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Direct Detection of (1-3)-β-Glucanase Isozymes in Isoelectrofocusing Gels Using a Dye-Labeled Substrate

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염료착색 기질을 이용한 IEF gel에서 (1-3)-β-glucanase 동위효소의 검출

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ABSTRACT : A procedure for the direct detection of (1-3)-β-glucanase isozymes in electrophoresis gels was developed. The procedure employed the commercial preparation of AZCL-pachyman as a chromogenic substrate for (1-3)-β-glucanases. The procedure detected the three basic isozymes which have been known to be expressed in germinating barley kernels. A major acidic and a minor isozymes were also detected in germinating kernels. The procedure was proved to be fast, simple and sensitive enough to be used for the analysis of the expression of (1-3)-β-glucanase isozymes in plant tissues. The detection limit of the procedure for the commercial preparation of *Penicillium* (1-3)-β-glucanase was estimated to be as low as 50μU. The procedure could be used for the investigation of (1-3)-β-glucanases in laboratories facilitated with ordinary equipments and reseach personnel.

Key word : (1-3)-β-glucanase, IEF, Direct detection

Plant (1-3)-β-glucanases have been studied extensively because of their possible involvement in pathogenesis-related responses[2,3]. The possibility of isozyme-specificity in the physiological role of the enzyme[15] has also stimulated investigations on the developmental regulation of isozyme expressions. (1-3)-β-glucanases specifically degrade (1-3)-β-glucans (pachyman) and (1-3,1-6)-β-glucans (laminarin)[1,5,7,8,10,11]. Therefore, (1-3)-β-glucanase activities have been assayed colorimetrically or viscometrically using laminarin and pachyman as substrates.

Assay procedures to directly detect (1-3)-β-glucanase isozymes in polyacrylamide gel electrophoresis (PAGE) or isoelectric focusing (IEF) gels have been developed. (1-3)-β-glucanase activity bands were developed by

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staining the undegraded laminarin substrate present in the separating gel with Aniline Blue[6]. (1-3)-β-glucanase activity bands were also detected in the separating gel after incubating the gel in the solution containing laminarin substrate and by staining reducing sugars released[14]. Activity bands were also detected in the overlay gel containing dye-labeled laminarin by the selective removal of the depolymerized dye-labeled substrate[16]. However, incorporation of substrate in the separating gels can cause migration artefacts of the enzyme due to enzyme-substrate interaction during electrophoresis[6]. Detection of (1-3)-β-glucanases by staining reducing sugar released from the (1-3)-β-glucan substrate also detected reducing sugars which are not the products of (1-3)-β-glucanase action on the substrate[14]. Although detection of (1-3)-β-glucanases on overlay gels containing dye-labeled laminarin is sensitive and specific, this method requires preparation of the dyed laminarin substrate, casting substrate gels and selective removal of the depolymerized dye-labeled substrate from overlay gels.

Here we describe a simple, fast, specific and sensitive method for the direct detection of (1-3)-β-glucanases in IEF gels using the commercially available dye-labeled pachyman.

MATERIALS AND METHODS

Enzyme and chemicals

Commercial preparation of *Penicillium* laminarinase [(1-3)-β-glucanase] was purchased from Sigma Chemical Co. (USA). The chromogenic substrate AZCL-pachyman was from Megazyme (Australia). Antibiotics and other chemicals were from Sigma Chemical Co. (USA).

Plant materials

Kernels of malting barley (*Hordeum distichum* L. cv. Jinkwang) at the different physiological stages were used in this study. Developing kernels were collected from plants grown in the field at 5 days after anthesis (DAA) and at maturity. For germination, kernels were surface-sterilized with 0.2% (w/v) silver nitrate solution for 20 min at room temperature, washed thoroughly with sterile water and steeped in sterile water containing 10 μg/ml chloramphenicol, 100 μg/ml neomycin, and 100 units/ml nystatin[17] for 16 hr at room temperature. Sterilized kernels were germinated on filter paper in sterile petri dishes at room temperature for 5 days. All samples were frozen immediately after collection and kept at −70℃ until enzyme extraction.

Enzyme extraction

One gram of sample tissue was ground in a mortar and pestle in liquid nitrogen into a fine powder and homogenized in 2.5 ml of 50 mM sodium acetate buffer, pH 5.2(containing 10 mM sodium azide, 10 mM EDTA, 3 mM mercaptoethanol, 3 mM phenylmethyl fluoride)[17] at 4℃. After 20 min at 4℃, the homogenate was centrifuged for 10 min at 10,000 × *g* at 4℃. The supernatant was used for enzyme assay and protein quantitation. Samples for activity staining were homogenized in water instead of the extraction buffer. Protein content was measured according to Bradford[4].

(1-3)-β-glucanase assay

(1-3)-β-glucanase activity was determined using the chromogenic substrate AZCL-pachyman (Megazyme, Austrailia). Enzyme extract (0.1 ml) was incubated with 0.5 ml substrate solution (10 mg substrate in 0.5 ml

of the enzyme extraction buffer) for 20 min in a shaking incubator at 37℃. The reaction was terminated by adding 2.5 ml of precipitant solution made as described[12]. The solution was filtered through a Whatman No. 1 filter paper before the measurement of absorbance at 590 nm[13].

(1-3)-β-glucanase activity staining

Proteins were separated by ultra thin-layer isoelectrofocusing (IEF) in polyacrylamide gels (0.8 mm thickness; ampholine: pH 3-10, LKB, Bromma, Sweden) on silanized polyester sheets in an Ultraphor flat bed apparatus from LKB. Separating gels were pre-run at 2 w for 30 min and run at constant watt for 1 or 2 hr at 5℃ after loading samples. Before activity staining the gels were incubated in 0.5 M sodium acetate (pH 5.2) for 5 min with slow shaking, and briefly air-dried to remove excess of solution on the gel surface. Gel solution for agar substrate overlay was prepared by mixing equal volume of agar (20 mg/ml in 0.5 M sodium acetate, pH 5.2) and AZCL-pachyman (20 mg/ml in 0.5 M sodium acetate, pH 5.2) solutions at 65℃. Agar substrate gel solution was directly poured on the separating gel and the complex was incubated at 37℃ until the degradation zones developed. The agar substrate overlay gel was peeled off from the separating gel and the separating gel was vacuum-dried at 70℃.

RESULTS AND DISCUSSION

A fast, simple and specific (1-3)-β-glucanase activity staining procedure employing the AZCL-pachyman substrate was developed. To check the sensitivity of the procedure serial dilutions of the *Penicillium* (1-3)-β-glucanase were separated in IEF gels and activity bands were detected in the gel as described above. An activity band was detected in the lane where as low as 10 μU of enzyme was loaded (Fig. 1). Considering the detection limit as the enzyme unit producing a readily visuable distinct band, the limit of detection for the *Penicillium* endo-(1-3)-β-glucanase used was estimated to be as low as 50 μU. Activity of the commercial preparation of *Penicillium* (1-3)-β-glucanase on the AZCL-pachyman was also assayed. Increasing amount of enzyme hydrolysed the substrate more rapidly showing a near linear relationship between the enzyme unit tested and the change in absorbance of

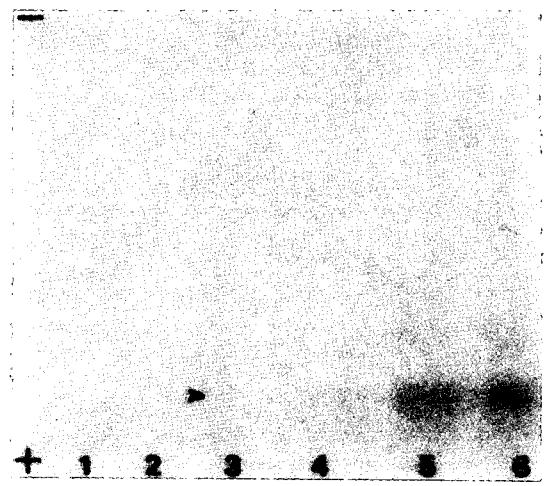

Fig. 1. Sensitivity of endo-(1-3)-β-glucanase detection by activity staining in the IEF separating gel. One-tenth (1), 1 (2), 10 (3), 100 (4), 300 (5) and 1000 (6) μU equivalents of the commercial preparation of *Penicillium* (1-3)-β-glucanase were separated by IEF (pH 3 to 10) and activity bands were detected by incubating the separating gel with the overlay substrate gel containing AZCL-pachyman (10 mg/ml agar and 10 mg/ml pachyman in 0.5 M sodium acetate buffer, pH 5.2) for about 3 hr at 37℃. A acidic activity band was detected in the land where as low as 10 μU enzyme was loaded (arrow-headed) in the separating gel.

the reaction supernatants. (data not presented). This reaction characteristic of the enzyme on the chromogenic substrate also indicated that the substrate could be used for the direct detection of (1-3)-β-glucanase isozymes in electrophoresis gels.

It has been recognized that (1-3)-β-glucanases have characteristic specificities to different substrates. The preferred substrate of (1-3)-β-glucanases is laminarin from *Laminaria digitata*[9]. Therefore, it was predicted that the susceptability of the AZCL-pachyman to the *Penicillium* (1-3)-β-glucanase could be lower than that of the dye-labeled laminarin. Sensitivity of the procedure was comparable with that of the procedure described by Pan et al. [14] but about thirty to fifty times lower than that of the procedure described by Sock et al[16].

It should be noted, however, that our procedure is simple, fast, specific and inexpensive. In the procedure described activity bands were detected in the separating gel after incubation with the substrate gel. The agar substrate gel was directly poured over the separating gel in a container and the substrate gel was simply peeled off from the separating gel after incubation of the complex about 1 to 3 hr depending on the levels of enzyme activity in samples. During the incubation period enzymes in the separating gel diffuse into the substrate gel and hydrolyse the dye-labeled substrate mainly to di- or trisaccharide[8]. The dyed enzyme-action products can diffuse into the separating gel developing activity bands in the gel. The agar substrate gel peeled off from the separating gel could be used at least three times by simply dissolving and pouring the gel on the separating gels. In Sock's procedure the agar substrate gel should be prepared by pouring the hot mixture between two glass plates separated by the spacer bars. Great care should be taken to overlay the substrate gel on the separating gel without trapping any air bubbles between the gels. Activity bands are developed in the substrate overlay gel after incubating the separating and substrate overlay gel sandwich for 4 to 5 hr and subsequent destaining of the overlay gel overnight. Furthermore, labeling laminarin with the dye requires extra facilities, toxic chemicals and special expertise[16]. In Pan's procedure the activity band was developed in the separating gel by sucessive incubation of the separating gel in the solutions containing laminarin substrate and triphenyltetrazolium chloride. Pan's procedure has disadvantages like the lack of specificity because the procedure could detect glycoproteins and reducing sugars which are not the products of the enzyme action on the substrate as enzyme activity bands[14].

After establishment of optimum substrate gel and incubation conditions investigations of (1-3)-β-glucanase isozymes were performed in barley kernels at different physiological stages. Different levels of (1-3)-β-glucanase activity were present in kernels at different physiological stages. Enzyme activity was highest in germinating kernels and lowest in developing kernels. Low level of activity was also present in dry mature kernels (Fig. 2A). The enzyme extracts used for the activity assay were used for the detection of (1-3)-β-glucanase isozymes in the IEF gel using the activity staining procedure developed. At least four major isozymes were clearly detected in barley kernels. Three major basic isozymes and a major acidic isozyme, designated as BI, BII, BIII and A, respectively, were detected in developing young kernels. The major isozyme present in dry ma-

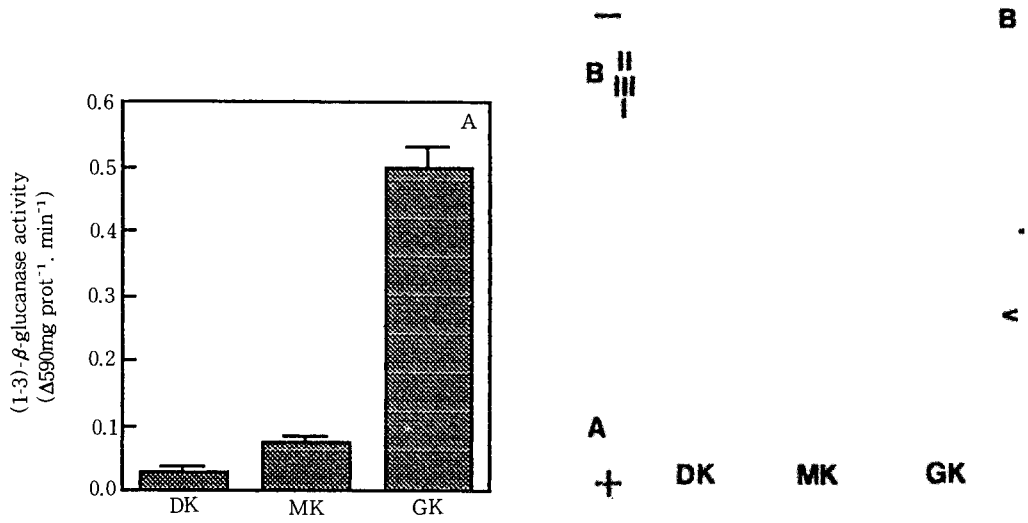

Fig. 2. A. (1-3)-β-glucanase activity in mature kernels (MK), developing kernels at 5 days after anthesis (DK), and germinating kernels at 5 days after germination (GK), respectively. Enzyme activity was determined by measuring changes in the absorbance at 590 nm after incubation of the protein extracts with the chromogenic substrate AZCL-pachyman for 20 min at 37℃. Vertical bars indicate standard deviations of three measurements. B. Multiple isoforms of (1-3)-β-glucanase in developing (DK), mature (MK) and germinating kernels (GK), respectively, detected by the procedure described. Protein extracts used for the enzyme activity determinations in A were separated by IEF (pH 3 to 10) and activity bands were detected by incubating the separating gel as in Fig. 1. Sixty (DK and MK) or thirty-five (GK) g of protein was loaded for each sample. Four major bands, one acidic and three basic bands, designated A, BI, BII and BIII, respectively, and a minor band (arrow-headed) were detected in the separating gel.

ture kernels was BII with the low levels of BIII and A isozymes. When dry kernels were germinated the four major isozymes increased rapidly. Drastic increase in the isozyme III expression was prominent in germinating kernels. A minor activity band was also detected in germinating kernels (Fig. 2B). Intensities of the activity bands were in good agreement with the levels of enzyme activity allowing qualitative explanation of the levels of enzyme activity in terms of the levels of isozyme expression. Three (1-3)-β-glucanase isozymes, GI, GII and GIII, with pI values ranging from 8.8 to 10.3 were identified as major basic isozymes expressed in germinating barley kernels[9]. The three basic isozyme BI, BII and BIII detected in the gel could be GI, GII and GIII, respectively, based on their pI values. This result also validated that the procedure described was specific and sensitive enough to be used for the quantitative and qualitative detection of (1-3)-β-glucanase isozymes in various plant tissues.

적 요

(1-3)-β-glucanase 동위효소의 발현 양상을 등전점 전기영동 젤에서 직접 검출 확인할 수 있는 방법을 개발하였다. 개발된 방법은 시판되고 있는

(1-3)-β-glucanase활성 측정용 염료착색 기질을 이용하였다. 본 방법은 신속, 간편하며 보리 종자에서 발현되는 것으로 알려져 있는 모든 (1-3)-β-glucanase 동위효소를 검출할 수 있을 정도로 민감하고 특이적이었다. 시판되고 있는 *Penicillium* (1-3)-β-glucanase에 대한 활성 검출 한계단위는 50 μU 정도로 추정되었다. 따라서, 본 방법은 특별한 시설이나 연구 인력을 확보하고 있지 않는 연구실에서 식물체의 (1-3)-β-glucanase발현에 대한 단백질 수준에서의 연구를 수행하는데 유용하게 이용될 수 있을 것으로 생각된다.

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