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Substrate-Baited Nanoparticles: A Catch and Release Strategy for Enzyme Recognition & Harvesting**

Michael A. Daniele[†], Yuriy P. Bandera[†], Deepti Sharma[‡], Parul Rungta[†], Ryan Roeder[†], Michael G. Sehorn[‡], and Stephen H. Foulger^{†,*}

[†]School of Materials Science and Engineering

[‡]Department of Genetics and Biochemistry

Abstract

The isolation of a single type of protein from a complex mixture is vital for the characterization of the function, structure, and interactions of the protein of interest and is typically the most laborious aspect of the protein purification process. In this effort, a model system was utilized to show the efficacy of synthesizing a “baited” nanoparticle to capture and recycle enzymes (proteins that catalyze chemical reactions) from crude cell lysate. Enzyme trapping and recycling was illustrated with the CARDO system, an enzyme important in bioremediation and natural product synthesis. The enzymes were baited with azide modified carbazolyl moieties attached to poly (propargyl acrylate) nanoparticles through a click transformation. MALDI-TOF and SDS-PAGE analysis indicated the single step procedure to immobilize the enzymes on the particles was capable of significantly concentrating the protein from raw lysate and sequestering all required components of the protein to maintain bioactivity. These results establish a universal model applicable to concentrating and extracting known substrate protein pairs, but it can be an invaluable tool in recognizing unknown protein-ligand affinities.

Keywords

nanoparticles; biological activity; enzyme substrate; click chemistry; protein purification

1. Introduction

There is widespread interest in developing robust, flexible platforms which can be employed in both the verification of proposed metabolic pathways for specific enzymatic chemical reactions and as a means to harvest these specific enzymes from a mixture. Ligand-immobilization techniques represent a powerful tool for downstream processing of enzyme biotechnologies, both in terms of enzyme identification and recovery.^[1-3] However, the most common methods to concentrate and deliver these enzymes use covalent immobilization of the enzyme to a bulk surface.^[4-6] Covalent binding of enzymes to the substrate aids in immobilization, but it hinders the recovery & recycling of the enzymes.^[7] Core-shell particles are also a common covalent-immobilization filter for low molecular weight proteins, but these methods require complex chemistries and fine control of shell porosity to allow proteins to access their specific ligand.^[8-9] In addition, many of these covalent immobilization methods employ an indiscriminant binding chemistry, *e.g.* thiol-

[†]Center for Optical Materials Science and Engineering Technologies Clemson University, Clemson, SC 29634-0971 (USA) (foulger@clemson.edu).

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reactivity, which results in a non-specific enzyme immobilization, resulting in the neglect of unknown members of the proteome.^[10]

An alternative approach would employ substrate or metabolite infused particles which would be capable of treating dilute solutions or mixtures containing only minute amounts of target molecules in the presence of other accompanying compounds. To our knowledge, the only report employing a metabolite-surface modified particle utilized commercial amine-reactive linkers with agarose beads to probe metabolic pathways.^[11]

Expanding on that effort, we present a general strategy that employs sub-100 nm particles on which a substrate for a unique protein is “tethered”. We demonstrate that these “baited” nanoparticles can immobilize a specific protein type then release them for subsequent analysis without a loss of bioactivity. The principle of this substrate-baited separation method is general and applicable to many systems. Particulate carriers bearing a general substrate or metabolite are mixed with a solution or mixture, *e.g.*, crude cell lysates, plasma, cultivation media, or environmental samples, containing the target or unknown metabolic enzymes. Following an incubation period during which the target compounds bind to the decorated particles, the particles with the immobilized target compounds are easily removed from the mixture using centrifugation. After washing out the contaminants, the isolated target protein can be eluted or reactivated from the particles and used for further work. Due to the simple synthesis and rapid decoration of the particles employed in this approach, this process can be tailored for a range of primary substrate/metabolites and their corresponding target proteins and produced in quantities appropriate for applications in large scale separation technologies, *e.g.*, fluidized bed systems.

Metabolic enzymes are a large class of proteins in which their biochemical functions are veiled and there is a need to establish their proposed functions as well as discover unforeseen activities.^[10, 12, 13] Xenobiotic metabolism is a good example of a complicated, unknown metabolic network that would elucidate the complications of metabolomics, specifically the inordinate number of metabolites as compared to metabolic enzymes.^[14, 15] Xenobiotic metabolism includes microbial biodegradation pathways^[16] and drug metabolism in mammals.^[17] The model system employed to demonstrate this strategy for extraction of xenobiotic metabolizing enzymes utilized *Pseudomonas resinovorans* CA10. This specific bacterial strain is a source of heterocyclic aromatic degrading enzymes,^[18] a critical biotransformation for numerous bioremediation and natural product synthesis processes.^[19-25]

In the current effort, an azide-modified carbazole was attached to crosslinked & inert poly(propargyl acrylate) (PA) particles following a previously presented procedure.^[26] Briefly, the preparation of aqueous-phase nanoparticles that are surface-functionalized with a carbazole substrate was achieved through a “click” chemistry approach.^[27-29] The carbazole decorated particles (PA/AC) were utilized to bind and harvest carbazole 1-9a dioxygenase (CARDO) from *P. resinovorans* CA10 lysate. The specificity of the PA/AC method was then compared to traditional nickel-bead methods. This method illustrates the power that can be harnessed from the diversity of a “clickable” protein harvesting substrate. Click transformations, specifically the copper(I)-catalyzed reaction between azides and terminal alkynes to form the stable heterocyclic linker, 1,4-disubstituted 1,2,3-triazole,^[30] have found widespread acceptance in the colloid community. Through this facile method of modifying the surface of aqueous-phase particles, a range of potential substrates and metabolites can be attached to particles at high grafting densities that are only limited by the steric interactions of the attached moieties. The subsequent steps of the enzyme recognition and harvesting can then take place in a single test tube, in which the immobilization of the

enzyme on the particle and release is studied to assess the affinity of the enzyme for the substrate and the ability to ultimately harvest and recycle the enzyme.

2. Results and Discussion

Figure 1 presents the schematic of the “catch and release” strategy for protein harvesting. The PA colloids were prepared using a standard aqueous emulsion polymerization technique. The copper catalyzed click transformations with the azide-terminated carbazole (AC) were done in water. Moieties which incorporate carbazolyl groups are blue emitters, which allows for spectroscopic measurement of their constitution.^[31, 32] The biotransformation of the small molecule 9H-carbazole (CAR) by *P. resinovorans* CA10 results in non-fluorescing intermediate metabolites which include 2'-aminobiphenyl-2,3-diol (ABP), 2-amino-benzoic acid (ABA), and pyrocatechol (PC).^[18, 33-35] We utilized these characteristics to monitor the degradation of carbazole by *P. resinovorans* CA10 (Supporting Information S1) and to analyze the interaction of PA/AC particles with *P. resinovorans* CA10 lysate.

Detailed synthesis procedures of the AC molecule and PA/AC particles are presented in the experimental section (cf. Figure 1a). The PA/AC particles underwent a 48 hour click transformation, which results in a surface grafting density of ca. 3.5 AC groups/nm² and corresponds to a 100 % coverage if the distance of a carbazole ring at its widest point (ca. 7 Å) can be assumed to define the diameter of a cylinder enclosing the moiety and attached to the PA surface;^[36] each particle (diameter = 83 ± 12 nm) has then ca. 76k AC moieties. The modified particles underwent multiple water washes with centrifugation to remove any remaining reactants or copper catalyst. The cleaned PA/AC colloids were then incubated at 5°C for 1 hour with the lysate of *P. resinovorans* CA10 (cf. Figure 1b).

Differing species of carbazole degraders (such as *P. resinovorans* CA10) all appear to follow a similar carbazole degradation pathway that begins with the oxidative cleavage of the heterocyclic nitrogen ring of carbazole, catalyzed by CARDO.^[33] This reaction results in the cleavage of one of the two carbon nitrogen bonds; however, subsequent biodegradation of carbazole by all characterized cultures involves the degradation of one of the aromatic rings, meaning these degraders also contain a carbon-carbon cleavage capabilities.^[37] For example, *P. resinovorans* CA10 has the capability to utilize carbazole as its sole source of carbon, nitrogen and energy, but the CARDO, present in all carbazole-degrading bacteria, also catalyzes diverse oxygenations of a variety of aromatic compounds, e.g. dioxin and fluorene, at reduced efficiency.^[33] These enzymes typically consist of two or three components that comprise an electron-transfer chain that mobilizes electrons from NADH or NADPH via flavin and the [2Fe-2S] redox center of the dioxygenating activation site. The activation of this catalytic chain can be slowed by temperature reduction. This was verified by an enzyme activity assay measuring 9H-carbazole degradation with purified CARDO at various temperatures (Supporting Information S2). Thus, low temperature incubation allows for the immobilization of the enzyme onto the particles through the bioaffinity of CARDO for the attached carbazole but reduces the kinetic rate in which this enzyme catalyzes the biotransformation.^[11]

First, the enzymatic degradation of the attached carbazole was performed at 30°C. Figure 2 presents the change in the photoluminescence (PL) spectra of PA/AC particles after a 12 hour incubation with various concentrations of *P. resinovorans* CA10 lysate. Initially, the spectral characteristics of the modified particles exhibit peaks at ca. 350 nm and 366 nm which are attributed to the monomeric emission of the carbazole rings. In addition, the particles exhibit two additional peaks centered at 405 nm and 430 nm and are routinely attributed to excimer emission stemming from carbazole ring dimers.^[38, 39] The appearance

of these lower energy peaks in the PL spectra suggest that the carbazoyl groups are in close proximity and can energetically couple. When the particles are incubated at a ratio less than 6.12×10^9 particles : $1 \mu\text{g}$ *P. resinovorans* CA10 lysate, the PL signature is completely destroyed and replaced by a broad & weak peak centered at 360 nm. Like the degradation of 9H-carbazole by CARDO, this enzymatic degradation can be slowed by reducing the incubation temperature. At 5°C , significant AC degradation has slowed beyond 1 h (Supporting Information S3). We can then take advantage of the slowed reaction to extract immobilized enzymes.

The bioavailability of the hydrophobic metabolites is also critical to understanding metabolic enzymes that bind to them.^[40] Prior efforts have indicated that *P. resinovorans* CA10 can degrade the small molecule 9H-carbazole.^[18, 41, 42] We show that the modification of this small molecule with an aliphatic chain attached to the nitrogen (9-(3-azidopropyl)-9H-carbazole) does not alter the ability of CA10 to employ it as a substrate. Figure 3 presents the proliferation of *P. resinovorans* CA10 with both 9H-carbazole and PA/AC particle based media during a 96 hour incubation. The consistent growth between free carbazole and PA/AC shows the flexibility of CA10 to degrade carbazoyl moieties sequestered to particles. The growth curves show that attachment of metabolites to PA colloids did not hinder bioavailability or activity.

The low temperature incubation of the particles with the lysate of *P. resinovorans* CA10 allows for the immobilization of the protein on the particle (cf. Figure 1b). Once immobilized, the particles can be centrifuged and effectively capture the enzymes, filtering them from non-specific proteins (cf. Figure 1c). To assess the specificity of the immobilization on the particles, the centrifuged particles were washed with a mild NaCl solution to remove non-specifically bound proteins and then were subjected to matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass analysis in order to identify the sequestered enzymes. Figure 4 presents the predicted mass spectrum of the neat CARDO protein constituents and the observed PA/AC particles with immobilized protein.^[1, 43] CARDO has been identified as a multicomponent enzyme system which consists of three components: CARDO-O (terminal oxygenase), CARDO-F (ferredoxin), and CARDO-R (ferredoxin reductase).^[44] The components CARDO-F (12-kDa monomer) and CARDO-R (37-kDa monomer) function as a ferredoxin and a ferredoxin reductase, respectively, to transport electrons from NADH to terminal oxygenase. The CARDO-O is a 132-kDa homotrimeric terminal oxygenase made up of 44-kDa monomeric units. The experimentally observed mass spectrum (cf. Figure 4b) exhibits high intensity peaks at 12, 24, 37, and 44 kDa, as well as two lesser peaks at 49 and 74 kDa. These majority peaks are consistent with the predicted mass spectrum, while the peaks at masses over 74 kDa are in the noise level of the instrument. To further demonstrate that correlation between the observed and predicted mass spectrum, the component proteins of CARDO were purified employing traditional multistep affinity purification methods using a polyhistidine-tag/nickel pair(cf. Experimental section) and their mass spectrum acquired. Figure 5 presents the MALDI mass spectrum of CARDO-O, CARDO-F, and CARDO-R. All of the peaks observed in these component proteins are observed in Figure 4b except for the masses greater than 74 kDa due to the signal to noise ratio in the spectrum.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was employed in order to assess the molecular weights of the immobilized proteins and visually confirm the extraction of the CARDO components from whole-cell *P. resinovorans* CA10 lysate. In Figure 6, the proteins immobilized on the PA/AC particles is presented in lane 3 while the raw lysate is presented in lane 2 (lane 1 is the molecular weight markers). The immobilized proteins produced two distinct bands corresponding to a molecular mass of 44 and 37 kDa. These molecular masses correspond to CARDO-O and CARDO-R respectively.^[44] This identification was

corroborated by comparison with the electrophoretic mobility of purified CARDO-O and CARDO-R as shown in lanes 4 and 5. The high intensity of the bands indicates that this methodology has advantages in the selective harvesting and concentrating of these proteins from the crude lysate in a single step. The purified CARDO-F protein (Figure 6 lane 6) exhibits multiple bands and appears to run slower than its actual molecular weight, characteristics which have been presented in previous reports.^[44, 45] Faint bands similar to the purified CARDO-F are present in the immobilized proteins. The presence of this protein can be verified by a bioactivity assay with the immobilized proteins because in the CARDO *P. resinovorans* CA10 system, CARDO-F is essential for electron transfer to CARDO-O and must be present for bioactivity.^[44] To assess the bioactivity of the proteins attached to the particles, a modified affinity assay was carried out in which the addition of NADH, FAD, and Fe²⁺ reconstituted the enzyme electron transport system and activity was promoted by the addition of carbazole and an increase in temperature to 30°C.^[44] Figure 7 presents the measurement of activity with incubation time for these harvested protein as well as CA 10 lysate incubated with PA/AC particles, neat CA 10 lysate, and non-specific proteins.

The ability of PA/AC particles to harvest desired enzymes was assessed by the conversion of NADH to NAD⁺. This conversion was monitored through the loss of an absorption peak at 340 nm of the supernatant over a specified incubation period. Under all activity studies, the carbazole concentration was kept constant. As was expected, the neat lysate exhibited the fastest conversion, with the NADH oxidized to NAD⁺ within a 2 hour incubation period. This is due to the high protein content of the neat lysate relative to the substrate concentration. Similarly, the lysate incubated with the PA/AC particles exhibited a high level of NADH to NAD⁺ conversion, confirming the PA/AC's ability to trap and remove carbazole degrading enzymes. The proteins immobilized on the particles were released and capable of the biotransformation of carbazole as determined through the oxidation of NADH (cf. Figure 1e). The degradation of carbazole by extracted proteins verifies the PA/AC particles capabilities to extract the entire CARDO complex, including CARDO-O, CARDO-R, and CARDO-F. Without each component of the enzyme, bioactivity could not be restored. Although, the activity of the proteins sequestered on the particles was similar to the neat lysate, it was reduced in rate due to the lower ratio of protein to substrate. In contrast, the non-specific proteins exhibited no activity towards the carbazole. Clearly, the immobilization of the CARDO proteins on the particles still allows them to function as a carbazole degrader once removed from the particles. In the CARDO *P. resinovorans* CA10 system, an unrelated reductase can be substituted for CARDO-R and still maintain activity, but CARDO-F is indispensable for electron transfer to CARDO-O.^[44] The observed activity of the sequestered proteins indicates that all three components have been harvested.

3. Conclusions

In this effort, a model system was utilized to show the efficacy of synthesizing a “baited” nanoparticle to capture and recycle enzymes from lysate. Enzyme trapping and recycling was illustrated with the CARDO systems, an enzyme important in bioremediation and natural product synthesis. The enzymes were baited with an azide modified carbazoyl moiety attached to a PA nanoparticle. The bait products is well dispersed in water and buffers, a property that is independent of selected ligand, but a result of their attachment to PA particles. These results establish a universal model applicable to concentrating and extracting known substrate protein pairs, but it can be an invaluable tool in recognizing unknown protein-ligand affinities. Despite the widespread availability of genome sequences, according to the sheer multitude of metabolites the selectivity of many metabolic enzymes are still veiled, this procedure goes a long way toward cultivating large banks of recyclable metabolic enzymes and probing enzyme selectivity.

4. Experimental Section

4.1 Reagents and solvents

All the commercial reagents were used without further purification. All the solvents were dried according to standard methods. Deionized water was obtained from a Nanopure System and exhibited a resistivity of ca. $10^{18} \text{ ohm}^{-1} \text{ cm}^{-1}$.

4.2 Characterization

^1H and ^{13}C NMR spectra were recorded on JEOL ECX 300 spectrometers (300MHz for proton and 76MHz for carbon). Chemical shifts for protons are reported in parts per million downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent (CDCl_3 : δ 7.26 ppm, DMSO-d_6 : δ 2.50 ppm). Chemical shifts for carbons are reported in parts per million downfield from tetramethylsilane and are referenced to the carbon resonances of the solvent (CDCl_3 : δ 77.16, DMSO-d_6 : δ 39.52 ppm). Electron impact (EI) (70 eV) ionization mass spectra were obtained using Shimadzu GC-17A mass spectrometer. LC/MS mass spectra were obtained using Finnigan LCQ spectrometer and HP 1100 (HPLC). Photoluminescence (PL) spectra were collected using a Jobin-Yvon Fluorolog 3-222 Tau spectrometer.

4.3 Materials

4.4 Synthesis of 9-(3-azidopropyl)-9H-carbazole (AC)

Figure 8: Reaction scheme for 9-(3-azidopropyl)-9H-carbazole (AC). *3-(9H-Carbazol-9-yl)propyl methanesulfonate* (2): Methanesulfonyl chloride (0.84 g, 7.32 mmol) was added dropwise at room temperature to a stirred solution of 3-(9H-carbazol-9-yl)propan-1-ol (1.5 g, 6.66 mmol) (1) (synthesized according to prior methods)^[46] and triethylamine (0.74 g, 7.32 mmol) in dichloromethane (25 mL). The solution was stirred for 8 hours and then washed with water two times. The organic layer was separated, dried with Na_2SO_4 and then filtered. The solvent was removed under reduced pressure to give the clear-yellow oil. Yield: 1.96 g (97%). ^1H NMR (CDCl_3) 2.36 (m, 2H, J 6.5, 5.9), 2.86 (s, 3H), 4.14 (t, 2H, J 5.9), 4.50 (t, 2H, J 6.5), 7.25 (m, 2H), 7.40-7.52 (m, 4H), 8.10 (d, 2H, J 7.9). *9-(3-Azidopropyl)-9H-carbazole* (AC) (3): A mixture of 3-(9H-carbazol-9-yl)propyl methanesulfonate (2) (1.96 g, 6.46 mmol) and sodium azide (0.46 g, 7.14 mmol) in dimethylformamide (25 mL) was heated and stirred at 90°C for 4 hours. After cooling to room temperature, the mixture was quenched with water and extracted with dichloromethane. The organic solution was washed with water, dried with Na_2SO_4 and filtered. The solvent was removed under reduced pressure to give the clear-brown oil, which was purified by flash chromatography (10% ethyl acetate/hexane; R_f = 0.4). A clear-yellow oil was obtained. Yield: 1.34 g (82%). ^1H NMR (CDCl_3) 2.13 (m, 2H, J 6.5, 6.2), 3.30 (t, 2H, J 6.2), 4.42 (t, 2H, J 6.5), 7.24 (m, 2H), 7.41-7.50 (m, 4H), 8.10 (d, 2H, J 7.6). ^{13}C NMR (CDCl_3 , 75.6 MHz) δ 28.3, 39.8, 48.8, 108.6, 119.2, 120.6, 123.1, 126.0, 140.4. EI-Mass (m/z; rel. intensity%) 251 (M^+ +1; 5), 250 (M^+ ; 35), 194 (12), 180 (100), 167 (65), 152 (46), 139 (21).

4.5 Preparation of the baited particles

Monodisperse poly(propargyl acrylate) (PA) particles were prepared using a modified emulsion polymerization procedure. The propargyl acrylate (PA) (4.6 ml) and divinylbenzene (DVB) (0.8 ml) were passed through a packed alumina column while all other materials were used as-received. A 500 mL three necked jacketed reactor was charged with 140 mL of deionized water and 0.08 g of sodium dodecyl sulfate (SDS, 99% Aldrich) was added and the solution was stirred for 1 h at 83°C under a nitrogen atmosphere. The PA and DVB were mixed and slowly dropped into the reaction vessel. Once the addition of the

PA:DVB mixture was completed, 0.2 mL of 3-allyloxy-2-hydroxy-1-propanesulfonic acid sodium salt (COPS-1, 40 wt% soln. Aldrich) in 5 mL deionized water was added dropwise to the solution. After the COPS-1 was completely added, the solution was stirred for an additional 5 min before 0.16 g potassium persulfate (KPS, 99+ % Aldrich), that was mixed with 5 mL deionized water, was added to the solution. The emulsion polymerization was carried out under a nitrogen atmosphere for at least 2 h. The resulting PA latex was dialyzed against deionized water for ca. 5 days at 60°C using a dialysis bag with a molecular weight cut-off of 50,000. The dialyzed dispersion was then shaken with an excess of mixed bed ion-exchange resin (Bio-Rad Lab AG 501-X8, 20-50 mesh) to remove excess electrolyte. After the cleaning procedures, the particle diameter was measured to be 83 ± 12 nm (average and standard deviation) with a Coulter N4Plus dynamic light scatter (DLS). Drying a known mass of the suspension in an oven at 90°C overnight and then in a vacuum oven for 2 days, resulted in a particle density of 3.43×10^{13} particles/mL.

For a typical surface modification of the particles, for example, the grafting of AC onto the particles, 1 mL PA particles and 4.5 mg AC were added to a 2 mL deionized water. Solutions of 0.0644 g copper(II) sulfate (99.999% Aldrich) in 10 mL deionized water and 0.17 g sodium ascorbate (99% Aldrich) in 10 mL deionized water were made. Initially, 0.2 mL of the Cu_2SO_4 solution was added to the PA/AC solution, followed by 0.3 mL of the sodium ascorbate solution. The resulting mixture was maintained at a temperature of ca. 28°C for 48 h. The resulting clicked particles were dialyzed against deionized water for ca. 3 days at 60°C using a dialysis bag with a molecular weight cut-off of 50,000.

4.6 Preparation of *P. resinovorans* CA10 lysate

P. resinovorans CA10 was grown in minimal media M9 minus glucose at 30°C for 48 hrs. The cells were harvested using a Beckman JLA 16.250 rotor at 10000 x g at 4°C. The cell pellet (20 g) was resuspended in five volumes of Buffer A (20 mM K_2HPO_4 (pH 7.4), 0.5 mM DTT, 10% sucrose, 0.250 M KCl, protease inhibitors: pepstatin, leupeptin, chymostatin and aprotinin 5 µg/ml final concentration, 1 mM PMSF and 1 mM EDTA) and incubated with 1 mg/ml lysozyme at 4°C. The resuspended cells were sonicated and subjected to ultracentrifugation using a T-1270 Sorvall rotor for 45 min at 100,000x g. The clarified supernatant was frozen in aliquots using liquid nitrogen and stored at -80°C. The total protein concentration was measured using Bradford's assay.^[47]

4.7 Purification of CARDO proteins

All the resins and chemicals were from GE healthcare and American Bioanalytical, respectively, unless otherwise mentioned.

4.7.1 Expression of CARDO-F, CARDO-O and CARDO-R—Plasmids harboring genes for CARDO-F-(HIS)₆, CARDO-O-(HIS)₆ and CARDO-R-(HIS)₆ vectors were a kind gift from Hideaki Nojiri. Each plasmid was transformed separately in the BL21(DE3) Rosetta strain of *Escherichia coli*. The transformed bacterial cells were grown in 2X LB media (yeast extract 5 g/L, tryptone 10 g/L and NaCl 5g/L) supplemented with kanamycin (50 µg/ml) at 37°C to an OD of 0.8. Isopropyl-D-1-thiogalactopyranoside (IPTG) was added to the cultures (final concentration of 0.4 mM) and the culture was further incubated for 16 hrs. The cells were harvested by centrifugation at 6000 rpm at 16°C for 10 min in a Beckman 8.1000 rotor.

4.7.2 Purification of CARDO-F, CARDO-O and CARDO-R—Lysate from bacterial cell pellets for each of CARDO-F, CARDO-O and CARDO-R was separately prepared using the same protocol at 4°C. A 30 g cell pellet of each bacterial culture was resuspended separately in 150 ml Buffer A and incubated at 4°C for 30 min in the presence of lysozyme

(1 mg/mL) followed by sonication. Each individual lysate was centrifuged at 40,000 rpm in a Beckman Ti-45 rotor for 1 hr at 4°C. The supernatant from each lysate was further subjected to affinity and conventional column chromatography as described below.

4.7.3 CARDO-F purification—The supernatant from the CARDO-F lysate was incubated with Ni-NTA sepharose (GE Healthcare) for 1 hr. The supernatant-Ni-NTA slurry was collected and poured into a column (0.7 cm inner diameter X 0.5 height). The Ni-NTA column was washed with Buffer B (20 mM K₂ HPO₄ pH 7.4, 300 mM KCl, 10% glycerol, and 1 mM EDTA) followed by elution of CARDO-F with Buffer B containing 500 mM imidazole. Peak fractions were diluted to match conductivity of Buffer C (20 mM K₂ HPO₄ pH 7.4, 10% glycerol, and 1 mM EDTA) containing 100 mM KCl and loaded on a 1 mL Source 15Q column (GE Healthcare). The bound protein was fractionated using Buffer C containing 100 mM - 800 mM KCl. The peak fractions containing the protein (~700 mM KCl) were pooled, diluted to match conductivity of Buffer C containing 100 mM KCl and loaded onto a 1 mL Source 15S column (GE Healthcare). The bound protein was fractionated using Buffer C containing 100 mM 800 mM KCl. The peak fractions (~150 mM KCl) containing CARDO-F were pooled, concentrated and stored at -80°C.

4.7.4 CARDO-O purification—The supernatant containing CARDO-O was subjected to Ni-NTA affinity chromatography as described for CARDO-F. The eluate from the Ni-NTA column was diluted to match conductivity of Buffer C containing 100 mM KCl then loaded on a 1 mL Source 15Q column. The bound protein was fractionated as described for CARDO-F. Peak fractions containing the CARDO-O (~360 mM KCl) were pooled, diluted to match conductivity of Buffer C containing 100 mM KCl and loaded onto a 1 mL Source 15S column. The bound protein was fractionated as described for CARDO-F. The peak fractions (~100 mM KCL) containing CARDO-O were pooled, concentrated and stored at -80°C.

4.7.5 CARDO-R purification—The supernatant containing CARDO-R was subjected to Ni-NTA affinity chromatography as described for CARDO-F. The eluate from the Ni-NTA column was diluted to match conductivity of Buffer C containing 100 mM KCl then loaded on a 1 mL Source 15Q column. The bound protein was fractionated as described for CARDO-F. Peak fractions containing the CARDO-R (~400 mM KCl) were pooled, diluted to match conductivity of Buffer C containing 100 mM KCl and loaded onto a 1 mL Source 15S column. The bound protein was fractionated as described for CARDO-F. The peak fractions (~100 mM KCL) containing CARDO-R were pooled, concentrated and stored at -80°C.

4.7.6 Denaturing polyacrylamide gel electrophoresis—The protein samples (whole cell lysate, 5 µg; purified lysate, 1 µg; CARDO-O, 1µg; CARDO-R, 1.5µg; and CARDO-F, 2µg) were loaded on a 12% SDS-polyacrylamide gel and subjected to electrophoresis for 50 min at 200V. The gel was stained using Commassie Brilliant Blue. The image was taken using Gel-Doc (Bio-Rad).

4.8 Enzyme trapping and affinity activity assay

The enzyme trapping and affinity of the bound proteins were assessed through the following procedures. Initially, 100 µL PA/AC particles in an aqueous buffer (3.43×10^{13} /cm³ and (100 µL in 0.8 mL water)) were added to 100 µL neat lysate of CA 10 and incubated at 5°C for 1 hour. This solution of particles and lysate was centrifuged (10,000 x g, 5°C, 10 min.) and the supernatant was decanted. The particles were washed with 1 mL of a 100 mM NaCl solution and the tube was inverted 10 times followed by incubation for 30 min at 5°C. After the incubation, the particles were centrifuged (10,000 xg, 5°C, 10 min) (PA/AC particles

with bound proteins) and the salt solution decanted. To assess the activity of the various components (PA/AC particles with bound proteins, incubated lysate, non-specific proteins, and neat lysate), each was individually added to an aqueous buffer in which 5 μ L of a 10 nmol carbazole / DMSO solution was added. To initiate bioactivity, 1 nmol flavin adenine dinucleotide (FAD), 100 nmol ammonium iron(II) sulfate, and 100 nmol NADH, were added, resulting in a total buffer volume of 1 mL, and the temperature raised to 30°C. All cofactors were added in excess. After either 30, 60, 120, 240, 320, or 960 min., the temperature of the samples was lowered to 5°C, the tubes centrifuged (10,000 $\times$ g, 5°C, 10 min), and the supernatant decanted and the oxidation of NADH to NAD⁺ assessed through the change in absorption of the supernatant at 340 nm.

4.9 MALDI/TOF measurements

All samples were analyzed in a saturated 3,5-dimethoxy-4-hydroxycinnaminic acid solubilized in 1:1 water:acetonitrile, 0.1% TFA. A sandwich method of 1 μ L matrix, followed by 1 μ L acid solubilized sample, capped with 1 μ L matrix was prepared on the plate. A desalting step of 1 μ L water was utilized for all samples. The samples were analyzed with the Bruker OmniFlex III.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic illustration of the baited-particle enzyme extraction method: (a.) the nanoparticles consist of poly(propargyl acrylate) (PA) and their surface modification with 9-(3-azidopropyl)-9H-carbazole (AC); (b.) after adding particles to the protein solution, the CARDO enzyme is attracted and binds to the bait; (c.) centrifugation is used to separate the particles with immobilized enzyme, and (d.) separate by decanting. After decanting and resuspension of the particles, the enzyme can be (e.) released and assayed for its carbazole-degrading activity by elevation of temperature and introduction of cofactors.

Figure 2.

Change in photoluminescence spectra of PA/AC particles after 12 hour incubation at 30°C with various concentrations of *P. resinovorans* CA10 lysate. Particle density was $3.43 \times 10^{13} / \text{cm}^3$ (diameter = 83 ± 12 nm) and (100 μL in 2.9 mL water) were combined with *P. resinovorans* CA10 lysate (5.6 $\mu\text{g}/\mu\text{L}$). Excitation wavelength at 295 nm.

Figure 3. Proliferation of *P. resinovorans* CA10 with small molecule CAR (○) and PA/AC particle (●) based media during 96 hour incubation at 23°C. Control growth performed with glucose medium (Δ).

Figure 4.
 (a) Predicted matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrum of neat CARDO protein and (b) observed PA/AC particles with immobilized protein.

Figure 5. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrum of purified (a) CARDO-R (b) CARDO-F, and (c) CARDO-O.

Figure 6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell lysate from *P. resinovorans* CA10 (2) and PA/AC particle immobilized proteins (3). For comparison, the purified CARDO components of CARDO-O (4, 1 µg), CARDO-R (5, 1.5 µg), and CARDO-F (6, 2 µg) obtained through traditional multistep affinity purification methods using a polyhistidine-tag/nickel pair is presented. Lane 1 is the molecular weight marker.

Figure 7. Measurement of activity with incubation time for proteins immobilized on PA/AC particles (●), CA 10 lysate incubated with PA/AC particles (▽), neat CA 10 lysate (Δ), and non-specific proteins (○). Activity measured by the oxidation of NADH to NAD⁺ and assessed through the change in absorption of the supernatant at 340 nm. See text for details.

Figure 8.
Reaction scheme for 9-(3-azidopropyl)-9H-carbazole (AC).