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Enzymatic Assay Method for Evaluating the Lipase Activity in Complex Extracts from Transgenic Corn Seed

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A colorimetric method was established to determine the activity of recombinant lipase in extracts from transgenic corn seed. The system was an oil-in-water emulsion that was stabilized by a surfactant to accommodate the organic phase substrate and aqueous phase enzyme. The lipase activity was measured by monitoring the release of nitrophenol at 346 nm from the substrate, 4-nitrophenyl butyrate. Emulsions prepared with various surfactant types and concentrations were tested. For each surfactant, the measured activity was greatest when the surfactant concentration was close to the critical micelle concentration, consistent with the changing trend of oil droplet size as a function of surfactant concentration. The optimal system, with 0.01% (w/w) Tween 80, demonstrated good reproducibility, high sensitivity, robustness, and a linear response to lipase concentration.

KEYWORDS: Enzyme assay; lipase; emulsion

INTRODUCTION

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are a group of enzymes that successively hydrolyze triacylglycerols to diacylglycerols and monoacylglycerols, with free fatty acids as coproducts (*1*). Lipases are generally water soluble, and catalytic reactions take place after the adsorption of enzymes onto an oil−water interface (*1, 2*).

There are ubiquitous natural sources of lipases, including animals (*3, 4*), plants (*5−7*), insects (*8*), fungi, and bacteria (*9*). Modern biotechnology has developed various lipases for applications in foods, detergents, and pharmaceuticals (*9*). As a therapeutic protein, recombinant human gastric lipase has been expressed and produced in insects (*10, 11*), yeast (*12*), and recombinant dog gastric lipase in transgenic tobacco (*13*). Transgenic corn has been used as a host to express and produce recombinant dog gastric lipase in seeds (*14*).

Along with the discovery of natural lipases and development of industrial processes for producing commercial lipases, numerous methods have been proposed to measure lipase activity. Beisson et al. (*1*) classified these methods into nine categories: titrimetry, spectroscopy (photometry, fluorimetry, infrared), chromatography, radioactivity, interfacial tensiometry, turbidimetry, conductimetry, immunochemistry, and microscopy. Two general principles underlying these methods included the measurements on the disappearance of substrate or release of product and the quantifications based on immunological responses.

Colorimetric methods are particularly attractive because of the availability of spectrophotometers in laboratories. Fletcher et al. (*15*) developed a water-in-oil (w/o) microemulsion system with well-characterized droplet size distributions modulated by the molar ratio of water to surfactant sodium bis-2-ethylhexyl sulfosuccinate (AOT), referred as the AOT system hereafter for simplicity. This method started with the establishment of a microemulsion by mixing an aqueous buffer with heptane containing AOT. During assays, the substrate (nitrophenyl esters of varying alkyl chain lengths) was introduced into the oil phase, followed by the addition of an aqueous lipase sample. Upon mixing, lipase was distributed in discrete aqueous buffer droplets. Lipase hydrolyzed nitrophenyl esters at the oil−water interface. The released nitrophenol was water soluble and monitored by UV absorbance.

The hydrolytic reaction in the AOT system was a second-order reaction, and the measured reaction rates were proportional to the enzyme or substrate concentration when a *Chromobacterium viscosum* lipase (CVL) was used. Similar characteristics were observed for *Humicola lanuginose* lipase and *Rhizomucor miehei* lipase (*16*). Our preliminary experiments showed a good reproducibility of this method when a commercial CVL was used. However, this method was not applicable for a lipase sample that included detergents commonly used for extracting a hydrophobic protein, e.g., 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), polyoxyethylene (20) sorbitan monolaurate (Tween 20), polyoxyethylene (20) monooleate (Tween 80), or polyoxyethylene mono-*p-tert*-octylphenyl ether (Triton X-100). The presence of these detergents in the sample may have led to the destabilization of the assay's water-in-oil emulsion, as a phase separation resulted on addition of the sample.

Crooks et al. (*16*) described an aqueous system in which a substrate *p*-nitrophenyl butyrate was first dissolved in acetone and then mixed in a buffer. The method, however, had a low reproducibility possibly due to a poorly defined surface area of

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oil (acetone) droplets in the absence of surfactants. Colorimetric methods using oil-in-water (o/w) emulsion systems have also been used to characterize lipase activity (*17*, *18*). However, we were unable to locate a method that characterized the physicochemical properties of the assay system and demonstrated a linearity of activity with enzyme concentration, similar to the AOT system.

The need for an assay compatible with detergent-aided extraction arises when a hydrophobic protein is to be extracted from an oil-containing solid. In our case, this was the task for extraction (and subsequent purification) of recombinant dog gastric lipase from transgenic corn seed. Our solution was to convert the AOT system developed by Fletch et al. (*15*) to an o/w emulsion system that would be compatible with the extraction detergents, eliminating the phase separation that occurred when the AOT system was used. The experiments were designed toward three sequential goals to develop an assay system, and the results were also presented in the same order. The first goal was to identify a surfactant type and concentration for measuring lipase activity and characterize physicochemical properties of the selected system. The second goal was to test the linearity and robustness of the identified assay system. The last goal was to compare the selected system with the AOT system. Since our samples had complex constituents from transgenic corn seed, a good tolerance to sample impurities was required, as well as a good sensitivity and reproducibility.

MATERIALS AND METHODS

Chemicals. CVL (type XII), 4-nitrophenyl butyrate (NPB), AOT, CHAPS, and L-histidine were purchased from Sigma (St. Louis, MO). Other chemicals were from Fisher Scientific (Pittsburgh, PA). Deionized water was used in all experiments.

Transgenic Corn Seed Meal. Transgenic corn seed expressed with recombinant dog gastric lipase (rDGL) was provided by Meristem Therapeutics (Clermont-Farrand, France). Corn meal from the same grain, size reduced by use of a Krups coffee grinder (Krups, Medford, MA) for 30 s, was provided by the Center for Crops Utilizations Research at Iowa State University. The meal was stored at 4 °C and used without any further size reduction.

Extraction of rDGL. Extracts were prepared by stirring 1 g of corn meal in 4 mL of extraction buffer. Unless noted otherwise, extraction buffer was 2% w/w CHAPS in 50 mM L-histidine at pH 5.5 as was optimal for recovery of lipase from the oil bodies of cotyledons from germinating sunflower seedlings (*19*). After stirring for 14 h (shorter times were not sufficient for this coarse meal), the slurry was centrifuged (10000*g*, 30 min, Sorvall RC5B Plus centrifuge, DuPont, Wilmington, DE). The supernatant was then syringe filtered through a 0.45 μm cellulose acetate membrane (Corning Incorporated, Corning, NY). The above operations were performed at room temperature, and the clarified sample was stored at −20 °C until analysis.

Assay Protocol. *Solutions.* Detergents were first dissolved at predetermined concentrations (w/w) in buffers (solution A). Buffers were constituted to include 50 mM citric acid (pH 3 and 4), sodium acetate (pH 5), L-histidine (pH 5.5 and 6), sodium phosphate (pH 7), tris(hydroxymethyl)aminomethane (pH 8), or sodium carbonate (pH 9 and 10). Buffers were filtered through a 0.22 μm cellulose acetate membrane (Corning Incorporated, Corning, NY) to remove any insoluble components. The substrate solution (solution B) was prepared by dissolving NPB in *n*-heptane at a concentration of 0.15 M and had a shelf life of up to 1 week when stored at 4 °C.

Assay. Upon addition of 6.7 μL of solution B into 1 mL of solution A, the mixture was vortexed (Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY, at the maximum setting) for 10 s to emulsify the oil phase. The lipase sample was then introduced into the emulsion at a volume of 6.7 μL, followed by a brief mixing. The emulsion was then transferred into a quartz cuvette (Fisher) immediately, and the absorbance at 346 nm (*15*) was recorded at 25 °C for 5 min (Ultraspec 4000 UV/visible spectrophotometer, Pharmacia Biotech, Piscataway,

NJ). All samples were analyzed in triplicate, and the results were expressed as averages with 95% confidence intervals (CI).

Calibration and Activity Determination. Background hydrolysis was measured for each pH in the assay protocol by monitoring the autohydrolysis of substrate in the emulsion. A significant autohydrolysis was observed at a pH higher than 7. The initial rate of absorbance increase was used as the measure of enzyme activity (*15*) and was calibrated after the subtraction of the background hydrolysis at the same pH. The absorbance was converted to nitrophenol concentration by establishing a standard curve for nitrophenol in each buffer. One unit of lipase activity was defined as the production of 1 μmol of nitrophenol per minute.

Characterization of Emulsion Droplets. Emulsions were characterized with a Malvern Mastersizer E (Southboro, MA) for droplet size and distribution for the Tween 80 based system. Emulsions, with 6.7 μL of heptane in each milliliter of surfactant solution at pH 5.5, were prepared without the addition of lipase samples by vortexing for 10 s, as in the assay protocol. Immediately after emulsification, emulsions were transferred into 18 mL of 50 mM L-histidine at pH 5.5 in the sample cell of particle sizer. The volume of emulsion added (3.0, 0.8, and 1.5 mL for 0.0005%, 0.01%, and 1.0% Tween 80, respectively) was adjusted to avoid coincident counting, and counts were taken immediately upon dilution to avoid any emulsion redistribution that might result from the dilution. Each Tween 80 emulsion was measured in triplicate with fresh emulsions under continuous mixing, and the results were presented as averages with 95% CI.

RESULTS AND DISCUSSION

Emulsion Effects on Measured rDGL Activity. As rDGL's reported maximal activity is at pH 5.5 (*14*), we used pH 5.5 in solution A to maximize sensitivity for the screening of detergents. Measurements from emulsions prepared with three surfactants (Tween 20, Tween 80, and Triton X-100), each dissolved at several concentrations, demonstrated different activities of rDGL even though the same extract was used (**Figure 1**). The changing trend was similar for each detergent type: rDGL demonstrated a maximum in the nominal activity when the detergent used to prepare emulsion was increased from 0.0001% to 5%. Tween 20, Tween 80, and Triton X-100 have a critical micelle concentration (cmc) of 0.049, 0.01, and 0.3 mM, respectively (*20*). While these levels are for clean systems of water and surfactant, the cmc of each detergent (converted to weight concentration) is indicated as a vertical dashed line in each figure. The maximum nominal activity corresponded to a concentration in the region of the cmc of the corresponding detergent. The variety of solutes, including surfactant, added for extraction would likely affect the point of formation of micelles.

Comparing the direct experimental data, the measured maximum activity values were similar for the emulsions prepared with Tween 20 and Tween 80, while that from the Triton X-100 based emulsion was slightly higher. Based on the sensitivity (maximum activity) and reproducibility (relative error, 95% CI divided by average, of 14.0%, 6.3%, and 3.7% for 0.01% Tween 20, 0.01% Tween 80, and 0.01% Triton X-100, respectively), 0.01% Tween 80 and 0.01% Triton X-100 were identified as two potential systems for further evaluation.

To understand why different results were obtained for emulsions prepared with different detergent concentrations at the same pH 5.5, droplet sizes of emulsions were characterized for Tween 80 based systems at three surfactant concentrations, 0.0005%, 0.01%, and 1%, using the procedures described under Materials and Methods. These three concentrations were selected to present a concentration well below, at, and well above the Tween 80 concentration (0.01%) corresponding to the maximum measured activity (**Figure 1b**). The droplet size distributions
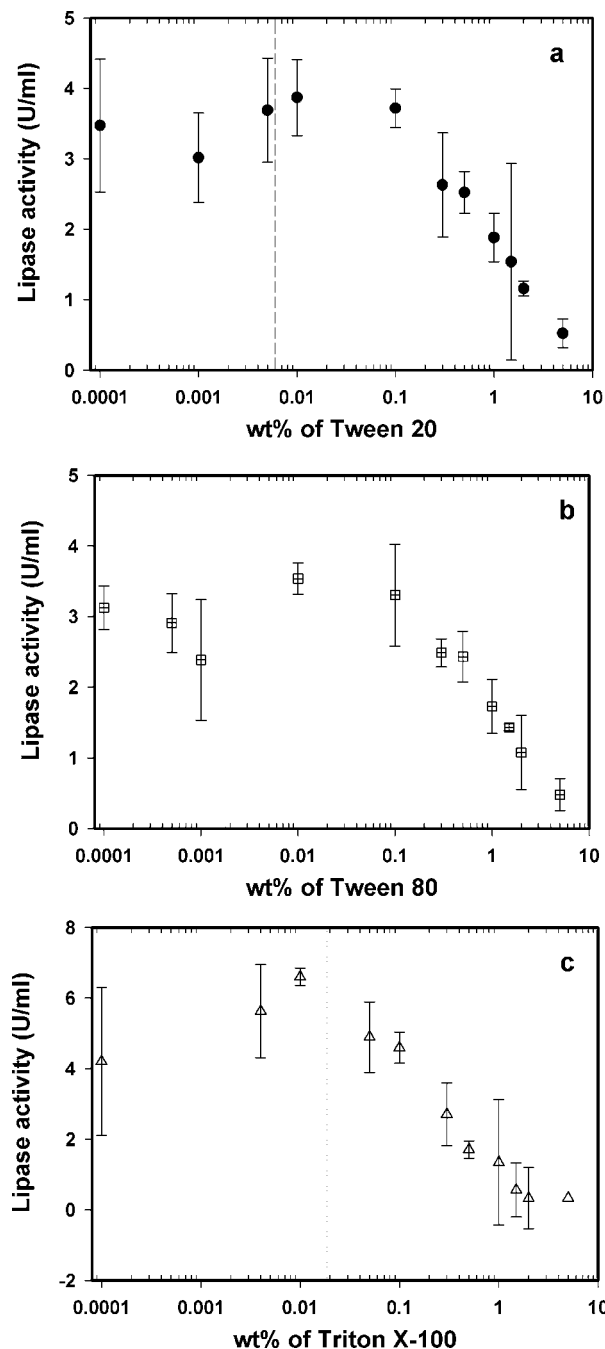
Lipase Activity in Complex Extracts from Transgenic Corn Seed

J. Agric. Food Chem., Vol. 54, No. 9, 2006 **3183**

Figure 1. Activity of rDGL at pH 5.5 measured in emulsions prepared with different surfactant concentrations: (**a**) Tween 20, (**b**) Tween 80, and (**c**) Triton X-100. The dashed vertical line in each panel is an indication of the critical micelle concentration for the corresponding surfactant in water. Error bars show the 95% confidence intervals.

Figure 2. Emulsion droplet size distributions as a function of Tween 80 concentrations at pH 5.5: (**a**) 0.0005, (**b**) 0.01, and (**c**) 1 wt %. $D_{4,3}$ is the volume-average diameter with 95% confidence interval.

droplet surface area (A) in assay emulsions can be estimated by

$$A = \frac{6\phi V}{\bar{d}} \qquad (1)$$

where ϕ is the volume fraction of oil phase (6.7 μL/1006.7 μL in all assay emulsions), V is the total volume (1006.7 μL in all assays) of assay emulsion, and $\bar{d}$ is the volume-average droplet diameter.

The total droplet surface area was thus greater at 0.01% Tween 80 than at 0.0005% and 1% Tween 80. Since the catalytic reaction of lipase occurs at the oil–water interface, the variation in particle size (**Figure 2**) and thus interfacial area corresponded to the measured lipase activities in emulsions prepared with different surfactant concentrations (**Figure 1b**). At a low surfactant concentration (below 0.01%), the interfacial area between oil droplet and continuous aqueous phase increased with an increase in surfactant concentration, which provided more interfacial area (due to smaller droplets) available for hydrolytic reaction catalyzed by lipase: an increase in apparent lipase activity. At a high surfactant concentration (above 0.01%),

are plotted in **Figure 2**, and the averages from three measurements are also included in the plot together with 95% CI. At 0.01% Tween 80, droplets had a narrower size distribution and a smaller average size than at the other two concentrations. The average size of droplets emulsified in the presence of 1% Tween 80 was greater than that in the presence of 0.01% Tween 80, which was unexpected. Microscopic observation of the emulsions showed that the size increase at 1% was the result of extensive particle aggregation rather than having a dispersed emulsion, resulting in less accessible area for the surface reaction.

Regardless of the mechanism determining the droplet size as a function of surfactant concentration, the amount of total
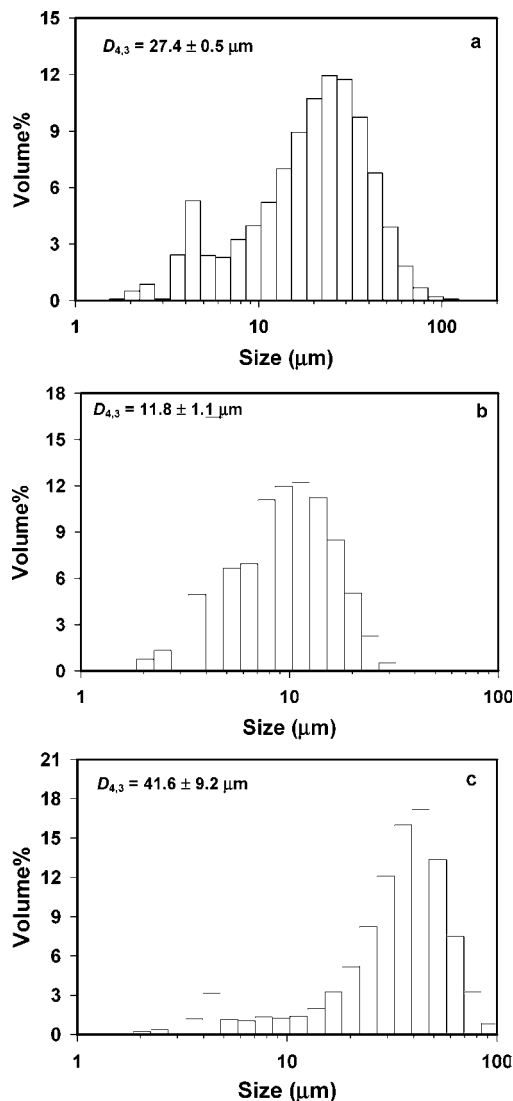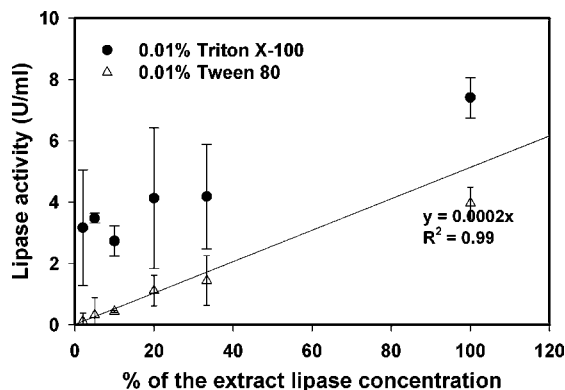
Figure 3. Linearity of activity as a function of rDGL concentration at pH 5.5: comparison of measurements from emulsions prepared with 0.01% Triton X-100 and those with 0.01% Tween 80. Concentration was varied by dilution of the original extract with 50 mM L-histidine, pH 5.5. Error bars show the 95% confidence intervals.
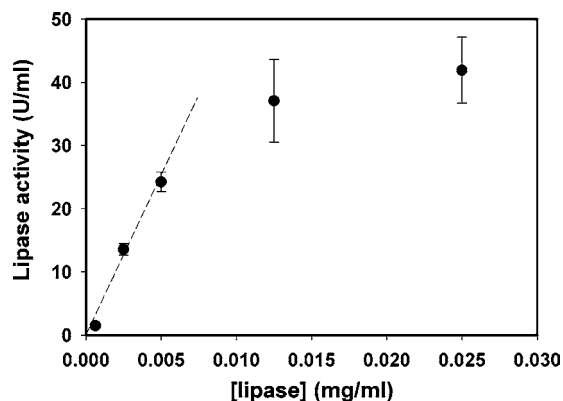

Figure 4. Activities of samples diluted from a *Chromabacterium viscosum* lipase solution (0.25 mg/mL), measured with the 0.01% Tween 80 system at pH 5.5. The line indicates the usable linear range of the assay. Error bars show the 95% confidence intervals.

the surface area available for reaction decreased as droplets became bigger at a higher surfactant concentration, corresponding to a lower measured lipase activity.

Linearity of the Measured Activity with Lipase Concentration. As a further comparison of the two selected conditions (0.01% Tween 80 and 0.01% Triton X-100), the extract containing rDGL was serially diluted with 50 mM L-histidine at pH 5.5 and each dilution was assayed. **Figure 3** shows that measured activities in the Triton X-100 system were higher than those from the Tween 80 system and in both systems the response was linear in rDGL concentration. However, since the slopes from both sets of data were similar and the regression of data from the 0.01% Triton X-100 system did not lead to an intercept of zero, there appeared to be a background response for the Triton system. Hence, 0.01% Tween 80 was selected for further study.

Defining the upper end of lipase concentration was needed to ensure the linearity of measurements with enzyme concentrations, and CVL was used for this purpose because the extract had a limited lipase concentration. At higher CVL concentrations, errors were bigger (**Figure 4**) because reactions were fast and time differences were greater when transferring emulsions to a cuvette and initiating a kinetics measurement. Linearity existed for the samples containing CVL less than 0.005 mg/mL or 24 U/mL.

Robustness of the Assay System. Since butyric acid is a weak acid with a pK_a of 4.83 at 25 °C (*21*), butyric acid
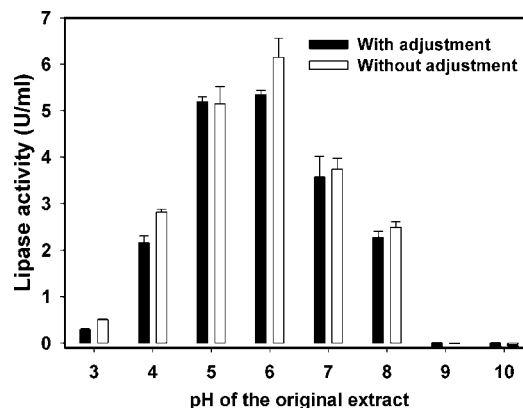

Figure 5. Measured rDGL lipase activity at pH 5.5 for the extracts obtained at different pH conditions with 4% Tween 20: comparison of results with and without adjustment to pH 5.5 before assaying. Error bars show the 95% confidence intervals.

produced during the hydrolysis of nitrophenyl butyrate could potentially change the solution pH. The need to ensure a stable pH during measurements also came from the fact that lipase activity was a strong function of pH (*14*). Additionally, samples to be tested may have a pH other than 5.5, and their addition may affect the pH of the emulsion. The robustness of the assay system was evaluated with emulsions prepared with 0.01% Tween 80 at pH 5.5, and samples were extracts prepared with 4% Tween 20 at a pH range from 3 to 10 using 50 mM buffer salts as in the assay protocol, following the steps in the section Extraction of rDGL. Two sets of assays at pH 5.5 were compared: those directly from extracts and those after a pH adjustment to 5.5. Results (**Figure 5**) did not show a significant difference between these two sets of data, and the emulsion pH was around 5.5 after sample addition regardless of the sample pH (data not shown). This set of experiments thus eliminated the concern of sample pH effects and demonstrated a good buffering capacity of the emulsion to accommodate sample compositions.

Comparison of the Methods. As the AOT system did not accommodate samples with CHAPS, Tween 20, Tween 80, or Triton X-100, pure CVL was used to compare the 0.01% Tween 80 o/w system of this work with the AOT w/o system of Fletcher et al. (*15*). When using the AOT system, there was no need for sample dilution at a CVL concentration of 0.25 mg/mL, and the measured CVL activity as a function of pH is plotted as the filled columns in **Figure 6**. However, when the Tween 80 system is used, a dilution (generally more than 100 times) was needed. The measured specific activity of CVL using the Tween 80 system was much higher than that from the AOT system (**Figure 6**), indicating that the Tween 80 system had a much higher sensitivity. At pH 3, there was no measurable CVL activity in the AOT system, while activity was very apparent in the Tween 80 system.

Conclusions. An assay has been developed to estimate the lipase activity in samples containing many impurities. The assay system was an oil-in-water emulsion stabilized by 0.01% Tween 80 at pH 5.5 with the following attributes: The standard deviations of all experiments were within 10%. The assay system was 100 times more sensitive than the literature method based on water-in-oil microemulsions. Measured activities showed linearity with lipase concentration up to 24 U/mL. The assay system also had a good robustness to tolerate sample impurity, sample pH difference, and the product from hydrolytic reaction.
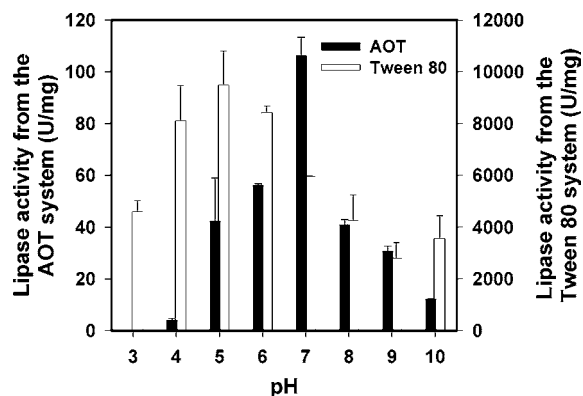
Lipase Activity in Complex Extracts from Transgenic Corn Seed

J. Agric. Food Chem., Vol. 54, No. 9, 2006 **3185**

Figure 6. Comparison of the measured *Chromabacterium viscosum* lipase activities using the 0.01% Tween 80 system with those using the AOT system at different pH conditions. Error bars show the 95% confidence intervals.

ACKNOWLEDGMENT

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