## A Microplate Assay for the Determination of Protein Deglycosylation

A common requirement for studying the effects of glycosylation on proteins, or when aglycosyl proteins are required for crystallization, is the ability to determine rapidly whether or not the protein under study is deglycosylated. Concanavalin A (Con A), a lectin that is capable of binding to the sugar moieties of glycosylated proteins, is used widely to probe protein glycosylation (1). The lack of binding to Con A is considered as indicative of a protein being non-glycosylated. However, the procedures currently available are largely based upon (lectin-) blotting methodologies, which can be time-consuming. We reasoned that since Con A is itself a protein, it should be possible to coat a 96-well microplate with Con A or other lectins using standard enzyme-linked immunosorbent assay (ELISA) procedures and use it to assess the glycosylation status of proteins as a function of their ability to bind to lectin-coated microplates.

To test the validity of this idea, we devised a micro-assay employing insect juvenile hormone esterase (JHE), a glycosylated and secreted enzyme important in insect metabolism, as a model glycoprotein. We used two different preparations of recombinant (baculovirus-expressed) JHE proteins, one fully glycosylated (2) and another rendered glycosylation-free by site-directed mutagenesis of the four putative N-linked glycosylation sites (3).

In a typical assay, Con A (Type IV; Sigma Chemical, St. Louis, MO, USA) was resuspended in 50 mM carbonate-bicarbonate buffer pH 9.6 at a final concentration of 10 mg/mL and dispensed in 175-µL aliquots in the wells of a 96-well microplate. After overnight incubation of the plate at 4°C to allow coating of the wells with Con A, the wells were washed three times with 0.1× phosphate-buffered saline (PBS) containing 0.05% Tween® 20. Then, 100 µL of either PBS, insect cell culture medium containing glycosylated wild-type JHE or insect cell culture medium containing non-glycosylated ($Gly^-$) mutant JHE were added in triplicate to the wells of the Con A-derivatized microplate. Samples of both wild-type JHE and $Gly^-$ JHE were normalized to contain the same total enzyme activity. Similar aliquots of PBS, JHE and $Gly^-$ JHE samples were added to wells that were not coated with Con A to serve as negative controls. Five-tenths microliters of 50 mM $MnSO_4$ and 0.5 µL of 50 mM $CaCl_2$ were then added to each well, and the assay plates were incubated for 1 h at 37°C before washing each well three times with 0.1× PBS containing 0.05% Tween 20.

To determine the enzyme activity of the test protein bound to the plate by Con A, a chromogenic substrate for JHE was added to each well (6), and the plates were incubated at room temperature for 90 min in the dark. The plates were then read at 405 nm in a Vmax® microplate reader (Molecular Devices, Menlo Park, CA, USA), and the results are shown in Figure 1. Although the assay was started using the same levels of enzyme activity in both wild-type and mutant samples (Figure 1a), the enzyme activities varied significantly at the end of the assay. Samples incubated in Con A-coated wells showed distinctly high levels of enzyme activity (Figure 1b). This is in sharp contrast to the samples incubated in the wells that were not coated with

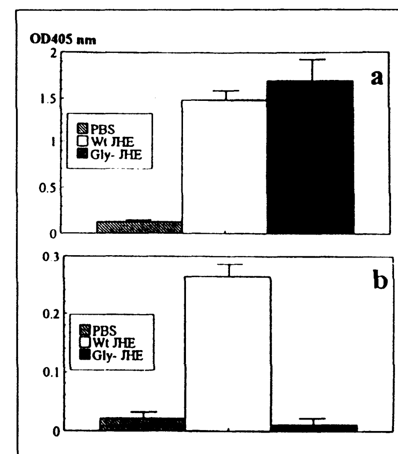


**Figure 1. Concanavalin A binding of JHE and aglycosyl JHE using a microplate-based format. a.** Catalytic activity of a 20 µL-aliquot of the JHE samples tested. **b.** Catalytic activity of JHE samples bound to concanavalin A, after subtraction of assay background.

Con A, which failed to show any enzyme activity, indicating the inability of the aglycosyl protein to bind to the Con A-coated microplate. The stability of the catalytic activity of glycosylated JHE samples throughout the procedure indicated that the observed loss of enzyme activity of the Gly⁻ JHE was not due to inactivation caused by any of the steps of the assay procedure, but rather due to its inability to bind the Con A-coated microplate matrix and thus be retained during the washing steps. JHE, deglycosylated completely by enzymatic means, had been shown to not bind Con A in previous studies using blotting procedures (4,5).

In its present format, our assay recognizes a protein as glycosylated as long as it has any glysyl moieties on it. This feature is an advantage when one is attempting to find out, as an all-or-none answer, whether or not a protein is glycosylated, rather than the extent of glycosylation. From the results presented here, it is evident that the assay clearly distinguishes the aglycosylated form of a protein from its glycosylated form. The assay is simple to perform relative to blotting procedures, and once a plate is coated with Con A, the time required to carry out the assay is minimal. The use of automated plate readers and plate washing systems simplifies this procedure further. In the example used in this study, since the protein being assayed is an enzyme, it could be assayed directly using its enzymatic activity. However, since the technique relies on a microplate-based format, the assay can be readily applied to assay any protein to which antibodies are available, even if it lacks a functional activity that can be assayed spectroscopically.

## REFERENCES


1. **Bittiger, H. and H.P. Schnebli (Eds.).** 1976. Concanavalin A as a Tool. John Wiley & Sons, New York.
2. **Hammock, B.D., B.C. Bonning, R.D. Possee, T.N. Hanzlik and S. Maeda.** 1990. Expression and effects of the juvenile hormone esterase in a baculovirus vector. Nature *344*:458-461.
3. **Hanzlik, T.N., Y.A.I. Abdel-Aal, L.G. Harshman and B.D. Hammock.** 1989. Isolation and sequencing of cDNA clones coding for Juvenile Hormone Esterase from *Heliothis virescens*: Evidence for a catalytic mechanism for the serine carboxylesterases different from that of the serine proteases. J. Biol. Chem. *264*:12419-12425.
4. **Hanzlik, T.N. and B.D. Hammock.** 1987. Characterization of affinity-purified juvenile hormone esterase from *Trichoplusia ni*. J. Biol. Chem. *262*:13584-13591.
5. **Ichinose, R., S.G. Kamita, S. Maeda and B.D. Hammock.** 1992. Pharmacokinetic studies of the recombinant Juvenile Hormone Esterase in *Manduca sexta*. Pesticid. Biochem. Physiol. *42*:13-23.
6. **McCutcheon, B.F., T. Uematsu, A. Szekacs, T.L. Huang, T. Shiotsuki, A. Lucas and B.D. Hammock.** 1993. Development of surrogate substrates for juvenile hormone esterase. Arch. Biochem. Biophys. *307*:231-241.


*The authors thank P.G. Schneider for isolating and making available the glycosylation-deficient JHE clone. The research was supported by grants from the US Environmental Protection Agency (CR-819047-01-0), Superfund Basic Research Program (2P-42-ESO-4699), Office of Research of the University of California at Davis, UC Davis - NIEHS Center for Affects of Agro-chemicals (IP30-ES-05707) and UC Davis - EPA Center for Ecological Health Research (CR 819 658).*

*Address correspondence to Prabhakara V. Choudary, Antibody Engineering Laboratory and Department of Entomology, University of California at Davis, Davis, CA 95616 USA.*

**Vernon K. Ward, Bruce D. Hammock and Prabhakara V. Choudary**

*University of California at Davis*

*Davis, CA, USA*