The zymogram gel was specifically stained with iodine solution to detect α-amylase inhibitor activity bands.

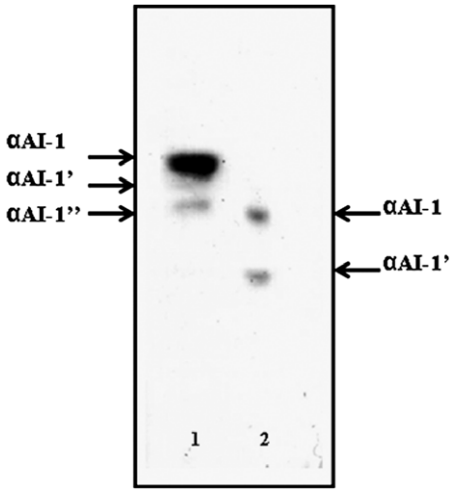

Fig. 2. Isoelectric focusing of α-amylase inhibitors from two different accessions of *P. coccineus* seeds. Lanes 1 and 2, α-AI zymograms showing the activity protein bands. Electrophoresis was performed on PhastGel IEF 3-9.

some characteristics of α-AI*s* from common and scarlet runner bean seeds are revealed.

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