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Concanavalin A-Based Sedimentation Assay to Measure Substrate Binding of Glucan Phosphatases

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Abstract

Glucan phosphatases belong to the larger family of dual specificity phosphatases (DSP) that dephosphorylate glucan substrates, such as glycogen in animals and starch in plants. The crystal structures of glucan phosphatase with model glucan substrates reveal distinct glucan-binding interfaces made of DSP and carbohydrate-binding domains. However, quantitative measurements of glucan-glucan phosphatase interactions with physiologically relevant substrates are fundamental to the biological understanding of the glucan phosphatase family of enzymes and the regulation of energy metabolism. This manuscript reports a Concanavalin A (ConA)-based *in vitro* sedimentation assay designed to detect the substrate binding affinity of glucan phosphatases against different glucan substrates. As a proof of concept, the dissociation constant (K_D) of glucan phosphatase *Arabidopsis thaliana* Starch Excess4 (SEX4) and amylopectin was determined. The characterization of SEX4 mutants and other members of the glucan phosphatase family of enzymes further demonstrates the utility of this assay to assess the differential binding of protein-carbohydrate interactions. These data demonstrate the suitability of this assay to characterize a wide range of starch and glycogen interacting proteins.

Introduction

Glucan phosphatases are members of a functionally diverse subfamily of dual specificity phosphatases (DSPs) within the protein tyrosine phosphatase (PTP) superfamily[1]. They have been found in most life forms, including widely divergent photosynthetic organisms, humans, vertebrates, and some invertebrates and protists[2,3,4]. Plants contain three known glucan phosphatases: Starch Excess4 (SEX4), Like Sex Four1 (LSF1), and Like Sex Four2 (LSF2)[5,6,7]. Plants that lack glucan phosphatases display decreased rates of transitory starch degradation and accumulation of starch in the leaves[8,9]. Laforin is the founding member of the glucan phosphatase family that dephosphorylates glycogen in vertebrates and humans[3,10]. The mutations of laforin result in neurodegenerative Lafora disease, a fatal autosomal

recessive form of epilepsy[11]. Glucan phosphatases are necessary for glycogen and starch metabolism and have emerged as important enzymes for modulating starch content in plants and treating neurodegenerative Lafora disease[12,13]. Recent X-ray crystallography studies on glucan phosphatases with model glucan substrates have shed light on substrate binding and the catalytic mechanism of glucan dephosphorylation[14,15,16,17]. However, the current understanding of how glucan phosphatases bind to their physiological substrates is incomplete.

Starch is an insoluble polymer of glucose made of 80%-90% amylopectin and 10%-20% amylose[18]. The substrates for plant glucan phosphatases are phosphorylated carbohydrate molecules, such as glycogen and starch granules. The phosphorylated glucosyl residues are present at a 1:600 phosphate:glucosyl residue ratio. Interestingly, the phosphates are present only on the amylopectin molecules[19]. The main plant glucan phosphatase SEX4 acts on the starch granule to dephosphorylate amylopectin molecules. The X-ray crystal structure of SEX4 combined with structure-guided mutagenesis studies has demonstrated the unique substrate specificities of SEX4 for different positions within a glucan structure[15]. We recently showed that the biologically relevant activity of SEX4 can only be observed when acting on its solubilized amylopectin substrates[20]. However, understanding glucan-SEX4 interactions has proven to be difficult due to the structural complexity of the substrate, broader binding specificities, and low binding affinities between the protein and its substrates. These issues have hindered the ability to utilize methods commonly used in protein-ligand interactions, such as isothermal titration calorimetry (ITC), nuclear magnetic resonance (NMR) spectroscopy, and enzyme-linked immunosorbent assay (ELISA)-based assays.

Interestingly, much of our understanding of carbohydrate-protein interactions have come from studying lectins. Concanavalin A (ConA) is a legume lectin family of proteins originally extracted from the jack bean. ConA binds carbohydrates with high specificity, which is advantageous for its use in drug targeting and delivery applications. The binding of ConA to a variety of substrates containing nonreducing α-D-mannosyl and α-D-glucosyl has been extensively studied[19,20]. Commercially available ConA-bound Sepharose beads are commonly used to purify glycoproteins and glycolipids[21]. ConA binds to these glucans *via* C3, C4, and C6 hydroxyl groups of the glucose residues. ConA-Sepharose beads have also been successfully used to measure the binding of glycogen-protein and starch-protein interactions[22,23]. In this study, we used ConA-Sepharose beads to develop a binding assay to measure the binding specificities of glucan phosphatase-amylopectin interactions.

Previously, a ConA-based sedimentation assay was employed to assess glucan phosphatase substrate binding ability[14,20,24]. In this study, the same strategy was used to develop a novel method to determine the binding affinity of glucan-glucan phosphatase and carbohydrate interactions. This method also has an advantage for investigating various solubilized carbohydrate-protein interactions.

Protocol

1. Preparation of ConA-Sepharose beads

1. Make 250 mL of a binding buffer containing 67 mM HEPES (pH 7.5), 10 mM $MgCl_2$, and 0.2 mM $CaCl_2$. Adjust the pH using 1 M NaOH solution.

2. Pipette 250 µL of ConA-Sepharose bead suspension into a 1.5 mL microcentrifuge tube. Centrifuge the contents at 10,000 x *g* for 30 s at 4 °C. Discard the supernatant.
   NOTE: 250 µL of ConA-Sepharose beads in a 1.5 mL microcentrifuge tube is needed for each amylopectin concentration used for the assay.
3. Add 750 µL of the binding buffer to each tube containing 250 µL of ConA-Sepharose beads. Centrifuge the tubes at 10,000 x *g* for 1 min at 4 °C. Remove the supernatant. Repeat this step 2x to ensure that the beads are appropriately washed and equilibrated with the binding buffer.

2. Preparation of amylopectin solutions

1. Make a stock solution of 10 mg/mL potato amylopectin. Amylopectin is water-insoluble and can be solubilized by heat. To solubilize, add 0.1 g of potato amylopectin to 10 mL of distilled water. Heat the suspension in a water bath at 80 °C for 1 h or until the solution is no longer cloudy.
2. Allow the solution to return to room temperature (RT), with repeated vortexing to avoid clumping.
3. Alcohol-alkaline treatment is an alternative method to solubilize amylopectin substrates. To solubilize using this method, follow the steps below.
   1. Suspend 0.5 g of amylopectin substrate in 5 mL of 20% ethanol and 5 mL of 2 M NaOH. Stir the contents vigorously for 15-20 min at RT.
   2. Next, add 10 mL of water, and adjust the pH of the solution to 6.5 by adding 2 M HCl. Bring up the volume of the resulting solution to 50 mL with distilled water to make a 10 mg/mL amylopectin solution.
4. Dilute the 10 mg/mL solubilized amylopectin solution to make a series of 2 mL of diluted amylopectin solutions. For example, perform half dilutions of 10 mg/mL to prepare a series of amylopectin concentrations (5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, 0.3125 mg/mL, 0.156 mg/mL, 0.078 mg/mL, 0.039 mg/mL, 0.019 mg/mL, and 0 mg/mL).

3. Preparation of ConA-Sepharose: amylopectin beads

1. Add 250 µL of each diluted amylopectin solution to 1.5 mL microcentrifuge tubes containing 250 µL of ConA-Sepharose beads pre-equilibrated in binding buffer. Mix the contents well. Label the tubes with the corresponding amylopectin concentration.
2. Incubate the contents on a rotating wheel at 4 °C for 30 min.
   NOTE: There is no change in the ConA-Sepharose:amylopectin bound complex over time after 20 min. The 30 min incubation time was chosen by varying incubation times from 10 min to 1 h to ensure equilibrium was reached.
3. Centrifuge the tubes at 10,000 x *g* for 1 min. Collect the supernatant in a newly labeled 1.5 mL microcentrifuge tube. Save these supernatant fractions to perform D-glucose assay[12] (acid hydrolysis of amylopectin followed by UV determination of glucose *via* enzymatic assay). This step is necessary to ensure all amylopectin is bound to the beads.
4. Add 750 µL of binding buffer to the ConA-Sepharose:amylopectin beads. Centrifuge the tubes at 10,000 x *g* for 1 min. Discard the supernatant to remove any unbound amylopectin molecules.

5. Repeat step 3.4 to ensure sufficient washing. Each tube now contains ConA-Sepharose beads bound to varying amounts of amylopectin substrates.

4. Incubating SEX4 with ConA-Sepharose:amylopectin beads

1. Mix 250 µL of ConA-Sepharose:amylopectin beads with 100 µL of the binding buffer which includes 10 µg of SEX4 protein, 10 mM dithiothreitol (DTT), and 10 µM protease inhibitor cocktail (PIC). Note that the total volume in each tube is 350 µL.

   NOTE: A protease inhibitor cocktail is added as a precautionary step to avoid any unnecessary SEX4 degradation. This is an optional step. In this assay, the recombinant protein *Arabidopsis thaliana* SEX4 (AtSEX4) is used. The purified protein contains an N-terminal histidine tag necessary for detecting the protein *via* chemiluminescence. Detailed information on glucan phosphatase purifications is described in previous publications[14,20,24].

2. Incubate the protein and ConA-Sepharose:amylopectin bead suspension at 4 °C for 45 min with gentle rotation.

   NOTE: The 45 min incubation time is chosen to ensure equilibrium is reached for the complex.

3. Centrifuge the tubes at 10,000 x *g* for 1 min. Pipette 50 µL of the supernatant carefully using a gel loading tip into a new 1.5 mL microcentrifuge tube. Add 20 µL of 4x SDS-PAGE dye and 10 µL of water to each tube containing 50 µL of the collected supernatant fractions. Heat the samples at 95 °C for 10 min. Save these samples for running the SDS-PAGE gels. Ensure 10 new tubes labeled "supernatant (S)" have the corresponding substrate concentrations.

4. Add 750 µL of the binding buffer to the ConA-Sepharose:amylopectin: SEX4 beads to remove any unbound protein from the beads. Centrifuge the tubes at 10,000 x *g* for 1 min. Repeat this step one more time to ensure proper washing. Discard the supernatant.

5. Add 20 µL of 4x SDS-PAGE dye and 80 µL of distilled water into the tubes containing washed ConA-Sepharose:amylopectin:SEX4 beads. Heat the samples at 95 °C for 10 min and centrifuge at 10,000 x *g* for 1 min.

6. Discard the pellet and save the supernatant for running the SDS-PAGE gels. Pipette 80 µL of the supernatant into new tubes and label them as "pellet (P)".

5. Running SDS-PAGE gels

1. Load 40 µL of the unbound protein samples (made in step 2.3, labeled S) into 4%-12% precast polyacrylamide gel wells from the lowest substrate concentration to the highest, but keep the first lane free to load the protein molecular weight marker. Use a second gel to load 10 bound protein samples made in step 2.5 (labeled as P).

2. Add freshly prepared 1x SDS-PAGE running buffer to both chambers of the apparatus. Run the gel at 150 V for 35 min or until the dye front reaches the bottom of the gel.

3. Remove the run gel from the apparatus and remove the spacers and glass plates. Use the separated gel to run a western blot analysis.

6. Western blotting for chemiluminescence detection[14,15]

NOTE: This method can be easily modified/adapted depending on the western blotting equipment that users have in their labs.

1. Make 1 L of transfer buffer containing 5.8 g of Tris base, 2.9 g of glycine, 0.37 g of SDS, and 200 mL of methanol.
2. Transfer the size-separated proteins from the polyacrylamide gel to a nitrocellulose membrane. Briefly assemble the sponges, filter papers, gel, and nitrocellulose membrane according to western transfer protocol[14,15]. Run at 70 V for 1 h.
3. To prevent nonspecific protein binding, incubatethe nitrocellulose membrane containing the protein solution of 1%-5% bovine serum albumin (BSA) or milk protein in 50 mL of TBST buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20) for 1 h. Wash the membrane 3x using TBST buffer to remove any unbound blocking solution.
4. Incubate the membrane with a horseradish peroxidase (HRP)-linked antibody specific for His-tagged protein for 1 h. Wash the membrane 3x in TBST buffer to remove any unbound antibodies. Use a 1:2,000 dilution of antibody to TBST for optimal reproducibility and sensitivity.
5. The HRP enzyme-linked antibody binds specifically to the histidine tag of the SEX4 protein, which yields a band in the presence of chemiluminescence reagents. For digital imaging, make a solution of equal parts of chemiluminescent substrate solutions (750 µL each) in a 1.5 mL tube. Incubate the membrane for at least 5 min in the solution.
6. Place the membrane protein side down on the blot scanner and run the acquisition software to quantify the protein in both the pellet and supernatant fractions.

7. Data analysis

1. Perform the quantitative signal measurements using the acquisition software with the blot scanner. Normalize all quantitative measurements in the supernatant and pellet fractions to the total protein loaded.

   NOTE: The software allows to quantify the intensity of each protein band in the supernatant and pellet fractions.
2. In the saturating binding experiment, plot the percentage of protein-bound versus amylopectin concentration. Fit the data to $Y = Bmax \times X/(K_D + X)$, using data analysis software to calculate K_D.

   NOTE: Bmax is the maximum specific binding, the Y-axis is the percentage of protein bound, the and X-axis is the amylopectin concentration.

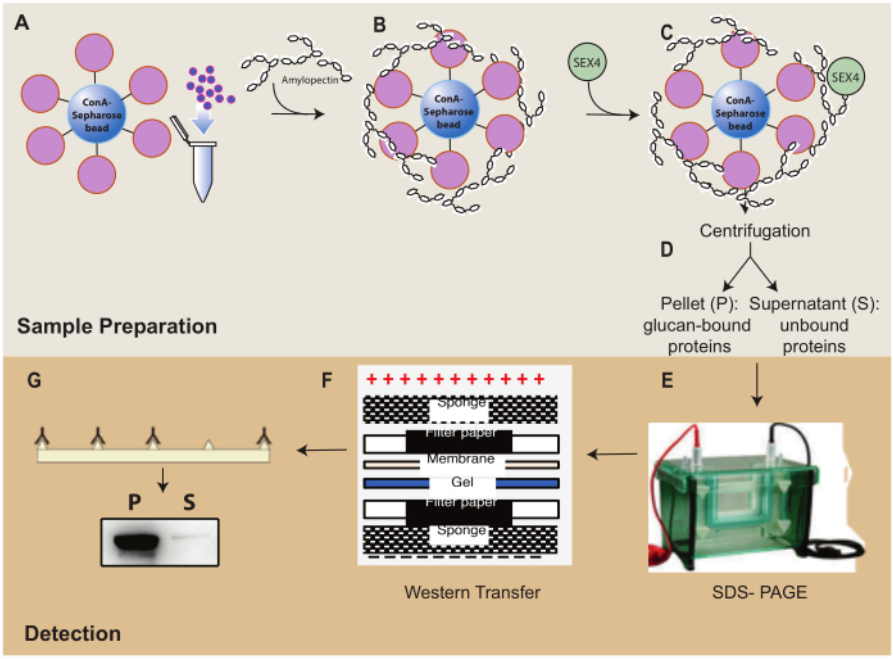

Figure 1: Overview of the ConA-Sepharose sedimentation assay workflow. (**A**) Preparation of ConA-Sepharose beads. (**B**) Incubation with amylopectin substrate. (**C**) Incubation with SEX4 protein. (**D**) Separation of bound and unbound protein fractions through centrifugation. (**E**) Separation of protein through SDS-PAGE. (**F**) Western-blot analysis. (**G**) Chemiluminescence detection of His-tagged SEX4 protein. Please click here to view a larger version of this figure.

Representative Results

One of the key features of the glucan phosphatase family of proteins is their ability to bind to glucan substrates. First, the binding capacity of SEX4 to ConA-Sepharose:amylopectin beads was analyzed using SDS-PAGE (**Figure 2A**). Bovine serum albumin (BSA) served as a negative control to detect any nonspecific binding of proteins to the ConA-Sepharose:amylopectin beads. The SDS-PAGE analysis of proteins showed the presence of SEX4 protein in the pellet fraction and BSA in the supernatant fraction. W278A, a known SEX4 mutant with significantly reduced glucan binding ability, was also included in the assay. The W278A mutant appeared in the supernatant fraction, indicating the utility and specificity of this assay for detecting the glucan binding ability of SEX4 proteins. While SDS-PAGE visualized the SEX4 proteins, there was concern about the sensitivity and use of small glucan phosphatases; For instance, the ConA lectin protein could potentially interfere with detecting small proteins such as LSF2. To test whether this method could detect only the glucan phosphatases, a western blot was performed using an antibody specific to the N-terminal histidine tag. Indeed, there was a significant increase in the detection of SEX4, and the method is specific for detecting glucan phosphatases with an N-terminal histidine tag (**Figure 2B**). Quantitative measurement of ligand-protein interaction experiments requires performing essential controls for establishing the appropriate ligand

and protein concentrations. Therefore, the co-sedimentation assay was tested next at three different SEX4 concentrations (**Figure 2C-E**). While 2 µg of SEX4 is sufficient to visualize *via* chemiluminescence detection, using 5 or 10 µg of SEX4 allows for a more accurate detection of the partial binding. The SEX4 binding against varying amylopectin concentrations (0.5 mg/mL, 1.0 mg/mL, and 5 mg/mL) was also tested. These results also indicate increased SEX4 binding with increased amylopectin concentration, with saturated binding at 5 mg/mL of amylopectin.

To determine the binding affinity of amylopectin and SEX4 wild-type protein, 10 µg of SEX4 was incubated with amylopectin (up to 5 mg/mL), and binding at each concentration was determined (**Figure 3A**). The fitting of percent protein-bound data to the specific, one-site binding model gave a K_D of 1.03 ± 0.23 mg/mL (**Figure 3B**). There is no available crystal structure of SEX4 wild-type protein; instead, the crystal structure of catalytically inactive SEX4 C198S mutant reveals the binding interface of SEX4, made of DSP and carbohydrate-binding molecule (CBM) domains. The binding affinity of SEX4 C198S and amylopectin interaction was determined to have a K_D of 0.11 ± 0.05 mg/mL for C198S mutant proteins; a ~10 times increased the binding affinity compared to SEX4 wild-type protein. While the exact mechanism of the increased binding of C198S is not understood, it is intriguing to see how a single-point mutation of SEX4 results in a significant increase in glucan binding ability.

Next, the applicability of this method to measure the binding of laforin, LSF2, corn (*Zea mays*) SEX4, and potato (*Solanum tuberosum*) SEX4 against potato amylopectin was assessed. Using the protocol presented here, the results demonstrated that laforin, LSF2, and SEX4 from agronomically important crops also differentially bind to amylopectin, suggesting different binding mechanisms for each protein (**Figure 4A,B**). Collectively, these findings revealed a successful method development for the determination of substrate binding of glucan phosphatase with amylopectin substrates.

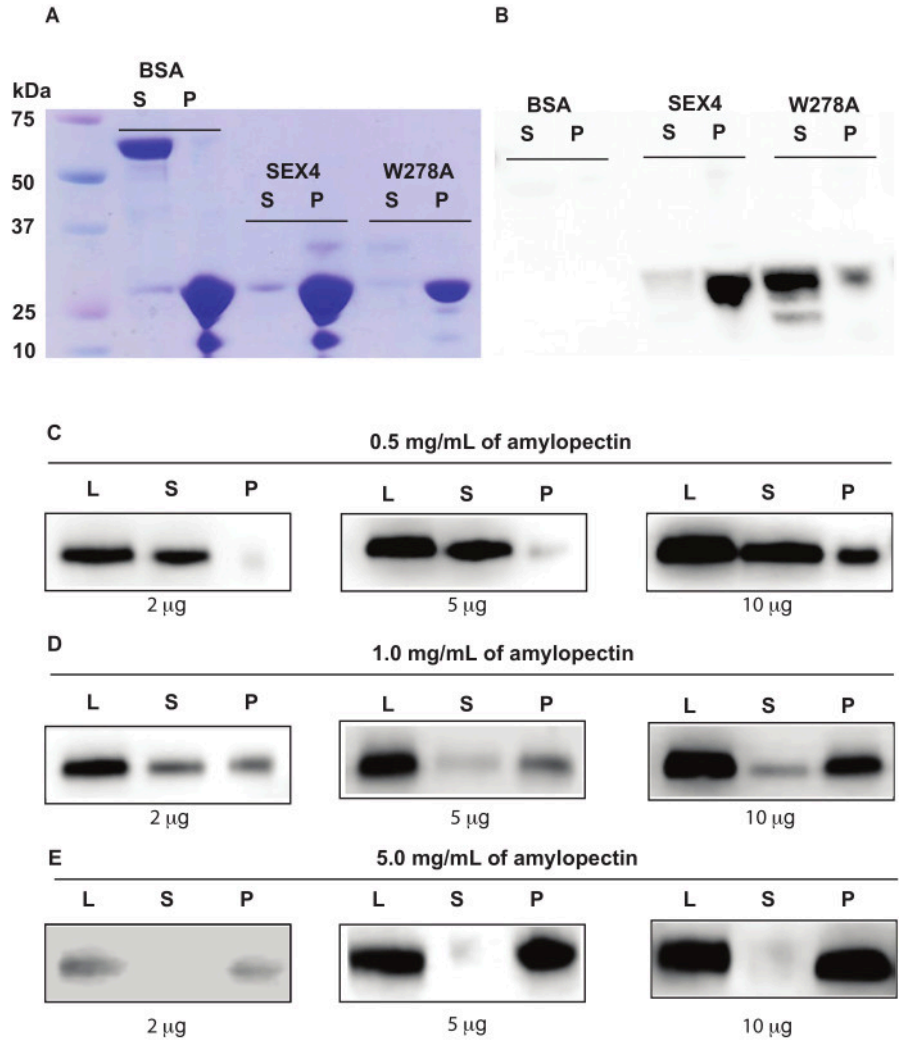

Figure 2: Visualization of pellet (P) and supernatant (S) SEX4 fractions and optimization of the assay parameters. (**A**) Visualization of 5 µg of SEX4 protein (34 kDa) in the pellet and supernatant fractions using SDS-PAGE and Coomassie staining. The protein bands at 25 kDa and below are for the ConA protein attached to Sepharose beads. (**B**) Visualization of SEX4 proteins in the pellet and supernatant fractions *via* western analysis. 5 µg of SEX4 protein was detected *via* an antibody developed for the histidine tag. (**C-E**) The western analysis was done using varying amounts of SEX4 protein (2 µg, 5 µg, and 10 µg) and amylopectin concentrations (0.5 mg/mL, 1.0 mg/mL, and 5.0 mg/mL). A volume of 250 µL of beads and an incubation time of 30 min were used for all experiments to ensure equilibrium binding. Please click here to view a larger version of this figure.

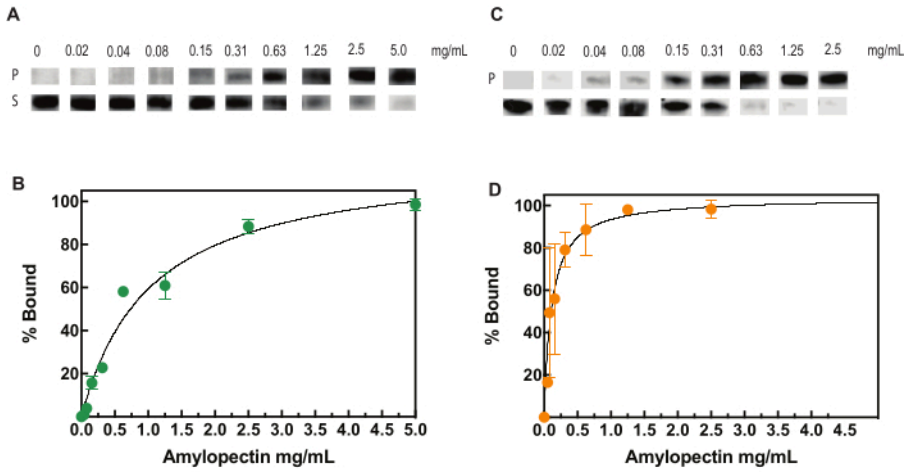

Figure 3: Quantitative *in vitro* sedimentation assay. (**A**) Representative data of supernatant depletion and the gradual increase of pellet signal of SEX4 wild-type protein as the amylopectin concentration increased from 0 mg/mL to 5 mg/mL. (**B**) The percentage of SEX4 wild-type protein bound against varying amounts of amylopectin immobilized onto ConA-Sepharose beads. The beads were pelleted at 10,000 x *g* for 1 min, and both the supernatant and pellet proteins were separated by SDS-PAGE, detected by western analysis, and quantified to measure the percent bound. Data represent the average ± SD of three replicates. (**C**) Representative data of supernatant depletion and the gradual increase of pellet signal of SEX4 C198S protein as amylopectin concentration increased from 0 mg/mL to 5 mg/mL. (**D**) The percentage of SEX4 C198S protein bound against varying amounts of amylopectin immobilized onto ConA-Sepharose beads. The beads were pelleted at 10,000 x *g* for 1 min, and both the supernatant and pellet proteins were separated by SDS-PAGE, detected by western analysis, and quantified to measure the percent bound. Data represent the average ± SD of three replicates. Please click here to view a larger version of this figure.

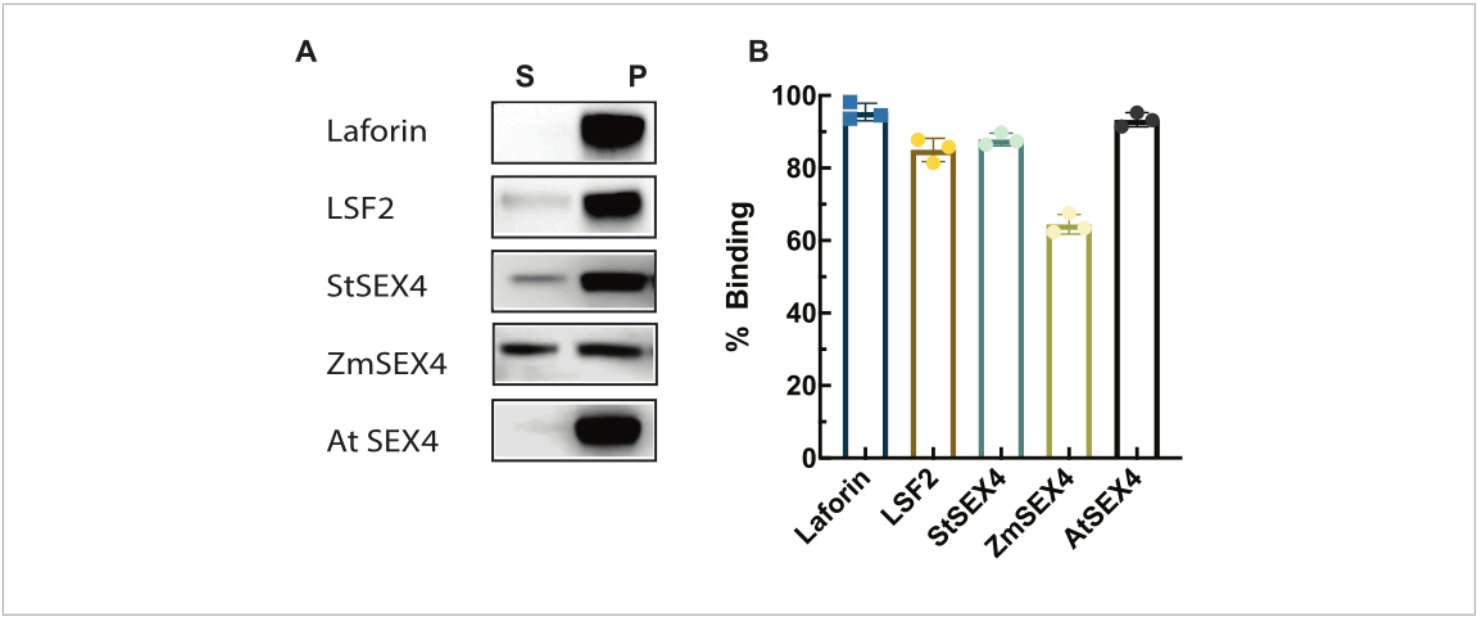

Figure 4: Substrate binding of SEX4 orthologs, laforin, LSF2 against solubilized amylopectin. (**A**) Representative binding of each protein with 5 mg/mL of amylopectin immobilized in ConA-Sepharose beads. (**B**) The percentage of bound protein was calculated using the normalized chemiluminescence signals obtained for three independent experiments. Data represent the mean ± SD of three replicates. Please click here to view a larger version of this figure.

Discussion

This study demonstrates the successful development of a novel *in vitro* sedimentation assay that allows determination of the binding affinity of glucan-glucan phosphatase interactions. The assay design takes advantage of the specific binding of lectin ConA to glucans *via* the hydroxyl residues of glucose to indirectly capture solubilized carbohydrate substrates onto Sepharose beads. This allows the separation of bound and unbound protein fractions *via* centrifugation and determination of the binding affinity of solubilized glucan substrates and glucan phosphatases. All of the glucan phosphatases tested were found to specifically bind to amylopectin, with no detectable binding to ConA-Sepharose beads in the absence of amylopectin.

The experiment was performed to measure the binding affinity with a small, fixed amount of SEX4 protein and a range of amylopectin concentrations attached to ConA-Sepharose beads. An excess of ConA-Sepharose beads was used to ensure the highest amylopectin concentration binding to beads. The complete binding of amylopectin was tested *via* a glucose assay. The protocol presented in the manuscript utilizes quantitative sedimentation to separate bound and unbound protein fractions, as well as gel electrophoresis and western analysis to measure the amount of SEX4 in the supernatant compared with the amount bound to the ConA-Sepharose:amylopectin beads. Quantifying the pellet fraction requires minimal washing to not disturb the binding equilibrium. To avoid any underestimation of pellet fraction, rather than examining the pellet, measuring the concentration

of free SEX4 in the supernatant and calculating the difference of the bound SEX4:amylopectin complex is sufficient.

The *in vitro* sedimentation assay described here determined the differential binding affinity of SEX4 wild-type protein. Interestingly, the determined binding affinity of SEX4 wild-type protein using the developed *in vitro* sedimentation assay falls within the higher range of the reported value determined with a gel shift assay[25]. The binding assay should be sensitive to lower substrate concentrations and straightforward, whereas the gel shift assay requires making individual native gels in the presence of varying amounts of glucan substrates. This time-consuming method limits the number of concentrations that can be tested, and a limited substrate concentration range can be tested. Instead, sample preparation is fast and reliable with our *in vitro* sedimentation assay. Comparing SEX4 wild-type protein and SEX4 C198S mutant reveals a tenfold binding affinity difference. In this regard, this assay is sensitive to determine the binding of SEX4 mutant proteins quantitatively and qualitatively.

Protein-carbohydrate interactions are vital for many important biological processes, including catalysis, cell signaling, and recognition. They are also gaining increasing attention due to their important roles in the pharmaceutical and food industries. X-ray crystallography is widely used to obtain protein structures at the atomic level. However, the structural heterogeneity and flexibility of carbohydrate substrates make it harder to obtain carbohydrate-bound protein structures. Recently cryo-EM and nuclear magnetic resonance (NMR) spectroscopy have also been employed to determine the structures of carbohydrate-binding proteins[26]. Despite the many advancements in high-resolution structural techniques, it is challenging to obtain structures of carbohydrate-protein complexes. As a result, only a limited number of protein-carbohydrate complex structures have been solved experimentally. Furthermore, crystal structures only provide a snapshot of a protein at a certain conformation, and not the dynamic nature of substrate binding. Alternatively, several biophysical techniques, including isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR), are used to measure protein-carbohydrate interactions[27]. However, these methods are also not without limitations. One main challenge in determining protein-carbohydrate interactions is their relatively weak interactions compared to other protein-ligand interactions. The interactions within the range of high micromolar to low micromolar range significantly reduces the usability of ITC and SPR. Indirect methods, such as gel shift and pull-down assays, have gained significant attention for determining interactions that are difficult to assess by other methods[25]. Therefore, this binding assay is a widely applicable approach for sensitive measurements of protein and soluble carbohydrate interactions.

Glycogen and starch are major carbohydrate storage molecules made of branched polymers of glucose. The branching pattern, flexibility, and the presence of different microdomains within the starch and glycogen require interacting proteins to adopt unique substrate binding mechanisms. Much understanding of substrate recognition mechanisms of SEX4 has come from X-ray crystallographic studies coupled with structure-guided mutagenesis. To dephosphorylate starch, SEX4 must interact with significantly different microdomains of the starch granule to locate phosphates for position-specific dephosphorylation. Experimentally, it remains a challenge to determine the binding affinities of glucan phosphatases, given the weak interactions between the protein and the glucans and the structural flexibility and heterogeneity of carbohydrates. This manuscript presents a method to determine binding affinities

of glucan phosphatase-carbohydrate interactions using a Concanavalin A (ConA)-based *in vitro* sedimentation assay. Glucan phosphatases employ distinct mechanisms to bind, locate, and dephosphorylate carbohydrates. Previously, ITC has been employed to measure the binding affinity of laforin and linear oligosaccharide chains. However, no study has revealed the binding affinities of glucan phosphatases with their physiological substrates *via* direct techniques such as ITC and SPR. To better understand the utility of this assay for determining substrate binding of glucan phosphatases, the binding of laforin, LSF2, and SEX4 against amylopectin was assessed. Future studies can be done to experimentally determine the binding affinity of all glucan phosphatases and their physiologically relevant glucan substrates. One of the critical steps of the method is to determine the optimum incubation times and substrate concentration range for the assay. Each glucan phosphatase binds differently with the substrate, and the method is optimized for SEX4. It is important to carry out initial optimization experiments to figure out these parameters. One other limitation is the use of histidine tagged protein for the assay. However, if antibodies are present for the study protein, the method can be easily optimized for any glucan-interacting protein.

Disclosures

The authors declare no conflicts of interest.

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References

1. Meekins, D. A., Vander Kooi, C. W., Gentry, M. S. Structural mechanisms of plant glucan phosphatases in starch metabolism. *The FEBS Journal.* **283** (13), 2427-2447 (2016).
2. Gentry, M. S. et al. The phosphatase laforin crosses evolutionary boundaries and links carbohydrate metabolism to neuronal disease. *The Journal of Cell Biology.* **178** (3), 477-488 (2007).
3. Worby, C. A., Gentry, M. S., Dixon, J. E. Laforin, a dual specificity phosphatase that dephosphorylates complex carbohydrates. *The Journal of Biological Chemistry.* **281** (41), 30412-30418 (2006).
4. Gentry, M. S., Pace, R. M. Conservation of the glucan phosphatase laforin is linked to rates of molecular evolution and the glucan metabolism of the organism. *BMC Evolutionary Biology.* **9**, 138 (2009).
5. Niittyla, T. et al. Similar protein phosphatases control starch metabolism in plants and glycogen metabolism in mammals. *The Journal of Biological Chemistry.* **281** (17), 11815-11818 (2006).
6. Kotting, O. et al. STARCH-EXCESS4 is a laforin-like Phosphoglucan phosphatase required for starch degradation in Arabidopsis thaliana. *The Plant Cell.* **21** (1), 334-346 (2009).

7. Comparot-Moss, S. et al. A putative phosphatase, LSF1, is required for normal starch turnover in Arabidopsis leaves. *Plant Physiology.* **152** (2), 685-697 (2010).
8. Zeeman, S. C., Northrop, F., Smith, A. M., Rees, T. A starch-accumulating mutant of Arabidopsis thaliana deficient in a chloroplastic starch-hydrolysing enzyme. *The Plant Journal: For Cell and Molecular Biology.* **15** (3), 357-365 (1998).
9. Kotting, O. et al. Identification of a novel enzyme required for starch metabolism in Arabidopsis leaves. The phosphoglucan, water dikinase. *Plant Physiology.* **137** (1), 242-252 (2005).
10. Tagliabracci, V. S. et al., Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen in vivo. *Proceedings of the National Academy of Sciences.* **104** (49), 19262-19266 (2007).
11. Gentry, M. S., Guinovart, J. J., Minassian, B. A., Roach, P. J., Serratosa, J. M. Lafora disease offers a unique window into neuronal glycogen metabolism. *The Journal of Biological Chemistry.* **293** (19), 7117-7125 (2018).
12. Brewer, M. K. et al., Targeting pathogenic lafora bodies in lafora disease using an antibody-enzyme fusion. *Cell Metabolism.* **30** (4), 689-705.e686 (2019).
13. Santelia, D., Zeeman, S. C. Progress in Arabidopsis starch research and potential biotechnological applications. *Current Opinion in Biotechnology.* **22** (2), 271-280 (2011).
14. Raththagala, M. et al. Structural mechanism of laforin function in glycogen dephosphorylation and lafora disease. *Molecular Cell.* **57** (2), 261-272 (2015).
15. Meekins, D. A. et al. Phosphoglucan-bound structure of starch phosphatase Starch Excess4 reveals the mechanism for C6 specificity. *Proceedings of the National Academy of Sciences.* **111** (20), 7272-7277 (2014).
16. Vander Kooi, C. W. et al. Structural basis for the glucan phosphatase activity of Starch Excess4. *Proceedings of the National Academy of Sciences.* **107** (35), 15379-15384 (2010).
17. Meekins, D. A. et al. Structure of the Arabidopsis glucan phosphatase like sex four2 reveals a unique mechanism for starch dephosphorylation. *The Plant Cell.* **25** (6), 2302-2314 (2013).
18. Smith, A. M., Zeeman, S. C. Starch: A flexible, adaptable carbon store coupled to plant growth. *Annual Review of Plant Biology.* **71**, 217-245 (2020).
19. Jane, J., Kasemuwan, T., Chen, J. F., Juliano, B. O. Phosphorus in rice and other starches. *Cereal Foods World.* **41** (11), 827-832 (1996).
20. Mak, C. A. et al. Cooperative kinetics of the glucan phosphatase starch excess4. *Biochemistry.* **60** (31), 2425-2435 (2021).
21. Campbell, K. P., MacLennan, D. H. Purification and characterization of the 53,000-dalton glycoprotein from the sarcoplasmic reticulum. *The Journal of Biological Chemistry.* **256** (9), 4626-4632 (1981).
22. Campbell, K. P., MacLennan, D. H., Jorgensen, A. O., Mintzer, M. C. Purification and characterization of calsequestrin from canine cardiac sarcoplasmic reticulum and identification of the 53,000 dalton glycoprotein. *The Journal of Biological Chemistry.* **258** (2), 1197-1204 (1983).

23. Davey, M. W., Sulkowski, E., Carter, W. A. Binding of human fibroblast interferon to concanavalin A-agarose. Involvement of carbohydrate recognition and hydrophobic interaction. *Biochemistry.* **15** (3), 704-713 (1976).

24. Meekins, D. A. et al. Mechanistic insights into glucan phosphatase activity against polyglucan substrates. *The Journal of Biological Chemistry.* **290** (38), 23361-23370 (2015).

25. Wilkens, C. et al. Plant α-glucan phosphatases SEX4 and LSF2 display different affinity for amylopectin and amylose. *FEBS Letters.* **590** (1), 118-128 (2016).

26. Atanasova, M., Bagdonas, H., Agirre, J. Structural glycobiology in the age of electron cryo-microscopy. *Current Opinion in Structural Biology.* **62**, 70-78 (2020).

27. Doyle, M. L. Characterization of binding interactions by isothermal titration calorimetry. *Current Opinion in Biotechnology.* **8** (1), 31-35 (1997).