Homogeneous Enzyme-Linked Binding Assay Mediated by the Interaction of Avidin with Biotin: Mistletoe Lectin I Assay

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We have examined the feasibility of using the specific interaction between mistletoe lectin I (ML I) and β-D-galactose instead of the anti-ML I antibody in developing a homogeneous type competitive binding assay for ML I. We also have examined the feasibility of adapting the biotin/avidin mediated homogeneous assay for this system. Alkaline phosphatase (AKP) was employed as a single substrate enzyme label. The dose-response curve shows a detection range of 1-25 μg/mL and a linear response with a correlation coefficient of 0.99. To demonstrate the analytical utility of this method, 10 μg/mL of ML I was spiked into distilled water. The results show that the mean recovery was 10.03 μg/mL with an SD of 0.18. The difference between the spiked value and the mean recovery was 0.03 μg/mL, with a relative error of 0.3 and 1.6 % of RSD.

Key Words : ELBA, Avidin/Biotin, ML I, Homogeneous assay

Introduction

Aqueous extracts of mistletoe are widely used in cancer prevention and as an adjuvant therapy in cancer treatment.[1-3] Their therapeutic activity[4] is mainly due to the immunostimulating and immunomodulating properties of one of the group of mistletoe lectins (ML), ML I. Mistletoe lectins type I, II and III consist of two subunits, a cytotoxic A-chain and a sugar binding B-chain, linked by disulfides.[5-7] Among the three types of ML, ML I is the major active constituent of mistletoe extracts and the β-D-galactose (gal) group-recognizing one, whereas ML II and ML III are specific to N-acetylgalactosamine group binding.[8] Quantitative determination of mistletoe lectins, especially ML I, has been examined using the enzyme-linked immunosorbent assay (ELISA)[9] and the enzyme-linked lectin assay (ELLA)[10] by using an anti-ML I antibody. Both methods are heterogeneous arrangements, which are too slow because of the time-consuming washing steps to separate bound and free enzyme labels. Also, another disadvantage of these methods[1] is the response of lectins, ML I, II, and III, to the anti-ML I antibody used for the ML I assay. The homogeneous type assays, such as the enzyme-multiplied immunoassay technique (EMIT),[11] are more rapid because there is no need for separation steps, and they are, additionally, amenable to automation.

In the present work, we examined the feasibility of using the specific interaction ML I with gal instead of the anti-ML I antibody in developing a homogeneous type competitive binding assay for ML I. We examined the feasibility of adapting the biotin/avidin mediated[12-14] homogeneous assay method to a new analyte system. Alkaline phosphatase (AKP), a single substrate enzyme, was employed to measure the enzyme activity at 405 nm. The proposed method utilized AKP-biotin conjugate as a signal generator and an avidin-gal conjugate as a signal modulator in the solution phase. AKP-biotin conjugate was inhibited in solution by the avidin-gal conjugate due to the recognition reaction of the avidin and biotin. In the presence of a gal-specific binder, ML I, the enzymatic activity of the conjugate was regained in an amount proportional to the concentration of ML I, since the enzyme inactivation by the sterical hindering of the binding between AKP-biotin and avidin-gal was released due to the binding of the ML I to the avidin-gal conjugate. Homogeneous competitive binding assay for ML I mediated by the avidin/biotin interaction was optimized and the dose-response behaviors were examined.

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Experimental Section

Enzymatic activities were measured with a Gilford Stassar-III spectrophotometer equipped with a vacuum-operated sampling system and temperature-controlled cuvette. This spectrophotometer was connected to a Syva CP-5000 EMIT Clinical Processor for automatically setting the reading intervals and recording the absorbance values.

AKP from bovine intestinal mucosa, bovine serum albumin (BSA), *N*-hydroxysuccinimidobiotin (NHS-biotin), avidin from egg white, and gal were obtained from Sigma (St. Louis, MO, USA). ML I was purchased from the Institute of Phytochemistry, University of Witten/Herdecke, Germany. Deionized water was used to prepare all buffers.

Preparation of AKP-biotin conjugates. AKP-biotin conjugate was prepared by reacting AKP with different amounts of NHS-biotin. The required amount of NHS-biotin dissolved in dimethylformamide (DMF) was added to 500 μL of coupling buffer containing a given amount of 200 units for AKP. The coupling buffer was 0.05 M sodium carbonate, pH 9.0. The reaction was run for 24 hr at 4 °C under stirring. After reaction, the conjugate was dialyzed against 0.05 M Tris-HCl, pH 7.8, and diluted to a final

volume of 2.0 mL with the dialysis buffer. The resulting enzyme conjugates were characterized by their residual activities and percent inhibition induced by an excess amount of avidin. All conjugates were kept at 4 °C until the additions of reagents for activity measurements.

Preparation of avidin-galactose conjugates. Three mg of avidin were dissolved in 2.5 mL of coupling buffer, 0.05 M sodium carbonate, pH 9.2. To protect the active site of avidin, 1 mg of 2-iminobiotin dissolved in 100 μL of DMF was added to the avidin solution. The required amount of *β*-D-galactopyranosylphenyl isothiocyanate was added to each 500 μL of the avidin/iminobiotin solution. The coupling reaction was run for 24 hr at 4 °C under stirring. The resulting avidin-gal conjugates were dialyzed against 0.05 M Tris-HCl, pH 7.8, and diluted to a final volume of 2.0 mL with the dialysis buffer.

Determination of enzymatic activity and percentage inhibition. The activity of AKP-biotin conjugates was determined by measuring the rate of appearance of *p*-nitrophenol, measured by the change in absorbance at 405 nm per unit time. The assay involves addition of 100 μL of *p*-nitrophenyl phosphate (10 mM) and 100 μL of AKP-biotin conjugate to a tube containing 800 μL of assay buffer. For each assay, after mixing and agitation (1-2 s), the reaction mixture was aspirated into the thermostatic flow cell of the spectrophotometer. The absorbance of the mixture in each tube was read over a 1-min period (ΔA/min) after an initial 10-s delay for AKP-biotin conjugates. To determine the percent inhibition value for each conjugate, 100 μL of assay buffer was replaced by 100 μL of avidin or avidin-gal (10 μg/mL) prepared in assay buffer. In addition, conjugates were first incubated with avidin or avidin-gal for 10 min before subsequent addition of substrate solution. The reversed percent inhibition was determined by incubating 100 μL of each avidin-gal conjugate (5 μg/mL) with 100 μL ML I (10 μg/mL) for 10 min, and with the addition 100 μL AKP-biotin3 (1 : 100 dilution; 0.1 units), and then incubating the mixture for 10 min. The working assay buffer for AKP-biotin conjugate was 0.05 M sodium carbonate (pH 9.5), containing 0.01% (w/v) NaN_3 and 0.01% (w/v) gelatin. And dilutions of avidin-gal conjugates, binders, standards, and sample solutions were made using 0.05 M Tris-HCl (pH 7.8), containing 0.01% (w/v) NaN_3, 0.5% (w/v) Tween 20 and 0.01% (w/v) gelatin.

Association kinetic study. The rate of binding between AKP-biotin and avidin-gal conjugates was measured by mixing 100 μL AKP-biotin (1 : 100 dilution) with 100 μL of avidin-gal (10 μg/mL) and by incubating the mixture for varying periods of time. The kinetics of association between avidin-gal and ML I was studied by first incubating a mixture of 100 μL of avidin-gal6 (5 μg/mL) and 100 μL of ML I (10 μg/mL) for varying periods of time, adding 100 μL of AKP-biotin3 (1 : 100 dilution) and incubating the mixture for 10 min. After incubation, the enzymatic activity was determined by adding the substrate solution as outlined above.

Dilution curve of avidin-gal conjugate. One hundred μL of solutions containing different amounts of avidin-gal6 were incubated with 100 μL AKP-biotin3 (1 : 100 dilution) and 700 μL of assay buffer for 10 min. The substrate was then added and enzymatic activity was measured as described above. A dilution curve for avidin-gal6 was prepared by plotting percent inhibition *vs.* the amount of avidin-gal6 used.

Dose-response curves. For a dose-response curve for ML I, AKP-biotin3 and avidin-gal6 were employed. 100 μL of solutions containing different amounts of ML I (100 μL) were incubated with 100 μL of avidin-gal6 and 600 μL of assay buffer for 10 min. After adding 100 μL of AKP-biotin3 conjugate (1 : 100 dilution), each mixture was incubated for an additional 10 min period. The enzymatic activity was measured as outlined above.

Results and Discussion

In the design of the homogeneous enzyme-linked binding assay (ELBA), the properties of the enzyme conjugate used exert an important effect on analytical performance. The enzyme conjugate in the homogeneous assay must be inhibited by an excess of binder in mixture and still possess high enzymatic activity. Thus, various AKP-biotin conjugates were prepared by reacting AKP with different molar ratios of NHS-biotin. Table 1 summarizes the characteristics of AKP-biotin conjugates. As can be seen, the residual activity decreased with an increasing initial molar ratio of biotin/AKP, whereas the percent inhibition of enzyme increased with an initial molar ratio up to 2250 (biotin/AKP) and started to decrease due to a given excess of avidin in all cases. These observations show that the number of biotins attached to the enzyme molecule increased with a higher biotin/enzyme ratio. However, the inhibition was reversed when too much biotin was attached (AKP-biotin4). Therefore, on the basis of the residual activity and maximum percentage inhibition, AKP-biotin3 was chosen for use in these subsequent homogeneous binding assay studies.

In the homogeneous ELBA for ML I mediated by avidin/biotin interaction, it was additionally necessary to synthesize avidin-gal conjugates. Here, the binding ability of avidin-gal to both AKP-biotin and ML I affects the detection capabilities of the assay. To accomplish a sensitive homogeneous assay, avidin-gal conjugates must retain high binding affinity toward biotin in AKP-biotin. And the observed inhibition needs to be reversed when ML I binds the gal attached to

Table 1. Characteristics of AKP-Biotin Conjugates

Conjugates	Initial biotin/AKP molar ratio	% Residual activity[a]	% Inhibition by avidin[b]
AKP-Biotin1	0	100	0
AKP-Biotin2	2000	70	72
AKP-Biotin3	2250	65	76
AKP-Biotin4	2500	64	74

[a]Percent residual activity was calculated by comparing the activities of the conjugates to the initial activity of the unconjugated enzyme. [b]Percent inhibition was determined by using 1 : 100 dilutions of the conjugates and 10 μg Avidin.

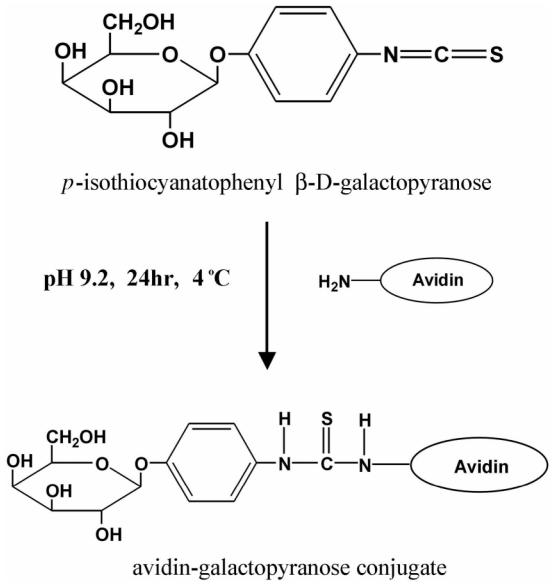

Figure 1. Reaction sequence used for preparing avidin-galacto-pyranose conjugate.

avidin. In this work, several avidin-gal conjugates were prepared by varying initial molar ratios in the conjugation reaction mixture, according to the reaction scheme shown in Figure 1. A 100-fold excess of 2-imminobiotin to avidin was used as described in the experimental section to prevent the attachment of galactose to the binding site of avidin during conjugation. And 2-immunobiotin can be removed readily during the dialysis process of avidin-gal conjugates. Table 2 summarizes the characteristics of the avidin-gal conjugates.

The percentage inhibition induced by avidin-gal and the reversed inhibition by ML I were estimated by using the AKP-biotin3 conjugate. It decreased due to the ability of the avidin-gal conjugate to inhibit the AKP-biotin conjugate, when the initial molar ratio of gal/avidin increased. The more gal attached to avidin, the more steric hindrance occurred on binding of avidin to the AKP-biotin conjugate. However, the reversed extent of the observed inhibition by a given excess ML I tended to increase as the initial molar ratio of gal/avidin increased, since Avidin-gal6 exhibits a high inhibitory effect, up to 59%, and more importantly,

Table 2. Characteristics of Avidin-galctose Conjugates

Conjugates	Initial gal/ avidin molar ratio	% Inhibition[a] (without ML)	% Inhibition[b] (with ML)
Avidin-gal1	0	100	100
Avidin-gal2	50	93	79
Avidin-gal3	100	86	66
Avidin-gal4	150	81	62
Avidin-gal5	250	80	53
Avidin-gal6	300	59	16
Avidin-gal7	350	55	23

[a]Percent inhibition was determined by using 1 : 100 dilution of AKP-biotin3 and 5 μg of each Avidin-gal conjugates. [b]Reversed percent inhibition was determined by using 10 μg of ML.

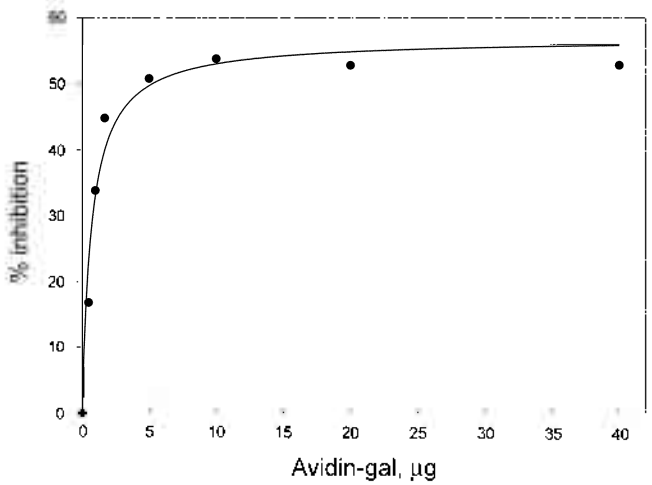

Figure 2. Effect of varying the levels of avidin-gal6 on the inhibition of AKP-biotin3 (0.1 units).

reverses the observed inhibition up to 84% with an excess ML I. The degree of such activity modulator is sufficiently high for use in homogeneous type assay arrangements. Thus, avidin-gal6 was chosen as the signal modulator.

This assay involved two types of binding interactions: AKP-biotin with avidin-gal, and avidin-gal with ML I. Information on their association kinetics was required to determine the optimal incubation periods for this assay. More than 95% of the maximum inhibition was achieved within 10 min of incubation in both cases. Thus, for the subsequent experiments, an incubation period of 10 min was chosen for each step.

Binder dilution studies were performed to determine the optimum concentration of binder for the homogeneous assay. A binder dilution curve was prepared for different amounts of avidin-gal6 incubated with a fixed amount of AKP-biotin3 (Figure 2). As shown in Figure 2, as the amount of avidin-gal increases in the assay mixture, the extent of inhibition increases. This figure shows that the use of avidin-gal6 in the range of 1-2 μg provided more than 60%

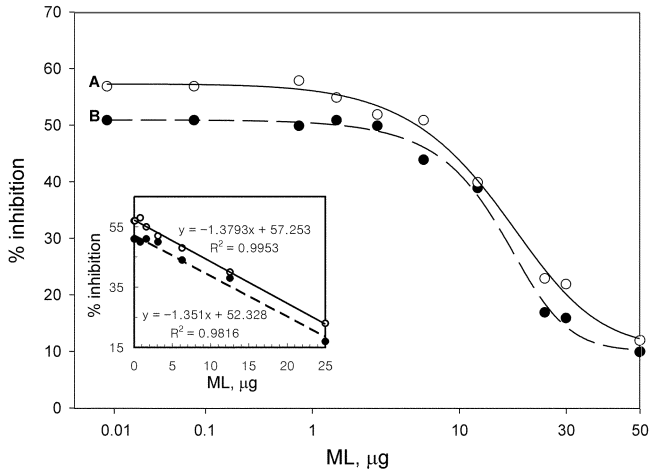

Figure 3. Dose-response curves for ML I on the inhibition of AKP-biotin3 conjugate (0.1 units): (A) 1 μg avidin-gal6 (○); (B) 0.67 μg avidin-gal6 (●): insert graph was calibration curves for ML I with avidin-gal6 conjugate.

of the maximum inhibition for the AKP-biotin3 conjugate.

Under the optimized conditions, a competitive dose-response curve for ML I was constructed by plotting percentage inhibition vs. the amount of ML I in the standards (Figure 3). Figure 3(A) and 3(B) were obtained from two different amounts (1 μg and 0.67 μg, respectively) of avidin-gal conjugate. It is known that the characteristics (detection limit, effective detection range, etc.) of the dose-response curve are affected by the ratio of binder/enzyme conjugate employed in the assay mixture.[15-17] The intrinsic detection capability of an assay system is almost completely dictated by the binding ability of the binder system. The proposed biotin-avidin mediated assay involves two different binder systems; avidin-biotin and ML I-gal systems. In this case, the detection ability of the assay system was influenced by the activity that included the weaker binder system; *i.e.* the ML I-gal system. The curves show a steepness over a detection range (1-25 μg/mL). Also, the inset shows the calibration curves for ML I, including good linearity with a correlation coefficient of 0.9953 (A) and 0.9816 (B), respectively.

Aqueous mistletoe extracts[18] are used in cancer prevention and as an adjuvant therapy in cancer treatment. Thus, to demonstrate the analytical utility of this method, a preliminary study was undertaken by determining the amount of ML I spiked into distilled water by 10 μg/mL. 100 μL of 10 μg/mL ML I aqueous solution was used instead of 100 μL of the standards, the percent inhibition was observed and, finally, the concentration of ML I was determined by the calibration curve obtained. The experiment was repeated five times. The results show that the mean value is 10.03 μg/mL and the SD is 0.18. The difference between the spiked value and the mean value is 0.03 μg/mL, with a relative error of 0.3%. Thus, this method has good reproducibility for trace analysis, such as 10 μg/mL of ML I with 1.6% of RSD.

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