An Affinity-Based Method for the Purification of Fluorescently-Labeled Biomolecules

Trung Nguyen, Neel S. Joshi, and Matthew B. Francis*

Department of Chemistry, University of California, Berkeley, California 94720-1460, and Materials Sciences Division, Lawrence Berkeley National Labs, Berkeley, California 94720. Received May 22, 2006; Revised Manuscript Received June 17, 2006

Due to the difficulty of separating mixtures of labeled and unlabeled biomolecules, a general new method for the affinity purification of modified proteins has been developed. A Sepharose-based solid support bearing β-cyclodextrin groups was used to capture chromophore-modified proteins selectively, while unmodified proteins remained in solution. After isolation of the resin, the modified proteins were released by treating the sample with a competitive cyclodextrin binder, such as adamantane carboxylic acid. This procedure was demonstrated for several dyes displaying a wide range of spectral characteristics and diverse chemical structures. Preliminary studies have shown that this method can also be used to enrich modified peptide fragments present in proteolytic digests. This technique is anticipated to accelerate the development of new protein modification reactions and could provide a useful tool for proteomics applications.

The attachment of chromophores to biomolecules is a common practice in chemical biology, affording fluorescent probes that can track protein localization (1a), detect the binding of substrates (1b), and measure distances through fluorescence resonance energy transfer (FRET) (1c,d). Typically these labels are introduced through the reaction of lysine residues with NHS-esters, isocyanates, or isothiocyanates, or through the modification of cysteine residues with maleimides or iodoacetamides (2). However, these reactions commonly do not reach full conversion, yielding inseparable mixtures of labeled and unlabeled proteins. Although this is acceptable in some instances, many applications (such as FRET studies) would benefit from the removal of unreacted proteins from the sample. Unfortunately, no general method exists to achieve this separation under conditions mild enough to maintain the folded, native structure of the protein targets. To address this, we report herein a general affinity-based method that can isolate biomolecules that are labeled with small organic chromophores.

Numerous strategies have emerged for the formation of host−guest complexes through molecular recognition events (3). Of these, complexes formed with cyclodextrins and their derivatives are arguably the most general (4). A wide range of organic molecules has been found to form complexes with cyclodextrins with binding constants that span 6 orders of magnitude (5), and, as a result, cyclodextrins have found use in applications ranging from the solubilization of pharmaceutical compounds (6) to chiral chromatography (7). Several studies have established the ability of cyclodextrins to form host−guest complexes with fluorescent chromophores (7b, 8, 9); in contrast, we have found that they have little-to-no affinity for most proteins. We envisioned using biocompatible resins functionalized with cyclodextrins to sequester labeled biomolecules selectively, while leaving unlabeled species in solution, Figure 1b. After isolation of the resin, it was anticipated that the captured proteins could be released through the addition of a competitive binder, such as 3.

A solid support suitable for this purpose was prepared through the exposure of commercially available NHS-ester functionalized Sepharose resin to amine-functionalized β-cyclodextrin (9a), yielding resin 2a, Figure 1a. The efficiency of the coupling reaction was monitored by UV analysis of the released NHS, indicating that a loading of 8.7 µmol of β-cyclodextrin/mL of dry resin was obtained. Following hydrolysis of any remaining NHS-esters using ethanolamine, the resin was thoroughly rinsed with water and stored in solution at 4 °C until use (10).

In light of the high cost of 1a, we have also developed a more practical route for the preparation of the resin. Activation of inexpensive β-cyclodextrin with carbonyl diimidazole followed by treatment with excess propylenediamine afforded carbamate 1b with only minimal amounts of doubly coupled product. This mixture was used to prepare resin analogue 2b, which performed equivalently in the studies described below.

The ability of the cyclodextrin resin to purify labeled biomolecules was evaluated in the context of a typical lysine conjugation experiment. After exposure of a solution of myoglobin to 1 equiv of Oregon Green NHS-ester (yielding conjugate 4) and subsequent removal of the small molecules using gel filtration, 320 µL of the resulting 1 mg/mL solution was exposed to 37 mg of resin 2a for 5 min. UV−vis analysis indicated the complete disappearance of the Oregon Green absorbance from the supernatant ($\lambda_{\text{max}} = 495$ nm), while unlabeled myoglobin ($\lambda_{\text{max}} = 410$ nm) remained in solution, Figure 2a. SDS−PAGE analysis of the supernatant indicated that no fluorescent protein remained (Figure 2b, lane 2), and ESI-MS confirmed that virtually all of the labeled protein had been captured (Figure 2c,d). After isolation via filtration, it was found that the resin could be washed with several portions of water without releasing the bound protein.

Several competitive binders have been used to liberate the modified protein from the resin. Adamantane carboxylic acid (3, 10 mM in 100 mM ammonium chloride, pH 7) has been particularly useful in this regard, resulting in nearly complete release in under 1 min, Figure 2a,b. ESI-MS analysis of the resulting solution confirmed that >90% of the eluted protein had been modified, Figure 2e (11). Under saturating conditions, UV−vis analysis indicated that 100 mg of resin was able to capture 40 nmol (0.67 mg, in this case) of labeled protein.
In terms of buffers, we have found that efficient capture of labeled proteins can be achieved in 50–100 mM HEPES (pH 7), although the use of increased amounts of resin was sometimes required to improve the amount of captured protein (Table 1, entries 2, 9). Other buffers, including phosphate, tris, and acetate, consistently afforded 60–90% yields of modified protein. The technique was also effective for chymotrypsinogen A labeled with FITC (5) in a variety of buffers (entries 8–12) and for subtilisin Carlsberg (entry 13). In all cases, MS analysis indicated that the fractions eluted with 3 contained only labeled proteins. Importantly, the chymotrypsinogen A was found to possess similar levels of proteolytic activity before and after the purification process (12). This technique has also been applied to the purification of TAMRA-(6), Cascade Blue-(7), and coumarin-labeled (8) proteins, Table 1, entries 15–17.

Consistent with cyclodextrin-dependent binding, resin pre-washed with 3 was unable to capture either labeled or unmodified protein. However, we have observed in several cases that
In conclusion, a new general procedure has been developed for the isolation of modified proteins and peptides. This technique features mild conditions for the capture and release of biomolecular targets, ease-of-use, and the potential to purify a large number of synthetic modification products. It is anticipated that this method will also find use as a tool for proteomic analysis and will facilitate the development of new protein modification reactions. Current efforts are exploring the use of this strategy to separate singly labeled proteins from multiply labeled proteins, as well as the adaptation of this technique to afford continuous flow LC-based methods.

**ACKNOWLEDGMENT**

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**Supporting Information Available:** Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

**LITERATURE CITED**


(3) An entire issue of *Chemical Reviews* has been devoted to this topic: (1997) *Chem. Rev.*, 97, 1321–1734.


(10) Resin samples have been stored at 4 °C for months with no loss in activity.

(11) Comparison of the heme absorbance at 409 nm to the Oregon Green absorbance at 495 nm was consistent with this observation. Control experiments have also confirmed that unlabeled protein has no affinity to resin 2a or 2b (see Supporting Information Figure S10).

(12) See Supporting Information for details.
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Supporting Information

General Procedures. Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification. All small molecule reactions were carried out under a nitrogen atmosphere in oven dried glassware unless otherwise noted. All organic solvents were removed under reduced pressure using a rotary evaporator. Pyridine was distilled under an inert atmosphere from calcium hydride. Water (ddH$_2$O) used in biological procedures or as a reaction solvent was deionized using a NANOpure™ purification system (Barnstead, USA). Myoglobin (M 1882) from horse heart, Subtilisin Carlsberg protease (P5380) from Bacillus licheniformis, and α-chymotrypsinogen A (P 5380) from bovine pancreas were purchased from Sigma (St. Louis, USA) and used without further purification. Size exclusion chromatography was performed using NAP™ 5 columns from Amersham Biosciences. Microspin™ G-25 columns were purchased from Amersham Biosciences. The pre-packed Sephadex resin was discarded and the columns were thoroughly washed prior to use. Dyes used for protein labeling experiments were purchased from Invitrogen and used without further purification. Samples were mixed using a Barnstead Thermolyne Labquake® Shaker Rotisserie. NHS-activated Sepharose™ 4 Fast Flow resin (17-0906-01) was purchased from Amersham Biosciences. 6-monodeoxy-6-monoamino-β-cyclodextrin (M 2314) and β-cyclodextrin hydrate (856088) were obtained from Aldrich Chemical Company.
Instrumentation and Sample Analysis Preparations. UV-Vis spectroscopic measurements were conducted on a Tidas-II benchtop spectrophotometer (J & M, Germany).

Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager-DE™ system (PerSeptive Biosystems, USA). All samples were co-crystallized using either an α-cyano-4-hydroxycinnamic acid or a sinapinic acid solution (10 mg/mL in 1:1 MeCN:ddH₂O with 0.1% TFA). Electrospray LC/MS analysis was performed using an API 150EX system (Applied Biosystems, USA) equipped with a Turbospray source and an Agilent 1100 series LC pump. Protein mass reconstruction was performed on the charge ladder with Analyst software (version 1.3.1, Applied Biosystems). Prior to MS analysis, biological samples were desalted and/or separated from small molecule contaminants using μC18 ZipTip® pipet tips (Millipore, USA), NAP-5™ columns (Amersham Biosciences, USA), Strata C-18E™ reversed-phase columns (Phenomenex, USA), or 3500 molecular weight cutoff Slide-A-Lyzer® Dialysis Cassettes (Pierce Biotechnology, Inc., USA) as indicated below. Intact protein chromatography was performed using a Jupiter 5u C5 300Å reversed phase column (2.0 mm x 150 mm) with a ddH₂O:MeCN gradient mobile phase containing 0.1% formic acid (250 μL/min). An Agilent Zorbax 3.5 μm 300SB-C8 reversed phase column (2.1 mm x 50 mm) was used for the analysis of trypsin digest fragments.

For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was accomplished on a Mini-Protean apparatus (Bio-Rad, USA), following the general protocol of Laemmli. Crude reaction mixtures were diluted by a factor of 5
(v/v) in ddH$_2$O and combined 1:1 (v/v) with gel loading buffer containing SDS, DTT, and bromophenol blue. Samples were then loaded onto the gel without heating. After removal of the completed gels from their cassettes, the bottom portions containing free dye were excised promptly, after which the gels were submerged in water for rinsing and imaging. Commercially available markers (Bio-Rad, USA) were applied to at least one lane of each gel for calculation of apparent molecular weights. Fluorescence visualization of gels was obtained by UV transillumination at 302 nm. Visualization of protein bands was accomplished by staining with Coomassie® Brilliant Blue R-250 (Bio-Rad, USA). Gel imaging was performed on an EpiChem3 Darkroom system (UVP, USA).

**General procedure for fluorescent labeling of proteins.** In a typical procedure, 3.5 mL of a 0.1 mM myoglobin solution in 0.1 M NaHCO$_3$ pH 8.3 aqueous buffer was transferred to a centrifuge tube. An amine reactive fluorophore (~1 mg) in 20-40 μL of DMF was then added. The solution was stirred on a laboratory mixer for 2.5 h and then quenched with 0.1 M H$_2$NOH pH 7.2 (80 μL, 8.0 μmol) aqueous solution. The reaction was then mixed overnight at room temperature. For specific examples of protein labeling reactions refer to Table 1. A portion of the crude reaction was purified by size exclusion chromatography. The purified solution was analyzed by UV-vis spectroscopy, MALDI-TOF-MS or ESI-MS, and SDS-PAGE. The sample used for many of the experiments presented in the supporting information will be referred to as S1 (see Table S1)
### Table S1. Fluorescent labeling of myoglobin with amine reactive dyes.

<table>
<thead>
<tr>
<th>Dye/fluorophore</th>
<th>Conjugate</th>
<th>Eq. of dye added</th>
<th>Conjugates detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oregon Green 488 carboxylic acid SE</td>
<td>4</td>
<td>1.0</td>
<td>Unmod, +1, +2</td>
</tr>
<tr>
<td>TAMRA-NHS</td>
<td>6</td>
<td>5.0</td>
<td>+2, +3, +4, +5</td>
</tr>
<tr>
<td>Cascade Blue acetyl azide</td>
<td>7</td>
<td>5.0</td>
<td>Unmod, +1, +2</td>
</tr>
<tr>
<td>Alexa Fluor 350 carboxylic acid SE</td>
<td>8</td>
<td>5.0</td>
<td>+2, +3, +4, +5</td>
</tr>
<tr>
<td>Oregon Green 488-X-succinimidyl ester</td>
<td>S1</td>
<td>5.0</td>
<td>Unmod, +1, +2, +3</td>
</tr>
</tbody>
</table>

1Extent of labeling was determined by MALDI-TOF or ESI-LC MS. Only predominant species present in the spectra were reported.

**Quantification of sepharose resin coupling reaction.** In order to quantify the degree of modification to the NHS-activated sepharose resin the Amersham literature procedure was employed. To analyze the amount of NHS hydrolyzed during a coupling experiment, a calibration curve was formed by monitoring the absorbance at 260 nm for serial dilutions of a 30 mM NHS aqueous solution in 0.2 M NaHCO₃/0.5 M NaCl pH 8 buffer. For coupling reactions to the NHS-activated resin, Amersham Biosciences literature values predicted concentrations of released NHS to be from 16-23 μmol/mL of resin.

**Synthesis of β-cyclodextrin modified sepharose resin (2a).** NHS-activated resin was transferred to a fritted column as a slurry and filtered to dryness. The resin occupied a volume of 17.5 mL. A solution of 6-monodeoxy-6-monoamino-cyclodextrin (0.44 g) in 10 mL 0.2 M NaHCO₃/0.5 M NaCl pH 8 aqueous buffer was added to the resin. The column was then capped and agitated on a laboratory shaker for 2 h. The reaction tube was drained and the eluting solvent was collected for subsequent analysis. Afterwards, the resin was subjected to the following series of washes:

- 3 washes of 0.2 M NaHCO₃/0.5 M NaCl pH 8 aqueous buffer (10 mL portions)
- 3 washes of 0.5 M ethanolamine/0.5 M NaCl pH 8.3 aqueous buffer (10 mL portions)
• 3 washes of 0.1 M acetate/0.5 M NaCl pH 4 aqueous buffer (10 mL portions)
• 1 wash of H₂O (10 mL portions)
• 2 washes of 25 mM phosphate buffer pH 7 (10 mL portions)
• 3 washes of H₂O (200 mL portions)

The resin was then stored as a slurry in H₂O at 4 °C.

The eluting fraction from the coupling reaction was determined to contain 153 
µmol of NHS. This corresponds to a cyclodextrin loading of 8.7 µmol/mL of dry resin. Expected values for NHS release during this reaction were 280-403 µmol according to Amersham loading values.

**Synthesis of amino β-cyclodextrin derivative (1b).** β-cyclodextrin monohydrate was placed in a flask as a solid and dried under vacuum at 50 ºC overnight. The dry cyclodextrin (1.0 g, 0.86 mmol) was transferred to a new flask and dissolved in freshly distilled pyridine (3.0 mL). A solution of carbonyldiimidazole (140 mg, 0.86 mmol) dissolved in 1 mL of freshly distilled pyridine was added to the reaction mixture dropwise over 2 minutes while stirring. The activation reaction was allowed to proceed at rt for 2 h. After this time, the activated cyclodextrin solution was added dropwise to a flask containing 1,3-diaminopropane (360 µL, 4.32 mmol) while stirring. After 1 h, the cyclodextrin mixture was precipitated by adding the reaction solution dropwise to a flask containing 500 mL of CH₂Cl₂ with vigorous stirring. The flaky white precipitate was isolated *via* suction filtration through filter paper, then placed under vacuum overnight. The cyclodextrin mixture was analyzed by MALDI-TOF mass spectrometry (Figure S1) and used without further purification.
**Figure S1.** MALDI-TOF spectrum of 1b using α-cyano-4-hydroxycinnamic acid matrix. Expected mass for β-cyclodextrin [M+Na]+ = 1158.0. Other peaks correspond to additions of the 100 Da carbamate fragment (see Figure 1 in main text).

**Synthesis of β-cyclodextrin modified Sepharose resin (2b).** Sepharose-NHS resin slurry (0.5 mL) was placed in a fritted tube and filtered. To the dry resin was added a solution of 1b (75 mg in 1.5 mL of NaHCO₃ (0.1 M), NaCl (0.5 M), pH 8). The resulting slurry was agitated overnight. The resin was then subjected to the following rinses:

- 3x 1 mL of 0.5 M ethanolamine/0.5 M NaCl pH 8
- 3x 1 mL 0.1 M acetic acid/0.5 M NaCl pH 4
- 2x 20 mL H₂O

The resin was stored at 4 °C as a slurry in water.

**General procedure for resin capture of proteins.** First, the crude protein solution was purified by size-exclusion chromatography using a NAP™ 5 column into pure H₂O or into the appropriate buffer solution. To a microcentrifuge tube containing 10-20 mg of dry CD modified sepharose resin, the purified protein (100-200 μL of 60 μM solution) was added. Additional water was added to the heterogeneous solution to ensure proper mixing of the resin and protein solution. The solution was then agitated on a laboratory mixer for approximately 5 min. The heterogeneous solution was then transferred to a fritted microspin column and spun dry.
The eluting solvent was collected in microcentrifuge tubes. It was observed that the color of the protein solution had been transferred to the resin. Water (0.5 mL) was then added to the column and mixed by manual inversion for 1 min. The resin was rinsed with additional amounts of H₂O. To elute the protein, 0.5 mL of 10 mM adamantane carboxylic acid/100 mM NH₄Cl pH 7 was added to the resin and inverted by hand for 1 min. The color of the eluting solution resembled that of the beginning protein solution. Additional washes with the eluting solvent mixture were performed to achieve higher levels of protein recovery. The eluting fractions were analyzed by UV-vis spectroscopy (Figure S2), and SDS-PAGE (Figure S3).

**Figure S2.** UV-vis spectrum of resin 2b capture of S1: before exposure to resin (blue), flow through after resin exposure (green), H₂O washes of resin (orange), elution off of resin with 10 mM adamantane carboxylic acid, 100 mM NH₄Cl, pH 7 (red).
Figure S3. SDS PAGE analysis of eluting fractions for a) TAMRA labeled myoglobin b) Oregon Green labeled myoglobin c) Alexa Fluor 350 labeled myoglobin d) Cascade Blue labeled myoglobin.

**SDS-PAGE quantification of modified protein recovery.** SDS-PAGE analysis was used to quantify the amount of modified protein recovered by the resin. The values in the “% fluorescence” column of Table 1 were calculated in the following manner:

Using the fluorescent images of the gels in Figure S3 as an example,

\[
\% \text{ fluorescence} = \frac{100 \times (\text{sum of fluorescence in elution lanes})}{(\text{sum of fluorescence in all lanes})}
\]

The fluorescence in each lane was quantified using ImageJ 1.34s, an image processing and analysis program available free from the NIH website (http://rsb.info.nih.gov/ij/download.html).
Figure S4. SDS-PAGE analysis of eluting fractions for Oregon Green-labeled myoglobin in the presence of a) 100 mM sodium phosphate buffer and b) 100 mM HEPES buffer. These samples correspond to entries 2 and 5 in Table S1.

**Protein binding in commonly used buffer solutions.** A solution of S1 was diluted into 4 different buffers (Tris, HEPES, NH₄OAc, and phosphate) to a final buffer concentration of 0.1 M, pH 7. These solutions were then added to centrifuge tubes containing resin 2a. The proteins were purified as described in the protein purification procedure. The eluting fractions were analyzed by SDS-PAGE and UV-vis spectroscopy (Figures S4 and S5).
Adamantane effects on protein binding. Resin 2a (71.4 mg) was suspended in 500 μL of 10 mM adamantane carboxylic acid/100 mM NH₄Cl pH 7 and then agitated for 15 min by a laboratory mixer. The solution was transferred to separate microspin columns and spun dry. A solution of S1 (250 μL) was diluted to 1 mL and added to the respective resin in a centrifuge tube. The heterogeneous solution was mixed for 5 min. Afterwards, the solution was transferred to microspin column and spun dry. The eluent was analyzed by UV-vis spectroscopy. The resin was then washed with 500 μL H₂O (2x). The collected washes were analyzed by UV-vis spectroscopy. A final wash was performed with 500 μL 10 mM adamantane carboxylic acid/100 mM NH₄Cl (2x). The collected washes were also analyzed by UV-vis spectroscopy (Figure S6).
Figure S6. UV-vis spectra of S1 exposed to resin 2a pre-washed with 10 mM adamantane carboxylic acid/100 mM NH$_4$Cl pH 7 solution.

MALDI-TOF MS analysis of the resin. To resin 2a (33.2 mg), 50 µL of myoglobin conjugate 4 and 250 µL of H$_2$O were added. After mixing for 5 min, the heterogeneous solution was spun dry in a microspin column. The resin was then washed with 500 µL of H$_2$O and a small portion of the dry resin was cocrystallized with sinapinic acid on a MALDI plate for analysis. The remaining resin was washed with 500 µL of 10 mM adamantane carboxylic acid/100 mM NH$_4$Cl pH 7 buffer. The aqueous fractions were then analyzed by MALDI-TOF MS (Figure S7).
Figure S7. MALDI-TOF spectrum of a) Oregon Green labeled myoglobin b) aqueous flow through solution c) cyclodextrin modified sepharose resin after exposing to a solution of Oregon Green labeled myoglobin d) the eluting solution from the 10 mM adamantane carboxylic acid/100 mM NH₄Cl pH 7 wash

Procedure for enrichment of modified trypsin digest fragments. 50 mL of a solution of myoglobin conjugate 6 (1 mg/mL) was combined with 50 µL of acetonitrile. DTT was added to the resulting solution to a final concentration of 10 mM. The protein was denatured at 65 ºC for 1 h. The denatured protein solution was diluted by the addition of 0.45 mL of 50 mM NH₄HCO₃, pH 7.8. Trypsin (Promega Sequencing Grade) was then added to the myoglobin solution in a 1:100 (w/w) ratio. After 5 hours of incubation at 37 ºC, the proteolysis solution was lyophilized overnight. The peptide mixture was dissolved in 200 µL of water and divided into two 100 µL portions. To one portion was added 5 mg of dry resin 2a. The heterogeneous mixture was agitated for 5 min before being transferred to an empty MicroSpinTM (Amersham) column and centrifuged at 14,500 rpm for 30 s. The dry resin, which appeared brown due to the capture of modified peptides, was washed twice with 200 µL aliquots of H₂O. The resin was then washed with one 100 µL aliquot of 10 mM adamantane carboxylic acid, 100 mM NH₄Cl, pH 7.
The brown color was transferred from the resin to the solution. Aliquots from before and after the resin capture procedure were both analyzed by LC-ESI MS (Figures S8).

**Figure S8.** LC-MS analysis of modified trypsin fragments from digestion of TAMRA modified myoglobin:

- **a** mass spectrum of trypsin fragments before resin capture. Starred masses represent unmodified trypsin digest fragments.
- **b** mass spectrum of trypsin fragments after elution from **2a** with adamantane-CO₂ solution. Modified N-terminal fragment (GLSDGEWQQVLNVWGK) expected m/z = 1115.5.

*mass spectrum of trypsin fragments before resin capture. Starred masses represent unmodified trypsin digest fragments. *mass spectrum of trypsin fragments after elution from **2a** with adamantane-CO₂ solution. Modified N-terminal fragment (GLSDGEWQQVLNVWGK) expected m/z = 1115.5.
Activity assay of resin captured chymotrypsinogen. After a typical resin capture procedure was performed on chymotrypsinogen modified with 5, the eluted fractions were assayed for chymotryptic activity. 20 uL of the released protein was treated with 2.9 uL of a 0.4 mg/mL trypsin solution in 50 mM acetic acid. The activation of the zymogen was allowed to proceed for 10 min at room temperature before the addition of 80 uL of 0.5 mM chymotrypsin substrate I, colorometric (Suc-GGF-pNA, Calbiochem 230912) in 50 mM Tris, 20 mM CaCl$_2$ buffer, pH 7.6. The hydrolysis of p-nitroaniline from the tripeptide substrate occurred at room temperature for 30 min. The progress of the hydrolysis reaction was followed by monitoring the absorbance at 390 nm (Figure S9).

**Figure S9.** Normalized spectra for chymotrypsin activity assay. Absorbance at 380 nm indicates the hydrolysis of p-nitroaniline from the assay substrate. The intact assay substrate has an absorbance maximum at 305 nm. Chymotrypsin shows similar levels of activity before (blue) and after (pink) resin capture.
Additional figures.

Figure S10. Complete recovery of unmodified myoglobin was obtained in the supernatant after exposure to resin 2a, confirming that the protein does not bind in the absence of the fluorescent label.

Figure S11. UV/vis spectra of modified protein resin capture: starting mixture (blue), flow through (green), water washes of resin (orange), elution with 10 mM adamantane carboxylic acid, 100 mM NH₄Cl, pH 7 (red). (a) Chymotrypsinogen modified with FITC. (b) Subtilisin Carlsberg modified with Oregon Green-succinimidyl ester.