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Prostate-specific antigen (PSA) glycan-binding profile analysis based on enzyme-linked lectin assay (ELLA) and storage effect of assay components

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Abstract

Changes in the prostate-specific antigen (PSA) glycosylation profile may be used to distinguish indolent from aggressive prostate cancer (PCa). This study aimed to obtain the glycan profiles of PSA isolated from a normal individual using the enzyme-linked lectin assay (ELLA) method. We used sialic acid-specific lectins (MAA I, MAA II and SNA) for PSA glycoprofiling and generated the binding curve of PSA-lectin interactions. Compared to MAA I and MAA II, *Sambucus nigra* agglutinin (SNA) had the highest binding (A_{450} = 3.59) with the normal individual PSA sample harbouring α2,6-sialic acid glycan used in this study. The binding signals for *Maackia amurensis* agglutinin (MAA) I were significant starting at 700 ng/mL PSA, implying that a small amount of α2,3-sialic acid glycan presented in the normal PSA. Binding saturation occurred at A_{450} = 3.60 and at a PSA concentration of 5×10^3 ng/mL (176 nM). In addition, the stability of assay components stored at room temperature (25 °C) for two months was assessed, and the results showed that the binding signals for PSA detection by SNA were still remarkably high (A_{450} = 2.37) at a PSA concentration of 1 µg/mL. Here, we established a simple and sensitive lectin-based assay for PSA glycoprofiling. The stability of the assay components during the storage test also revealed their potential to be utilised and stored for longer periods at room temperature.

Keywords: *glycoprofiling; lectin assay; MAA; PSA; sialic acid; SNA; storage*

1. Introduction

Prostate cancer (PCa) is a major health concern in men, with 1,276,106 new cases and 358,989 related deaths recorded worldwide in 2018 (Bray et al., 2018). This number is expected to increase by 79.7% in 2040 (Rawla, 2019). Screening for PCa is performed by measuring the level of a serological biomarker, known as prostate-specific antigen (PSA), despite its low specificity in the range of 4 to 10 ng/mL (Catalona et al., 1991). However, this screening method, together with the digital rectal examination (DRE)—approved for prostate cancer diagnostics by the US Food and Drug Administration (FDA) in 1994—caused overdiagnosis, resulting in unnecessary biopsies and treatment of non-aggressive cancers (Sandhu & Andriole, 2012), given that the elevation of serum PSA levels are associated with other non-cancerous prostate disorders (e.g. prostatitis and benign prostatic hyperplasia) (Oesterling et al., 1995). Due to the substantial social and economic burdens that emerged from overdiagnosis, the FDA approved various approaches to improve the specificity of PCa screening, such as the prostate health index (PHI) (Loeb et al., 2015) and prostate cancer antigen 3 (PCA3) (Wei et al., 2014), in 2012. However, these approaches are not sensitive enough to discriminate the aggressive forms from

the indolent form of the disease, especially in cases where the PSA concentration falls within the grey zone (4.0–10.0 ng/mL) (Vlaeminck-Guillem, Ruffion, André, Devonec, & Paparel, 2010).

Recent advances in glycomics studies have shown that PSA glycoprofiling is a promising approach in terms of specificity during PCa screening. A study from Ferrer-Batallé et al. (2017) has shown that combining PHI with the detection of α2,3-sialic acid PSA glycoforms results in 100% sensitivity and 94.7% specificity in distinguishing high-risk PCa from low- and intermediate-risk PCa and BPH patients. Another free PSA (fPSA) glycoprofiling analysis by Bertok et al. (2020) has shown that using the prostate glycan index (PGI) would avoid 63.5% of unnecessary biopsies compared to using fPSA (17.5%) and PHI (33.3%). Therefore, these studies support the use of PSA glycoforms in clinical PCa screening as a promising approach to discriminate between aggressive and indolent PCa.

Previous PSA glycoprofiling studies have highlighted several changes in PSA glycan structures that could be used as a hallmark of PCa, including α2,3-sialylation (Meany, Zhang, Sokoll, Zhang, & Chan, 2009), α2,6-sialylation, mannose (Ohyama et al., 2004), core fucosylation (Kekki et al., 2017) and LacdiNAc (GalNAc-GlcNAc) (Hagiwara et al., 2017). Instrumental-based approaches that employ high-performance liquid chromatography, mass spectrometry and nuclear magnetic resonance spectroscopy when characterising the PSA glycoprofile are not suited to a clinical setting because they run at high operational costs and require professional expertise. Moreover, analysis involving the enzymatic digestion of peptides (Haga et al., 2019) or the removal of glycans from glycoproteins (Thompson, Creavin, O'Connell, O'Connor, & Clarke, 2011), together with glycoprotein enrichment, makes the process complex and not ready for application in high-throughput clinical screening.

By contrast, lectins are oligomeric proteins that can bind specifically and reversibly to carbohydrates (Sharon & Lis, 2013). Lectins (also known as glycans-specific proteins) can directly bind to the target glycans on the protein of interest without requiring pre-treatment of PSA, as previously mentioned. The most popular lectins used in PSA glycan studies are sialic acid-specific lectins, such as *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), which can be specifically recognise α2,6-linked sialic acids and α2,3-linked sialic acids, respectively (Bhanushali et al., 2016; Pihikova et al., 2016). Based on the glycan structure on the PSA surface, these lectins are capable of distinguishing healthy individuals with high α2,6-sialic acid levels (Pihikova, Kasak, Kubanikova, Sokol, & Tkac 2016) from PCa patients with high α2,3-sialic acid levels (Yoneyama et al. 2014). Enzyme-linked lectin assays (ELLAs) is a labelling technique widely applied in studies of glycosylation, including PSA glycoprofiling (Dwek, Jenks, & Leathem, 2010; Meany et al., 2009). This method is similar to enzyme-linked immunosorbent assay (ELISA), except that lectin is used instead of antibodies to detect glycoproteins (Wu & Liu, 2019). It also has considerable potential for the clinical diagnosis of PCa as it is a simple, high-throughput technique that does not require highly skilled personnel.

In previous studies, the glycoprofiling of PSA was performed using lectin, together with other detector molecules such as antibodies, which are costly yet more sensitive than using lectin alone. However, a simple, indirect assay without antibodies can be applied to study the protein-carbohydrate interactions and glycoanalysis of targeted proteins. A few studies have reported that the sample range for glycoprofiling starts at 0.1 ng/mL up to 200 µg/mL (Damborský, Koczula, Gallotta, & Katrlík, 2016; Mohseni et al., 2019; Piao, 2002). Most glycoprotein samples from commercial kits are available in small quantities and usually come together with antibodies in a sandwich format, which is costly.

In this fundamental study, we focused on determining the presence of sialic acid linkage or isoforms in normal PSA as the first step towards further research in assay development using clinical samples. We aimed to obtain the PSA glycoprofiling using an indirect ELLA-based approach, which can be applied to differentiate sialic acid linkage in PSA glycoforms. Commercialised PSA purified from a normal individual was used as the model protein. The binding profile of PSA was obtained using sialic acid-specific lectins, and the binding curve was examined to identify the binding saturation between lectins and PSA at varying concentrations. In addition, we investigated the effect of the storage conditions on assay-binding to observe the stability of the assay components at room temperature (25 °C).

2. Objectives

1. To obtain the sialic acid linkage binding pattern in PSA from a normal individual using ELLA.
2. To determine the effect of room-temperature storage (at 25 °C) on the stability of the assay components.

3. Materials and methods

3.1 Materials

A phosphate-buffered saline (PBS) tablet, sodium bicarbonate ($NaHCO_3$), sodium carbonate decahydrate (Na_2CO_3), sodium chloride (NaCl), calcium chloride dihydrate ($CaCl_2 2H_2O$), sulfuric acid (H_2SO_4) and Tween® 20 were purchased from Sigma Aldrich (St. Louis, MO, USA). Biotinylated lectins—soybean agglutinin (SBA), *Maackia amurensis* agglutinin I (MAA I), *Maackia amurensis* agglutinin II (MAA II), and *Sambucus nigra* agglutinin (SNA)—were obtained from Vector Laboratories (Burlingame, CA, USA). Neutravidin-HRP conjugate, Nunc® MaxiSorp™ 384-well plates, and 1-Step™ Ultra TMB-ELISA Substrate Solution were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Carbo-free blocking solution was purchased from Vector Laboratories (Burlingame, CA). Prostate-specific antigen isolated from human semen was purchased from Lee Biosolutions (Maryland Heights, MO) in the free form. All other chemicals used in this study were commercially available and of the highest quality grade.

3.2 Enzyme-linked lectin assay (ELLA)

Enzyme-linked lectin assay was performed as previously described by Thompson, Creavin, O'Connell, O'Connor and Clarke (2011), with some modifications. The PSA was diluted to concentration levels ranging from 1–5000 ng/mL (10 mM carbonate buffer; pH 9.0), and 100 µL was pipetted into each well of a 384-well MaxiSorp™ microtiter plate. The microtiter plate was sealed with Parafilm® and incubated overnight at 4 °C. The wells were emptied and washed four times with 100 µL PBS-T. Carbo-Free blocking reagent (100 µL) was added to each well, and the plate was incubated for 2 hours at 25 °C. Then, 100 µL of 1 µg/mL biotinylated lectins was added to each well, and the plate was incubated for 1 hour at room temperature, followed by the addition of 100 µL of NeutrAvidin™-HRP conjugate (1:10 000) for 1 hour at room temperature. Finally, 100 µL of Ultra TMB-ELISA substrate was added to each well, and the mixture was incubated for 15 minutes before the reaction was stopped by adding 100 µL of 2 M H_2SO_4. The intensity of colour developed in each well was measured at a 450-nm wavelength using a Multiskan™ FC plate reader (Thermo Fisher Scientific, Waltham, MA). All of the incubation steps mentioned above were conducted at room temperature with the exception of the PSA coating. A washing step was included after each incubation. All lectins used in this study were subjected to a hemagglutination test and corresponding sugar inhibition test in order to ensure optimal activity.

3.3 PSA binding assay

The saturation binding of the detected molecule and the target protein were determined using the absorbance values as reported by Gupta, Massinick, Garrett and Hazlett (1997). The PSA binding data were generated using 1 µg/mL each of SNA and MAA I, and a range of PSA concentrations (ng/mL): 1, 3, 5, 7, 10, 30, 50, 70, 100, 300, 500, 700, 1,000, 3,000 and 5,000. Binding assays were performed using the ELLA procedure. The binding curves were analysed and fitted to a sigmoid dose-response model, according to the method described by Legardinier et al. (2005).

3.4 Storage effect

The assay components were initially stored at the recommended temperature, i.e. 4 °C for N-HRP and TMB-ELISA, and −20 °C for PSA and lectins. The temperature was monitored using a Fisherbrand™ refrigerator and freezer thermometer (Thermo Fisher Scientific, Waltham, MA). A glycoprofiling study by Li et al. (2011) used a similar ELLA-based technique with 1 µg/mL PSA. The initial absorbance readings for lectins MAA I, MAA II, and SNA were recorded. All assay components were stored at room temperature (25 °C) for two months. The final absorbance readings were recorded and compared.

3.5 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.4.3 (GraphPad Software, Inc., San Diego, CA). All data are presented as the mean ± SD. The means were compared using one-way analysis of variance (ANOVA) followed by Sidak's *post-hoc* test. Differences were considered statistically significant at $p < 0.05$, with the significant indicators being * ($p < 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$). Non-significant data were noted as "ns".

4. Results and discussion

4.1 Determination of the PSA glycan-binding profile

Glycan binding studies were carried out using an indirect format of ELLA, with PSA as the analyte. The PSA contains only one *N*-glycosylation site at Asp69, with the sialic acid group at the end of each glycosylation site (Bélanger et al., 1995; Drake, Jones, Powers, & Nyalwidhe, 2015). Therefore, sialic-acid specific lectins were used in this study. Detection of the target protein is typically carried out at a wavelength of 450 nm when performing enzyme assays that use PSA (Dwek et al., 2010; Liang et al., 2015; Paul, Dhir, Hitchens, & Getzenberg, 2005). In the present study, SBA served as a negative control due to its sugar specificity to terminal *N*-acetylgalactosamine (GalNAc), which is not essentially present as a PSA epitope glycan. The highest absorbance reading for SBA was at A_{450} = 0.07 (Figure 1), which was lower than the standard measurement of the absorbance value ranging from 0.1 nm to 0.8 nm (Wenzel, 2021). This finding indicates that the binding of SBA and PSA were undetected, thus making SBA a suitable negative control in this study.

As shown in Figure 1, the binding profile revealed that the highest specific binding of PSA was with SNA; binding with MAA I was also relatively high. The binding signal increased for both SNA and MAA I, corresponding to increasing concentrations of PSA. The cut-off absorbance value for this assay was at A_{450} = 0.1, where significant values for PSA-lectin binding were determined. Detection of PSA at concentrations above 50 ng/mL showed a significant increase in binding to SNA compared to SBA; the highest reading was noted at 5,000 ng/mL, with A_{450} = 3.59. This result was expected as SNA recognised α2,6-sialic acid glycan on PSA from a normal source, in concordance with previous studies that used lectin affinity chromatography (Gratacós-Mulleras et al., 2020) and chronopotentiometric stripping (CPS) (Belicky et al., 2017). Binding of MAA I significantly increased at PSA concentrations of 700 ng/mL (A_{450} = 0.10) and above, with the highest reading obtained at 5,000 ng/mL (A_{450} = 0.14), thereby indicating lower binding compared to SNA. Our findings are consistent with those of Peracaula et al. (2003) and Pihikova et al. (2016), who used mass spectrometry and reported a small amount of α2,3-sialic acid in normal PSA compared to α2,6-sialic acid glycan. MAA II showed non-significant binding at all PSA concentrations used.

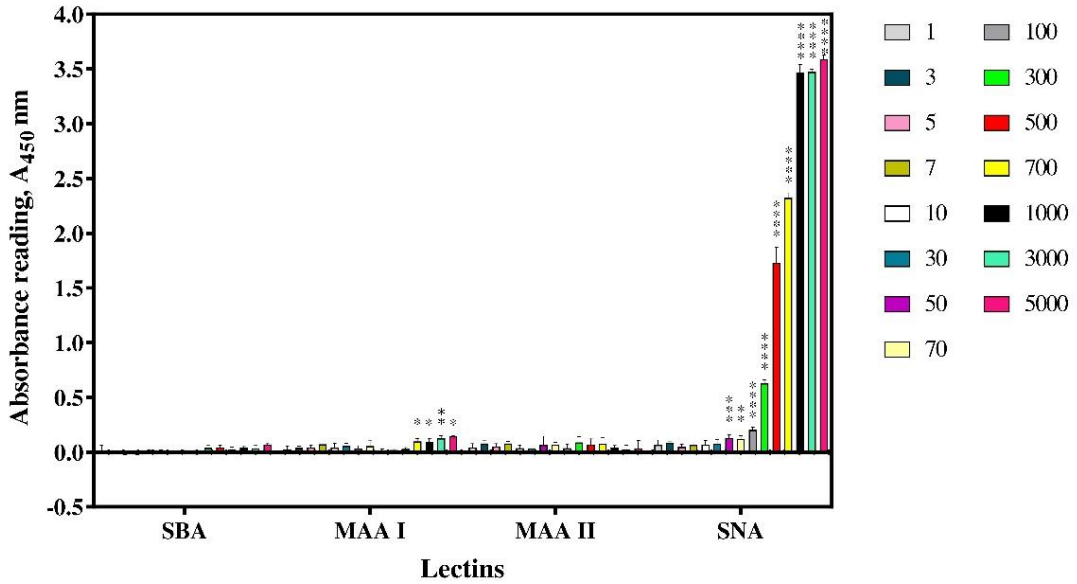

Figure 1 PSA binding profile using sialic acid-specific lectins. Each sample was measured in triplicate, and the error bar represents the mean of the absorbance reading (n = 3 ± SD, $p < 0.05$). Each bar shows PSA concentrations in ascending order from 1–5000 ng/mL. Statistically significant data were based on the negative control (SBA) and are denoted by asterisks (*). * ($p < 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$).

Additionally, the absorbance value could reflect the specificity and saturation binding of the detector molecule and target protein, as previously reported (Gupta et al., 1997). Figure 2a shows the binding curves of SNA and MAA I with PSA at various PSA concentrations (1 ng/mL to 5×10^3 ng/mL). For SNA, the maximum binding (B_{max}) value was measured at $A_{450} = 3.60$, showing that binding was saturated at a PSA concentration of 5×10^3 ng/mL (176 nM). Compared to a cancerous PSA sample, which contains a mixture of biantennary, triantennary, and possibly tetra-antennary oligosaccharides structures, a PSA sample from a normal individual only has a biantennary oligosaccharide structure that exposes the α2,6-sialic acid linkage (Prakash & Robbins, 2000). SNA has a strong preference for α2,6-linked sialic acids. Here, the interaction between the SNA and PSA glycoproteins was due to the weak hydrogen bond and hydrophobic interaction, as well as the amino acid residue essential for ligand binding. However, molecular recognition is poorly understood (Shang & Van Damme, 2014; Sharon & Lis, 2002). By contrast, the B_{max} value for MAA I was noted at $A_{450} = 0.13$, which was saturated at 4×10^3 ng/mL (141 nM) (Figure 2b). The indirect ELLA-based method used in the present study detected higher concentrations of PSA than commercially available kits (pg/mL) because it uses only one type of detector molecules (lectins). However, similar sensitivity has been reported to differentiate PSA glycosylation patterns using an in-house lectin microarray (Sonawane, Nimse, Song, & Kim, 2016).

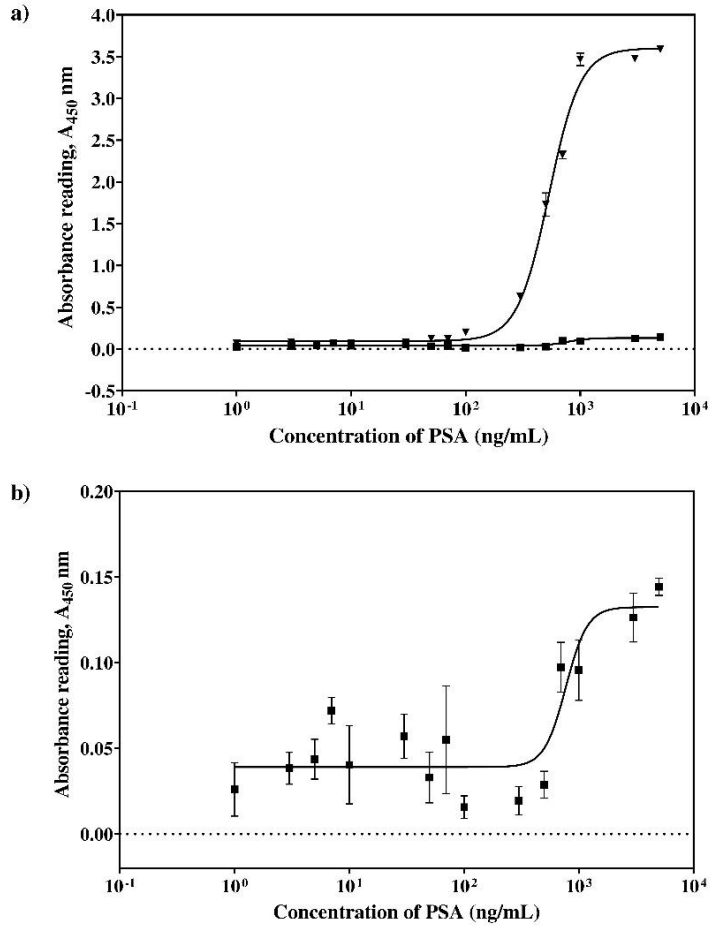

Figure 2 Using the same experimental data, a) generated binding curves, showing a significant increase in binding affinity by SNA and MAA I, indicated by (▼) and (■), respectively; b) an enlarged graph of the PSA binding curve for MAA I. Each sample was measured in triplicate, and the error bar represents the mean absorbance reading ($n = 3 \pm SD$, $p < 0.05$).

4.2 Storage effect of the assay components at 25 °C (room temperature)

This study was extended to determine the stability of the assay components, such as PSA, lectins, N-HRP and TMB-ELISA substrate, after they were stored at room temperature (25 °C) for two months. Figure 3 shows the binding of PSA to sialic acid-specific lectins before and after two months under the tested condition. The highest absorbance reading was recorded for SNA (A_{450} = 3.47; light grey bar) during the initial storage period. PSA and SNA binding were successfully detected after storing the assay components for two months, producing an absorbance reading of A_{450} = 2.37, as shown by the dark grey bar. Although the mean reading of the PSA-SNA binding in the final storage was significantly decreased by 1.5-fold, or 31.84%, compared to the initial storage period, the mean reading, A_{450} of PSA by SNA, remained markedly high (A_{450} = 2.37) compared to other lectins (A_{450} = 0.03 for MAA I; A_{450} = 0.02 MAA II) (Figure 3). These results were expected as the activity of the proteins would gradually decrease at a high temperature, thus affecting the binding activity between the PSA and lectins after two months under the tested condition. Meanwhile, MAA I and MAA II displayed non-significant binding before (A_{450} = 0.10) and after ($A_{450} < 0.03$) the storage period. The longest test period for this experiment was two months due to practical restrictions; thus is this study's limitation.

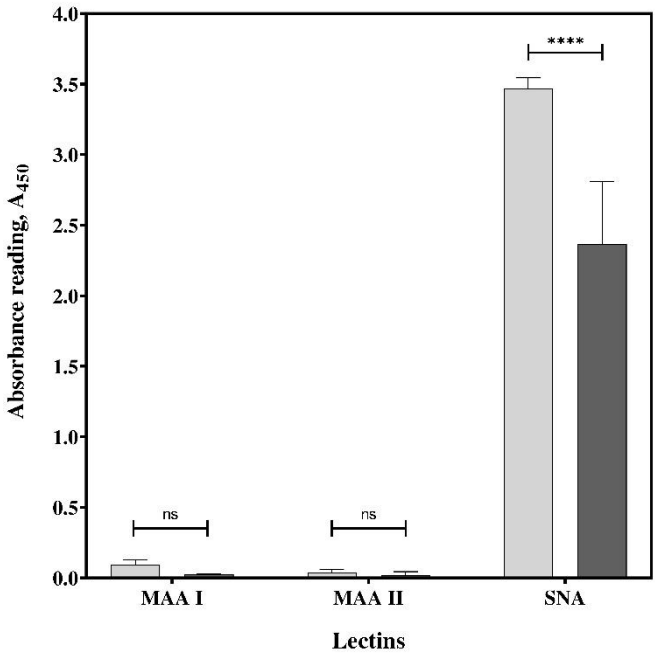

Figure 3 Determining the PSA binding profile using components of the assay stored at room temperature (25 °C) for two months. The light grey and dark grey bars indicate the initial and final absorbance readings, respectively, during the storage period. Each error bar represents the mean of the absorbance reading (n = 3 ± SD, $p < 0.05$). ns: non-significant; four asterisks (****) denote $p \leq 0.0001$.

An earlier study investigating PSA stability reported that free PSA purified from seminal plasma showed excellent stability following storage for four weeks at 35 °C (Pettersson et al., 1995). However, previous studies reported that free PSA (from PSA purified from blood samples) showed activity loss when stored at 23 °C (Woodrum, French, & Shamel, 1996) and decreased even more when stored for two weeks at −20 °C (Arcangeli, Smith, Ratliff, & Catalona, 1997). Similar to our findings, the free PSA used in these previous assays was obtained from seminal plasma and showed adequate binding activity with the lectins, suggesting that the PSA was stable even when stored for longer intervals. Additionally, our analysis focused on the interaction between the PSA glycoprotein and lectin, wherein the glycoprotein could be more stable than the protein itself due to

the stability of the carbohydrate epitope. Moreover, the glycoprotein helps to maintain the structure and stability of proteins (Sheng et al., 2017). Glycans, such as sialic acid (Neu5Ac), was reported as stable at 20 °C after 12 days of storage, but only if stored at a neutral pH (Siegert, Tolkach, & Kulozik, 2012). Furthermore, Montoya and Castell (1987) reported that the storage of HRP conjugates precipitated in 50% saturated ammonium sulphate at 25 °C only showed significant activity loss after 40 days. Another study reported on non-methanol-treated silk films containing 1 wt% HRP, in which the enzyme activity decreased significantly during the first month of storage and remained at 20% of the initial residual activity when stored at room temperature (25 °C) for five months (Lu et al., 2009). Ramachandran, Fu, Lutz, and Yager (2014) reported that, although HRP activity decreased after being stored for over five months at 45 °C, the enzyme nevertheless retained a relatively high level of activity. To summarise, our assay still exhibited a high binding signal because the assay components were thermally stable when stored at room temperature (25 °C) for a long period of time.

5. Conclusion

The present study was designed to generate the profile of normal PSA glycan using sialic acid-specific lectins (MAA I, MAA II, and SNA) and a simple and economical ELLA-based method. Among the candidate lectins, SNA showed a significant increase in binding activity at PSA concentrations greater than 50 ng/mL and displayed the highest binding affinity with the normal sample of free PSA harbouring α2,6-linked sialic acids. The PSA sample also contained a small amount of α2,3-linked sialic acids, as indicated by MAA I. By contrast, the binding of SNA to PSA remained markedly high despite a decrease in the activity of the assay components (PSA, lectins, N-HRP and TMB-ELISA substrate), implying that the assay components retained adequate stability following storage at room temperature (25 °C) for two months. Therefore, this fundamental study will serve as a base for future studies focusing on assay development using a clinical sample, as it has provided a framework for choosing suitable lectins. Future work should include the use of cancer samples to further differentiate the PSA glycoprofile using sialic acid-specific lectins and should be combined with a PSA-specific secondary detector molecule, such as an anti-PSA antibody or anti-PSA aptamer, to evaluate specificity. Additionally, the storage period test should be extended for at least one year to confirm our preliminary data on the storage effect on assay components. Here, we established a reliable PSA glycoprofile and highlighted the potential of this assay to be used and stored long-term at room temperature (25 °C).

6. Acknowledgement

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