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Detection of electrophoretically separated amylase inhibitors in starch–polyacrylamide gels

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Abstract

A method for detection of electrophoretically separated proteinaceous amylase inhibitors is described. Seed powders of pigeon pea, sorghum, chick pea and pearl millet were extracted with 0.1 *M* HCl and the amylase inhibitors present in the extract were analyzed after ammonium sulfate fractionation. The inhibitors were separated in polyacrylamide gel containing 0.5% soluble starch by electrophoresis and visualized by incubation of the gel in salivary amylase solution and staining with iodine. Starch in the gel is hydrolyzed by amylase during incubation, but the starch in vicinity of amylase inhibitor is protected from hydrolysis and appears as a blue band after staining. Using this protocol, pigeonpea, sorghum, chick pea and pearl millet seed extracts were found to contain at least three inhibitors of salivary amylase. These inhibitors had no activity against bacterial and fungal amylases. The method can be used to screen specificity of individual amylase inhibitors against various amylases.

Keywords: Amylase inhibitors; Enzymes; Detection; Electrophoresis

1. Introduction

α-Amylase inhibitors in a variety of plants are being studied for their possible use in strengthening plant defense against insect and microbial pests (for review see [1]). Transgenic pea (*Pisum sativum*) expressing bean (*Phaseolus vulgaris*) α-amylase inhibitor in developing seeds has been found to be resistant to pea weevil, *Bruchus pisorum* [2] and storage pests *Callosobruchus maculatus* and *C. chinensis* [3]. Limitations in the use of amylase inhibitors as tools in strengthening plant defense stem largely from lack of information on properties of amylase inhibitors in various plants. Most of the plants have several amylase inhibitors, differing widely in their properties, including toxicity against specific insects [4–7].

The earlier method for detection of amylase inhibitors in biological materials [8] was not suitable for study of the individual amylase isoinhibitors unless purified. It takes more time, is liable to the interference of endogenous amylases and does not permit detection of electrophoretically resolved inhibitors. The method of Siepmann and Stegemann [9] for electrophoretic separation and visualization of amylases in starch–polyacrylamide gels was there-

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fore extended to enable detection of amylase inhibitors in crude extracts. We describe here a simple, inexpensive and sensitive method for analysis of electrophoretically separated amylase inhibitors. Using this method, we have studied the amylase inhibitors in seeds of pigeon pea (*Cajanus cajan*), sorghum (*Sorghum bicolor*) chick pea (*Cicer arietinum*) and pearl millet (*Pennisetum typhoideum*).

2. Experimental

2.1. Materials

Chick pea (decorticated and split seeds-Dahl) was obtained from local market. Pigeon pea, sorghum and pearl millet seeds were obtained from Marathwada Agricultural University Research Station at Badnapur, Dist. Jalna, India. Starch was from Qualigen Fine Chemical Company, Bombay, India. Bacterial α-amylase type II A, *Aspergillus oryzae* α-amylase type X-A and polyvinylpolypyrrolidone (PVP) were from Sigma, St. Louis, MO, USA. Human saliva was diluted and used as salivary amylase. All other chemicals were of the highest purity available.

2.2. Extraction of amylase inhibitors

Decorticated seeds of chick pea (Dahl) and seeds of sorghum, pearl millet and pigeon pea were washed, dried and ground in a blender to obtain fine flour. The flour was defatted with hexane, dried and ground again. Defatted seed powder was stirred with six volumes of 0.1 *M* HCl containing 0.1 *M* NaCl and 1% PVP for 2 to 2.5 h.

Inclusion of PVP helped in removal of phenolics from the extract [10]. The suspension was centrifuged at 12 000 *g* and the pH of the pooled supernatant was adjusted to 7.0 with 1 *M* NaOH [11,12]. The suspension was centrifuged again and supernatant was subjected to ammonium sulfate fractionation. The extracts were made 40% saturated with ammonium sulfate. The precipitated proteins were dissolved in and dialyzed against distilled water and centrifuged. The clear supernatant contained amylase inhibitor(s), and was used for analysis (hereafter mentioned as partially purified extract).

2.3. Amylase and amylase inhibitor assay

Amylase activity was assayed by measuring liberated maltose [13]. Amylase inhibitory activity was assayed by measuring reduction in maltose liberated by salivary amylase using dinitrosalisylic acid reagent (DNS) [8,13]. One amylase activity unit is defined as activity resulting into liberation of 1 mg of maltose from starch at pH 6.9 at 37°C in 3 min. One amylase inhibitor unit is one amylase-unit inhibited under the given assay conditions. Protein was estimated by using Folin phenol reagent [14].

2.4. Electrophoretic separation of amylase inhibitors

Amylase inhibitors in the partially purified extracts of sorghum, pearl millet, chick pea and pigeon pea were analyzed on a vertical slab gel electrophoresis system in 7% polyacrylamide gels containing 0.5% soluble starch using Davis buffer system [15] and sometimes without stacking gel, or with Tris–glycine (pH 8.9) both in gel and electrode tanks. Protein bands were stained with Coomassie Brilliant Blue R-250.

2.5. Visualization of amylase inhibitors

After electrophoresis gels were placed in 20 m*M* phosphate buffer (pH 6.9) containing 6.7 m*M* NaCl for 5–10 min for equilibration and incubated in salivary amylase (20 units/ml) in 20 m*M* phosphate buffer (pH 6.9) containing 6.7 m*M* NaCl for 30 min at 37°C, after incubation, the gels were rinsed briefly in distilled water to remove excess amylase, and placed in iodine solution (10 m*M* iodine in 14 m*M* KI) for 4 to 5 min. The gel was washed to remove excess iodine solution and photographed.

2.6. Sensitivity of amylase inhibitor detection

Electrophoretically purified sorghum amylase inhibitor-1 (SAI-1) was used for determination of sensitivity of the method.

3. Results and discussion

Salivary amylase inhibitors in seed extracts of pigeon pea, sorghum, chick pea and pearl millet separated on non-denaturing, basic (pH 8.3), discontinuous, starch–polyacrylamide gel and visualized as described above are shown in Fig. 1. The seed extracts of pigeon pea, sorghum, chick pea and pearl millet contain at least three amylase inhibitors (designated as PAI-1 through PAI-3, SAI-1 through SAI-3, CAI-1 through CAI-3 and MAI-1 through MAI-3, respectively). Additional fast moving minor band of sorghum, chick pea and pigeon pea (PAI-4) may be detected at higher concentrations. The PAI-2 and PAI-3 were found in protein fraction precipitated at 80–100% ammonium sulphate saturation. These inhibitors appear as broad bands and may contain more than one form (result not shown).

Amylase inhibitor bands were not found when the gels were incubated in bacterial and fungal amylases. The inhibitor activity was also not detected in solution assay with bacterial and fungal amylases. This is consistent with the reports that pearl millet inhibitor [11] and sorghum inhibitor [12] inhibited salivary amylase but did not inhibit bacterial and fungal amylases.

The inhibitors purified by Chandrasekhar and Pattabiraman [11] and Moiden and Pattabiraman [12] are probably MAI-2 and SAI-1 respectively (which are major activity bands), whereas the other inhibitors were either lost during purification or remained undetected. The lowest amount of sorghum inhibitor (SAI-1) detectable using this method under the conditions described here was 0.10 units (Fig. 2). Amylase inhibitor protein in these gels could not be detected by Coomassie Brilliant Blue R-250 staining.

During incubation, amylase hydrolyzes starch as it enters the gel. However, starch in the vicinity of amylase inhibitor bands is protected from hydrolysis due to inhibition of amylase and appears as blue bands after staining with iodine. The size and intensity of blue bands correspond to the extent of inhibition of amylase which depends upon the concentration and activity of amylase inhibitor protein in the gel. Resolution and contrast between the amylase inhibitor bands and background depend upon amylase activity and starch content in the gel. Low starch concentration in the gel and low amylase activity in the incubating solution is necessary for the detection of inhibitor bands having low activity.

Besides being sensitive, this method is simple, convenient and inexpensive. The entire procedure takes about one hour after electrophoretic run. Simultaneous incubation of gel strips in solutions of desired amylases allows determination of specificity of inhibitors. Amylase solutions can be reused several times. Hydrolysis products of starch and proteins (including amylase inhibitors) may diffuse in incubation buffer and amylase solution but not affect reuse of amylase solution. The losses in amylase activity are negligible. The presence of starch in polyacrylamide gels did not alter the mobility of amylase inhibitors. The stained gels may also be washed and stored in refrigerator and restained for visualization, photography or scanning.

Presence of amylases in biological sample, par-

Fig. 1. Starch–PAGE (discontinuous) of amylase inhibitors. Lanes a and b: 20 μl, 50 μl of pigeon pea seed extract (protein 15 mg/ml); Lanes c, d and e: 10 μl, 20 μl, 50 μl of sorghum seed extracts (6 mg/ml); Lanes f, g and h: 10 μl, 20 μl, 40 μl of chick pea seed extract (21 mg/ml); Lane i and j: 90 μl, 80 μl of pearl millet seed extracts (3.6 mg/ml). Lane k: 1 AIU of electrophoretically purified SAI-1. Fast moving minor AI bands of pigeon pea, sorghum and chick pea were not shown. For details see Section 2.4.

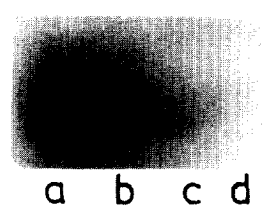

Fig. 2. Sensitivity of amylase inhibitor detection on starch–PAGE (discontinuous). Lane a, 10 amylase inhibitor units (AIU); Lane b, 2 AIU; Lane c, 0.1 AIU; and Lane d, 0.01 AIU.

ticularly from plants pose a major problem in detection of amylase inhibitors. The detection of amylase inhibitors in samples containing endogenous amylases is possible with our method. Usually the endogenous amylases appear as lighter bands and inhibitors appear as dark blue bands on faint blue background. Partial purification of samples by ammonium sulfate fractionation reduced this interference and also helped in enriching amylase inhibitory activity.

Fossum and Whitaker [8] in their method for detection of amylase inhibitor activity in biological samples, proposed incubation of the samples at 60–70°C for 5 to 20 min to overcome the interference of endogenous amylases. Most of the amylase inhibitors are heat sensitive [11,12] and are destroyed to a considerable extent during such treatment. The method is crude, requires 8 to 20 h and is useful in detection of amylase inhibitor activity or semiquantitative determination of aggregate amylase inhibitor activity in biological material.

Albumins in the seed extracts also inhibit starch–iodine complex formation by sequestering iodine and may be confused for amylase activity bands in the gel [16]. Staining part of the gels in iodine immediately after elctrophoresis enables identification of albumin bands. Some starch preparations have a higher tendency to form clumps and precipitate during polymerization of gels. Slow polymerization and overnight keeping of gels prior to run helps in even distribution of starch in the gel.

Some proteins separated by SDS–PAGE have been reported to regain their activity after thorough washing with non ionic detergents like Triton X-100. We have successfully used this technique to determine the molecular mass of protease isoinhibitors of pigeon pea [17]. However, our attempts of visualizing amylase inhibitor bands in starch–SDS-polyacrylamide gels were unsuccessful. In spite of extensive washing with and without Triton X-100, the starch iodine complex was not produced in the gel. This may be due to irreversible inactivation of inhibitor or due to stability of starch–SDS complex.

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