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Technical note

Development of fast and simple chromogenic methods for glucan phosphatases in-gel activity assays

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ABSTRACT

Glucan phosphatases are essential for normal starch degradation in plants and glycogen metabolism in mammals. Here we develop two chromogenic methods for the detection of glucan phosphatase activity *in situ* after non denaturing poliacrylamide gel electrophoresis; one method uses *p*NPP and the second one applies BCIP/NBT. The assays are sensitive, fast, simple, reliable and cost-effective preventing the use of radioactive or fluorogenic compounds.

Taking advantage of an efficient separation method combined with the reported assays it is possible to obtain information about oligomeric state of the active enzymes as well as to simultaneously detect glucan substrate binding and phosphatase activity.

Glucan phosphatases have recently emerged as essential enzymes for normal starch degradation in plants as well as glycogen metabolism in mammals. *Arabidopsis thaliana* phosphoglucan phosphatases starch excess 4 (SEX4) and like-SEX4 2 (LSF2) and human Laforin are the fundamental representatives of the atypical Dual Specificity Phosphatases (DSPs), which belongs to the larger Protein Tyrosine Phosphatase superfamily [1–5].

In-gel assay techniques are widely used for detection of various functional enzymes following electrophoretic separation in gels [6–10]. However, to our knowledge, there are no previous reports on in-gel assays for glucan phosphatase detection. Thus, the aim of this work was to develop an assay to easily visualize glucan phosphatases in-gel after native polyacrylamide gel electrophoresis (PAGE) using *A. thaliana* SEX4 as model.

SEX4 and *E. coli* alkaline phosphatase (AP) (used as control) were purified by a single purification step using a HiTrap Chelating column (Amersham Biosciences, UK.). Detailed protocols for methods reported in this paper can be found in Supplemental Material. Both enzymatic preparations showed a single protein band with the expected molecular mass when analyzed by SDS-PAGE: 38 kDa for SEX4 and 50 kDa for AP (Fig. 1A) and, when assayed in solution with *p*NPP (Sigma-Aldrich, St. Louis, MO, USA (N-3254)) as a substrate (in a buffer containing DTT since it is optimized for SEX4 activity [11]), the specific activities determined were: 3.42 μmol/min mg for SEX4 and 1.51 μmol/min mg for AP. SEX4 identity was confirmed by western blotting with a-His and a-SEX4 antibodies (Fig. S1).

The same enzyme preparations were then analyzed by native PAGE. Following electrophoresis, the gels were either stained with Coomassie Blue (Fig. 1A) or developed for phosphatase activity using *p*NPP or the couple BCIP/NBT (Figs. 1B and 2). The two analyzed enzymes migrate as unique protein bands reflecting a particular oligomerization state in the assayed conditions, presumably a dimeric quaternary structure [12,13] (SEX4 PDB ID code 3NME) (Fig. 1A, Native PAGE). When tested for *p*NPP or BCIP hydrolysis (Fig. 1B, Native PAGE), the two phosphatases assayed showed a single, clear, easily visible chromogenic band that is coincident with the Coomassie Blue staining (Fig. 1A, Native PAGE).

For *p*NPP phosphatase activity detection, the electrophoresed gels were directly soaked in 5 mL of reaction mixture containing 50 mM Bis-Tris HCl (pH 8.0), 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$, 2 mM DTT and 25 mM *p*NPP. Gels were incubated at 37 °C until the yellow bands began to appear. In case of BCIP/NBT phosphatase activity detection, after electrophoresis gels were pre-incubated for 5 min in a medium containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 2 mM DTT. Since DTT interferes with the colorimetric oxidation of NBT, it must be removed from

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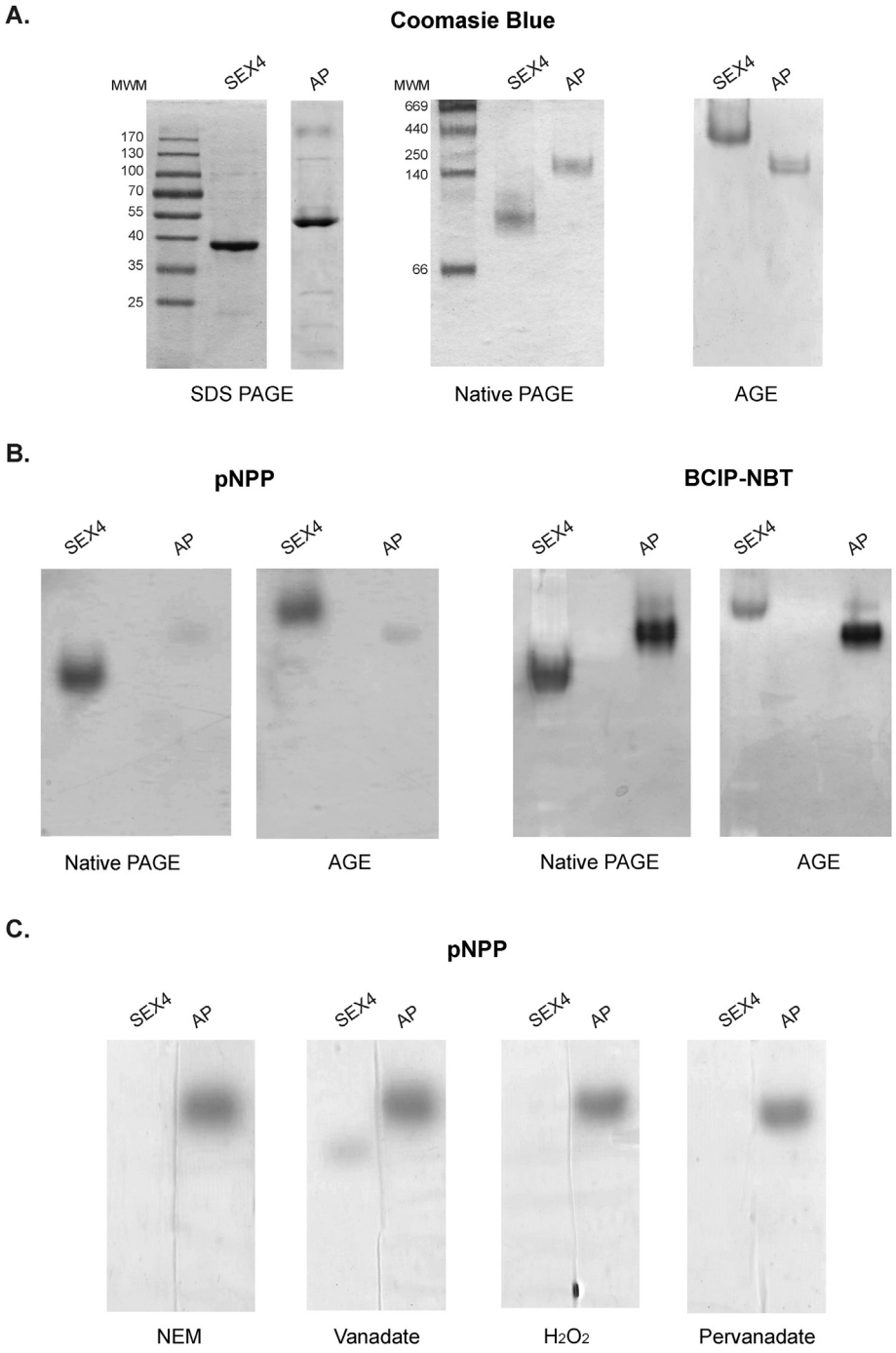

Fig. 1. Coomassie Blue staining and In-gel *p*NPP or BCIP/NBT assays of phosphatases after electrophoretic analysis. A) Coomassie Blue staining of a SDS PAGE, a Native PAGE and an AGE of SEX4. For SDS-PAGE 1 μg of proteins were loaded while 2 μg of proteins were used for Native PAGE and AGE analysis. For AGE, amylopectin (5 mg/mL) was included in gel. Numbers indicate the molecular masses of markers in kDa. For SDS-PAGE, Page Ruler Prestained Protein Ladder (range 10–170 kDa was used (Thermo Fisher Scientific, Waltham, MA USA) and, for native electrophoresis, the markers were Amersham High Molecular Weight Calibration Kit (range 66–669 kDa). MWM, molecular weight markers. **B) AGE revealed by pNPP or BCIP/NBT assays**. SEX4 (2 mU) and AP (0.3 mU) were run on native PAGE without or with amylopectin (5 mg/mL) simultaneously under the same conditions. Gels were revealed either by pNPP or BCIP/NBT assays. **C) Inhibitors effect over SEX4 activity**. SEX4 (7 mU) and AP (3 mU) μg) were run on native PAGE. Gels were then revealed by pNPP assay but being previously incubated for 10 min with NEM (50 mM), H_2O_2 (2 mM), vanadate (10 μM) or pervanadate (vanadate plus H_2O_2). SEX4, *A. thaliana* SEX4; AP, *E. coli* AP; AGE, affinity gel electrophoresis and NEM, N-ethylmaleimide.

the medium prior exposure to both substrates. Thus, then, gels were washed in the same buffer but without DTT and finally transferred to the final reaction mixture containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.165 g/l BCIP (#34035, Promega, WI, USA) and 0.33 g/l NBT (#34035, Thermo Fisher Scientific, Waltham, MA USA). The gel was incubated in darkness at room temperature until the colored bands began to appear.

It is important to note that no gel washing prior to scanning is needed and reaction can be followed despite being the gel out of the reaction medium. The revealed gel can be scanned at different times as the reaction proceeded using a scanner without any

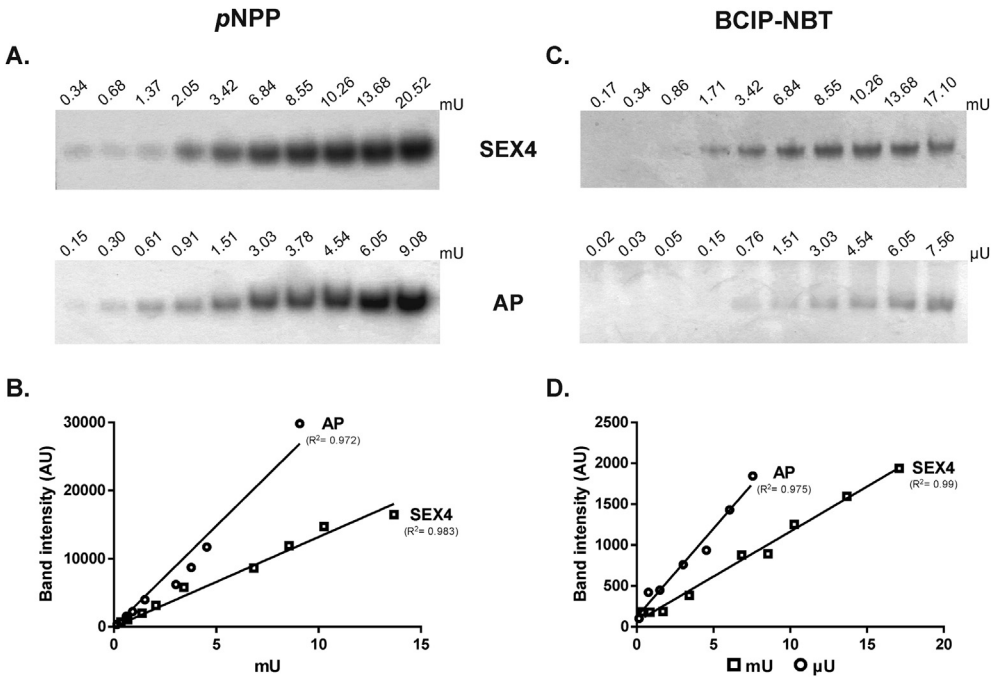

Fig. 2. In-gel *p*NPP (A) or BCIP/NBT (C) assays of phosphatases after separation by Native PAGE. Linear dependence of the band intensity (AU) on the enzymatic activity (mU or µU) assayed by *p*NPP (B) or BCIP/NBT (D). SEX4 and AP were resolved on native PAGE. After electrophoresis at 4 °C, each gel was incubated with the reaction mixture containing either *p*NPP or BCIP/NBT. The mU or µU of enzyme loaded are indicated above the respective lane (the units correspond to the specific activity of the enzymes assayed in solution with pNPP as substrate: 3.42 µmol/min.mg for SEX4 and 1.51 µmol/min.mg for AP). Bands quantification was performed as described in material and methods. Coefficients of determination (R^2) of each linear regression are presented in the graphs.

particular optical filter. Long incubations with *p*NPP are not recommended, since it will cause diffusion of the *p*-nitrophenol chromogenic product formed in gels. Besides, the medium composition was optimized in order to visualize both phosphatases activities at the same time. Whereas SEX4 requires DTT, AP needs the presence of divalent cations (0.1 mM $ZnCl_2$, 1 mM $MgCl_2$) to evidence activity [14].

As it is generally known, glucan phosphatases efficiently binds polysaccharides by means of a glucan binding platform composed exclusively or mainly by carbohydrate binding domains (CBMs) or surface binding sites (SBSs) [5]. A method commonly used to quantify the affinity of glucan phosphatases for different substrates is affinity retardation gel electrophoresis (AGE) [15]. Thus, we furthermore decided to probe if the methods reported here are also applicable after AGE. To test this hypothesis we subjected SEX4 to amylopectin mediated AGE and consecutively probed the gel to glucan phosphatase activity with *p*NPP or BCIP/NBT as substrate. As it is shown in Fig. 1B, SEX4 showed a retardation by 5 mg/mL amylopectin compared to *E. coli* AP and maintain *p*NPP and BCIP hydrolysis capability indicating that is possible to combine AGE and in gel assay to simultaneously measure binding and activity of a glucan phosphatase.

Glucan phosphatases as being members of PTPs possess at the active site an invariant catalytic cysteine making enzymes susceptible to certain inhibitors and probably involved in redox regulation as probed for SEX4 [16]. Enzyme inhibitors and effectors are very important as research tools for enzymology. Thus, we tested if the methods reported here are also suitable to study different effectors on in gel glucan phosphatase activity. To this end, after native PAGE and previous to being revealed, gels were incubated for 10 min in 50 mM NEM (Sigma-Aldrich, E-3876), 10 µM vanadate (Sigma-Aldrich, 205559), pervanadate (vanadate plus H_2O_2) (general inhibitors for PTPs since inhibitors that are selective for a particular PTP have been elusive) or 2 mM H_2O_2 (Fig. 1C). As shown in Fig. 1C, all compound tested inhibits in gel SEX4 activity excepting vanadate perhaps because of the competitive characteristic of this effector [17]. As expected no effect was found over AP activity (Fig. 1C).

In order to compare the sensitivity of the methods, increasing amount of the enzymes were loaded to native gels and revealed for phosphatase activity using *p*NPP (Fig. 2A) or BCIP/NBT (Fig. 2C). For both methods, we found that band intensity is directly proportional to the mU of SEX4 enzyme loaded in each lane since a good linear fit was obtained between band intensity and the mU loaded (Fig. 2B and D). As shown in Fig. 2A, using the *p*NPP phosphatase assay it is possible to detect up to 0.34 mU of SEX4 activity on the gels ($R^2 = 0{,}983$, Fig. 2B) while for BCIP/NBT, it is possible to detect up to 0.86 mU (Fig. 2C) with a R^2 of 0.99 (Fig. 2D).

As it is also shown in Fig. 2, the amount of *p*-nitrophenol or tetrazolium-based product formed increased in parallel with the amount of AP loaded in gels being possible to detect AP with a much higher sensitivity in comparison to SEX4: up to 0.15 mU with *p*NPP (Fig. 2A and B) and 0.76 µU with BCIP/NBT (Fig. 2C and D).

In summary, in the present work we demonstrated that colorless *p*NPP, known to be converted to bright yellow soluble *p*-nitrophenol and, BCIP/NBT, pairwise substrate that yields an intense, insoluble black-purple precipitate when reacted with alkaline phosphatase, are very good substrates for visualization of glucan phosphatase activity enzymes after native PAGE gels since *A. thaliana* SEX4 enzyme was easily detected. *A. thaliana* SEX4 protein showed clear chromogenic bands with BCIP/NBT, but it showed more significant bands when *p*NPP was used as substrate (Fig. 2) reflecting the fact that *p*NPP would be a better substrate for detection of glucan phosphatases. Taking into consideration the fact that *p*NPP and the couple BCIP/NBT are low molecular weight compounds, it seems likely that both assays can be extrapolated to

test other glucan phosphatases. For example, the present methods would be suitable to study human Laforin since SEX4 and Laforin are functionally equivalent and Laforin can partially complement mutations in SEX4 [3]. The procedures are simple, rapid, and reliable requiring only low-price chemicals. Another advantage is that they allow the direct observation of the chromogenic bands after the completion of electrophoresis thus eliminating the need of a transilluminator with a particular excitation wavelength.

Moreover, the detection of enzyme activities *in situ* in native polyacrylamide gels employs the power of the efficient separation method to obtain information on molecular masses and activity of enzymes, often guiding their identification and characterization of native active oligomerization state. Thus, the method could demonstrate differences in activity between monomer, dimer, and multimers as well as mutants, truncations or chimeric glucan phosphatase constructs. Even more, the assays reported could be performed in conjunction with Affinity Gel Electrophoresis to simultaneously detect glucan substrate binding and phosphatase activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2016.11.005.

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