研究简报

Micropreparation of a Native PHGPx Protein From Radish Seedlings by Immunoaffinity Chromatography*

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Abstract Radish phospholipid hydroperoxide glutathione peroxidase (RsPHGPx) was identified as a mitochondrion-targeting PHGPx in previous work. To determine its cleavage site of the targeting peptide, the immunoaffinity chromatography (IAC) purification approach was carried out to isolate the native RsPHGPx protein. Polyclonal antibodies directed against recombinant RsPHGPx were raised in rabbit. Monospecific anti-RsPHGPx antibodies were isolated by means of affinity chromatography using the recombinant RsPHGPx as affinity ligand, and employed in assembling an IAC column. A single-step, highly specific and easy-to-use protocol was developed for purification of the active RsPHGPx protein through the assembled IAC column. Using this approach, a specific protein of the expected molecular size was obtained from the mitochondrial fraction of radish seedlings. Western blot analysis showed that it could be specifically recognized by anti-RsPHGPx antibodies, and an enzyme activity assay indicated that it exhibited significant PHGPx activity, suggesting that the purified protein should be the desired native RsPHGPx. These results will lead to clarification of the targeting peptide and the active mature protein of RsPHGPx and will be helpful to further probe the intracellular localization mechanism and biological function of this plant PHGPx.

Key words immunoaffinity chromatography, native protein, polyclonal antibodies, phospholipid hydroperoxide glutathione peroxidase, radish

Phospholipid hydroperoxide glutathione peroxidase (PHGPx), a subclass of glutathione peroxidases, is considered to be the primary enzymatic defense against oxidation for biomembranes in mammals[1,2]. Although the presence of PHGPx in the plant kingdom has been an unarguable fact[3~5], current studies on plant PHGPx are mainly concentrated on gene cloning and expression characterization at the transcriptional level[6~11], and little detailed information about physiological functions as well as subcellular localization is available. This contrasts to extensively investigated mammalian PHGPx[1,2]. Recently, a novel PHGPx gene (*RsPHGPx*) isolated from radish was found to encode an active PHGPx (RsPHGPx)[12,13] in our laboratory, and it was demonstrated to be a previously uncharacterized mitochondrion-localized PHGPx (unpublished data). In plants, mitochondrion-targeting proteins contain N-terminal targeting peptides (mTP)[14], which are responsible for targeting the precursors to mitochondria and are commonly cleaved off to form active mature proteins after import into destinations. To exactly known the probable mTP cleavage site and the mature form of RsPHGPx, purification of the native RsPHGPx protein from radish plants is the first step.

Unlike purification of nucleic acids, which is usually easier to succeed by rigidly following standard procedures, each purification procedure for a given protein is unique [15]. Therefore, isolation of trace proteins from plants is a challenging work in most cases. To meet these challenges, selection of an appropriate purification method is the prerequisite step. Considering specificity and sensitivity of all techniques available, immunoaffinity chromatography (IAC), a kind of affinity chromatographic approach in which the stationary phase consists of an antibody or antibody-related reagent[16], is most worthy of trying for purification of the native RsPHGPx protein.

The key point of the IAC method is the preparation of antibody[16]. Owning to high specificity, homogeneity, and reproducibility, monoclonal

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antibodies (mAbs) have been used extensively for IAC purification [17]. However, the time-consuming, cost-high and the inherent frustrations in the production of mAbs sometimes limit their utilization [18]. In contrast, polyclonal antibodies (pAbs) are less applied to immunoaffinity chromatography due to their lower specificity, poor consistency between different batches, and the affinity inhomogeneity of different antibody molecules [19]. Nevertheless, the large advantages of pAbs, including lower cost, faster production, and easier availability[18], prompted our efforts to employ them in IAC purification.

In this study, we raised polyclonal antibodies (pAbs) directed against recombinant RsPHGPx protein and purified the monospecific antibodies by affinity chromatography. An efficient IAC column was assembled by covalently coupling the purified monospecific antibodies to the column support and a suitable IAC protocol was developed by using recombinant RsPHGPx as the target protein. Finally, the native RsPHGPx protein was successfully purified from radish seedlings through this IAC column. Some valuable experiences about the IAC method are also discussed.

1 Materials and methods

1.1 Production and purification of anti-RsPHGPx antibodies

N-terminally truncated RsPHGPx protein was expressed in *E.coli* cells, and pAbs directed against the recombinant RsPHGPx were raised in rabbit and well characterized by Western blot analysis [13]. To purify monospecific anti-RsPHGPx antibodies from the antiserum, a HiTrap NHS-activated HP 1 ml column (Amersham, USA), a preactivated set for covalent binding of ligands containing primary amines, was used according to the manufacturer's instructions with slight modifications. Briefly, pure recombinant RsPHGPx protein was purified by nickel affinity chromatography (Ni-NTA agarose) from *E.coli* cell lysates[13] and 5 mg of this protein was coupled to the preactivated sepharose matrix to assembly an affinity chromatography column. Dilution of the antiserum (1∶1) in phosphate-buffered saline (PBS, 50 mmol/L NaH_2PO_4, 15 mmol/L NaCl, pH 7.4) was cleared by filtration through a 0.45 μm filter and then loaded onto the prepared column at a flow-rate of 0.2 ml/min. After successive washing with PBS until the eluent had no optical absorbance at 280 nm, the pure antibodies were eluated by 0.1 mol/L citrate buffer (pH 3.0) with a flow-rate of about 0.5 ml/min and immediately frozen at −70℃. The frozen antibodies were then lyophilized, redissolved in coupling buffer (50 mmol/L NaH_2PO_4, 0.5 mol/L NaCl, pH 8.0), and dialyzed against the coupling buffer at 4℃ for 24 h. Following centrifugation at 1 2000 ×*g* for 15 min, the concentration of the final antibody sample was determined by the Bradford method [20]. SDS-PAGE was performed to check the homogeneity of the purified antibodies, and standard enzyme-linked immunosorbent assay (ELISA) [21] was carried out to measure their immunoreactivity.

1.2 Preparation of IAC column

The purified monospecific antibodies (3 mg) were coupled to a new HiTrap NHS-activated HP 1 ml column as described above. The coupling efficiency was calculated according to the formula recommended by the manufacturer. This column coupled with the monospecific anti-RsPHGPx antibodies was the final IAC column that would be utilized for the purification of native RsPHGPx from radish seedlings.

1.3 Protocol establishment for the IAC column

To develop a practical protocol, the recombinant RsPHGPx protein was first tried out to evaluate the functionality of the IAC column. The lysate of RsPHGPx-expressing *E.coli* cells (approximately 2 mg protein) was loaded and purified as described above. Then the effluent and eluent (200 μl in each collection tube) were subjected to SDS-PAGE to evaluate capabilities of the immobilized antibodies to specifically adsorb and release RsPHGPx protein. To assess the effect of the IAC purification course on the enzymatic activity of the target protein, 60 μg of pure recombinant RsPHGPx protein purified through nickel affinity chromatography was applied to the IAC column. The eluted protein fraction was immediately measured for PHGPx activity and subsequently adjusted with 3 mol/L NaOH solution from pH 3.0 to approximately pH 8.0. Additional activity measurements were conducted to check stability of this activity. All enzymatic activity assays were performed as reported previously[3].

1.4 Purification procedure of the native RsPHGPx protein

Since the RsPHGPx protein is localized in mitochondria (unpublished data), the total mitochondrial protein extract was used as the starting sample for purification. Isolation of radish mitochondrial fraction was performed by the method of Sakamoto[22] with slight modifications. Four-day-old radish (*Raphanus sativus* cv. Meinong) seedlings were

harvested and gently homogenized with muller in extraction buffer (400 mmol/L sucrose, 50 mmol/L Tris-HCl pH 7.5, 3 mmol/L EDTA, 0.1% BSA, 1 mmol/L PMSF, 5 mmol/L 2-mercaptoethanol). The homogenate was first filtered through 4 layers of pledget to remove large contaminants, and subsequently centrifuged at 2 000 *g* for 10 min to remove small cell debris, nuclei and plastids. Then the crude mitochondrial fraction was pelleted from the supernatant by centrifugation at 16 000 *g* for 20 min. After washing in wash buffer (400 mmol/L sucrose, 50 mmol/L Tris-HCl pH 7.5, 3 mmol/L EDTA, 1 mmol/L PMSF, 5 mmol/L 2-mercaptoethanol) with recentrifugation at 16 000 *g* for 20 min, the precipitate of crude mitochondria was resuspended in lysis buffer (50 mmol/L NaH_2PO_4 pH 7.4, 15 mmol/L NaCl, 3 mmol/L EDTA, 1 mmol/L PMSF, 10 mmol/L 2-mercaptoethanol) and lysed by sonication on ice for 5× 60 s. To remove insoluble impurities, the lysed sample was centrifuged at 20 000 *g* for 20 min. The final clear supernatant was loaded onto the equilibrated IAC column at a flow-rate of 0.2 ml/ min, followed by reloading of the first eluent in the same condition. Then successive washing with PBS buffer was performed until the eluent had no optical absorbance at 280 nm. Finally, the desired fraction was eluted from the column as described above, and the eluate was collected with tubes (200 μl per tube). The fractions containing proteins were immediately incorporated together and adjusted to approximately pH 8.0 as mentioned above. After ultrafiltration performed in a Nanosep® centrifugal device (Pall, USA), measurements of protein concentration and activity, SDS-PAGE, and Western blot analysis were performed as described above.

2 Results

2.1 Purification of monospecific antibodies and assembly of an IAC column

For development of an efficient IAC column, the purification of monospecific anti-RsPHGPx antibodies from the serum consisting of a mixture of immunoglobulins with different specificities was indispensable. Because of specific antigen recognition by antibodies, affinity purification with an immobilized antigen as affinity ligand was used to purify the monospecific antibodies. 5 mg of pure recombinant RsPHGPx protein was subjected to the HiTrap NHS-activated HP 1 ml column for coupling. After stringent washing and complete blocking, approximately 4 mg protein was tightly immobilized on it. When the purification of monospecific antibodies was carried out through this column, approximately 0.2 mg protein was obtained from 1 ml of original serum (approximately 20 mg protein). The result of SDS-PAGE analysis of the purified pAbs was in good agreement with the molecular composition of a typical rabbit immunoglobulin G with two similar heavy chains and two variable light chains, and demonstrated perfect homogeneity of the purified antibodies (Figure 1). An ELISA test showed that the specific immunoreactivity was retained in the purified antibodies (data not shown), indicating that the purified anti-RsPHGPx antibodies were active.

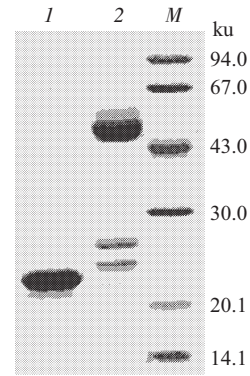

Fig.1 SDS-PAGE analysis of the purified anti-RsPHGPx antibodies

1: the antigen (recombinant RsPHGPx protein); *2*: the purified fraction; *M*: protein marker.

3 ml of the purified antibodies (1 g/L) was applied to a new NHS-activated column and approximately 2.5 mg antibodies were successfully coupled to it, corresponding to a coupling efficiency of 83% . Thus, the IAC column with immobilized monospecific anti-RsPHGPx antibodies as the ligand was established.

2.2 Practicable protocol suitable for the IAC column

To test the IAC column, the lysate of RsPHGPx-expressing *E.coli* cells was first used. As shown in Figure 2, after the lysate was loaded onto the IAC column, the recombinant RsPHGPx protein was specifically adsorbed from the complex protein mixture and efficiently eluted with the citrate buffer, indicating the specific and reversible adsorbility of the ligand to the recombinant RsPHGPx protein. When 60 μg of pure recombinant RsPHGPx protein was applied, approximately 53 μg of protein could be adsorbed and eluted, corresponding to a recovery efficiency of 88%. An enzyme activity assay showed

that the eluted fraction maintained 84% activity at the beginning (Figure 3). However low pH rendered the protein inactivate in a short time and the enzyme activity decreased to half within 60 minutes (data not shown). To resolve this problem, immediate pH adjustment after elution to approximately pH 8.0 was performed, and thereby the purified protein could maintain higher activity (79%) for a long time (Figure 3). These results showed that the IAC column could be used for specific purification of active RsPHGPx protein from a complex sample and that the steps described above constitute a practicable protocol for purification by the IAC column.

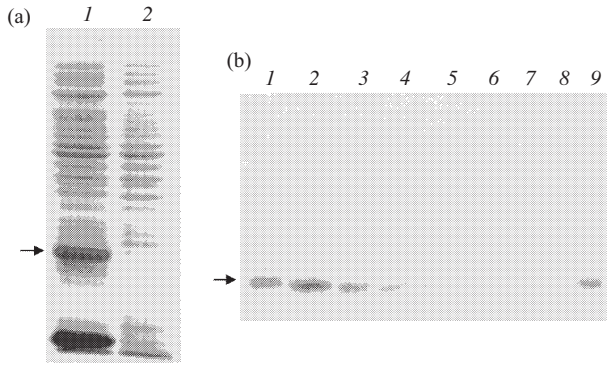

Fig.2 SDS -PAGE analysis for assessment of specific adsorption and release of the IAC column

(a) Total protein of RsPHGPx-expressing *E.coli* cells before loading (lane *1*) and the collected effluent after loading (lane *2*) were shown. (b) The eluated fractions from (a) (lane *1*~ *8*) and the pure recombinant RsPHGPx as the control (lane *9*) were shown. Arrows in (a) and (b) indicate the recombinant RsPHGPx protein.

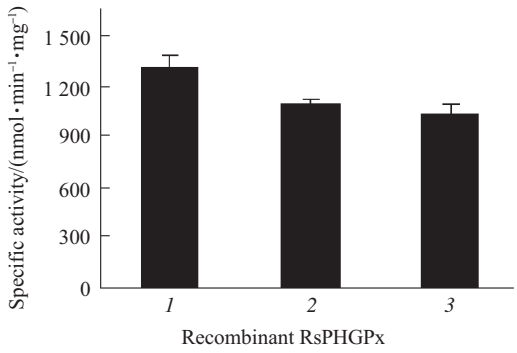

Fig.3 PHGPx activity assay on the recombinant RsPHGPx protein during the IAC purification

Results of three independent experiments are shown. *1*:the recombinant RsPHGPx protein before loading; *2*: the eluted RsPHGPx protein in acidic buffer was immediately measured; *3*:the eluted RsPHGPx protein was measured after it was adjusted to approximately pH 8.0 for 4 h.

2.3 Purification of the native RsPHGPx protein

In our previous report, we predicted that RsPHGPx was a mitochondrion-targeting protein [13]. Recently, we confirmed this prediction by multiple parallel methods including Western blot analysis of mitochondrial fraction (unpublished data). Based on this result, crude mitochondrial fraction was isolated from radish seedlings in order to purify the native RsPHGPx protein. Total mitochondrial protein extract was loaded onto the well-characterized IAC column. To avoid rapid denaturation of the target protein in the acidic solution, the eluent was immediately adjusted to a neural pH value and subsequently concentrated and desalted by ultrafiltration. Generally, 1~2 μg of protein was obtained from about 30 mg total mitochondrial protein. SDS-PAGE analysis of the concentrated eluent yielded a single band of expected size (21.5 ku) and this protein could be specifically recognized by anti-RsPHGPx antibodies in Western blot analysis, just as the native RsPHGPx protein present in total mitochondrial protein extract could (Figure 4). Furthermore, enzymatic activity assay demonstrated significant PHGPx activity from the concentrated eluent and an obvious increase of specific activity during the course of purification (data not shown). These results indicated that the specific protein obtained should be the desired native RsPHGPx protein.

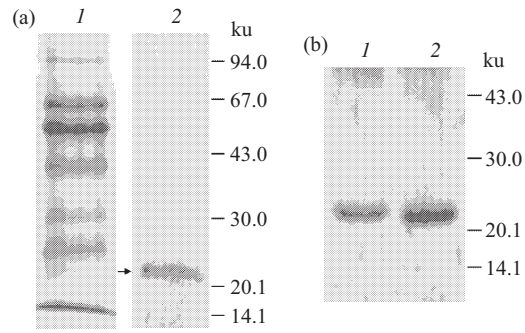

Fig.4 Identification of the purified protein from radish mitochondrial fraction

(a) SDS-PAGE analysis of the total mitochondrial protein extract (lane *1*) and the purified protein (lane *2*). (b) Western blot analysis of the two protein samples in (a) with the anti-RsPHGPx serum.

3 Discussion

To get insight into the probable cleavage event happened to the RsPHGPx after import into mitochondria, the native protein purification becomes an essential first step. In this study, we prepared and purified the pAbs directed against RsPHGPx,

assembled an IAC column by immobilizing the monospecific anti-RsPHGPx, and estalished a practicable protocol for purification by this IAC column. Moreover, the native RsPHGPx protein was successfully purified from the mitochondrial fraction of radish seedlings.

With respect to purification of native plant PHGPx proteins, only one report is available so far[3]. A partially purified PHGPx protein (Cit-SAP) was obtained from cultured citrus cells by affinity chromatography on a Sepharose-GSH column, giving the first evidence of PHGPx protein present in plants [3]. Since GSH, as a substrate with an affinity to PHGPx[1], also interacts with other proteins, an affinity chromatography using GSH as the ligand could result in failure to obtain pure targeting protein. In this study, we chose monospecific antibody, a more specific ligand than GSH, to develop an affinity chromatography column and conducted the IAC purification. It is the high specificity of the antibodies in their interactions with the antigen that ensured the ultimate success in purification of native RsPHGPx protein. This suggests that monospecific antibody is better than GSH for purification of a target protein using an affinity chromatography technique.

As far as the IAC technique is concerned, mAbs are commonly preferred [17]. However, pAbs, which have unique advantages in production [18], can be economical and effective alternatives. It should be noted that one vital step of an IAC technique using pAbs as the ligand is the purification of antibodies. In this study the purification of monospecific antibodies should be the most key point of the successful IAC application. It is well known that serum consists of enormous immunoglobulins and other components, and that monospecific antibodies to the corresponding antigen only account for a minute ratio of total immunoglobulins. If general purification methods, such as precipitation with ammonium sulfate [23] and protein-A-chromatography [24], are chosen, other immunoglobulins will be obtained in addition to monospecific antibodies. Development of an IAC column with such antibodies could significantly reduce the capacity and specificity of the column and even abort the purification. Here, an affinity chromatography using antigen as affinity ligand was employed to select the monospecific antibodies. By this method, it is easy to get the monospecific antibodies and remove other antibodies. Application of monospecific antibodies would lead to better purification effect in specificity and productivity.

As expected to some extend, the productivity of the IAC column for purification of native RsPHGPx was much lower than that for purification of recombinant RsPHGPx. On the one hand, this might result from the much lower abundance of native RsPHGPx in the starting material. On the other hand, the structure difference of native RsPHGPx from recombinant RsPHGPx maybe weakened the interaction of the antibodies with native RsPHGPx. Anyway, to increase purification times or enlarge the purification scale, for instance, to employ a 5 ml column, would obtain more target protein for further analysis.

With the identification of different targeting isoforms of PHGPxs, increasing evidence has suggested different organelle localizations of plant PHGPxs, such as plastids [7,8], mitochondria [13], and peroxisomes[8]. However, where the cleavage occurs on these precursor proteins after import into the destinations and what the resulting active mature proteins are like need to be elucidated. To our knowledge, this is the first report to purify a native organelle-localized PHGPx in plants. Investigations on the purified RsPHGPx protein will exactly illuminate its mitochondrial targeting peptide and functionally active mature protein and will be helpful to further understand its intracellular targeting mechanisms as well as possible functions in cells.

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用免疫亲和层析法纯化萝卜 PHGPx 天然蛋白 *

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摘要 萝卜磷脂氢谷胱甘肽过氧化物酶 (RsPHGPx) 是一个定位于线粒体的蛋白质. 为了阐明该蛋白质线粒体定位信号的准确切割位点，采用了免疫亲和层析方法纯化天然的 RsPHGPx. 用重组 RsPHGPx 蛋白免疫兔子获得了抗 RsPHGPx 的多克隆抗血清，以重组 RsPHGPx 蛋白为配体，采用亲和层析技术对抗血清进行了纯化，得到了单特异性的抗 RsPHGPx 的抗体. 将纯化好的抗体偶联到一个 N- 羟基琥珀酰亚胺 (NHS) 预先激活的琼脂糖柱子上，装配成一个以单特异性的抗 RsPHGPx 抗体为配体的免疫亲和层析柱. 经过对纯化条件的摸索和优化，形成了一个简单、特异的一步法纯化方案. 按照该方案，从萝卜幼苗线粒体总蛋白质提取物中纯化到一个分子质量与预期值相一致的特异蛋白质. 免疫印迹分析表明，该蛋白质被抗 RsPHGPx 的抗血清特异识别. 酶活性分析表明，该蛋白质具有显著的 PHGPx 活性. 这些结果表明，纯化到的特异蛋白质是萝卜的 RsPHGPx 天然蛋白. 这是首个关于定位于植物细胞器的 PHGPx 蛋白纯化的报道. 这一结果为准确测定 RsPHGPx 信号肽的切割位点奠定了基础，并将有助于对植物 PHGPx 的亚细胞定位机制及其生理功能的深入研究.

关键词 免疫亲和层析，天然蛋白质，多克隆抗体，磷脂氢谷胱甘肽过氧化物酶，萝卜
学科分类号 Q943

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