Detection of Protease Activity Using a Fluorescence-Enhancement Globular Substrate

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ABSTRACT

Bovine serum albumin (BSA) highly derivatized with fluorescein isothiocyanate (FITC, isomer I) served as a fluorescent enhancement substrate to measure protease activity. In the native globular BSA structure, the fluorescence of the lysine-conjugated fluorescein moieties was quenched 98%. Proteolytic digestion of highly derivatized BSA with Pronase® resulted in fluorescence enhancement of 4300%. Both α-chymotrypsin and proteinase K yielded lower but similar fluorescence enhancement values of 2880% and 2800%, respectively. Digestion of the fluorescein-BSA substrate with trypsin, which required basic amino acids for activity, showed fluorescence enhancement of 1480% reflecting the fluorescein-lysine thiocarbamyl linkage. When derivatized substrate was pretreated with a thiol-reducing agent prior to incubation with proteases, a relatively small increase in fluorescence was noted relative to the untreated substrate except in the case of Pronase. The minimum sensitivity of proteolytic activity, based on a comparison of untreated and reduced FITC₂₅-BSA was 32 × 10⁻⁶ units for 1 ng proteinase K, 1 × 10⁻³ units for 1 ng α-chymotrypsin and 10 × 10⁻³ units for Pronase and trypsin (1 ng each). The fluorescence enhancement assay was suited for sensitive intensity measurements or as an endpoint assay to detect protease activity.

INTRODUCTION

The synthesis, production and purification of naturally occurring or genetically engineered recombinant proteins in industrial research and development, quality assurance and/or academic research laboratories often necessitates testing for intrinsic proteolytic activity. Contaminating protease activity directly impacts product or reagent stability and biological efficacy. Assays designed to test a broad spectrum of protease activities generally (i) require multiple steps including fractionation procedures (e.g., trichloroacetic acid [TCA] precipitation, sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE] or HPLC analytical assays), (ii) lack sensitivity and quantitation (e.g., casein-gel test) or (iii) necessitate generation of radioactive substrates with varying specific activities due to differential decay times. Such procedures are time-consuming, and when performed quantitatively, generally possess intrinsically high standard deviations.

Fluorometric assays for detection of protease activity have been developed as replacements for radioactive labels. For example, Twining (6) used fluorescein-labeled casein as a fluorescent substrate to detect protease activity. Proteolytic hydrolysis of the fluorescent substrate was measured using TCA precipitation to resolve small soluble peptides from non-hydrolyzed (or partially degraded) casein. Bolger and Checovich (2) adapted the Twining procedure to a fluorescence polarization format to detect enzyme-mediated generation of relatively small peptides. The principles governing fluorescence polarization measurements, including a direct correlation with the volume of a macromolecule, are consistent with requirements for a protease detection assay. However, fluorescence polarization (or anisotropy) measurements require the use of appropriate instrumentation and are limited to a relatively restricted quantitative scale that is based on the theory of polarization of macromolecules (8) and selection of a fluorescent probe with the appropriate fluorescence lifetime.

In this report a sensitive fluorometric intensity assay is described to detect protease activity. The assay is based on the development of a unique probe possessing important spectral and biochemical properties that facilitate the measurement of large increases in fluorescence intensity. Specifically, fluorescein-derivatized serum albumin served as a soluble protein substrate for sensitive detection of protease activity. The general principle inherent in the use of FITC₂₅-BSA as a substrate was that the densely conjugated fluorescein moieties rendered the substrate relatively nonfluorescent and significant fluorescence was generated upon enzymatic hydrolysis of the peptide backbone of bovine serum albumin (BSA). A fluorescence intensity-based assay requires only the basic fluorometer for instrumentation and provides relatively simple measurements that are (i) performed in a single-phase solution reaction mixture, (ii) performed without separation or transfer steps and (iii) sensitive because of significant increases in the quantum yield of fluorescence relative to the initial quenched state.

MATERIALS AND METHODS

Reagents

BSA and trypsin (bovine pancreas, 10,000 U/mg) were obtained from Sig-
ma Chemical (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC, isomer I [FITC (I)]) was purchased from Molecular Probes (Eugene, OR, USA) and proteinase K (Tririchardum album, 32 U/mg) was from Stratagene (La Jolla, CA, USA). Pronase® (Streptomyces griseus, 10 000 U/mg) and α-chymotrypsin (bovine pancreas, 1000 U/mg) were obtained from Calbiochem (La Jolla, CA, USA).

**Fluorescein Conjugation of Carriers**

BSA (10 mg/mL) was dissolved in water with an equal weight of K2CO3 to adjust the pH to approximately 10.5. FITC was added (2 mg/mL) and reacted at 37°C with mild stirring for 24 h in an amber bottle. The derivative product was purified using Dowex 1-x8 chromatography (Bio-Rad, Hercules, CA, USA) in water or dialyzed against water with frequent changes until no free fluorescein was evident by spectrophotometric analysis of the dialysate at 492 nm. Final products were analyzed for the degree of substitution (7), and an average value of 25 fluorescein residues per BSA molecule (67 kDa) was obtained.

**Physical Properties of FITC25BSA**

Molecular-sieve chromatography (ACA-34; Spectrum, Los Angeles, CA, USA) in a 100- × 2-cm column equilibrated in 0.1 M phosphate buffer, pH 8.0, was used to determine the relative molecular weights of native BSA and FITC25BSA. One-milliliter fractions were collected and optical densities determined at either 278 or 492 nm. SDS-PAGE was performed as described by Laemmli (5).

**Quantitation of Fluorescence Quenching of Fluorescein in FITC25BSA**

The FITC content in FITC-BSA was determined by absorption (scan 400–600 nm) in 0.1 M phosphate, pH 8.0, or Tris-HCl-CaCl2 buffer, pH 8.0, at room temperature. FITC (I) was dissolved in pH 8.0 buffer to the same optical density at 492 nm (λmax). The relative fluorescence intensities of the two preparations normalized to the same optical densities at 492 nm were determined in a spectrofluorometer with excitation at 480 nm and emission at 530 nm using a Corning Costar 3-69 cutoff filter (Cambridge, MA, USA).

The relative fluorescence properties of FITC25BSA in 10 mM Tris-HCl, pH 8.0, and 1 mM CaCl2 were examined under various conditions at room temperature: (i) untreated, (ii) incubated in 1% SDS, (iii) reduced with 1 mM diithiothreitol (DTE), (iv) reduced with 1 mM DTE and alkylation with 2 mM iodoacetic acid and (v) reduced with 1 mM DTE followed by addition of 1% SDS.

**Preservation of FITC25BSA as a Substrate**

Because fluorescein fluorescence in FITC25BSA was quenched 98%, it was important that the substrate was preserved and no degradation occurred during storage. The latter was essential because the protease-induced fluorescence-enhancement values were directly dependent on starting with a standard substrate in which the fluorescein fluorescence was quenched 98%. Storage of the substrate as a precipitate or slurry in 50% saturated ammonium sulfate (4°C) was determined to preserve the substrate for several months. When proteases were added to FITC25BSA samples in 50% saturated ammonium sulfate, no degradation was observed over several weeks. Since highly fluorescent proteolytic products derived from FITC25BSA proved soluble in 50% saturated ammonium sulfate, this storage procedure served as a retardant of proteolytic activity and as a general purification step. Prior to use of the substrate, a specific volume was withdrawn from the precipitated stock and centrifuged at 10000 rpm (16000× g) for 10 min at 4°C. The precipitate (FITC25BSA) was dissolved to a known concentration (usually 1.0 mg/mL) and used immediately in the protease assay. The substrate concentration was determined at 492 nm (18 OD U/mg protein).

**In Vitro Protease Digestion of FITC25BSA Monitored with Time**

FITC25BSA was examined for susceptibility to enzymatic proteolysis under various conditions: (i) untreated, (ii) thiol reduction with 1 mM DTE and (iii) four different proteases. Each reaction was performed in sequential steps at room temperature. First, 1.0 mL of the Tris-HCl-CaCl2, pH 8.0, buffer was added to a fluorescence cuvette, and the background fluorescence was measured in a fluorometer (Amino-Bowman; SLM Instruments, Urbana, IL, USA). One microliter or 1 microgram of FITC25BSA (untreated or reduced) was added and the fluorescence measured. The cuvette was washed and the experimental enzymatic reaction mixture constituted. FITC25BSA (treated or untreated) was added (1 μg in 100 μL of buffer) to the cuvette. Nine hundred microliters of Tris-HCl-CaCl2 containing the protease were flushed into the cuvette through a small hole in the cuvette chamber lid. Instant flushing based on a 9:1 vol/vol ratio resulted in total mixing in 1.0 s. Fluorescence units were monitored continuously from zero time through 10 min.

Increased fluorescence with time was monitored using an Amino-Bowman spectrophotofluorometer at room temperature. Samples were excited at 480 nm, and fluorescence emission was monitored through a 510 nm 3-69 cutoff filter at 530 nm. Fluorescence units were monitored continuously by direct-feed digital readout to an IBM® PC. Data were plotted and analyzed using the computer program OFFRATES Version 1.2 (Interactive Software, Urbana, IL, USA). The OFFRATES software was written to determine dissociation constants based on increased fluorescence with time (1.4).

**Endpoint Assay for Detection of Protease Activity**

The previously described kinetic assay was modified for endpoint determinations as follows: FITC25BSA (1 μg) was added to 100 μL of the Tris-HCl-CaCl2 buffer after the initial background fluorescence had been measured and adjusted to 10 or less fluorescence units. Various concentrations of enzyme samples were added to the buffer and the final volumes normalized to 900 μL. The total volume was flushed into the cuvette containing the fluorescence substrate for instantaneous mixing, and the reaction was allowed to proceed for the desired time (e.g., 10 min). The final fluorescence-intensity reading was compared to the initial reading as a measure of prote-
RESULTS

Properties of Fluorescein-Conjugated BSA

FITC-BSA used throughout these studies had an average density of 25 fluorescein moieties per BSA molecule. Of the 59 lysine residues and 1 α-amino terminal residue constituting BSA, an average of 41.6% was covalently conjugated with FITC (I). Thus, fluorescein was substituted on a density basis of 1 FITC per 23.4 residues (585 amino acid residues in BSA).

Highly substituted FITC-BSA serially diluted from 1.0 mg/mL to 1.0 µg/mL in a neutral buffer showed that the fluorescein fluorescence was quenched 98% at all concentrations tested. This indicated that the observed fluorescein autoquenching (98%) was a function of the density of fluorescein moieties per globular BSA molecule and not protein concentration. A 98% fluorescence-quenching value also appeared to be a function of the three-dimensional and multiple domain structure of globular BSA. The latter was evident based on various treatments of the FITC<sub>25</sub>BSA substrate molecule. As shown in Table 1, when the fluorescein-conjugated molecule was treated with a thiol-reducing agent (DTE), a modest 15%–16% increase in fluorescence was measured relative to the untreated molecule. Similarly, when nonreduced conjugate was treated with 1% SDS, only a 3% increase was observed. However, thiol reduction followed by alkylation or treatment with 1% SDS resulted in significantly increased fluorescence of 28.7% and 159.7%, respectively. Thus, unfolded or extended BSA (reduced domain structure) produced a degree of spacing between the covalently attached fluorescein molecules to partially alleviate the fluorescence quenching observed in the folded protein.

Physical Properties of FITC<sub>25</sub>BSA and Proteolytic Digests

Using molecular-sieve chromatography (ACA 34), native BSA was analyzed relative to FITC<sub>25</sub>BSA. On a column (100 × 2 cm) equilibrated in the pH 8.0 buffer and calibrated with molecular weight markers in the range of 150 kDa (IgG) to 20 kDa (trypsin inhibitor), the FITC<sub>25</sub>BSA showed a molecular weight of 76 to 77 kDa relative to BSA at 67 kDa based on the volume of elution plotting the fraction with maximum optical density. There was no evidence of aggregates larger than 77 kDa. The elution pattern reflected an increased molecular weight due to the conjugated fluorescein moieties (based on an average of 25 groups). If the BSA molecule had unfolded to any significant degree because of extensive derivatization with FITC, a long axis would have preferentially influenced the elution profile and FITC<sub>25</sub>BSA would have exceeded the observed value of 77 kDa. The FITC<sub>25</sub>BSA elution peak was distinctly sharp and symmetrical indicating a relatively homogeneous molecular weight. The latter suggested that 25 fluoresceyl residues was a representative value with limited variation around the average. Highly fluorescent peptides of FITC<sub>25</sub>BSA, obtained after a 10-min digestion with either Pronase or proteinase K, applied to the column were significantly retarded in their elution and displayed low molecular weights (data not shown). There was no evi-

### Table 1. Relative Fluorescence Properties of the FITC<sub>25</sub>BSA Substrate Under Various Treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Increase in Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0%</td>
</tr>
<tr>
<td>Nonreduced and SDS (1%)</td>
<td>3.0%</td>
</tr>
<tr>
<td>Reduced (1 mM DTE)</td>
<td>15.7%</td>
</tr>
<tr>
<td>Reduced and alkylated&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.7%</td>
</tr>
<tr>
<td>Reduced and SDS&lt;sup&gt;d&lt;/sup&gt;</td>
<td>159.7%</td>
</tr>
</tbody>
</table>

<sup>a</sup>FITC<sub>25</sub>BSA preparation was diluted to 1.0 µg/mL in 10 mM Tris-Cl, pH 8.0, and 1 mM CaCl<sub>2</sub> for fluorescence measurements. The reported fluorescence values were stable and independent of time of incubation.

<sup>b</sup>Based on triplicate measurements with three separate formulations in 10 mM Tris-Cl, pH 8.0, and 1 mM CaCl<sub>2</sub>. The background fluorescence value was then normalized to 0%.

<sup>c</sup>Reduction with 1 mM DTE and 2 mM iodoacetic acid.

<sup>d</sup>Thiol reduction with 1 mM DTE and 1% SDS.
idence of undigested BSA after a 10-min digestion period with either protease. These results were verified using SDS-PAGE analyses with appropriate molecular weight markers.

**Proteolysis Studies with FITC₂₅BSA**

Figure 1 shows the comparative results of proteolysis studies with untreated FITC₂₅BSA incubated with Pronase, α-chymotrypsin, proteinase K and trypsin on a weight basis. Digestion with Pronase resulted in a fluorescence enhancement of 4300%. Digestion with α-chymotrypsin and proteinase K yielded lower but similar values of 2880% and 2800%, respectively. In contrast, digestion with trypsin yielded a fluorescence increase of 1480%. The lower yield of fluorescence enhancement upon trypsin digestion was consistent with the specificity of FITC conjugation for the ε-amino groups of lysine residues, which along with arginine residues are required for trypsin activity.

The 4300% fluorescence enhancement upon digestion of FITC₂₅BSA with Pronase was a function of the degree of fluorescein substitution and the 98% fluorescence-quenching value. Similar results were obtained with FITC₂₂BSA suggesting that when the density of fluorescein groups is high (>20), the fluorescence quenching is similar and therefore the fluorescence-enhancement values based on proteolysis are nearly equivalent. Further evidence for this was apparent when Pronase digestion of FITC₁₀BSA yielded only a 450% fluorescence enhancement, directly reflecting an initial fluorescein fluorescence-quenching value of 80%. Finally, fluorescein moieties constituting FITC₃BSA were not quenched, and Pronase digestion yielded no fluorescence enhancement.

When a similar experiment was repeated under the same conditions with thiol-reduced FITC₂₅BSA, enhanced results were obtained with all proteases tested except Pronase, which appeared to yield complete digestion of the untreated substrate. Proteinase K was selected for these studies as a protease showing average activity in the assay.

<table>
<thead>
<tr>
<th>FITC₂₅BSAᵃ</th>
<th>Enzyme</th>
<th>Percent Increase in Fluorescenceᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Untreated</td>
<td>proteinase K</td>
<td>2800 (±53)</td>
</tr>
<tr>
<td></td>
<td>trypsin</td>
<td>1480 (±38)</td>
</tr>
<tr>
<td>B. Reduced</td>
<td>proteinase K</td>
<td>3300 (±57)</td>
</tr>
<tr>
<td></td>
<td>trypsin</td>
<td>1740 (±42)</td>
</tr>
<tr>
<td></td>
<td>proteinase K + trypsinᶜ</td>
<td>3450 (±59)</td>
</tr>
</tbody>
</table>

ᵃIn all reactions, 1.0 µg of FITC₂₅BSA was reacted with 100 ng of the protease.
ᵇAll fluorescence enhancement values are reported after an incubation period. Standard deviations for triplicate measurements are indicated in parentheses.
ᶜIn the combined experiment, 1.0 µg of FITC₂₅BSA was reacted with a mixture of 100 ng of proteinase K and 100 ng trypsin.
Trypsin was selected as representative of an inefficient protease in terms of the substrate. As shown in Table 2, incubation of the reduced substrate with proteinase K resulted in an increase in fluorescence to 3300%. Incubation with trypsin with the reduced substrate resulted in a 1740% increase in fluorescence. Finally, simultaneous incubation with both proteinase K and trypsin at equal molar concentrations produced a 3450% increase in fluorescence. All values were reported at an incubation time of 10 min.

When the reduced FITC-BSA substrate was alkylated (iodoacetamide), a reduction in fluorescence-enhancement values was noted with the proteases. Thus, studies based on alkylated substrate were not pursued in detail.

Sensitivity of Protease Detection

Untreated and reduced FITC_{25}BSA were compared as substrates in enzyme-sensitivity studies. As shown in Figure 2, incubation of 1.0 µg of FITC_{25}BSA (reduced or untreated) with varying concentrations of proteinase K at room temperature showed that a significant increase (ca. 200%) in fluorescence could be obtained with 1.0 ng of proteinase K in either form of the substrate in 10 min. The sensitivity of proteolytic activity for the 4 proteases was 32 \times 10^{-6} \text{ units for proteinase K, 1 \times 10^{-3} units for } \alpha\text{-chymotrypsin and 10 \times 10^{-3} units for Pronase and trypsin (all at 1 ng).}

DISCUSSION

Use of a globular substrate, such as the BSA protein, provided specific advantages over protein substrates devoid of a higher-ordered structure. As shown in Table 1, 98% fluorescence quenching of fluoresceyl residues was directly attributable to the homofluorophores being brought sufficiently close in the context of the three-dimensional BSA globular structure. The observed 98% fluorescence quenching within FITC_{25} BSA was undoubtedly due to auto-quenching or fluorescence energy transfer of the excited-state energy between neighboring fluorescein molecules covalently attached to BSA. Rates of energy transfer depend upon (i) the extent of overlap of the emission and absorption spectra characteristic of fluorescein directly correlating with a relatively small Stoke’s shift, (ii) the relative orientation of the donor and acceptor transition dipoles and (iii) the distance between neighboring fluorescein moieties on the same BSA molecule. These requirements are accounted for in Förster’s mathematical treatment of energy transfer processes (3). Results of molecular-sieve chromatography showed that despite the additional molecular weight attributable to 25...
FITC residues (calculated increase of 9.7 kDa) native BSA and conjugated BSA eluted at a volume consistent with the appropriate molecular weight (in kDa). These data indicated that the higher-ordered structure was largely intact which was further verified by protein treatment analyses presented in Table 1. This was verified through observations that when the untreated FITC$_{25}$BSA molecule was incubated with 1% SDS only a modest increase (3.0%) in fluorescence was noted (Table 1). This suggested that the multiple domains characteristic of BSA contain multiple FITC groups which remain quenched. It was further shown that after thiol reduction, the conjugated BSA molecule retained most of the native configuration (Table 1). However, if the reduced protein was placed in 1% detergent (SDS), the molecule unfolded and a significant increase (159.7%) in fluorescence was noted. It is important to note that the substrate characterization and protease reactions were performed at room temperature. Temperature, especially elevated temperatures, may be a variable influencing the degree of fluorescence quenching in untreated FITC-BSA substrate.

The importance of using a protein carrier with a higher-ordered structure was verified in the proteolysis experiments. Figure 1 showed significant increases in fluorescence with Pronase, proteinase K, α-chymotrypsin and trypsin. Pronase and proteinase K exhibit similar specificities in their proteolytic activity cleaving at ser/thr residues. α-Chymotrypsin specifically hydrolyzes carboxyl groups of aromatic l-amino acids (i.e., try/tyr, phe residues). Even in the case of trypsin, which requires basic amino acids (lys/arg) for activity, a significant increase of 1480% was measured. Thus, despite a lower percentage of fluorescence enhancement relative to Pronase, proteinase K and α-chymotrypsin, the assay detected protease activity specific for basic amino acids. Reduced FITC$_{25}$BSA proved to be a more sensitive probe when using proteases other than Pronase. The difference in Pronase activity appeared to correlate with the fact that Pronase digestion of untreated FITC$_{25}$BSA yielded the maximum fluorescence enhancement from the 98% quenched state. Therefore thiol-reduction does not facilitate any further susceptibility with Pronase. Proteinolysis of reduced FITC$_{25}$BSA with proteinase K showed an increase in fluorescence of 3300% and 1740% with trypsin. However, when the two enzymes were combined, a fluorescence enhancement of 3450% was observed. Thus, the activity of the two enzymes combined was not additive. The exceptional degree of fluorescence enhancement can be accounted for in the protease-mediated proteolysis process that results in simultaneous alleviation of the distance (spacing) and orientation factors involved in the energy transfer and quenching processes of the covalently attached fluorescein moieties.

The FITC-BSA substrate fulfills many essential requirements for a sensitive protease assay. The sensitivity of detection of protease activity at 1 ng ranged from $32 \times 10^{-6}$ units to $10 \times 10^{-3}$ units with intermediate sensitivity fluorometer settings (Figure 2). Thus, it is reasonable to assume that the fluorescent assay can be modified to be even more sensitive than described in this report. Although this report was based on FITC$_{25}$BSA as a model substrate, fluorescein densities of 15–25 groups per BSA molecule all provide highly quenched substrates for sensitive protease detection assays. In addition, the assay is amenable to fast and accurate measurements that do not require sophisticated instrumentation. Finally, the assay can be extended to include design of synthetic polypeptides that include a known specific proteolytic cleavage site (e.g., amino acid sequence for signal peptidase activity) surrounded by quenched fluorescein-derivatized lysyl residues. Such substrates could be used to search for specific peptidase activities in various situations.

REFERENCES


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