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Glutathione transferases immobilized on nanoporous alumina: Flow system kinetics, screening, and stability

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ABSTRACT

The previously uncharacterized *Drosophila melanogaster* Epsilon-class glutathione transferases E6 and E7 were immobilized on nanoporous alumina. The nanoporous anodized alumina membranes were derivatized with 3-aminopropyl-triethoxysilane, and the amino groups were activated with carbonyldiimidazole to allow coupling of the enzymes via ε-amino groups. Kinetic analyses of the immobilized enzymes were carried out in a circulating flow system using CDNB (1-chloro-2,4-dinitrobenzene) as substrate, followed by specificity screening with alternative substrates. A good correlation was observed between the substrate screening data for immobilized enzyme and corresponding data for the enzyme in solution. A limited kinetic study was also carried out on immobilized human GST S1-1 (also known as hematopoietic prostaglandin D synthase). The stability of the immobilized enzymes was virtually identical to that of enzymes in solution, and no leakage of enzyme from the matrix could be observed.

Immobilization of enzymes offers several practical and economical benefits. Application of immobilized enzymes in flow-through reactors enables a continuous process. Furthermore, immobilization on granular or fibrous supports allows easy enzyme recovery in batch processes [1,2]. It is also observed that immobilization may improve the enzyme stability in some cases [2]. The use of immobilized enzymes in a fixed reactor will also lead to good reproducibility in, for example, substrate specificity screening. A large selection of protocols is available for immobilization of enzymes and other proteins on both organic and inorganic matrices [3]. An earlier example of glutathione transferase (GST)[1] immobilized on a silica matrix using 3-glycidoxypropyltrimethoxysilane was reported by Paddeu and coworkers, who immobilized a nonspecified GST [4].

The use of nanoporous materials as a template for immobilized enzymes provides several advantages. First, it provides a very large effective surface area available for immobilization, allowing a high activity in a given volume. Nanoporous anodized alumina, in particular, provides a membrane structure with remarkably straight and parallel pores stretching all the way through the membrane, resulting in excellent flow-through properties. This material is commercially available in a standard filter design with a typical pore diameter of 100 to 200 nm. As a consequence of this, the interior of a membrane or a stack of membranes is kept sterile, and the substrate diffusion distances are within the same range. This affords mass transfer properties that are superior to many other materials [5,6]. Thus, the product eluate from a flow-through design will automatically be cleared of bacteria and larger viruses. Proteins are conveniently immobilized on alumina with protocols similar to those that are developed for silica, and several authors have reported the successful design of enzyme reactors based on alumina [1,7,8].

GSTs are multifunctional enzymes that are universally distributed in most eukaryotes and prokaryotes [9]. They play a pivotal role in the metabolism and detoxification of numerous endogenous and exogenous electrophiles, including drugs, by conjugating them with the ubiquitous tripeptide glutathione (GSH) [10–12]. In addition to the typical detoxifying reactions, GSTs also catalyze other types of chemical transformations, including isomerization and reduction [10]. Their roles in both prostaglandin processing and steroid biosynthesis have been established for human GSTs [13,14]. The Sigma-class enzyme GST S1-1 catalyzes the formation of prostaglandin D_2, a mediator of allergy and inflammation from prostaglandin H_2, and therefore is also known as prostaglandin D synthase (PGDS) of the hematopoietic type [15]. In insects, GSTs

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[1] *Abbreviations used:* GST, glutathione transferase; GSH, glutathione; PGDS, prostaglandin D synthase; CDI, *N,N′*-carbonyldiimidazole; APTES, 3-aminopropyltriethoxysilane; TEA, triethylamine; ACN, acetonitrile; GuHCl, guanidine–HCl; MQ water, water purified in a Milli-Q water system; CDNB, 1-chloro-2,4-dinitrobenzene; PrITC, propyl isothiocyanate; PEITC, phenylethyl isothiocyanate; EtOH, ethanol.

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play prominent roles in the resistance to insecticides [9]. These examples show that GSTs are of interest as pharmacological targets and in the control of insects. Therefore, we have investigated immobilized GSTs in order to develop assays that could facilitate the screening of alternative substrates as well as inhibitors. Two homologous Epsilon-class GSTs, E6 and E7 from *Drosophila melanogaster* [16], as well as human PGDS [15] were immobilized on nanoporous alumina and functionally characterized.

Materials and methods

Chemicals and materials

Anodisc membranes (13 mm, 0.2 μm) were purchased from Whatman/GE Healthcare, and Swinnex 13-mm polypropene membrane holders were purchased from Millipore. *N,N′*-Carbonyldiimidazole (CDI), 3-aminopropyltriethoxysilane (APTES), triethylamine (TEA), and GSH were purchased from Sigma–Aldrich. Acetonitrile (ACN) was purchased from J. T. Baker, and NaH_2PO_4 was purchased from Merck. Guanidine–HCl (GuHCl) was obtained from Fluka. All other chemicals were of analytical grade.

Expression and purification of PGDS, E6, and E7

GSTs were prepared as recombinant proteins heterologously expressed in *Escherichia coli* and purified by standard procedures [17]. The enzymes E6 (gene CG17530) and E7 (gene CG17531) have been identified in *D. melanogaster* [18]. The third enzyme was human Sigma-class GST, commonly known as PGDS. The coding sequences were chemically synthesized by DNA 2.0 (Menlo Park, CA, USA) with a codon usage optimized for high-level expression in *E. coli* [19]. E6 and PGDS were tagged with a hexa-His sequence following the initiator Met in order to allow purification by immobilized metal (Ni^{2+}) affinity chromatography. Homogeneity of the purified enzymes was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Membrane derivatization and enzyme immobilization

Anodisc membranes were silanized by treatment with 235 μl of APTES and 100 μl of TEA in 10 ml of dried ACN for 1 h at room temperature on a rocking table in a polypropene beaker covered with parafilm. The membranes were subsequently rinsed several times with ethanol and acetone and then dried at 110 °C. Activation was carried out in the same equipment by allowing 100 to 120 mg of CDI and 100 μl of TEA in 10 ml of dry ACN to react with the silanized membranes for 1 h, followed by repeated rinsing with Milli-Q water (MQ water), ethanol, and acetone. The membranes were subsequently dried in a vacuum desiccator for 30 min. A water jet pump provided evacuation of air, and solvent. silica gel served as water adsorbent. Sets of 10 activated membranes were incubated with 2.5 mg of enzyme in 25 mM sodium borate buffer (pH 9.0) in a 20-ml glass vial with a plastic lid in an orbital shaking incubator for 20 h, followed by thorough rinsing with 25 mM Na–phosphate (pH 6.5), and stored at 4 °C. The stability under immobilization conditions was evaluated by incubation of the enzymes in the corresponding buffer at room temperature for 24 h. Aliquots were withdrawn and assayed under the conditions given above at selected time intervals.

Enzyme quantification by amino acid analysis

After completed activity assays, the membranes were first washed for 30 min with 6 M GuHCl followed by MQ water to remove any adsorbed protein. Immobilized GST was then quantified by amino acid analysis of nine membranes carrying E6 and E7 on a Biochrome 30 analyzer after hydrolysis in 6 M HCl for 24 h at 110 °C.

The molar amount $n_{protein}$ bound to a membrane was calculated from the sum of the amino acid analysis values n_{obs} for Arg, Asp + Asn, Leu, Phe, Ser, Thr, and Tyr and the corresponding sum of residues N_{seq} in the following sequence:

$$N_{protein} = \frac{1}{9}\frac{\sum n_{obs}}{\sum N_{seq}}$$

Enzyme activity assays

GSH conjugation to 1-chloro-2,4-dinitrobenzene (CDNB) was monitored spectrophotometrically by the increase in absorbance at 340 nm ($\Delta\varepsilon_{340nm}$ = 9600 M^{-1} cm^{-1}) accompanying the reaction, whereas the corresponding reaction with the isothiocyanates was detected at 274 nm with a $\Delta\varepsilon$ at 8350 and 8890 M^{-1} cm^{-1} for propyl isothiocyanate (PrITC) and phenylethyl isothiocyanate (PEITC), respectively.

Kinetic analysis in solution

The reaction rate with a series of CDNB concentrations was measured at room temperature using a fixed GSH concentration of 1 mM. The enzyme concentration was 129 nM for E7 and 51 nM for E6. Blank experiments were carried out under identical conditions in the absence of enzyme.

Kinetic analysis of immobilized enzymes

A set of nine membranes with immobilized enzymes was placed in a membrane holder coupled to an Amersham P-50 pump with a stirred 50-ml sample container (Fig. 1). To obtain kinetic parameters, different CDNB concentrations ranging from 0.1 to 1 mM in 25 mM sodium phosphate buffer (pH 6.5) with 5% (v/v) ethanol (EtOH) and 1 mM GSH were used. The samples were pumped through the reactor at a flow rate of 2 ml/min for 316 s in single pass mode to equilibrate the reactor before the system was set to circulating mode. The absorbance at 340 nm was continuously monitored for 1000 s in circulating mode on a Shimadzu UV-1601 spectrophotometer using a quartz flow cuvette with a 70-μl volume and a circular window (ϕ = 3 mm). Data were collected and processed using Shimadzu UVProbe software (version 2.31). The slope of the absorbance time course between 600 and 850 s was used to determine the reaction rate. All data were corrected for nonenzymatic background reactions. The standard Michaelis–Menten model was fitted to the reaction rate data using Graphpad Prism 5.01. The k_{cat} value was calculated from V_{max} and the enzyme subunit quantity as determined by amino acid analysis.

Substrate specificity screening for immobilized enzymes

The experiments were carried out as described above using a fixed concentration of 1 mM CDNB with monitoring at 340 nm and 0.5 mM of the isothiocyanates with monitoring at 274 nm. All experiments were carried out in triplicate.

Control experiments

Nonenzymatic blank reactions were determined using a set of nine silanized and CDI-treated membranes in the same way as for the enzyme-carrying membranes. Residual activity from possible adventitiously released enzymes in the buffer was investigated