Oil Bodies of Transgenic *Brassica napus* as a Source of Immobilized β-Glucuronidase

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ABSTRACT: The process of immobilizing enzymes is a major cost factor in the utilization of heterogeneous catalysts on an industrial scale. We have developed a new strategy, based on plant genetic manipulation, for the production of foreign peptides associated with the oil body in plant seeds. Seeds of transgenic rapeseed can be produced on a large scale at relatively low cost. Furthermore, oil bodies are readily isolated from seeds by floatation centrifugation. In this paper, we describe some physical and operational properties of an oil body--fusion protein complex and its suitability as a heterogeneous catalyst. Oil bodies from rapeseed, corn, and flax aggregate at pH 5, which facilitates their recovery by floatation. Oil bodies from transgenic rapeseed, carrying the reporter gene β-glucuronidase or the pharmaceutical peptide, hirudin, also aggregate in the same range. This aggregation is reversible. Oil bodies are resistant to a wide range of pH, with some lysis occurring (<10%) at the extremes. They are resistant to shearing forces, such as stirring. The thermal and pH stabilities, as well as the catalytic activity of β-glucuronidase expressed on the oil body surface, are comparable to those of free β-glucuronidase enzyme. 

KEY WORDS: β-Glucuronidase, immobilized enzyme, oil body, oleosin, transgenic plants.

Immobilized enzyme technology has applications in clinical, pharmaceutical, chemical, and food industries (1). Economic factors, such as the cost of enzymes, immobilization, capital investment and cleanup, as well as overall system performance, are determinants in utilization of immobilized enzymes (2). Conventional immobilization of enzymes employs a solid support matrix, such as acrylamide, cellulose and alginate, onto which the enzyme is attached by adsorption, entrapment, or covalent binding.

In recent years, rapid progress has been made in the area of plant genetic engineering. This was made possible by the development of plant transformation techniques by a variety of biological (*Agrobacterium*-mediated) and physical (e.g., biolistics, polyethylene glycol-mediated) methods. This research has made it possible to investigate the use of transgenic plants for large-scale production of foreign peptides and proteins (3–7). Farming costs associated with growing and harvesting of plants are relatively small compared to the scale-up costs associated with microbial, fungal, or mammalian cell cultures. Plant-based production systems, therefore, provide an attractive alternative for the production of enzymes or peptides.

Recently, our laboratory has developed a novel strategy for the production and purification of foreign proteins and peptides in seeds (8,9). This strategy involves the fusion of the protein of interest to oleosin. Oleosins are structural proteins that are tightly associated with the oil body, the natural oil storage organelle of the plant seed (10). The amino and carboxy termini of the oleosins are exposed on the surface of the oil body (9,10), while the highly conserved hydrophobic central domain is proposed to be embedded into the oil body matrix, securely anchoring the oleosin molecule to the oil body (Fig. 1). Oil bodies from a variety of oilseeds, including *Brassica napus*, are 0.5 to 2.5 μm in diameter (10).
Oil bodies, and proteins associated with them, can be easily separated from the majority of other seed cell components by floatation centrifugation, which facilitates the purification of the desired protein. Our previous studies demonstrated that foreign proteins that are expressed as oleosin fusion proteins in transgenic plants of *B. napus* are correctly targeted into the oil body and remain on the oil body surface (8). When the foreign protein is fused to the oleosin via a linker that comprises a protease recognition site, this system allows for facilitated purification of the produced protein. Using this strategy, we have been able to produce and purify the blood anticoagulant in transgenic plants of *B. napus* are correctly targeted into the oil body and remain on the oil body surface (8). The bacterial enzyme GUS catalyzes the cleavage of a wide variety of β-glucuronides. Its absence in higher plants and its specific and sensitive activity assay render it an ideal reporter enzyme in plants. Here, we describe some of the physical properties of the oil body and the kinetic properties of the GUS enzyme attached to it by following the Guidelines for the Characterization of Immobilized Biocatalysts (11).

**MATERIALS AND METHODS**

*Transgenic Brassica napus* (rapeseed) with an oleosin-β-glucuronidase (GUS) fusion protein on the oil body surface (8). The GUS enzyme GUS catalyzes the cleavage of a wide variety of β-glucuronides. Its absence in higher plants and its specific and sensitive activity assay render it an ideal reporter enzyme in plants. Here, we describe some of the physical properties of the oil body and the kinetic properties of the GUS enzyme attached to it by following the Guidelines for the Characterization of Immobilized Biocatalysts (11).

**Isolation of oil bodies.** Oil bodies were isolated by floatation centrifugation as previously described (8). Dry, mature seeds were ground in Tricine buffer B (0.15 M Tricine-KOH pH 7.5, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 0.6 M sucrose) with mortar and pestle for 5 min. The homogenate was filtered through a layer of miracloth and centrifuged at 16,250 × g for 15 min. The fat pad containing the oil bodies was removed, resuspended in fresh Tricine buffer B, and centrifuged at 16,250 × g for 15 min. This procedure was repeated twice. Purified oil bodies were resuspended in Tricine buffer A for storage or in double distilled water for pH experiments.

**Fractionation of oil body preparations.** One mL of oil body suspension was centrifuged in a TLS-55 swinging bucket rotor in the TL-100 tabletop ultracentrifuge (Beckman, Mississauga, Ontario, Canada) at 100,000 × g for 1 h at 4°C. The fat pad containing the oil bodies was removed with a spatula and resuspended in 500 μL Tricine buffer A. The supernatant was transferred into a fresh tube. The tube containing the pellet was drained, and the pellet was resuspended in 100 μL Tricine buffer A. GUS activity was standardized to reflect the total activity of the fraction.

**Absorbance measurements.** Optical density at 650 nm (OD₆₅₀) was measured as follows: 5 to 10 μL oil body suspension in water was added to 1.5 mL buffer of a specific pH in a 1.5-mL cuvette. The cuvette was covered with parafilm, inverted three times, and absorbance was measured at 650 nm to yield t₀ value. The cuvettes were left for 18 h at room temperature with minimal disturbance and measured again for t₁₈ value. Relative OD₆₅₀ was calculated as (OD₆₅₀ at t₁₈/OD₆₅₀ at t₀) × 100%. The buffers used were KCl/HCl (pH 1.4–2.4), citric acid/Na₂HPO₄ (pH 2.8–7.6), and boric acid/NaOH (pH 8–10) (13). For the inverse experiments, described in the section *Effects of pH on oil body aggregation*, oil bodies were incubated in citric acid/Na₂HPO₄ buffers of pH 2.6, 5.1, and 7.9, respectively, in a 50-mL flask. At t = 0, 1.5 mL of the suspension was taken for OD measurements in a 1.5-mL cuvette. After 18 h, the OD of the suspension in the cuvettes was measured again with minimal disturbance. The flasks were agitated, and the pH of the flask at 5.1 was decreased with concentrated HCl or increased with concentrated NaOH. Again, absorbance of 1.5-mL aliquots was measured at 650 nm at time 0 and 18 h.

**Expression of bacterial β-glucuronidase.** The GUS gene was cloned into the bacterial expression vector pKK233-2 (Clontech, Palo Alto, CA) and transformed into *Escherichia coli* strain DH5α. Bacteria carrying this plasmid were grown o/n in LB media with ampicillin (50 μg/mL). The cells were pelleted by centrifugation, washed with STE (100 mM NaCl, 10 mM Tris.Cl pH 7.5, 1 mM EDTA), and lysed with lysozyme (4 mg/mL) in GUS extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM DTT, 1 mM EDTA, 0.1% Sarkosyl, 0.1% Triton X-100) (14) at 37°C. After centrifugation, the nonviscous top layer was removed and used as a source of "free" enzyme.

**GUS assay.** The GUS assays were performed in GUS extraction buffer with 1 mM MUG (4-methyl umbelliferyl glucuronide; Jersey Lab Supply, Livingston, NJ) as described by Jefferson (14). The GUS enzyme catalyses the conversion of MUG to MU (4-methyl umbelliferone). The accumulation of MU was measured on a Hitachi F-2000 fluorescence spectrophotometer (Tokyo, Japan) with excitation wavelength at 365 nm and emission at 455 nm.

**Determination of K_m and V_max of free and immobilized β-glucuronidase.** Dilutions of "free" GUS, produced in *E. coli*, and oil body-bound GUS, produced in *Brassica* seeds, were made to generate maximum fluorescence reading at 3-h incubation and 1 mM substrate. Reactions (300 μL) with increasing MUG concentrations were incubated at 37°C. 100-μL aliquots were taken at 5, 30, 90, and 180 min. To obtain the Lineweaver-Burk plot, slope values of generated trendlines...