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Picogram detection levels of asialofetuin via the carbohydrate moieties using the light addressable potentiometric sensor

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Fetal calf serum asialofetuin was assayed in the sandwich format using biotinylated and fluoresceinated ricin toxin (B-RCA and F-RCA). The sandwiched species was captured on a biotin-BSA coated nitrocellulose membrane with streptavidin. Anti-fluorescein antibody-urease conjugate was bound to the complex, and detected and quantitated under microvolume conditions using the light addressable potentiometric sensor. As little as 250 pg of asialofetuin was detectable whereas fetuin gave no response at conditions as high as 32 ng. Using a competitive inhibition assay, we established that the binding constant for the asialofetuin-ricin complex was 3.6×10^8 M^{-1}. This is in good agreement with data published using glycopeptides derived from asialofetuin, and RCA and the ricin agglutinin, RCA_{120}.

Keywords: asialofetuin, fetal calf serum, ricin toxin, light addressable potentiometric sensor

Introduction

Carbohydrates are one of the most common moieties found in nature and are prevalent on cell surfaces where they act as cell markers, antigens, cell structural support, and receptors in the form of polysaccharides, glycolipids, and glycoproteins [1, 2]. Detection of specific carbohydrate sequence and structure are made difficult because of broad lectin or antibody specificity and poor binding constants, which require methods that necessitate relatively large quantities of lectin/antibody. Some of the problems mentioned above can be overcome by using methods that reduce the level of detection.

The Light Addressable Potentiometric Sensor (LAPS) has many of the advantages for overcoming the problems mentioned above [3, 4]. First of all, all the binding events occur in solution phase, thereby eliminating anomalous results due to liquid/solid phase interactions. Secondly, the filtration capture system provides a mechanism for utilizing samples that have extremely low analyte concentrations. It is currently commercially available and has a proven track record for the detection of DNA to the two picogram level. Furthermore, detection of ricin (as an analyte) has also been made possible to the pg level and can be theoretically lowered in order to detect 500 000 molecules (attomole levels) [5].

The Threshold Immunoassay system (see Materials and methods) has been used to establish binding constants for antigen-antibody molecules to limits of 1×10^{12} M^{-1}. This is possible because of the solution assay format and the sensitivity of the system which allows data to be obtained at concentrations below the K_d value. To this end we have used a competitive inhibition binding method to determine the binding constant of the ricin-asialofetuin complex that is well within literature values determined using glycopeptides and other methods [6, 7].

Materials and methods

Fetuin (gel filtration purified from fetal calf serum), asialofetuin (Type II from fetal calf serum), biotinylated ricin (B-ricin; 2.2 biotin/ricin), FITC-ricin (F-ricin; 1.7 fluorescein/ricin) and D-galactose (ultrapure) were purchased from Sigma Chemical Company (St Louis, MO). The wash buffers, assay buffers, anti-fluorescein antibody-urease conjugate, capture reagent (streptavidin, SA), and the biotin-BSA coated nitrocellulose capture membranes are products of Molecular Devices Corp.

The assay format was as follows: For the detection of asialofetuin or fetuin, an assay volume of 200 µl per test

was used. Each test contained 20 ng each of B-ricin and F-ricin, and varying quantities of either fetuin or asialofetuin. Finally, 2 μg per test streptavidin was added and the mixture was incubated for 1 h at room temperature to ensure equilibrium binding. The sample was then captured by vacuum filtration through a prewashed biotin-BSA coated nitrocellulose membrane (filtration capture) mounted on the Threshold Immunoassay manifold/vacuum system (Molecular Devices Corp.). The captured sample was then rinsed with 0.5 ml of wash buffer, via filtration, and then followed by 200 μl of anti-fluorescein antibody-urease conjugate solution (2 μg of conjugate). Lastly, the membrane was rinsed once more with 0.5 ml wash buffer. Each membrane contained eight assay spots so that eight individual fetuin/asialofetuin concentrations could be assayed at one time. Typically for the standard detection curves, three spots were used per fetuin/asialofetuin concentration (each sample in triplicate).

Detection of the complex was made possible by placing the membrane ('sticks') containing the assay spots into a silicon chip-based reader filled with wash buffer and the substrate (urea). A plunger pushes the membrane against the silicon chip to create a microvolume chamber (0.6 μl) and the rate of pH change is detected, with the signal-output given in $\mu V s^{-1}$ [4, 8]. The rates are directly related to the quantity of complex captured and, hence, the amount of glycoprotein present.

For the galactose inhibition experiments, the following format was used. Twenty ng each of F-ricin and B-ricin were incubated with 16 ng of asialofetuin in 225 μl volume. To each sample, varying amounts of D-galactose was added and the mixture allowed to incubate for 1 h. Enough sample was prepared at each galactose concentration so that at least three data points could be obtained. The capture procedure, binding of conjugate, and detection of the complex is the same as that detailed above. The inhibition curve was analysed using the equation given below, which is a modified version of an equation used for the analysis of pH titration data [9].

$$\text{Sig (Theoretical)}_i = \text{Sig (No Gal)} + \frac{\Delta 10^{(pK_I - pK_{Gal})}}{1 + 10^{(pK_I - pK_{Gal})}}$$

In this equation, Δ represents the maximum signal difference from a sample in the presence of a large excess of galactose and a sample devoid of galactose. Sig (Theoretical)$_i$ represents the theoretical signal based upon the specific galactose concentration and pK_I value. Sig (No Gal) is the signal observed when no galactose is present. The term pK_{Gal} is defined as -log [Gal]. The % inhibition can then be calculated based on the relative signal for that sample concentration of galactose and Δ. The best fit is obtained when $\sum_i$ [Sig (Theoretical)$_i$ – Sig (Observed)$_i$]^2 is minimized.

Results and discussion

Ricin is a lectin that has an affinity for terminal galactose and it is composed of two subunits [6, 7, 10]: One contains the carbohydrate binding site (actually one high affinity site and one low affinity site) while the other is enzymic and it inhibits protein synthesis. Although both units are not required for the assay, we nonetheless used the entire lectin. Figure 1 shows the format of the sandwich assay. Both B-ricin and F-ricin are required to form the sandwich with asialofetuin, so that the complex may be captured on the membrane giving rise to a signal in the LAPS based detection system. The streptavidin is necessary in order to bind the biotinylated-ricin to the biotin-coated membrane. F-ricin provides the binding site for the signal generator, the antibody-urease conjugate. The attached urease hydrolyses urea to generate carbon dioxide and ammonia, thus increasing the pH of the sample solution at the silicon chip surface which is then detected by the LAPS system [8].

Fetuin contains six oligosaccharide chains [11–14]. Asialofetuin is identical to fetuin except that the terminal sialic acid residues have been removed. Three of the oligosaccharide chains are simple, identical tetrasaccharides linked to Thr/Ser [12, 13]. Removal of sialic acid gives rise to the disaccharide, shown below, which is also known as the T antigen and found on human carcinoma cells.

Galβ1 → 3GalNAcα → Thr(Ser) [T antigen]

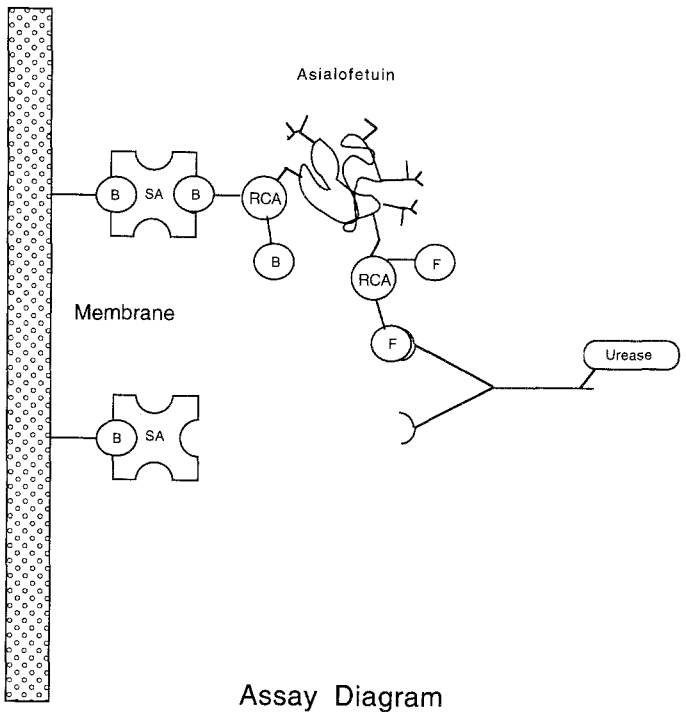
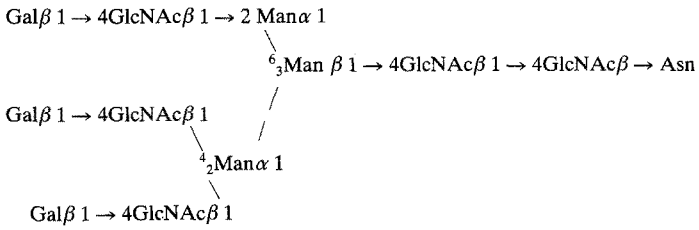


Figure 1. Diagram of the sandwich assay format. The abbreviations used in the diagram are as follows: B, biotin; SA, streptavidin; F, fluorescein; RCA, ricin toxin.

The three remaining oligosaccharide chains are also thought to be identical in structure (micro heterogeneity may exist) but are of the 'complex carbohydrate' variety [13–15]; the asialo-form of this complex oligosaccharide is shown below.



As can be seen, the asialo-disaccharide contains only one terminal galactose residue whereas the triantennary asialo-form of the 'complex carbohydrate' contains three terminal galactose residues. All together then, one molecule of asialofetuin would have exposed 12 terminal galactose residues.

Figure 2 shows the standard curve for the detection of fetuin and asialofetuin using the procedure described in the Materials and methods section. Fetuin appears to elicit no response in this assay. However, asialofetuin gives a linear response as the quantity of asialofetuin in the assay is increased. As seen, asialofetuin is readily detectable at the ng level. The limit of detection, based upon two times the standard deviations above the zero analyte value, is 250 pg of asialofetuin. The limit of detection could be decreased substantially if the background signal (nonspecific binding) for this assay could be lowered. As shown earlier, that B-ricin and F-ricin molecules are necessary to form the sandwich for capture and detection in the LAPS based system. Should a glycoprotein have only one terminal galactose available for binding, then a sandwich would not be formed and detection made impossible.

It is known that galactose binds to the active site of ricin toxin ($K_a = 1.1 \times 10^4$ for the high affinity site) [7, 16] as well as the ricin agglutinin [6]. The lectin appears to have no anomeric preference for the galactose residues [17]. Addition, then, of free galactose to our samples containing B-ricin, F-ricin and asialofetuin should inhibit formation of the ricin-asialofetuin complex. The inhibition value obtained can be used to deduce a 'relative' binding constant for the ricin-fetuin complex. Absolute ricin-asialofetuin affinity values could be obtained since the ricin-galactose binding constant is known [16]. A plot of signal inhibition (0% to 100%) vs log of galactose concentration ($-pK_{Gal}$) is shown in Fig. 3. The Materials and methods section shows the equation used to ascertain the pK_I values and to generate the theoretical curve shown in Fig. 3. The midpoint of this apparent titration is the pK_I from which a binding constant can be obtained.

In order to obtain the binding constant for the ricin-fetuin complex, we made the following assumptions

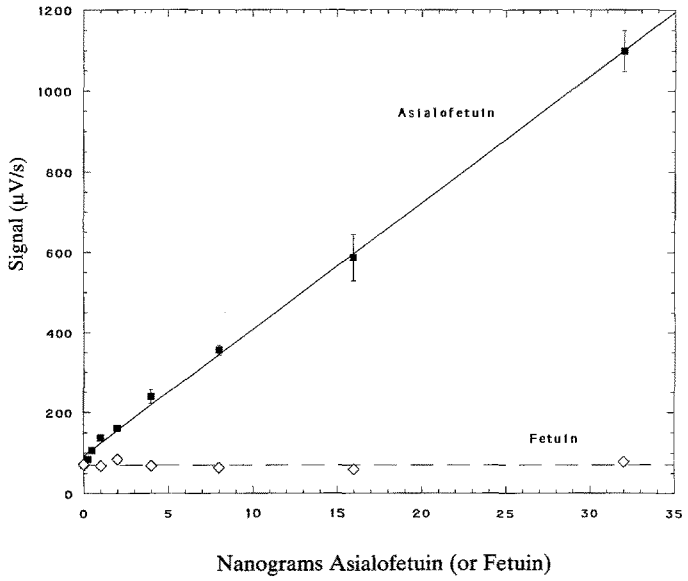


Figure 2. Plot of the LAPS response as a function of increased concentrations of fetuin or asialofetuin. Each sample (in 200 μl buffer) contained 20 ng of B-ricin and F-ricin, 2 μg of streptavidin, and varying quantities of fetuin or asialofetuin. Error bars are standard deviations derived from the triplicate samples assayed for each asialofetuin/fetuin concentration. Details are given in the Materials and methods section.

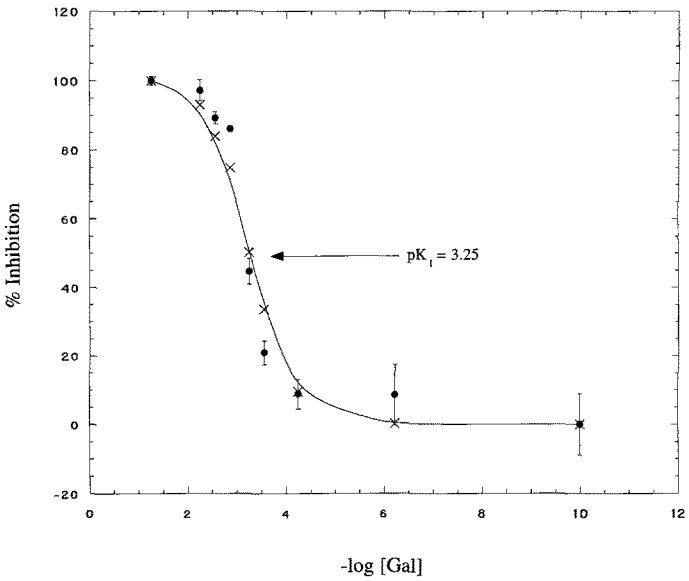


Figure 3. Inhibition of the signal generated from the asialofetuin sandwich assay with D-galactose (•). Concentrations of B-ricin and F-ricin are those given in Fig. 2. Asialofetuin concentration was 16 ng in each sample assay. The theoretical plot (x) and pK_I of the curve were determined using the equation given in the Materials and methods section.

and deductions: F-ricin and B-ricin have the same affinity for terminal galactose residues. There are 12 terminal galactose residues present on asialofetuin and they are accessible and would bind 12 ricin molecules. This is more than likely correct because recent studies with a complex carbohydrate glycopeptide derived from asialofetuin, showed that the three terminal galactose residues were all accessible to the much larger ricin agglutinin (RCA_{120}) [6]. The loss of signal would result from either the displacement of the B-ricin or F-ricin by free galactose. From the data and the assumptions, we obtain a K_a value of $3.6 \times 10^8\ M^{-1}$ for the ricin-asialofetuin complex; this is an average value based upon the different terminal galactose sites available. This value is in close agreement with $1.6 \times 10^8\ M^{-1}$ published by Shinohara *et al.* [6] for the binding of RCA_{120} to a complex-carbohydrate glycopeptide derived from asialofetuin. Furthermore, the value is only two-fold greater than the approximate affinity value obtained by Yamamoto *et al.* [7] for the binding of RCA to a glycopeptide derived from asialofetuin. Fetuin, like other glycoproteins, may have several glycoforms present [14]. The ricin-asialofetuin binding constant obtained above would not be altered significantly unless a large part of the galactose residues were blocked (nonterminal) or missing.

The literature values mentioned above were obtained using kinetic techniques where the glycopeptide was attached to a solid phase; this means that one binding component was in the solid phase, while the lectin was in the liquid phase. Our immunoassay and detection system is based upon solution binding of the various species and the bound-complex is then captured while flowing through the membrane. Our method is not skewed due to solid phase/liquid phase interactions required for the binding process to occur [3].

We have shown that the Threshold system can be used to detect picogram quantities of glycoproteins, quickly and accurately. Furthermore, it can be used to assess binding constants of glycoprotein-lectin complexes. There are several advantages of using this type of system for gathering data. Firstly, this system typically requires concentration ranges of picogram to nanogram per ml, whereas other methods, such as the kinetic approach referred to in the previous paragraph, typically use microgram per ml concentrations. This is of great importance in obtaining solution phase binding constants for systems where the affinity constant may be large since the concentration of the constant species must conform to the following condition for Scatchard data analysis: $[X] \ll K_d$. Furthermore, Threshold allows 'in solution phase' inhibition studies, thus determining dynamic-solution binding phenomena.

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