Purification and Analysis of Abscisic Acid-Specifically-Inducible Proteins from Rice Callus

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Abstract: Two ABA-specifically-inducible proteins from rice callus were isolated and purified by precipitation with 65-100% saturated (NH4)2SO4, followed by the DEAE-sepharose, TSK-gel, and two-dimension electrophoresis. Iso-electric points (pI) of the proteins with the same molecular mass (24.5 kD) were 6.1 and 6.9, respectively. The Western blot analysis indicated that the proteins expressed in different tissues were obviously different. The A1 (pI 6.1) protein was only detected in calli treated with ABA and seed embryos (SE). However, the A2 (pI 6.9) protein was found not only in the calli treated with ABA and SE, but also in the white dry callus. Thus it suggested that the two proteins might play some important roles in the processes of seed embryo (or somatic embryo) formation.

Key words: rice; callus; abscisic acid; specifically-inducible protein; purification; expression

The abscisic acid (ABA) played an important role in embryogenic callus formation [1], somatic embryo development [2] and maintenance of embryogenic callus in subculture [3]. In our previous studies, we had noted that exogenous ABA influenced callus structure, somatic embryo development and plant regeneration in rice [4], and found that ABA changed saccharide metabolism and related enzyme activities in rice callus [5]. All these results suggested that the rice callus might produce some differential proteins or enzymes responded to exogenous ABA. This hypothesis was consistent with Xu et al [6]. They found four specific proteins in the long-term cultured callus, with molecular mass of 45, 24.5, 18.5, and 14 kD, respectively. According to our knowledge, the functions of these proteins on the rice callus differentiation, somatic embryo formation and physiological characteristics have not been studied yet. To get enough purified proteins was vital for studying the bioactivity of the proteins, and preparing antibodies in immunoreactions. In this study, a technique via different types of ion-exchange materials and two-dimensional electro- phoresis was designed to purify the 24.5 kD proteins in rice callus induced by ABA. According to this method, two proteins were purified, with iso-electric points (pI) of 6.1 and 6.9, respectively. In order to analyze the expression of these proteins in different rice tissues, we prepared the antibodies of these proteins and performed the Western blot analysis. This work might establish some basic procedures for purifying other proteins and studying their functions in rice callus induced by ABA.

MATERIALS AND METHODS

Rice callus preparation

The mature seeds of rice (Oryza sativa subsp. japonica cv. Nipponbare) were used in this experiment. The seeds were dehusked and sterilized by immersion in 70%(V/V) ethanol for 2-3 min. After rinsing with pure water for three times, seeds were sterilized in 0.5-1.0% sodium hypochlorite solution and supplemented with a few drops of Tween-20 for 30 min, and rinsed 4-5 times with sterile pure water. Then the seeds were inoculated and subcultured in the induction medium, which contains NB basal medium (macronutrients of N6 basal medium, micronutrients and organics of B5 basal medium), 0.03% (W/V) casein hydrolysate (Sigma), 0.05% (W/V) L-proline (BBL), 0.025% (W/V) L-glutamine (Amresco), 3%
(W/V) sucrose, and was solidified with 0.75% (W/V) agar (BBI). The medium for callus initiation and proliferation was supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and adjusted pH to 5.7-5.8. After cultured for 180 d successively, the calli were transferred to the medium (MSB5, 2 mg/L 2,4-D, 3% sugar, pH 5.7-5.8) containing 0-12 mg/L ABA, and subcultured for 21 days.

**Extraction of the crude protein**

Weighed rice calli were blotted up the water with filter paper, and ground in liquid nitrogen, and then homogenated in cold extraction buffer (containing Tris-HCl, EDTA-Na, β-mercaptoethanol, pH 7.4) with a ratio of 2:1 for the buffer volume to the weight of rice calli in ice bath. The homogenates were centrifuged at 13 000 r/min for 20 min. The supernatant was centrifuged again at 13 000 r/min for 15 min to get the crude protein. The protein in seeds was extracted using the methods by Qu et al [7].

**Purification of the 24.5 kD protein**

The pre-cooled crude protein was added with (NH₄)₂SO₄ to get 65% saturation, and then agitated for 4 h. The mixture was centrifugated at 10 000 r/min for 10 min at 4°C. The supernatant was supplemented with (NH₄)₂SO₄ to 100% saturation and centrifuged at 13 000 r/min for 10 min. The precipitate was subsequently dissolved in a 0.05 mol/L Tris-HCl buffer (pH 7.4), and dialyzed completely. The solution was applied to a column of DEAE-Sepharose (2.0 cm × 1.5 cm, 0.15 mL/min) which previously equilibrated with the same buffer. After that, three NaCl solutions of different concentrations (0-0.2 mol/L, 0.2-0.4 mol/L, 0.4-0.75 mol/L) were used to elute the proteins from the DEAE-Sepharose column at the flow rate of 2.0 mL/min. The eluted liquid was collected and briefly applied to SDS-polyacrylamide gel electrophoresis (PAGE). The eluted liquids with target electrophoretic patterns were adjusted to 50% saturation of (NH₄)₂SO₄, and applied to a column of TSK-gel (Toyo Pear, 2.5 cm × 2.5 cm, 2.0 mL/min) which had been equilibrated with the buffer [0.05 mol/L Tris-HCl, 50% (NH₄)₂SO₄, pH adjusted to 7.4]. Then the eluted liquid was eluted from the TSK-gel column with the saturated (NH₄)₂SO₄ of 20%, 10% and distilled water at a flow rate of 2.0 mL/min and dialyzed for further experiments.

**Determination of the soluble proteins and electrophoresis**

The protein quantities of the samples were determined by Coomassie brilliant blue (G-250) staining [9] and the molecular weight of protein was determined by comparing the electrophoretic mobility using bovine serum albumin (BSA) as a standard protein. SDS-PAGE was conducted according to the method of Laemmli et al [8] with the separating gel and stacking gel concentrations at 12.5% and 3.0%, respectively. The two-dimensional (2-D) electrophoresis was performed according to Kim et al [10].

**Collection of the 24.5 kD proteins**

The Coomassie brilliant blue stained proteins (spots) were manually excised out of gels (1-2 mm in length). After washed several times the gel slice was transferred into 0.9% NaCl and vibrated for 5 min to eliminate the impurity and non-portentous objects from the surface, and rinsed with distilled water for three times. The gel was then ground in a glass homogenizer by adding double volume of buffer (10 mmol/L Tris-HCl, 5% SDS, pH 7.4) as much as the gel weight, and the mixture was shaken for 5-8 h at 40°C, then centrifuged at 5 000 r/min for 10 min at 4°C. After the supernatant was added by acetone (-20°C) to adjust the concentration to 70% (V/V), the mixture was stored at -20°C overnight, and then centrifuged at 5 000 r/min for 10 min. The precipitate was rinsed with 70% acetone for three times to remove the rudimental SDS from the proteins and air dried, then the proteins were dissolved with double distilled water by an ultrasonic cleaner, and the solution was centrifuged at 10 000 r/min for 15 min. The supernatant was adjusted to 1 mg/mL of concentration with 0.9% NaCl and then stored at -80°C until used.

**Preparation of antiserum and Western-blot analysis**

Pure 24.5 kD proteins were injected into male New Zealand rabbits for three times. For the first time, 45 µg proteins emulsified by mineral oil was injected into the gluteus muscle, 10 days later, 90 µg emulsified...
proteins were injected by the same method, and another 10 days later, 90 µg protein solution was injected into the ear vein. Ten days after the last injection, 30 mL blood samples were taken from the rabbit ear and recovered the serum for the antiserum of object protein. At the same time, the serum of rabbit which was not dealt with the protein, was also recovered. Immunoreactions were followed by the method of Li et al \[11\] and Western blot was performed according to Sambrook et al \[12\].

**RESULTS**

The diversity of two-dimensional electrophoretic patterns of soluble protein and its elution with (NH₄)₂SO₄

The differences of the soluble proteins in rice calli treated with and without 10 mg/L ABA according to 2-D electrophoresis (Coomassie brilliant blue staining) were compared. In the ABA-treated calli, seven types of proteins at molecular weights of 93, 45, 32, 24.5, 21, 18.5 and 14 kD, respectively, were induced (Fig. 1-B, marked with ‘○’). However, these types of proteins were not found in the calli without ABA treatment (Fig. 1-A). Therefore, to verify these proteins were induced by the ABA treatment, it was necessary to further purify the proteins. First, the crude protein from the calli treated with ABA was eluted with (NH₄)₂SO₄. The result showed there were obvious diversity when different concentrations of (NH₄)₂SO₄ were used to elute the protein (Fig. 2, Lane 2). Direct separation by SDS-PAGE electrophoresis could not distinguish the 24.5 kD protein from others, but the 18.5 kD protein was clear (Fig. 2, Lane 1). Salting-out with 25-65% of saturated (NH₄)₂SO₄ made the patterns be hardly separated (Fig. 2, Lane 2), while after salting-out with 65-100% of saturated (NH₄)₂SO₄, the patterns disappeared, only the protein of 24.5 kD was in focus (Fig. 2, Lane 3). In view of the above mentioned phenomena, we first want to purify the proteins of 24.5 kD, just because there were few proteins in Lane 3, and it was easy to eliminate the impurity possibly. Meanwhile, it was suitable to salt-out proteins with 65-100% of saturated (NH₄)₂SO₄ before applied to the column for ion exchange.

**SDS-PAGE of the protein sample after passing through DEAE-Sepharose column**

The soluble proteins salted-out by 65-100% of saturated (NH₄)₂SO₄ was applied to the column of DEAE (0.05 mol/L Tris, pH 7.4, 0.15 mL/min). An obvious difference was noted among the eluting effects with different concentrations of NaCl in SDS-PAGE electrophoresis (Fig. 3). The protein of 24.5 kD was clear in Lane 3. However, there were no target pattern in Lanes 2 and 4-6, suggesting that it was better to elute the protein of 24.5 kD by using...
double distilled water (DDW) and it was unpropitious to elute the object protein when the concentration of NaCl higher than 0.2 mol/L. From the result of SDS-PAGE, the protein of 24.5 kD could be obtained after eluted with DDW and applied the sample to the column of DEAE. To further validate the result of purification, the proteins from the sample treated by ABA were compared with those untreated by ABA in 2-D electrophoresis (Fig. 4). The result showed that the protein of 24.5 kD was clear in the section of pI 5.2 in the samples without ABA treatment (Fig. 4-A, marked with ‘△’), while three types of proteins in the same extent with the same molecular weight of 24.5 kD (Fig. 4-B) was obtained in the samples with ABA treatment, and the pI was 5.2, 6.1 and 6.9, respectively. The present result suggested that the protein of pI 5.2 (marked with ‘△’) was the constitutive protein of rice callus, but those of pI 6.1 and pI 6.9 (marked with ‘○’) were the specific proteins induced by ABA.

**SDS-PAGE of the protein sample after passing through TSK-gel column**

The above experiments showed that there were some non-object proteins in lanes after applied the

**Fig. 4. Two-dimension electrophoresis of non-adsorbed proteins from DEAE-gel ion exchange.**

A. Protein from calli without ABA treatment; B. Protein from calli treated with 10 mg/L ABA.
soluble protein to column of DEAE and eluted with double distilled water. To solve the problem, we tried to further purify the protein of 24.5 kD by passing through the column of TSK-gel and elution with DDW, finally got the clear protein of 24.5 kD (Fig. 5-A, Lane 2, marked with ‘▽’). At the same time, the sample was eluted with different concentrations of saturated (NH₄)₂SO₄ after passing through the column of TSK-gel, and it was found that there were lots of inseparable proteins around the protein of 24.5 kD when eluted with 10% saturated (NH₄)₂SO₄ (Lane 3), and could not elute the object protein by using 20% saturated (NH₄)₂SO₄ (Lane 4). This indicated that higher concentration of the salt could increase the adsorbability of protein in the column. The protein of 24.5 kD could be eluted completely with DDW after passing through the column of TSK-gel. There were two clear protein spots at iso-electric points of 6.1 and 6.9, respectively, in 2-D electrophoresis (Fig. 5-B), with the higher quantity of protein pI 6.9 than pI 6.1.

**Detection by SDS-PAGE**

To improve the specific immunoreaction, the two types of proteins were injected into the rabbit separately. During this research, the spot of protein was cut from the 2-D electrophoretic lane to get the protein according to the method of *Collection of the 24.5 kD proteins*. SDS-PAGE result showed two single patterns with different pI values (Fig. 6, Lanes 1 and 2). According to the methods abovementioned, the ABA-specially-induced proteins with the same molecular weight and different pI values were obtained, which could meet the requirement for immunoreaction, and the proteins at pI 6.1 and pI 6.9 were named A1 and A2, respectively.

**Fig. 5.** SDS-PAGE patterns of proteins by TSK-gel (A) and two-dimension electrophoresis and patterns of proteins eluted with DDW and TSK-gel from calli treated with 10 mg/L ABA (B).

Lane 1, Soluble proteins from calli treated with 10 mg/L ABA; Lane 2, DDW eluate of TSK-gel; Lane 3, 10% (NH₄)₂SO₄ eluate of TSK-gel; Lane 4, 20% (NH₄)₂SO₄ eluate of TSK-gel.

**Fig. 6.** SDS-PAGE patterns of proteins purified by two-dimension electrophoresis purification method.

Lane 1, Purified protein (pI 6.1); Lane 2, Purified protein (pI 6.9); Lane 3, Soluble proteins from calli treated with 10 mg/L ABA; Lane 4, Soluble proteins from calli without ABA treatment.
Western blot analysis

The antibodies with the proteins A1 and A2 were analyzed by Western blot, and the different expressions were shown in Fig. 7. A1 protein was detected in calli treated with ABA and in seed embryos (Fig. 7-A, Lane 3, 4), while the expression level in seed embryos was higher than that in calli and it was not detected in rice calli without ABA treatment and in white dry callus (WDC) (Fig. 7-A, Lanes 1, 2). A2 protein was detected in calli treated with ABA, WDC and seed embryos (Fig. 7-B, Lanes 2, 3 and 4), but not be detected in rice calli without ABA treatment (Fig. 7-B, Lane 1), and the expression level in WDC was lower obviously than those in rice calli treated with ABA and in seed embryos.

DISCUSSION

During the preparation of callus some specific proteins could be induced and expressed by adding exogenous ABA in the medium [13-14], most of which were related to the plant reaction to adverse circumstances [15]. However, the mechanism of specific protein induced by ABA and the physiological characteristics are not yet very clear [16]. In previous work, we had discussed the effects of ABA on saccharide metabolism and related enzymes [5], i.e., exogenous ABA induced production of specific proteins in rice callus, and these proteins regulated and synthesized the substances of rice callus, and further affected the rice callus, including the development of somatic embryo and plant regeneration [4]. Some studies had shown that ABA served in the function of modulating rice development and growth [17], and the expression was coincident in callus and seed embryo dealt with ABA. In present study, the Western blot analyses showed that A1 and A2 expressed in rice calli and seed embryos, which was consistent with the previous study [18-19]. Moreover, a significant difference was noted in A2 and A1, the expression of A2 could be detected not only in calli dealt with ABA and seed embryos but also in WDC (Fig. 7-B), a kind of callus with higher differentiation capability [4]. The A2 protein could be expressed in WDC, but could not in the calli without ABA treatment. Some research indicated that exogenous ABA could improve the rice callus redifferentiation obviously [3-4]. However, further study should be done to reveal the relationship between exogenous ABA and the rice callus redifferentiation. As we known, most of the proteins in seed embryo were storage proteins, the expression level of A2 in callus tissue dealt with ABA and seed embryo was unanimous, suggesting that A2 might had the function in regulating and synthesizing the storage protein or the enzymes of storage protein. Mundy and Chua [20] had obtained similar specific protein with the same molecular weight as got in our research, but no further report about this protein was available. Therefore, further study was needed to make clear whether they belong to the same protein. However, the A1 and A2 were the two specific proteins induced by ABA in rice calli, which owned the same molecular weight but with different iso-electric points and expression levels, suggesting that the functions of them might be greatly different. We are further studying these two proteins in cellular localization and the amino acid sequence.
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REFERENCES


