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Indirect Homologous Competitive Enzyme-Linked Immunosorbent Assay for the Detection of a Class of Glycosylated Dihydrochalcones

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Supporting Information

ABSTRACT: Hesperetin dihydrochalcone 4′-glucoside, **1**, and phloretin 4′-glucoside, **2**, belong to a family of dihydrochalcone glycosides that exhibit flavorant properties. In this study was developed a competitive, indirect homologous ELISA for the detection of targets **1** and **2** in fermentation media. Immunogen and coating antigen were prepared by conjugating hapten, 4-(3-oxo-3-(2,6-dihydroxy-4-glucoside phenyl)propyl) benzoic acid, to thyroglobulin and bovine serum albumin, respectively. Antibodies raised in rabbits *M6122*, *M6123*, and *M6124* and the coating antigen were screened and characterized to determine their optimum concentrations. The optimized ELISA, developed with antibody *M6122*, gave IC_{50} values of 27.8 and 21.8 ng/mL for **1** and **2**, respectively. Selectivity of the assay was assessed by measuring cross-reactivity of antibody *M6122* to related congeners such as aglycones and the 2′-glycosides of hesperetin dihydrochalcone, **5** and phloretin, **6**. Antibody *M6122* showed very low recognition of **5** and virtually no recognition of the aglycones and **6**.

KEYWORDS: *dihydrochalcone glycoside, microbial fermentation, hapten, polyclonal antibodies, ELISA*

INTRODUCTION

Flavonoids[1−5] are a large and diverse group of polyphenol compounds. They are present in many plants. Flavonoid patterns are normally characteristic of individual plants. They often are ubiquitous in leaves, stems, roots, and fruits and thus form an important part of the human diet. Structurally, they contain a 15-carbon atom core, with a chroman ring (rings A and C) coupled to a second phenyl ring (ring B) through its C2, C3, or C4 position. Figure 1 depicts the structure of a citrus flavanone, neohesperidin, as an example. Some of these compounds exhibit taste properties. One of the most active areas of flavonoid research is on their beneficial health effects, especially their antioxidant activities. The high antioxidant activity of some flavonoids is attributed to the presence of specific structural synthons that drive the inhibition of lipid peroxidation, as evidenced by in vitro analyses.[6,7] Epidemiological studies point to an inverse relationship between dietary flavonoid intake and incidence of coronary heart disease,[8] explained by their inhibitory effects on free radical oxidation of low-density lipids.[9] They act as natural ultraviolet radiation filters, by scavenging oxygen free radicals generated by UV irradiation.[10] Some phenolics can serve as good topical photoprotective agents. Hydroxyflavones are well-known for their broad-spectrum antimicrobial function[11] that not only aids in protecting plant life but also is useful for the treatment of human diseases. One notable example is 5,6,7-trihydroxyflavone 7-glucuronide (baicalin), which has an inhibitory effect on the human immunodeficiency virus (HIV).[12]

Dihydrochalcones[13] are a subclass of flavonoids. The unique feature that distinguishes the dihydrochalcones from other flavonoids is the open-chain three-carbon structure linking the A- and B-rings in place of a heterocyclic C-ring (Table 1). This close structural correlation accounts for the co-occurrence of dihydrochalcones and flavanones as natural products. Dihydrochalcones are reported to exhibit a wide spectrum of bioactivities.[14−17] Simple dihydrochalcones such as phloretin, phloridzin, and the recently discovered sieboldin[18] possess anti-inflammatory and antihypertensive properties and have important ramifications on cardiovascular disease and diabetes.[19] Some dihydrochalcones and their glycosylated derivatives are found to be sweet. They are usually derived from bitter ingredients found in plants such as apple, citrus, or tea leaves. Studies suggest a strong structure−"sweet" taste correlation for the dihydrochalcones.[20]

Hesperetin dihydrochalcone 4′-glucoside, **1**, and phloretin 4′-glucoside, **2**, also commonly referred to as trilobatin (Table 1), are dihydrochalcone glycosides that are capable of imparting sweetness to food products, when used at high levels, but to our knowledge are not currently used commercially for this purpose. The 4′-glucoside targets elicit interesting taste properties when used at levels below their detection threshold.[21] There is an interest in the large-scale synthesis of the target compounds **1** and **2** by microbial fermentation. This synthetic procedure may also yield other structurally similar, but undesirable dihydrochalcones, such as the corresponding

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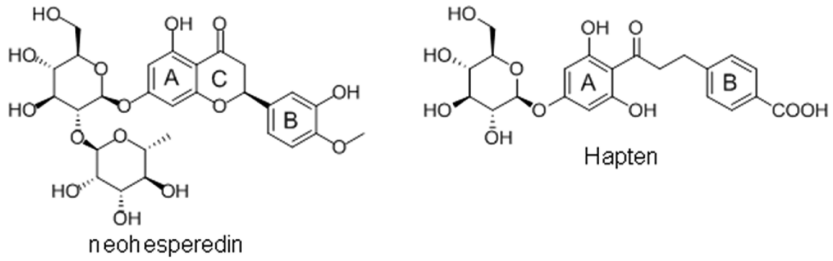
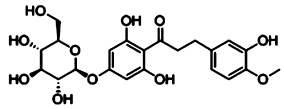
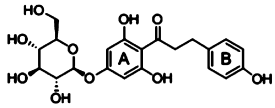
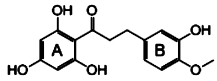
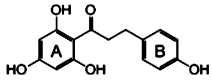
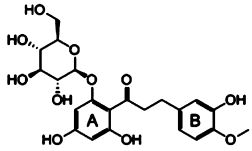
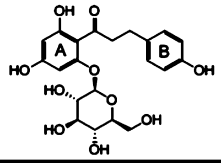


Figure 1. Chemical structures of neohesperedin, a flavanone glycoside, and hapten 4-(3-oxo-3-(2,6-dihydroxy-4-glucoside phenyl)propyl)benzoic acid.

Table 1. Cross-Reactivity (CR)[a] of Ab *M6122* to Structurally Related Compounds

Compound	CR (%)	Structure
hesperetin dihydrochalcone 4'-glucoside (**1**)	100	
phloretin 4'-glucoside (**2** or trilobatin)	127	
hesperetin dihydrochalcone (**3**)	NI[b]	
phloretin (**4**)	NI[b]	
hesperetin dihydrochalcone 2'-glucoside (**5**)	14	
phloretin 2'-glucoside (**6**)	< 0.01	

[a]CR (%) was calculated as (IC_{50} of the target analyte/IC_{50} of the tested compound) × 100. [b]Not inhibited at 50000 ng/mL. Three experiments each with 4-well replicates.

dihydrochalcone 2′-glycosides (**5**, **6**) and the aglycones (**3**, **4**). Therefore, there is a need for developing a rapid and accurate method for the exclusive detection and measurement of the target 4′-glucosides in the desired fermentation broth. Currently, the method used for the measurement of compounds **1** and **2** is high-performance liquid chromatography (HPLC). Despite the HPLC method being sensitive and selective, limitations still exist; it is expensive and time-consuming and requires large quantities of solvent. In contrast, the enzyme-linked immunosorbent assay (ELISA) technique is a rapid, sensitive, and cost- and time-effective tool, amenable to high-throughput, on-site screening tests for process development.[22] The assay will allow a "real-time" on-site detection and measurement of the targets, which will speed process optimization. The assay will be useful in two ways; one will be to test the fermentation media to measure the levels of targets **1** and **2** being produced during pathway optimization and process development. The other use could be to screen large enzyme libraries for specificity of the glycosylation step during enzyme discovery or optimization to narrow the search for an appropriate enzyme to develop a process. To the best of our knowledge, a polyclonal antibody-based ELISA for detection of dihydrochalcone glycosides such as **1** and **2** has not been previously reported, except for a single report on the production of monoclonal antibodies for quercetin flavonoid glycoside and its corresponding glucuronide.[23] Herein, we report the design and development of an indirect, homologous competitive ELISA for measurement of the desired target compounds in fermentation media.

MATERIALS AND METHODS

Chemicals and Instruments. The hapten coupling reagents, bovine serum albumin (BSA), thyroglobulin (Thy), Tween 20, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma-

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Aldrich. Co. (St. Louis, MO, USA). Goat anti-rabbit IgG peroxidase conjugate (GAR-HRP) was purchased from Abcam (Cambridge, MA, USA). ELISA was performed on 96-well polystyrene microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Molecular Devices, Sunnyvale, CA, USA) in dual wavelength mode (450−650 nm).

Buffers. All buffers were prepared with ultrapure deionized water: phosphate-buffered saline (1× PBS, pH 7.5), wash buffer (PBST), 1× PBS containing 0.05% Tween 20; coating buffer (pH 9.6), 15 mM Na_2CO_3, 34.88 mM $NaHCO_3$, 3.08 mM NaN_3; blocking agent, 1% BSA−PBS; substrate buffer (pH 5.5), 0.1 M sodium acetate/citrate buffer. For the substrate solution, 0.4 mL of 0.6% TMB (in DMSO w/v) and 0.1 mL of 1% H_2O_2 were added to 25 mL of sodium acetate−citrate buffer. Stop solution was 1 N H_2SO_4.

Hapten Synthesis. Figure 1 depicts the structure of hapten 4-(3-oxo-3-(2,6-dihydroxy-4-glucoside phenyl)propyl)benzoic acid. The hapten was prepared by an enzyme-catalyzed (naringinase) reaction of 4-(3-oxo-3-(2,6-dihydroxy-4-neohesperidoside phenyl)propyl)-benzoic acid. A jacketed flask equipped with a magnetic stir bar was equilibrated to 65 °C using a temperature-controlled water circulator. To the flask was added a buffer solution (50 mL, 0.1 M KH_2PO_4, 0.2 M Na_2HPO_4, pH 6.6) and naringinase (1.5 g). The reaction was stirred for 2 h at 65 °C. 4-(3-Oxo-3-(2,6-dihydroxy-4-neohesperidoside phenyl)propyl)benzoic acid (1.2 g, 0.002 mol) was dissolved in 10 mL of distilled water and added to the stirred reaction. The reaction was cooled to 45 °C with stirring for 24 h or until complete by TLC analysis (BuOH/PrOH/H_2O, 10:5:4 (v/v)). The reaction was heated to 90 °C for 15 min to deactivate the enzyme. The enzyme was removed from the reaction solution by vacuum filtration. Purification was performed with HP-20 resin (0−60% MeOH in water) followed by HPLC (5−50% MeOH with 0.01% formic acid in water). Fractions were identified by NMR analysis and lyophilized to provide a light yellow powder (0.092 g, 9.9% yield). NMR analysis provided an estimated 90% pure product, which was deemed acceptable for use in this assay.

^{1}H NMR (300 MHz, CD_3OD) δ 7.86−7.84 (2H, d, J = 8.0 Hz), 7.26−7.23 (2H, d, J = 8.0 Hz), 5.96 (2H, s), 4.94−4.91 (1H, d, J = 7.0 Hz), 3.91 (1H, d, J = 11.0 Hz), 3.76−3.73 (1H, m), 3.44−3.40 (4H, m), 3.32−3.30 (2H, q, J = 7.0 Hz, J = 1.5 Hz), 3.00−2.96 (2H, t, J = 15.0 Hz, J = 7.5 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 206.22, 165.71, 164.98, 148.39, 148.23, 130.81, 129.42, 106.82, 105.24, 101.12, 96.44, 78.23, 77.86, 74.60, 71.14, 64.36, 62.34, 46.49, 31.76.

Preparation of Immunogen and Coating Antigen. The carbodiimide method[24] was employed for activation of the carboxylic acid group in the hapten. The hapten was coupled to porcine thyroglobulin (as the immunizing antigen) and bovine serum albumin (as the coating antigen) using the *N*-hydroxysulfosuccinimide ester method. Hapten was dissolved (3 mg, 0.0065 mmol) in 0.9 mL of 0.1 M PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)) buffer (pH 6.1). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; 1.4 mg, 0.0072 mmol) and *N*-hydroxysulfosuccinimide sodium salt (1.6 mg, 0.0072 mmol) were added for preparation of the activated ester intermediate. After 1 h, 2-mercaptoethanol (0.5 μL, 0.0072 mmol) was added with stirring for 1 min. This latter step scavenges the unreacted EDC, which, if present, could cross-link with the protein, resulting in unwanted conjugates in solution. Thyroglobulin (Thy; 5 mg) was dissolved in 1 mL of a 0.25 M borate buffer (pH 8.8). The activated ester solution (0.6 mL) was added to the Thy solution with rapid stirring. A similar procedure was followed for preparation of the coating antigen by conjugating hapten to BSA (dissolving 2.5 mg of BSA in 0.5 mL of borate buffer (pH 8.8)). Then, the activated ester solution (0.3 mL) was added to the BSA solution. The solutions were stirred at room temperature for 1.5 h, after which they were transferred to 4 °C for overnight incubation. Unreacted small molecules were removed by dialysis. The reaction mixtures containing the immunogen and coating antigen were dialyzed (10000 MW cutoff) under stirring against PBS (0.01 M, pH 7.5), for 3 days, with frequent changing of the PBS solution to remove the unconjugated free hapten. Polyacrylamide gel electrophoresis and subsequent visualization with a SYPRO Ruby gel stain for glycoproteins showed that the haptens had coupled to the proteins. The resulting conjugates were stored at −20 °C for future use.

Immunization and Antiserum Preparation. The immunization procedure followed a previously published protocol.[24,25] Three female New Zealand white rabbits, *M6122*, *M6123*, *M6124*, were each immunized with 500 μg of immunogen hapten−Thy (concentration of 5 mg/mL) in the initial immunization (preimmune bleeds were collected from each rabbit prior to immunization). Following this, each rabbit received 300 μg of immunogen in the four subsequent boosts. A single test bleed was collected a month after the first immunization and as were two boosts separated by a 1 week interval. A week after the test bleed collection, the third and fourth boosts were given, separated by a 2 week interval. Production bleeds (20 mL from each rabbit) were collected over the 3 months following the first immunization and all of the four boosts. Antiserum (from the production bleed) was obtained by centrifugation, stored at −20 °C, and used without further purification.

Development of Indirect Competitive ELISA. Although there are several different configurations for competitive ELISAs, we chose to develop the ELISA using the antigen immobilization format, following a previously reported procedure. In this assay format, the coating antigen was directly immobilized on the ELISA plate. Fixed amounts of unlabeled primary antibody and labeled secondary antibody (tracer) were added. This implies an indirect detection method, which is contrary to direct detection wherein an enzyme-conjugated primary antibody is employed. Use of nonpurified and single antigen-specific primary antibody and commercial availability of a wide variety of labeled secondary antibodies are major advantages of the indirect ELISA approach. In the indirect method, the labeled secondary antibody binds to different epitopes (antigenic determinants) of the primary antibody, providing greater signal amplification than can be achieved with the direct method. The indirect ELISA is competitive when it is based on competition between target analyte in the sample and the immobilized or the coating antigen−hapten for a fixed amount of the primary antibody. As the concentration of the target analyte increases, the amount of coated antigen captured by the primary antibody decreases.

Details of the ELISA procedure followed for the reported assay are given in the subsequent sections.

Standard Curve Preparation. To evaluate the sensitivity and range of linearity of the ELISA, standard curves of both target compounds **1** and **2** were prepared in a 10% dimethyl sulfoxide (DMSO)−PBS dilution buffer (final dilution in the plate wells being 5% DMSO−PBS). In addition to two negative controls (no antibody blank wells in PBS and no coating antigen wells in 5% DMSO−PBS), a positive control in 5% DMSO−PBS with zero standard analyte was chosen. Nine standard concentrations were prepared from a 5-fold serial dilution of a 50000 ng/mL preparation. The limit of detection, set as IC_{10}, the assay sensitivity, represented by the IC_{50} value and the linear range of detection, set as IC_{20} − IC_{80}, were obtained from the calibration curve data. The data were fitted to a four-parameter logistic equation, using Igor Pro 6.22A (Wavemetrics, Inc.).

Cross-Reactivity Determination. ELISA plates were coated with coating antigen at 0.1 μg/mL in coating buffer (100 μL/well) by overnight incubation at 4 °C. The plates were washed five times with 1× PBS containing 0.05% Tween 20. Plates were blocked for 0.5 h at room temperature with 1% BSA−PBS (350 μL/well) and washed five times. For the competitive inhibition, 50 μL/well of competing analytes (targets **1** and **2**; cross-reactants hesperetin dihydrochalcone, **3**; hesperetin dihydrochalcone 2′-glucoside, **5**; phloretin, **4**; and phloretin 2′-glucoside, **6**; glucose and *p*-coumaric acid) were added starting at 50000 ng/mL, followed by a 5-fold serial dilution, ending at 0.128 ng/mL. Each ELISA plate had two competitors: target **1** and one cross-reactant, in two sets, with quadruplicate wells for each concentration. The competitor was co-incubated with 50 μL/well of primary antibody (at a dilution of 1 in 5000) for 1 h. After washing, secondary antibody, GAR−HRP, was added at a dilution of 1 in 25000, followed by an hour of incubation. This was followed by a 5× wash and addition of 100 μL of a solution containing TMB and 1% H_2O_2 in citrate buffer in each well, yielding a blue color when oxidized.

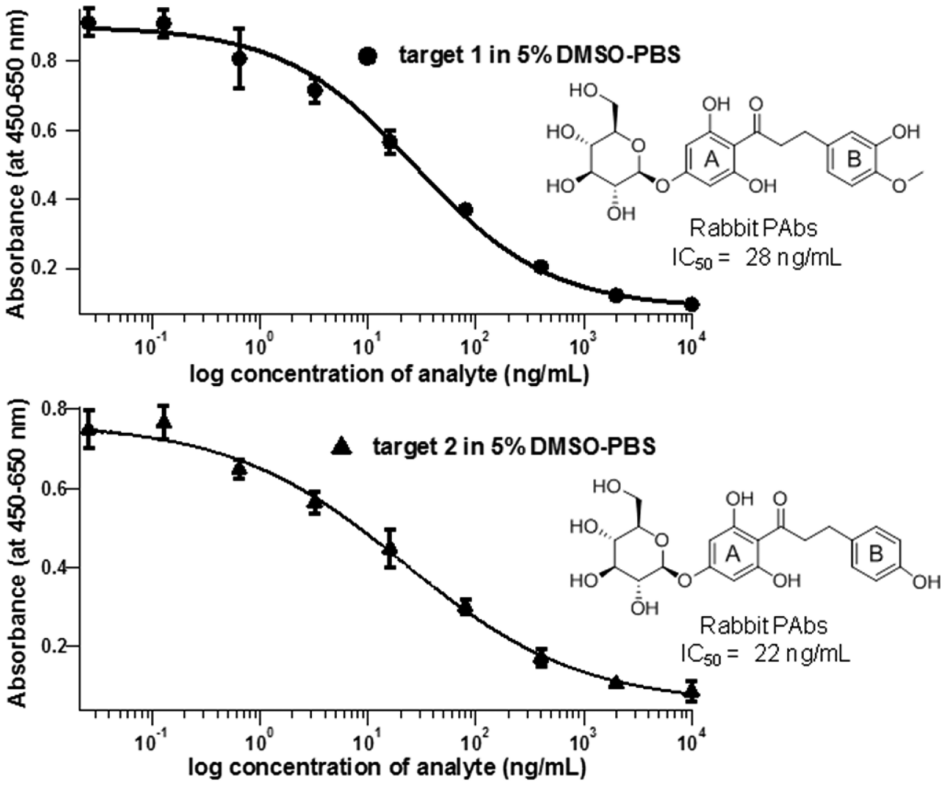

Figure 2. ELISA inhibition curves for target compounds: (A) hesperetin dihydrochalcone 4′-glucoside, **1**; (B) phloretin 4′-glucoside, **2**. Each data point represents the mean of quadruplicates.

The plate was incubated for 15 min before the reaction was stopped by the addition of 50 μL of 1 N H_2SO_4 in each well. The absorbances were recorded at 450 nm, with background subtraction at 650 nm. The cross-reactivity was calculated as [(IC_{50} of target)/(IC_{50} of cross-reactant)] × 100.

Matrix Effect Determination. Two commonly used culture media, LB broth and SOC, were used in these studies. Bacteria (*Escherichia coli* K12 strain) were grown in each broth to an $OD_{600} \geq 4.0$. The medium was centrifuged, and the supernatant was used for the matrix effect study. To determine interference effects from the matrix, neat fermentation media supernatant samples were spiked with the standard solution in 10% DMSO−PBS. Competitive curves with final concentrations of the target compounds **1** and **2** (5-fold serial dilution starting from 50000 ng/mL to 0.128 ng/mL) were run in 5% DMSO−PBS (final dilution in plate wells) and in various dilutions of fermentation media (1:5, 1:20, and 1:100). IC_{50} values obtained from each diluted curve were compared with those generated from the assay buffer (5% DMSO−PBS).

RESULTS AND DISCUSSION

Rationale for Design of Hapten. Hapten 4-(3-oxo-3-(2,6-dihydroxy-4-glucoside phenyl)propyl)benzoic acid (Figure 1) was chosen for the competitive assay development. The hapten with a −COOH functional group conjugated to Thy was designed for immunizing to raise antibodies. Immunizing haptens typically are a close structural mimic of the target analyte having a functional group that can be used to couple the hapten to a protein. The carboxylic acid substituted hapten was chosen because it would likely generate antibodies that will detect both compounds **1** and **2**, but not compounds **3**, **4**, **5**, and **6**. The hapten was coupled to the protein at a point most distal to the sugar (providing discrimination between 2′- and 4′-glucosides) and close to the B phenyl ring farthest from the glycoside moiety, thus minimizing discrimination between compounds **1** and **2** and, at the same time, precluding antibody detection of the aglycones **3** and **4**. In a competitive ELISA, the target analyte competes with the immobilized coating antigen−hapten for a fixed amount of antibody. The carboxylic acid group substituted hapten was used as both the immunogen and coating antigen hapten in a homologous competitive ELISA format. In the following discussion, results are reported for the homologous ELISA developed with this hapten for target **1**, with target **2** tested as a cross-reactant in the assay.

Antibody Characterization. Antisera collected from three rabbits, *M6122*, *M6123*, and *M6124*, after the first immunization and two boosts separated by a 1 week interval were subjected to titration by the homologous competitive ELISA. All three antisera showed significantly high titers (data not shown) indicative of the rabbits' response to the immunogen. Checkerboard titrations (CBTs) were performed to determine the optimum concentrations of the antibody raised against hapten−Thy and coating antigen (hapten−BSA) with a 2-fold serial dilution of the antibody, starting from a 1000× dilution up to a 128000× dilution, screened against a 5-fold serial dilution of coating antigen (2×10^4 to 1.3 ng/mL). The determined optimum concentrations of antibody and coating antigen in the assay had minimum variations among the three rabbits tested. The IC_{50} values obtained with antisera from rabbits *M6123* and *M6124*, for target **1** as the inhibitor, were 243.8 and 114.5 ng/mL, respectively (Figure S5 in the Supporting Information). These values were at least an order of magnitude higher than that obtained with Ab *M6122*, demonstrating much lower assay sensitivities. Following this, the antiserum obtained from rabbit *M6122* was used for further immunoassay development. A CBT was performed with concentrations (250, 25, and 0 ng/mL) of the target analyte **1** with four different antiserum dilutions, 5000×, 10000×,

15000×, and 20000×, screened against a 5-fold serial dilution of coating antigen concentrations (from 4000 to 32 ng/mL). The combination of Ab *M6122* and coating antigen, hapten−BSA, that had >80% inhibition at 250 ng/mL analyte concentration was screened using nine concentrations of target **1** ranging from 0.128 to 5×10^4 ng/mL. This dilution series was also used to assess the linear range of the assay. Wells with zero coating antigen and with zero primary antibody served as negative controls. Wells with zero analyte concentration served as positive controls. For targets **1** and **2** as inhibitors in the assay, IC_{50} values of 27.8 and 21.8 ng/mL, respectively, were obtained (Figure 2).

Assay Optimization. The optimum concentrations of coating antigen hapten−BSA and Ab *M6122* were 100 ng/mL and 1:10000 dilution in the well, respectively. Because both targets **1** and **2** were only partially soluble in water, a series of cosolvents were screened and DMSO was selected to improve solubility. PBS buffer containing DMSO at different concentrations was tested to assess changes in assay sensitivity. A reduction in the optical densities and a decrease in assay sensitivity were observed with increasing DMSO concentrations (Figure S6 in the Supporting Information). Thus, a 10% DMSO-PBS in 0.15 M PBS, pH 7.4, buffer was selected to prepare the standard solutions and spiked fermentation medium samples for assay optimization. The linear range of detection was 2−300 ng/mL. The fermentation media supernatant samples were diluted as necessary to stay within the linear range of detection. Intra- and interplate variations (assays run on the same day as well as on different days) were determined by calculating the percent coefficient of variation (% CV) in the assay for both **1** and **2** The % CV was <10%, implying minimum variations between wells and limited day-to-day variability.

Cross-Reactivity (CR). To determine antibody selectivity, compounds **3**−**6** (Table 1) with structural similarity to targets **1** and **2** and which are probable metabolites or precursors to the target compounds in the microbial system of production were investigated for relative cross-reactivity. With hapten−BSA as the competing coating antigen, Ab *M6122* showed >100% binding to target **2**, phloretin 4′-glucoside (CR 127%, IC_{50} = 21.8 ng/mL). As expected, the antibody exhibited virtually no recognition of the aglycones hesperetin dihydrochalcone, **3**, and phloretin, **4**, because they do not inhibit even at 50000 ng/mL. With the 2′-glucosides, the antibody exhibited a very low recognition of hesperetin dihydrochalcone 2′-glucoside, **5** (CR 14%, IC_{50} = 202 ng/mL, an order of magnitude greater than that observed for target **1** with an IC_{50} of 27.8 ng/mL, indicating >10× lower sensitivity). There was limited reactivity with phloridzin, **6** (CR <0.01%). Antisera obtained from rabbits *M6123* and *M6124* were also tested for cross-reactivity to hesperetin dihydrochalcone 2′-glucoside, **5** (see Supporting Information Figure S2). Both antibodies displayed a very small level of cross-reactivity to **5**: CR 15% for Ab *M6123* and CR 21% for Ab *M6124*. Also, no cross-reactivity was observed with glucose and *p*-coumaric acid, other probable byproducts in the microbial fermentation.

Matrix Effects. Variations in analyte reactivity in the presence of the matrix were tested by measuring the standard curves in fermentation media spiked with the target compounds **1** and **2**. The standard curves in spiked fermentation media supernatant obtained at different dilutions of the media were compared with that obtained in the assay buffer (5% DMSO−PBS final dilution in wells). A difference in the values of percentages of control for the standard curve and corresponding dilution of the media (LB broth supernatant), observed at the IC_{50} value of the standard curve in 5% DMSO−PBS, was taken as a measure to estimate the matrix effect. For target **1**, at 1 in 5 and 1 in 20 dilutions of the media, differences in the percents of control of 4.66 ± 1.68 and 5.74 ± 1.68, respectively, between the standard curves for assay buffer and spiked media were obtained. At a 1 in 100 dilution of the media, the standard curve of **1** (Figure 3) was parallel to the one in 5% DMSO−

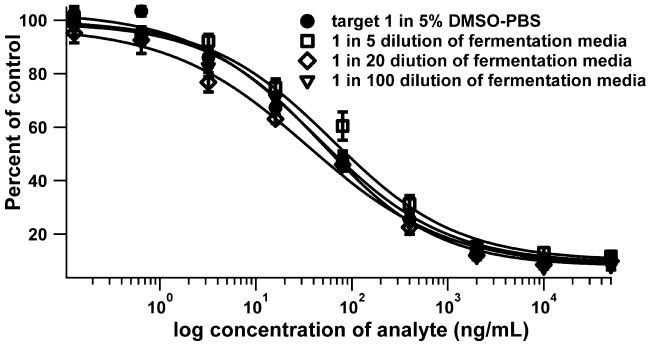

Figure 3. Matrix effects evaluation for fermentation media supernatant (LB broth) spiked with target **1**, hesperetin dihydrochalcone 4′-glucoside. Each data point represents the mean of quadruplicates. The standard curve in 5% DMSO−PBS was generated by taking the average of values from eight ELISA plates.

PBS buffer, with a much lower difference of 0.26 ± 1.68. Correspondingly, for target **2** (Figure 4), 6.47 ± 1.66 at a 1 in 5

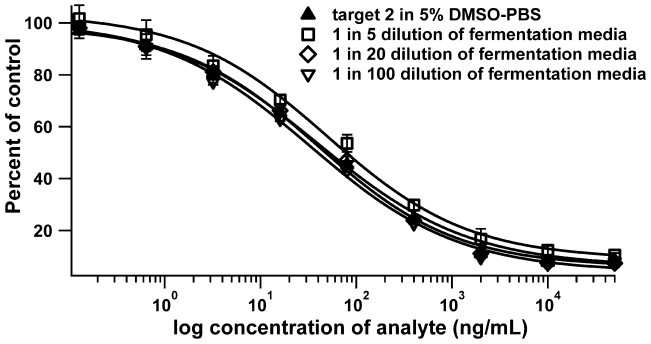

Figure 4. Matrix effects evaluation for fermentation media supernatant (LB broth) spiked with target phloretin 4′-glucoside, **2**. Each data point represents the mean of quadruplicates. The standard curve in 5% DMSO−PBS was generated by taking the average of values from three ELISA plates.

dilution and a difference of 0.5 ± 1.66 at a 1 in 20 dilution were observed, whereas at a 1 in 100 dilution, the difference was 1.5 ± 1.66. These small differences essentially signify the absence of any matrix interference. Also, no matrix effect was observed with the SOC media supernatant at the tested dilutions (1 in 5, 1 in 25, and 1 in 125). For the recovery study, hesperetin dihydrochalcone-4′-glucoside, **1**, dissolved in DMSO was spiked at 0.128 to 5×10^4 ng/mL in 10 μL of clear fermentation media supernatant and analyzed in triplicate. Table 2 shows the mean percent recovery data for target **1**, given for four different analyte concentrations. A recovery of >80% points to minimal interference from the fermentation media.

Table 2. Recovery of Hesperetin Dihydrochalcone-4′-glucoside, 1, from Spiked Fermentation Media

spiked concn[a] (ng/mL)	detected (ng/mL)	mean recovery (%) (SD)
2000	2747.3	137.4 (39.5)
400	384.9	96.2 (13.7)
80	67.5	84.3 (17.1)
16	16.2	100.8 (27.1)

[a]Different amounts of hesperetin dihydrochalcone-4′-glucoside, **1** (in DMSO), were added to the fermentation media supernatant. The spiked media samples were diluted 100-fold with PBS (5% DMSO) (n = 3).

In summary, the new competitive indirect, polyclonal antibody-based ELISA presented here was designed to serve as a class-selective immunoassay for the preferential detection of the target 4′-glucosides, hesperetin dihydrochalcone 4′-glucoside, **1**, and phloretin 4′-glucoside, **2**. The homologous assay has IC_{50} values of 27.8 and 21.8 ng/mL for target compounds **1** and **2**, respectively. There are many criteria for successful production of a target compound by fermentation or other approaches, one of which is reasonable yield. For this work, we selected a concentration of ∼1 mg/mL of the target compounds. This is considered to be a reasonable minimum yield for the high-throughput screening process using whole cells or purified enzyme. Thus, even with the necessary dilutions of the fermentation media to avoid matrix effects, the sensitivity of the assay is more than adequate for use in monitoring during optimization of fermentation conditions. The immunoassay can be utilized on-site, further reducing the time and cost of the optimization process. It can also be used for continued monitoring during production.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, NMR spectra, ELISA screening, and related data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

Ab, antibody; BSA, bovine serum albumin; CBT, checkerboard titration; CR, cross-reactivity; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; GAR-HRP, goat anti-rabbit IgG peroxidase conjugate; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline−Tween 20; TMB, 3,3′,5,5′-tetramethylbenzidine; Thy, thyroglobulin

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