Characterization of Protein Phosphatase 2A Acting on Phosphorylated Plasma Membrane Aquaporin of Tulip Petals

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A protein phosphatase holo-type enzyme (38, 65, and 75 kDa) preparation and a free catalytic subunit (38 kDa) purified from tulip petals were characterized as protein phosphatase 2A (PP2A) by immunological and biochemical approaches. The plasma membrane containing the putative plasma membrane aquaporin (PM-AQP) was prepared from tulip petals, phosphorylated in vitro, and used as the substrate for both of the purified PP2A preparations. Although both preparations dephosphorylated the phosphorylated PM-AQP at 20 °C, only the holo-type enzyme preparation acted at 5 °C on the phosphorylated PM-AQP with higher substrate specificity, suggesting that regulatory subunits are required for low temperature-dependent dephosphorylation of PM-AQP in tulip petals.

Key words: aquaporin; low temperature; okadaic acid; protein phosphatase; tulip petals

Reversible protein phosphorylation has a pivotal role as a mechanism in the control of many cellular functions such as hormonal, pathogenic, and environmental stimuli responses, cell cycle events, growth factor responses, metabolic control, and developmental processes.1–3) Many homologs of animal Ser/Thr phosphatases (PP1, PP2A, PP2C, and PP5) have been detected in plants.3,6) PP2As from plants and animals have been characterized using commercially available substrates such as phosphorylase a, phosphorylated histone, or p-NPP.6–9) It is necessary to elucidate the biochemical or physiological regulation of PP2As using endogenous phosphorylated proteins as the substrate. There have been no reports on the plant protein phosphatase acting on phosphorylated AQP, the water channel protein,10,11) despite the fact that some AQPs are regulated by phosphorylation.12) In our previous study, we found that the putative PM-AQP of tulip petals was phosphorylated at Ser residues when whole plants were incubated at 20 °C, and correspondingly dephosphorylated by further incubation at 5 °C.13) In the present study, we focused on the dephosphorylation of this P-PM-AQP by PP at 5 °C.

To assay protein phosphatase activity during the course of purification from tulip petals, and for the characterization of the PP, we used p-NPP as the substrate because PP2As from plants and animal tissues exhibited higher activity to p-NPP than to phosphorylase a.5,14) We purified the catalytic subunit from tulip petals after dissociating it from the endogenous regulatory subunit by an ethanol precipitation step.4,9) We used MC-Sepharose affinity chromatography as the final purification step, because MC is a highly selective inhibitor for the PP1 and PP2A holoenzymes as well as their catalytic subunits and MC-Sepharose is used for the purification of these types of PPs.6,15,16) The final 748-fold purified enzyme showed specific activity of 449 U/mg with a yield of 11%. SDS-PAGE (Fig. 1A) revealed the presence of only one protein band of 38 kDa. The molecular masses of PP2A/c from Arabidopsis thaliana and from maize seedlings were reported to be around 38 kDa.7,17) On the other hand, the final 623-fold purified enzyme prepared without ethanol precipitation, designated a holo-type enzyme, had specific activity of 380 U/mg with a typical yield of 13% (Table 1). This preparation, obtained after MC-Sepharose chromatography, had 3 protein bands of 75, 65, and 38 kDa (Fig. 1B), indicating that 65 and 75 kDa were the regulatory subunits. But the intensities of the stained 65 and 75 kDa bands were less than 40% of that of the 38 kDa band. These results suggested the presence of a free catalytic subunit in the holo-type enzyme preparation because the number of each subunit might be 1 in the interaction between catalytic and regulatory subunits. The anti-PP2A/c raised against rabbit PP2A/c (Sigma) clearly reacted with the 38 kDa protein in both

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Abbreviations: AQP, aquaporin; MC, microcystin; OA, okadaic acid; PM, plasma membrane; PM-AQP, plasma membrane aquaporin; p-NPP, p-nitrophenyl phosphate; PP, protein phosphatase; PP2A/c, PP2A free catalytic subunit; P-PM-AQP, phosphorylated plasma membrane aquaporin
preparations, as shown in Fig. 1C, suggesting that tulip 38 kDa has homology with animal PP2A/c.18) Figure 2A showed that the p-NPP phosphatase activities of the holo-type enzyme as well as the catalytic subunit were inhibited almost completely by 1 nM concentration of OA, the specific inhibitor of PPs.14) The calculated IC$_{50}$ values for the holo-type enzyme and for the catalytic preparations were approximately 0.11 and 0.09 nM respectively. These values were comparable to those of other PP2As.7,18) PP1 is unaffected by 1 nM OA, because the IC$_{50}$ of OA for PP1 ranges from 10–100 nM,4,18,19) suggesting the absence of PP1 in our preparations. PP2B is inhibited by micromolar concentrations of OA, whereas PP2C is resistant to OA.14,19) Moreover, the p-NPP phosphatase activities of the purified enzyme preparations were inhibited negligibly (4 ± 2%) by 200 nM concentration of protein phosphatase inhibitor-2 (Sigma), a specific inhibitor of PP1.4,18) As shown in Fig. 2B, 25 μg/ml of protamine, a pronounced stimulator of PP2As,4,20) stimulated the p-NPP phosphatase activities of the holo-type enzyme and the free catalytic subunit by 5.5- and 4-fold, respectively, in the presence of 16 mM (NH$_4$)$_2$SO$_4$, but insignificant stimulation by protamine was observed in both preparations without (NH$_4$)$_2$SO$_4$. The stimulatory effect of protamine on the holo-type enzyme may indicate that the 65 and/or 75 kDa subunits not only play a structural role as a modulator scaffold, but also may allosterically modulate the enzymatic properties of the PP2A/c. From the immunological reactivity, responses to inhibitors, effect of protamine, and affinity for MC-Sepharose, we concluded that the purified phosphatase was PP2A. Both preparations, however, might contain other types of PP2A present in tulip petals, because multi-forms of PP2A have been reported in other plant tissues.7)

We were prompted to elucidate the physiological roles of the purified PP2A, especially the dephosphorylation of the phosphorylated endogenous proteins, by comparing the properties of the catalytic subunit as well as the holo-type enzyme. We focused on the phosphorylated water channel protein, AQP, in the PM of tulip petals as the substrate, because the putative PM-AQP was phosphorylated at 20 °C and dephosphorylation was observed at 5 °C.13) Figure 3A shows the protein profile of the PM prepared from tulip petals. The 31 kDa protein band clearly reacted with the anti-PM-AQP raised

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**Table 1. Purification of PP2A Holo-type Enzyme from Tulip Petals**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% (NH$_4$)$_2$SO$_4$ ppt.</td>
<td>337</td>
<td>204</td>
<td>0.61</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>103</td>
<td>159</td>
<td>1.54</td>
<td>2.5</td>
<td>78</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>5.7</td>
<td>56</td>
<td>9.8</td>
<td>16.1</td>
<td>27</td>
</tr>
<tr>
<td>MC-Sepharose</td>
<td>0.071</td>
<td>27</td>
<td>380</td>
<td>623</td>
<td>13.2</td>
</tr>
</tbody>
</table>

PP2A holo-type enzyme was purified from 500 g of tulip petals. The data shown are typical of the three preparations. Assays were performed using the p-NPP as the substrate in the presence and absence of OA in order to attribute PP2A activity. One unit of phosphatase activity was defined as the amount of enzyme producing 1 μmol of p-nitrophenol per min at 25 °C.
against the conserved amino acid sequence in many plant PM-AQPs (Fig. 3B), and this suggested the presence of the putative PM-AQP in this band. The putative PM-AQP may be one of the constituents in the 31 kDa band because the stained band was broad. To investigate the dephosphorylation of P-PM-AQP by the holo-type enzyme and by the catalytic subunit from tulip petals, prepared PM was labeled with \( [\gamma-^{32}P]ATP \) at 20 °C,\(^{13}\) and then used as the substrate. Figure 3C shows the autoradiogram indicating many phosphorylated PM proteins (lane 1) including the phosphorylated 31 kDa band. We have identified P-PM-AQP in the 31 kDa protein band, as reported previously.\(^{13}\) The labeled PM was incubated with the holo-type enzyme (lane 2) and PP2A/c (lane 4) at 20 °C. Dephosphorylation, shown by a decrease in intensity with both enzyme preparations, occurred at 31 kDa, 52 kDa, and other phosphorylated proteins at 20 °C. Surprisingly, the reaction performed at 5 °C with the holo-type enzyme showed that the phosphorylated 31 kDa band was dephosphorylated almost completely (lane 3). Although other protein bands seen between 31 and 52 kDa were weakly or completely dephosphorylated as in the case at 20 °C by the holo-type enzyme, the phosphorylated 52 kDa protein remained entirely unchanged (lane 3). Moreover, the rate of dephosphorylation of the phosphorylated 31 kDa band with the holo-type enzyme appeared to be higher at 5 °C than at 20 °C. But no dephosphorylation of the phosphorylated 31 kDa band or other phosphorylated PM proteins, especially the 52 kDa protein, occurred by means of the PP2A/c at 5 °C (lane 5). The autoradiograms (Fig. 3C, lanes 6 and 7) showed that 1 nM OA abolished dephosphorylation of phosphorylated proteins by the holo-type enzyme at both temperatures.

Dephosphorylation of the putative P-PM-AQP was further analyzed immunologically using anti-phospho-Ser (Sigma). The 52 kDa and the 31 kDa containing putative PM-AQP reacted with the anti-phospho-Ser, but some of the phosphorylated PM proteins observed in the autoradiogram were not detected by immunoblot (Fig. 3D). These may be phosphorylated at amino acid residues other than Ser. The immunoblot revealed that at 5 °C, some phosphorylated PM proteins, including the phosphorylated 31 kDa protein, were dephosphorylated at the Ser residue by the holo-type enzyme only (lane 3), but not by the PP2A/c (lane 5). Almost all of the phosphorylated PM proteins, including the phosphorylated 31 kDa protein, were dephosphorylated at 20 °C by the holo-type enzyme and the PP2A/c (lanes 2 and 4). OA completely abolished the dephosphorylation of the phosphorylated PM proteins by the holo-type enzyme at both temperatures (Fig. 3D, lanes 6 and 7). These results obtained immunologically were almost the same as those obtained by autoradiography, and the results, shown in Fig. 3D, suggested that the purified PP2A from tulip petals belongs to the class of Ser/Thr phosphatase. At 5 °C, the dephosphorylation of 31 kDa containing P-PM-AQP by the holoenzyme proceeded in a time-dependent manner and was almost complete after 60 min (Fig. 3E). The 52 kDa protein remained entirely at the same stage as before the reaction whereas the other bands distributed between 31 and 52 kDa became weaker after 60 min of incubation.
Although post-translational activation by phosphorylation of AQP has been reported, the dephosphorylation of the activated water channel is less well understood. The present study indicates that tulip P-PM-AQP was dephosphorylated at 5°C only by the holo-type enzyme, although its dephosphorylation with lower substrate specificity at 20°C (acted on 52 kDa also) proceeded to some extent with both preparations. The results in this study also support the notion that the PP2A holoenzyme of tulip petals may serve as the sensor protein for low temperatures, and show a higher specificity to some of the phosphorylated PM proteins, including the putative P-PM-AQP. At present, 35 and 31 different AQPs have been identified in Arabidopsis and maize respectively, and some AQPs are regulated by phosphorylation and possibly by dephosphorylation. It is necessary to identify the molecular species of petal PM-AQP that is phosphorylated/dephosphorylated in response to changes in temperature. Additional research is also necessary to prepare intact holoenzyme and to identify and specify the significant function(s) of the regulatory subunit(s) in tulips by comparing activity, substrate specificity, and molecular structure at 5°C and 20°C.

References


