Detection of Chitinolytic Enzymes in *Ipomoea batatas* Leaf Extract by Activity Staining after Gel Electrophoresis

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A non-denatured SDS-PAGE followed by in-gel activity staining using embedded glycol chitin as a substrate was used to identify the proteins with chitinolyitc activities from sweet potato leaf extract. At least two chitinase activity zones can be clearly identified on the gel at positions with estimated molecular weights of 54.1~55.6 kDa and 39.6 kDa. Furthermore, our data also indicate that the activity of the larger one can withstand the standard SDS-PAGE sample preparation. Both of these chitinases, however, are different from that of the previously identified chitinase in sweet potato leaves, which has a molecular weight of 16 kDa. By using an embedded substrate, our method has superior sensitivity in detecting chitinases with higher molecular weights. It is a simple, affordable way and may aid in the future discovery of new chitinases.

Keywords: Chitinase; Glycol chitin; Activity staining; Sweet potato.

INTRODUCTION

Chitinases (EC 3.2.1.14) belong to family 18 and family 19 *O*-glycoside hydrolases, which hydrolyze the glycosidic bonds between two or more carbohydrates. Chitinases catalyze the hydrolysis of chitin, a β-1,4-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc), which is the main structural component of fungal cell walls and arthropod integuments. By means of endochitinase activity, chitinolytic enzymes hydrolyze chitin to produce chito-oligosaccharides with 2 to 6 GlcNAc units. Chitinases are classified into various classes, such as class I, II, III, or IV chitinases, based on their amino acid sequences.[1,2]

To date, chitinolytic activities have been reported in microorganisms,[3] plants,[4] and humans.[5,6] It is reasonable that all microorganisms that make chitin also make use of chitinases to sustain their morphogenesis, growth and nutrition.[3,7-8] Intriguingly, chitinases have also been identified in some organisms that do not synthesize chitin, such as plants and humans. The discovery of chitinases and chitinolytic enzymes in these organisms therefore suggests other functions of these enzymes. It has been proposed that chitinases may play a role in the defense against pathogens.[1-2,9-10] For example, in plants, chitinolytic enzymes are secreted in response to the attack of microorganisms and insects.[11-14] Therefore, these enzymes are also known as pathogenesis-related protein (PR-protein) families. Furthermore, families 3, 4, and 8 of the PR-proteins are known to be associated with food allergies.[15]

Chitinases are useful in the production of many biomedical and biotech products. They can be used in the production of chitooligosaccharides, glucosamines and GlcNAc, which have an immense pharmaceutical potential.[16-17] Other applications such as mosquito control and plant pathogenic fungi control have also been investigated.[18-21] The applications have stimulated the discovery and research of new chitinases from many organisms.

The sweet potato is one of the major vegetable crops worldwide. In Taiwan, the storage roots, young leaves and shoots are consumed. Many of the properties and physiological functions such as trypsin inhibitors, proteolytic systems, and specific proteases have been extensively studied.[22-26] However, it was not until 1998 that chitinase activity was found in sweet potatoes.[27] Here we report the identification of novel chitinases in sweet potato leaves by electrophoresis using glycol chitin-embedded SDS-polyacrylamide gel.

RESULTS AND DISCUSSION

The crude protein extracts from the sweet potato were separated by SDS-PAGE. (Fig. 1(A)) In lanes 1 and 2, pro-

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tein staining revealed that, by heating the sample with β-mercaptoethanol, the protein bands in the lower molecular weight region appeared to be relatively noticeable, although the major protein bands were still located near 54-55 kDa. It seems that, by breaking the disulfide linkage between the subunits of a larger protein complex, the proteins with lower molecular weights were released and thus resulted in the enhanced detection in the lower molecular weight region on the gel.

While using the SDS-glycol chitin polyacrylamide gel, we are able to detect the chitinase activities by silver staining (Fig. 1(B)). The chitinolytic activity in the gel can be visualized by detecting the clearance of embedded glycol-chitin, which resulted in bright zones after the silver staining. As shown in lane 3, several light zones were detected, in which the two with higher molecular weights apparently have higher chitinolytic activity. In lane 4, the sample was heated with β-mercaptoethanol before electrophoresis. It appeared that only the activity at 55 kDa survived the treatment of β-mercaptoethanol and heat. The activities detected at the lower molecular weight range seemed to be abolished by the treatment.

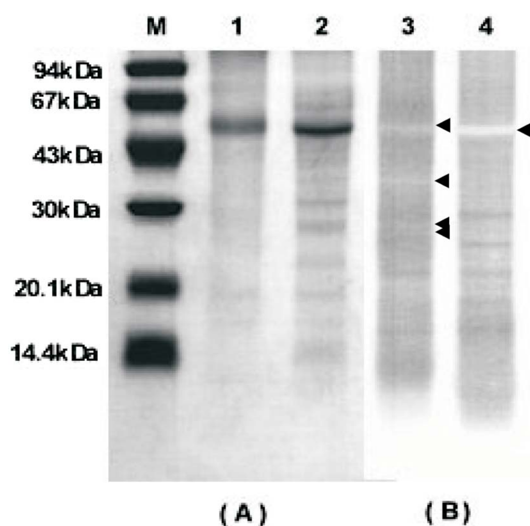

Fig. 1. Protein staining (A) on 15% SDS-PAGE by Coomassie Brilliant Blue R250 and activity staining (B) on 15% SDS-glycol chitin PAGE using the silver staining. Lane M, Commercial SDS-PAGE standards (Phamarcia, Amersham Biosciences Ltd.): phosphorylase b (94,000 Da), albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da); lanes 1 and 3, crude extract of sweet potato leaves loaded with sample loading buffer without β-mercaptoethanol and are not heat-treated; lanes 2 and 4, crude extract of sweet potato leaves, heat-treated with sample loading buffer containing 100 mM β-mercaptoethanol. Arrows indicate the positions with chitinolytic activity in lanes 3 and 4.

The molecular weights of the major activity zones detected in lane 3 were preliminarily determined at 55.6 kDa and 39.6 kDa according to the calibration curve of the mobility of standards on SDS-PAGE (Fig. 2). The thermo stable chitinolytic activity in lane 4 was determined to be 54.1 kDa. The discrepancy of the estimated molecular weights of the largest chitinase detected in lanes 3 and 4 were almost indistinguishable. It is reasonable that different sample treatment may have slightly changed the migration pattern of the PAGE, as the slight difference seen between lanes 1 and 2. However, since it is unlikely that heating a protein sample with β-mercaptoethanol can possibly shift the 39.6 kDa band in lane 3 to 54 or 55 kDa, we conclude that the two chitinolytic zones in lane 3 were the results of two enzymes. On the other hand, the activities detected at 55.6 kDa and 54.1 kDa, respectively, in lanes 3 and 4 were the results of the same enzyme.

Both of the 39.6 and 54/55 kDa activities were apparently different in molecular weights from the previously identified chitinase in sweet potato, which has a molecular

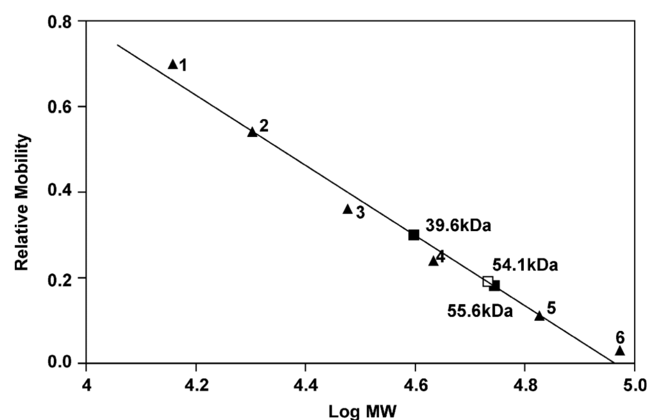

Fig. 2. Molecular weight determination of chitinases on SDS-glycol chitin PAGE. (▲) Commercial standards: 1, α-lactalbumin (14,400 Da), 2, trypsin inhibitor (20,100 Da), 3, carbonic anhydrase (30,000 Da), 4, ovalbumin (43,000 Da), 5, albumin (67,000 Da), 6, phosphorylase b (94,000 Da); (—) The calibration curve of the mobility of commercial standards; (■) Non-denatured extracts on SDS-glycol chitin PAGE; (□) Denatured extracts on SDS-glycol chitin PAGE.

weight of 16 kDa as reported by Hou et al.[27] In their study, the chitinase activity in the crude extract of sweet potato leaves was detected by overlaying another gel with incorporated glycol chitin onto the resolving gel. It is worth mentioning that several lesser clear zones were also detected in our SDS-glycol chitin PAGE analysis at lower molecular weight regions (Fig. 1(B), lane 3). It is plausible that the former studies fail to detect chitinases with higher molecular weights simply because the substrate they used in the activity staining was overlaid on the resolving gel rather than castled inside the gel. In our study, by embedding the glycol chitin in the resolving gel, direct interaction between the substrate and enzymes was assured. This allowed better detection of chitinolytic enzymes with higher molecular weights, which may have poorer mobility in the gel. Silver staining also allowed the direct visualization of clear zones with higher resolution than the previous methods.[30]

In conclusion, our analyses have identified new chitinolytic enzymes in sweet potato leaves. Further investigations on these chitinases are valuable in the understanding of their biochemical properties as well as their roles in host defense. The SDS-glycol chitin PAGE combined with silver stain is an excellent method in the detection of chitinolytic enzymes. The method is a simple and affordable way in the future discovery of new chitinases from different organisms.

EXPERIMENTAL SECTION

Plant materials

Fresh storage roots of the sweet potato (*Ipomoea batatas* [L.] Lam var. Tainong 57) were purchased from a local market in Taipei. The roots were placed in a thermostated (30 °C) growth chamber in the dark and water was sprayed twice a day. Sprouted roots of the sweet potato were planted in the open air and watered once every day. Leaves were collected and weighed immediately, put in an envelope and frozen in liquid nitrogen immediately. The frozen samples were kept at -70 °C for further use.

Crude extract from sweet potato leaves

Sweet potato leaves were milled to extremely fine powder in liquid nitrogen with a mortar and pestle. Powders were suspended in extraction buffer (20 mM sodium acetate buffer, pH 5.0, containing 0.7 mM β-mercaptoethanol) with a volume of 4 times the leaf weights and then homogenized by Polytron homogenizer (Kinematic, Switzerland) at 7,000 rpm for 3 min. The homogenate was centrifuged at 12,000 rpm for 20 min (Sigma 2K15, Rotor Nr. 12139). The supernatant was collected and designated as crude extract.

Electrophoresis

A discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate sample proteins under denatured conditions. 15% polyacrylamide gel was prepared in a vertical mini-gel system (Bio-Rad Laboratories, Richmond, CA) with a thickness of 1.0 mm. The gel, buffers and solutions were prepared as described by Weber and Osborn.[28] Samples (typically containing 10-15 μg total protein) for SDS-PAGE were preheated at 100 °C for 15 min in the SDS sample loading buffer (50 mM Tris-HCl, 2% (w/v) SDS, 100 mM β-mercaptoethanol, pH 6.8), run for 1 h at 120 V and then stained by Coomassie Brilliant Blue R-250. For molecular mass calibration, a subset of the following standards was included: phosphorylase b (94,000 Da), albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da).

SDS-glycol chitin polyacrylamide gel electrophoresis and activity staining of chitinase

The SDS-glycol chitin polyacrylamide gel was used for the in-gel detection of chitinase activity. The electrophoresis was performed in a 15% discontinuous SDS-glycol chitin PAGE containing 0.01% (w/v) glycol chitin. The sample loading buffer is the same as that used in SDS-PAGE but β-mercaptoethanol was absent. After electrophoresis, gels were first immersed in a 0.1 M sodium acetate buffer, pH 5.0, containing 1% (v/v) deionized Triton X-100, placed on a shaker, and incubated for 30 min. The gels were then transferred to a fresh 0.1 M sodium acetate buffer, pH 5.0, and incubated in a 37 °C thermostated chamber for 12 h. After incubation, the activity staining was executed according to the method adopted from Marek et al.[29] Fixation was performed in a liquid solution containing 50% (v/v) methanol, 12% (v/v) acetic acid, and 0.0185% (v/v) formaldehyde. Gels were then incubated on a shaker for 10 min in 40% (v/v) ethanol and for 10 min in 30% (v/v) ethanol. Pretreatment, rinsing, and silver impregnation were performed as reported by Marek et al.[29] The developer solution diluted at 1:4 with water was used to prevent staining of proteins within the clear zone and to provide better contrast over development. The development time, which usually takes 1 to 2 min, could be slightly varied upon visual inspection. The time that the gel was rinsed by

pure water between the developer and stop solutions was shortened to 10 s. After the development was stopped, gels were washed in 30% (v/v) methanol for 20 min and 10% (v/v) methanol for 20 min and were then stored in 10% (v/v) methanol at 4 °C before drying. Silver-stained gels were dried with a gel dryer (Bio-Rad Laboratories, Richmond, CA) and preserved at room temperature.

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