# Purification, Characterization, and Amino Acid Composition of Rabbit Pulmonary Bleomycin Hydrolase†

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ABSTRACT: Bleomycin (BLM) hydrolase, a protective enzyme that inactivates the antitumor antibiotic BLM, was purified (6000-fold) to homogeneity from rabbit lungs by DEAE-Sephacel, phenyl-Sepharose chromatography, BLM–Sepharose affinity chromatography, and Mono Q fast protein liquid chromatography. The enzyme had a molecular mass of 250 000 daltons as demonstrated by Superose gel permeation chromatography and polyacrylamide gel electrophoresis (PAGE) under native conditions. Sodium dodecyl sulfate–PAGE revealed a single band of 50 000 daltons, suggesting a pentameric structure. The $K_m$ and $V_{max}$ for BLM $A_2$ were 1.3 mM and 5.9 $\mu$mol $mg^{-1}$ $h^{-1}$, respectively. BLM hydrolase activity was labile, had a half-life of 25 min at 56 °C, 10 h at 37 °C, and 5 days at 4 °C, and was stabilized by 2 mM dithiothreitol. The enzyme had a pH optimum of 7.0–7.5 and was inhibited by *N*-ethylmaleimide, leupeptin, puromycin, and divalent cations such as $Cu^{2+}$, $Cd^{2+}$, $Zn^{2+}$, and $Co^{2+}$ but was unaffected by chelating agents. On the basis of Mono P chromatofocusing chromatography, three isoforms of BLM hydrolase (apparent p*I*'s of 5.3, 4.5, and 4.3) were present in rabbit pulmonary cytosol. The elution profiles of BLM hydrolase from phenyl-Sepharose and Mono P chromatofocusing indicated that this enzyme is hydrophobic and acidic. This was confirmed by amino acid composition analysis, which demonstrated that 48% of the total amino acids of bleomycin hydrolase were hydrophobic and 37% were acidic.

Bleomycin (BLM) is an antitumor antibiotic routinely used in the treatment of human cancers (Umezawa, 1980, 1979). Although BLM lacks the bone marrow toxicity frequently seen with anticancer agents, it can induce lethal pulmonary fibrosis (Carter, 1985).

BLM hydrolase is a ubiquitous cytosolic enzyme that converts BLM to its inactive metabolite, deamido-BLM, by hydrolyzing the carboxamide bond of the $\beta$-aminoalaninamide moiety on the BLM molecule to a carboxylic acid (Umezawa, 1980, 1979). This metabolic inactivation is believed to protect cells from BLM-induced toxicity (Lazo & Humphreys, 1983). Both in vivo and in vitro studies demonstrated that tissues with high levels of BLM hydrolase are resistant to damage produced by BLM while organs with low levels of this enzyme, such as lung and skin, are targets for injury (Lazo & Humphreys, 1983; Umezawa, 1980). Furthermore, BLM hydrolase appears to have an important role in the responsiveness of tumors to BLM (Umezawa, 1980). Tumors that are resistant to BLM have been reported to have high levels of BLM hydrolase whereas those that are sensitive to BLM had less of this enzyme activity (Umezawa et al., 1972).

Although BLM hydrolase activity was detected in tissue homogenates from a wide variety of organs over a decade ago (Umezawa et al., 1972), little is known about the biochemical characteristics of this biologically relevant enzyme. Umezawa et al. (1974) developed a bioassay to measure BLM hydrolase activity and partially (27-fold) purified this enzyme. It was found to be cytosolic with a molecular mass greater than 25 000 daltons. The authors suggested that BLM hydrolase was an aminopeptidase B like enzyme on the basis of its ability to cleave substrates with arginine or lysine but not leucine on their amino termini and the ability of BLM $B_2$ to competitively inhibit the aminopeptidase B reaction. These studies, however, were carried out on a partially purified preparation, and further purification and characterization of BLM hydrolase were hindered by the highly unstable character of this enzyme and the lack of a rapid and sensitive assay (Umezawa et al., 1974). Sebti and Lazo (1987) recently developed a high-speed liquid chromatography assay for BLM hydrolase and used this system coupled to fast protein liquid chromatography to demonstrate that BLM hydrolase was distinct from pulmonary aminopeptidases. In this paper we describe the purification to homogeneity, the biochemical characterization, and the amino acid composition of this BLM-inactivating enzyme.

## Materials and Methods

*Materials.* BLM $A_2$[1] was either prepared from Blenoxane (Bristol Laboratories, Syracuse, NY) as described by Lazo et al. (1984) or obtained as a gift from Dr. S. Hecht (University of Virginia). BLM $dA_2$ was enzymatically synthesized from metal-free BLM $A_2$ as described by Lazo and Humphreys (1983). Leupeptin, glutathione, and bestatin were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). NEM and puromycin were purchased from Calbiochem Brand Biochemicals (San Diego, CA). [Met]-enkephalin, arginine-$\beta$-naphthylamide, [Lys]bradykinin, EDTA, EGTA, PMSF, sucrose, $CdSO_4$, $FeSO_4$, $MnCl_2$, and $MgCl_2$ were purchased from Sigma Chemical Co. (St. Louis, MO). Cysteine hydrochloride was purchased from Fisher Scientific Co. (Fairlawn, NJ). Glycerol, $CuSO_4$, $ZnSO_4$, $CoCl_2$, $Na_2Cr_2O_7$, and $CaCl_2$ were purchased from Mal-

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[1] Abbreviations: BLM $A_2$, bleomycin $A_2$; BLM $dA_2$, deamido-bleomycin $A_2$; DTT, dithiothreitol; HSLC, high-speed liquid chromatography; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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linckrodt, Inc. (Paris, KY). 2-Mercaptoethanol was purchased from Bio-Rad (Richmond, CA). DEAE-Sephacel, phenyl-Sepharose, epoxy-activated Sepharose 6B, and Polybuffer 74 were purchased from Pharmacia (Piscataway, NJ). The following columns were also purchased from Pharmacia: Mono Q HR 5/5 (0.5 cm × 5 cm), Mono P HR 5/20 (0.5 cm × 20 cm), Superose 6 HR 10/30 (1 cm × 30 cm), and Superose 12 HR 10/30 (1 cm × 30 cm). PM-30 ultrafiltration membranes were purchased from Amicon (Lexington, MA).

*High-Speed Liquid Chromatography (HSLC) Assay for BLM Hydrolase Activity.* Aliquots of BLM hydrolase preparations from the various purification and characterization steps were incubated with BLM $A_2$ at 37 °C in a shaking water bath. All reaction mixtures were carried out in 10 mM Tris buffer (pH 7.5) in a final volume of 250 μL unless otherwise specified. The final BLM $A_2$ concentration and the time of incubation are indicated in the corresponding Materials and Methods section and/or in the legends to the figures and tables. After incubation at 37 °C, 200 μL of methanol and 50 μL of 7.5 mM $CuSO_4$ were added to the reaction mixture. The samples were then vortexed and spun at 12000 *g* for 10 min, and 200 μL of the supernatant was injected into the HSLC system as described by Sebti and Lazo (1987). The amount of BLM $dA_2$ generated was calculated from the relative fluorescence of known amounts of BLM $dA_2$ standards analyzed by HSLC.

*Preparation of Rabbit Lung Cytosol.* Sixty lungs (Pel-Freez, Rogers, AR) from New Zealand white male rabbits were homogenized at 4 °C in 10 mM Tris, pH 7.5, buffer as described by Lazo et al. (1984). The homogenate was then centrifuged (20800*g*) at 4 °C for 45 min and the supernatant centrifuged (105000*g*) at 4 °C for 60 min. The 105000*g* supernatant fractions were then frozen at −70 °C and thawed as needed.

*DEAE-Sephacel Chromatography.* The DEAE-Sephacel gel (200 mL) was washed with 4 L of 20 mM Tris/0.2 mM DTT/1 M NaCl, pH 7.5 (buffer B), followed by 4 L of the same buffer without NaCl (buffer A). The 105000 *g* supernatant fraction (470 mL, 64.5 mg of protein/mL) from rabbit lung homogenates was then added to the washed DEAE-Sephacel gel and shaken for 30 min at room temperature. The gel was then centrifuged (500*g*) at 4 °C for 5 min, and the supernatant was removed. This was followed by washing the gel 6 times with 800 mL of buffer A each time. An aliquot (230 μL) of each supernatant was incubated with BLM $A_2$ (60 μM) for 90 min at 37 °C and assayed for BLM hydrolase by HSLC as described above. The absorbance at 280 nm of each supernatant fraction (diluted 1:100) was measured. The DEAE-Sephacel gel was then packed into a column (2.6 cm × 40 cm, Pharmacia) that was attached to an FPLC system (Pharmacia) equipped with two P-500 pumps, an LCC 500 controller, and a UV-1 monitor with a 280-nm filter. The column was eluted at 0.5 mL/min with a 200-mL linear gradient of 0.1–0.3 M NaCl in buffer A followed by 100 mL of 0.3 M NaCl in buffer A and 100 mL of 1 M NaCl in buffer A. Ten microliters of each 10-mL fraction was diluted (1:100), and the absorbance at 280 nm was measured. An aliquot (20 μL) of each fraction was incubated with BLM $A_2$ (60 μM) for 90 min at 37 °C and assayed for BLM hydrolase activity by HSLC as described above.

*Phenyl-Sepharose CL-4B Chromatography.* The DEAE-Sephacel column fractions with BLM hydrolase activity were pooled, and 40% (16 mL) of the total volume was diluted up to 28 mL with 20 mM Tris/0.2 mM DTT/1.7 M $(NH_4)_2SO_4$ buffer, pH 7.5. The 28-mL sample (23.8 mg of protein/mL) was loaded onto a phenyl-Sepharose CL-4B column (1.6 cm × 40 cm) previously washed with 20 mM Tris/0.2 mM DTT/0.85 M $(NH_4)_2SO_4$, pH 7.5 (starting buffer). The column was attached to the FPLC system described above and eluted at 0.5 mL/min with 100 mL of starting buffer, followed by a 400-mL linear gradient of 0.85–0 M $(NH_4)_2SO_4$ in 10 mM Tris/0.2 mM DTT, pH 7.5, and 200 mL of 10 mM Tris/0.2 mM DTT, pH 7.5. Ten-milliliter fractions were collected, and the absorbance at 280 nm was monitored. An aliquot (20 μL) of each fraction was incubated with BLM $A_2$ (30 μM) for 120 min at 37 °C and assayed for BLM hydrolase activity by HSLC as described above.

*Blenoxane–Sepharose 6B Affinity Chromatography.* Blenoxane–Sepharose 6B was prepared by covalently coupling clinical grade BLM (Blenoxane) (132 mg) to epoxy-activated Sepharose 6B (10 g) as described by Sebti and Lazo (1987). The gel was then poured into a column (1.6 cm × 20 cm) and equilibrated with 10 mM $Na_2HPO_4$, pH 7.2 (starting buffer). The BLM hydrolase fractions from the phenyl-Sepharose column were pooled, and an aliquot (11 mL, 1.7 mg of protein/mL) was concentrated to 1 mL with a PM-30 ultrafiltration membrane in an Amicon concentrating chamber. The sample was diluted with 9 mL of starting buffer and concentrated to 1 mL. The process was repeated, and the final concentrate (4 mL, 4.8 mg of protein/mL) was loaded onto the Blenoxane–Sepharose column attached to the FPLC system. The column was then eluted at 0.5 mL/min with 90 mL of starting buffer, a 150-mL linear gradient of 0–0.2 M NaCl in starting buffer, and 50 mL of 0.5 M NaCl in the same buffer. The absorbance at 280 nm was monitored, fractions of 5 mL were collected, and an aliquot (50 μL) of each fraction was incubated with BLM $A_2$ (60 μM) for 60 min at 37 °C and assayed for BLM hydrolase activity by HSLC as described above.

*Mono Q Chromatography.* Column fractions from the Blenoxane–Sepharose 6B affinity column containing BLM hydrolase activity were pooled, and an aliquot (15 mL, 0.14 mg of protein/mL) was concentrated to 1 mL with a PM-30 ultrafiltration membrane. The concentrate was diluted up to 10 mL with 10 mM Tris, pH 7.5 (buffer A), and concentrated to 1 mL. This process was repeated, and the final concentrate (2 mL, 0.98 mg of protein/mL) was filtered through a 0.2 μm pore size ACRO filter and loaded onto a Mono Q anion-exchange column previously equilibrated with buffer A. The column was then eluted at 1 mL/min with 3 mL of buffer A, 7 mL of buffer A containing 0.1 M NaCl, and a 60-mL linear gradient of 0.1–0.25 M NaCl in buffer A followed by 5 mL of 0.4 M NaCl in buffer A, 10 mL of 1.0 M NaCl in buffer A, and 5 mL of buffer A. Fractions of 1 mL were collected, and the absorbance at 280 nm was monitored. An aliquot (50 μL) of each fraction was incubated with BLM $A_2$ (60 μM) for 12 h at 37 °C and assayed for BLM hydrolase activity by HSLC as described above.

*Mono P Chromatography.* The phenyl-Sepharose column fractions with BLM hydrolase activity were pooled, and an aliquot (4 mL, 1.7 mg/mL) was concentrated to 1 mL with a PM-30 ultrafiltration membrane. The concentrate was diluted (1:10) with 25 mM methylpiperazine buffer, pH 5.7, and concentrated to 2 mL (3.5 mg of protein/mL). The sample was filtered through a 0.2 μm pore size ACRO filter and loaded onto a Mono P chromatofocusing column previously equilibrated with 25 mM methylpiperazine, pH 5.7. The column was then eluted at 0.5 mL/min with 3 mL of 25 mM methylpiperazine, pH 5.7, followed by 30 mL of Polybuffer 74, pH 4. The absorbance at 280 nm was monitored, fractions

of 0.5 mL were collected, and an aliquot (50 μL) of each fraction was incubated with BLM $A_2$ (60 μM) for 12 h at 37 °C and assayed for BLM hydrolase activity by HSLC as described above.

*Tandem Superose 6 and 12 Chromatography.* To separate BLM hydrolase from other proteins on the basis of molecular size properties, we found it necessary to design a tandem of gel permeation columns. A Superose 6 column (1 cm × 30 cm) was connected in tandem to a Superose 12 column (1 cm × 30 cm). Both columns were then attached to the FPLC system described above. The Superose 6 column top was connected to the injection valve of the FPLC system, and the bottom of the Superose 12 column was connected to the UV-1 monitor of the FPLC system. The columns were then equilibrated with 150 mL of eluting buffer (50 mM $Na_2HPO_4$/150 mM NaCl, pH 7.2). The BLM hydrolase fractions from the phenyl-Sepharose column were pooled, and an aliquot was concentrated with a PM-30 ultrafiltration membrane in an Amicon concentrating chamber. An aliquot (200 μL, 4.5 mg of protein/mL) was filtered through a 0.2 μm pore size ACRO filter and loaded onto the tandem Superose 6 and 12 columns. The sample was eluted at 0.1 mL/min with 57.5 mL of eluting buffer, and the absorbance at 280 nm was monitored. Fractions of 0.5 mL were collected, and an aliquot (50 μL) was incubated with BLM $A_2$ (60 μM) for 6 h at 37 °C and assayed for BLM hydrolase activity by HSLC as described above.

*Determination of pH Optimum of BLM Hydrolase.* The BLM hydrolase fractions from the phenyl-Sepharose column were pooled, and aliquots (20 μL) were incubated with BLM $A_2$ (60 μM) in the presence of 210 μL of different buffers at various pHs. The samples were incubated at 37 °C for 2 h, and the BLM hydrolase activity of each sample was measured by HSLC as described above. The following buffers were used for the specified pH ranges: 50 mM ammonium formate for samples at pH 3.0, 3.5, and 4.0; 50 mM acetic acid for samples at pH 4.5, 5.0, and 5.5; 50 mM sodium phosphate for samples at pH 6.0, 6.5, and 7.0; 20 mM Tris for samples at pH 7.5 and 8.0; 50 mM diethanolamine for samples at pH 8.5, 9.0, 9.5, and 10.0. All buffers contained 0.2 mM DTT.

*Polyacrylamide Gel Electrophoresis (PAGE).* SDS–PAGE was carried out in 10% gels according to the method of Laemmli (1970). Native PAGE was carried out in 7.5% gels at 4 °C under identical conditions as SDS–PAGE except that SDS and 2-mercaptoethanol were omitted from all solutions. Gels were routinely stained with Coomassie Brilliant Blue. The molecular weight of the purified enzyme was determined by graphic comparison to a commercial set of standard proteins as specified in the figure legends.

*Amino Acid Composition Analysis.* The BLM hydrolase fractions from the Mono Q column were pooled, and an aliquot was hydrolyzed with 6 N HCl and 0.2% phenol at 110 °C for 16 h. The resulting amino acid mixture was analyzed by a Beckman 121M system as described preivously by Stone and Williams (1986).

*Kinetics Studies.* The BLM hydrolase fractions from the Mono Q column were pooled, and aliquots of 20 μL were incubated with various concentrations (0.35, 0.70, 1.0, 1.4, and 2.8 mM) of BLM $A_2$ for 30 min at 37 °C. The samples were then assayed for BLM hydrolase activity and the kinetic parameters determined as described by Lazo et al. (1984).

*Inhibition Studies.* Aliquots (20 μL, 1.7 mg/mL) from the BLM hydrolase fractions from the phenyl-Sepharose column were preincubated with various concentrations, specified in the tables, of the putative inhibitors for 5 min at room temperature. The samples were then incubated with BLM $A_2$ (60 μM) for 60 min at 37 °C and assayed for BLM hydrolase activity by HSLC as described above.

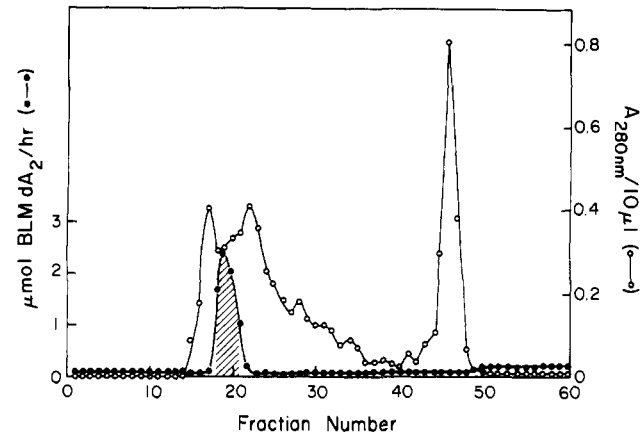


FIGURE 1: DEAE-Sephacel anion-exchange chromatography. An aliquot of the 105000*g* supernatant from rabbit lung homogenates was chromatographed on DEAE-Sephacel as described under Materials and Methods. An aliquot (10 μL) of each fraction was diluted (1:100), and its absorbance at 280 nm (open circles) was measured. BLM hydrolase activity (closed circles) was assayed as described under Materials and Methods.

*Stability Studies.* Aliquots (20 μL, 1.7 mg of protein/mL) from the fractions containing BLM hydrolase activity obtained from the phenyl-Sepharose column were incubated for various lengths of time at different temperatures (4, 37, and 56 °C). The samples were then incubated with BLM $A_2$ (60 μM) for 60 min at 37 °C and assayed for BLM hydrolase activity by HSLC as described above.

## RESULTS

*Purification of BLM Hydrolase.* An aliquot of the 105000*g* supernatant fractions from rabbit lung homogenate was incubated with DEAE-Sephacel gel. Unbound material (75% of total) was removed by several washes with Tris buffer as described under Materials and Methods. No BLM hydrolase activity was detected in these washes. The DEAE-Sephacel gel was then packed into a column and eluted with a linear gradient of NaCl as described under Materials and Methods. BLM hydrolase was eluted with 0.21 M NaCl in Tris buffer (Figure 1, closed circles), and the fractions with BLM hydrolase activity (hatched area) were pooled. Approximately half of this sample was loaded onto a phenyl-Sepharose column and eluted with decreasing $(NH_4)_2SO_4$ concentrations in Tris buffer. A large proportion of the total protein was eluted as the concentration of $(NH_4)_2SO_4$ decreased from 0.85 to 0 M (Figure 2). There was no detectable amount of BLM hydrolase activity in this area of the chromatogram (Figure 2, closed circles). BLM hydrolase was eluted with Tris buffer without $(NH_4)_2SO_4$, and the fractions with BLM hydrolase activity (Figure 2, hatched area) were then pooled. An aliquot was concentrated, loaded onto a Blenoxane–Sepharose affinity column, and eluted with various NaCl concentrations in $Na_2HPO_4$ buffer. BLM hydrolase activity (Figure 3, closed circles) was resolved from the bulk of the proteins and eluted at 0.22 M NaCl in phosphate buffer. The BLM hydrolase fractions (Figure 3, hatched area) were then pooled, and an aliquot was concentrated, injected into an FPLC Mono Q column, and eluted with various NaCl concentration gradients in Tris buffer. Upon elution of the column with a shallow NaCl linear gradient in Tris buffer, BLM hydrolase (Figure 4, closed circles) was resolved in a sharp peak with 0.23 M NaCl, and the fractions with BLM hydrolase activity (Figure

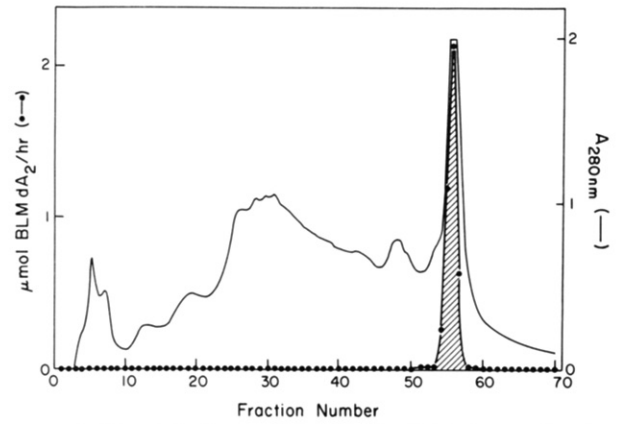


FIGURE 2: Phenyl-Sepharose CL-4B hydrophobic interaction chromatography. The BLM hydrolase fractions from the DEAE-Sephacel column (Figure 1, hatched area) were pooled, and an aliquot was chromatographed on a phenyl-Sepharose column and analyzed for proteins (solid line) and BLM hydrolase activity (closed circles) as described under Materials and Methods.

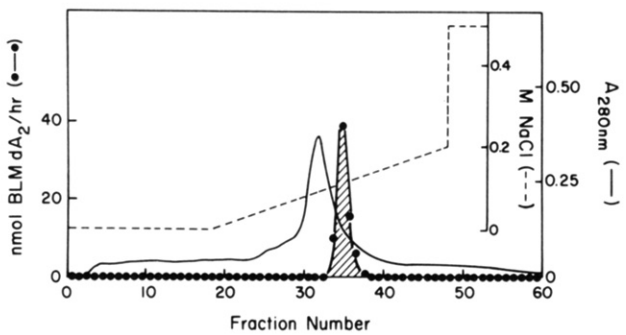


FIGURE 3: Blenoxane–Sepharose 6B affinity chromatography. The BLM hydrolase fractions from the phenyl-Sepharose column (Figure 2, hatched area) were pooled, and an aliquot was concentrated and chromatographed on a Blenoxane–Sepharose column as described under Materials and Methods. The absorbance at 280 nm (solid line) was monitored, and BLM hydrolase activity (closed circles) was assayed as described under Materials and Methods.

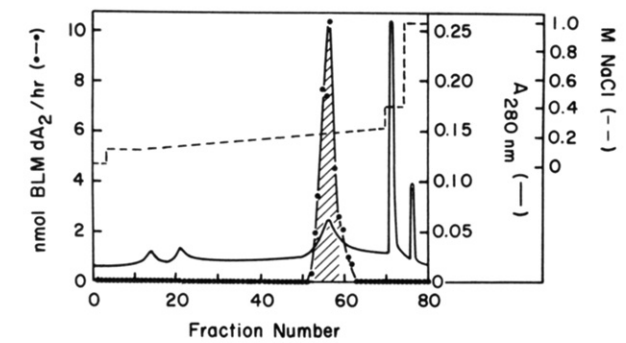


FIGURE 4: Mono Q anion-exchange chromatography. The BLM hydrolase fractions from the Blenoxane–Sepharose affinity column (Figure 3, hatched area) were pooled, and an aliquot was concentrated and chromatographed on a Mono Q column as described under Materials and Methods. The absorbance at 280 nm (solid line) was monitored, and BLM hydrolase activity (closed circles) was assayed as described under Materials and Methods.

4, hatched area) were pooled.

*Determination of Purity of BLM Hydrolase.* Aliquots from the homogenate, the 20800*g* supernatant, the 105000*g* supernatant, and the pooled BLM hydrolase fractions from DEAE-Sephacel, phenyl-Sepharose, BLM-Sepharose and Mono Q columns were assayed for BLM hydrolase by HSLC. The homogenate formed 0.10 nmol of BLM $dA_2$ per absorbance unit (280 nm) per hour whereas the BLM hydrolase fractions from the Mono Q column formed 633 nmol of BLM

Table I: Purification of BLM Hydrolase from Rabbit Lungs[a]

| fraction | sp act. (nmol of BLM $dA_2$ $A_{280}^{-1}$ $h^{-1}$) | purification ($x$-fold) |
|---|---|---|
| crude homogenate | 0.10 | 1 |
| 20800*g* supernatant | 0.61 | 6 |
| 105000*g* supernatant | 1.34 | 13 |
| DEAE-Sephacel | 7.12 | 71 |
| phenyl-Sepharose | 111.73 | 1117 |
| Blenoxane–Sepharose | 219.31 | 2193 |
| Mono Q | 633.06 | 6331 |

[a] Aliquots of homogenate (1 mg), 20800*g* supernatant (1 mg), 105000*g* supernatant (1 mg), DEAE-Sephacel (0.1 mg), phenyl-Sepharose (0.017 mg), Blenoxane–Sepharose (0.010 mg), and Mono Q (0.002 mg) fractions were incubated with BLM $A_2$ (60 μM) for 3 h at 37 °C and assayed for BLM hydrolase activity by HSLC as described under Materials and Methods.

Table II: Amino Acid Composition of BLM Hydrolase

| amino acid | no. of residues | amino acid | no. of residues |
|---|---|---|---|
| Asx | 33.7 | Ile | 15.2 |
| Thr | 22.8 | Leu | 31.5 |
| Ser | 75.0 | Tyr | 14.1 |
| Glx | 54.4 | Phe | 18.5 |
| Pro | 27.2 | His | 15.2 |
| Gly | 63.1 | Lys | 39.1 |
| Ala | 29.4 | Arg | 19.6 |
| Val | 29.4 | total | 500 |
| Met | 12.0 | | |

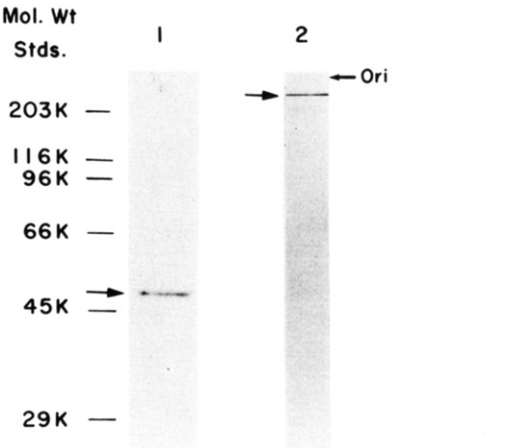


FIGURE 5: Polyacrylamide gel electrophoresis (PAGE). The BLM hydrolase fractions from the Mono Q column were pooled, and aliquots (6 μg) were loaded onto native PAGE (lane 1) or onto SDS–PAGE under reducing conditions (lane 2) as described under Materials and Methods. The arrows designate the position of BLM hydrolase. The following proteins were used as standards (Stds): carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine plasma albumin (66 kDa), rabbit muscle phosphorylase B (97 kDa), *Escherichia coli* β-galactosidase (116 kDa), and rabbit muscle myosin (203 kDa). Ori is origin of the gel.

$dA_2$ per absorbance unit (280 nm) per hour (Table I). Thus, the purification scheme resulted in more than a 6000-fold purification (Table I).

The purity of the Mono Q preparation was also judged by PAGE. The BLM hydrolase fractions from the Mono Q column were pooled, and aliquots (6 μg) were analyzed by SDS–PAGE and native PAGE (Figure 5, lanes 1 and 2, respectively). SDS–PAGE under reducing conditions revealed one single band with a molecular mass of 50 000 daltons (Figure 5, lane 1), whereas native PAGE revealed one single band with molecular mass of greater than 203 000 daltons (Figure 5, lane 2). Thus, BLM hydrolase appears to be composed of several subunits of molecular mass 50 000 daltons each.

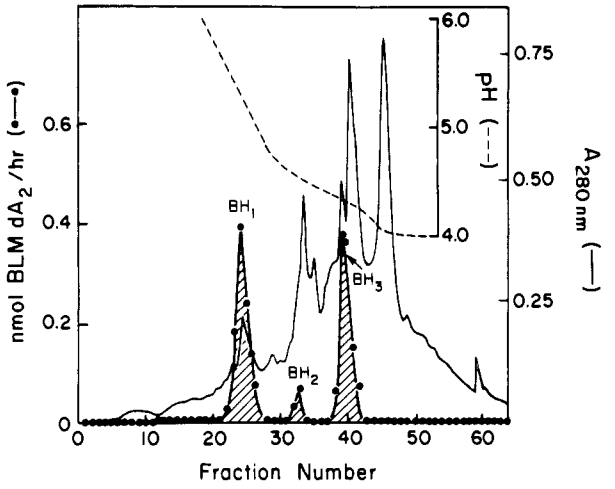


FIGURE 6: Mono P chromatofocusing chromatography. The BLM hydrolase fractions from the phenyl-Sepharose column (Figure 2, hatched area) were pooled, and an aliquot was concentrated and chromatographed on a Mono P column as described under Materials and Methods. The absorbance at 280 nm (solid line) was monitored, and BLM hydrolase activity (closed circles) was assayed as described under Materials and Methods.

*Amino Acid Composition and Kinetic Parameters of BLM Hydrolase.* The purified enzyme was acid hydrolyzed as described under Materials and Methods. Table II shows that BLM hydrolase is composed of approximately 500 amino acid residues. The analysis method used, however, does not detect Cys and Trp. The number 500 is only an approximation since BLM hydrolase appears to exist in three isoforms (see below), which may have different amino acid compositions. Almost half (48%) of the total amino acids of the protein were hydrophobic (Table II), confirming the previous observations from phenyl-Sepharose chromatography. Of all the amino acids detected, 37% were acidic and 16% were basic (Table II), again confirming the results from Mono P chromatography (see below), which indicated that BLM hydrolase was an acidic protein.

The $K_m$ and $V_{max}$ of the purified enzyme were determined by using BLM $A_2$ as a substrate. On the basis of Lineweaver–Burk analysis, the $K_m$ was calculated to be 1.3 mM and the $V_{max}$ was 5.9 $\mu$mol mg$^{-1}$ h$^{-1}$. These results are in agreement with our previously reported $K_m$ and $V_{max}$ values from crude homogenates (Lazo et al., 1984).

*Characterization of BLM Hydrolase. (A) Molecular Weight Determination by Gel Permeation Chromatography on Tandem Superose 6 and 12 Columns.* PAGE under native conditions indicated that BLM hydrolase has a molecular mass greater than 203 000 daltons. In order to confirm this, we chromatographed the BLM hydrolase fractions from the phenyl-Sepharose column on tandem Superose 6 and 12 columns as described under Materials and Methods. Also chromatographed on the same system was a set of commercial standards. The void volume of the two columns in tandem was $V_0$ = 14.65 mL (146.5 min), and BLM hydrolase had a retention time of 271.0 min. The retention times of the different standards were as follows: ferritin (450 kDa), 251.5 min; catalase (240 kDa), 275.9 min; aldolase (152 kDa), 279.3 min; BSA (68 kDa), 295.6 min; ovalbumin (45 kDa), 308.2 min; cytochrome *c* (12.5 kDa), 342.0 min. The molecular mass of BLM hydrolase was 250 ± 10 kDa as determined by graphic comparison with these molecular weight standards.

*(B) Isoelectric Point Estimation by Chromatofocusing on a Mono P Column.* The BLM hydrolase fractions from the phenyl-Sepharose column were chromatographed on a Mono P column as described under Materials and Methods. As the pH of the eluting buffer gradually decreased, several protein peaks were resolved (Figure 6, solid line). BLM hydrolase activity (Figure 6, closed circles and hatched area) was associated with three different peaks (BH1, BH2, and BH3) that were well resolved. The first BLM hydrolase peak (Figure 6, BH1) was eluted at pH 5.3, the second peak (BH2) at pH 4.5, and the third peak ($BH_3$) at pH 4.3. Thus, BLM hydrolase exists in three forms that differ in their apparent isoelectric points.

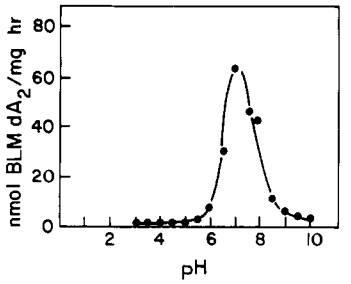


FIGURE 7: pH optimum of BLM hydrolase activity. The BLM hydrolase fractions from the phenyl-Sepharose column were pooled, and aliquots were incubated with various buffers at different pHs. The BLM hydrolase activity was assayed as described under Materials and Methods.

*(C) pH Optimum of BLM Hydrolase.* The BLM hydrolase fractions from the phenyl-Sepharose column were pooled, and aliquots were incubated with BLM $A_2$ in the presence of various buffers with pHs from 3.0 to 10 as described under Materials and Methods. No BLM hydrolase activity was detected from pH 3.0 to pH 5.0 (Figure 7). As the pH was increased, the enzyme activity increased, reaching a maximum at pH 7.0 and decreasing thereafter with little activity above pH 8.5 (Figure 7). BLM hydrolase activity was maximal at pH 7.5 in Tris buffer (data not shown) as compared to pH 7.0 in phosphate buffer (Figure 7). The concentration of Tris and phosphate buffers also affected the activity. BLM hydrolase activity increased with increasing concentration of phosphate and reached a maximum at 100 mM while the activity increased with decreasing Tris concentration reaching a maximum at 10 mM (data not shown).

*(D) Stability of BLM Hydrolase.* Aliquots of the BLM hydrolase fractions isolated from the phenyl-Sepharose column were preincubated at 4, 37, or 56 °C for various lengths of time as described under Materials and Methods. The samples were then incubated with BLM $A_2$, and the BLM hydrolase activity was assayed by HSLC. The half-life of BLM hydrolase activity was 5 days at 4 °C, 10 h at 37 °C, and 25 min at 56 °C. DTT (2 mM) stabilized BLM hydrolase at 4 °C; storage of this enzyme in the presence of 2 mM DTT for up to 10 days at 4 °C did not diminish its activity. Thus, BLM hydrolase can be stabilized by 2 mM DTT.

*(E) Effect of Divalent Cations on BLM Hydrolase Activity.* Aliquots from the BLM hydrolase preparation derived from the phenyl-Sepharose column were preincubated with various concentrations of different cations followed by incubation with BLM $A_2$ as described under Materials and Methods. Control samples that were preincubated with Tris buffer without cations had an average specific activity of 115 nmol of BLM $dA_2$ per absorbance unit (280 nm) per hour. Concentrations as low as 2 $\mu$M $CuSO_4$ inhibited BLM hydrolase activity by 98% (Table III). $CdSO_4$ was also a potent inhibitor of BLM hydrolase activity; 20 $\mu$M decreased the activity by 87%. Concentrations of 20 $\mu$M $ZnSO_4$, $CoCl_2$, and $FeSO_4$ inhibited 50% of the BLM hydrolase activity whereas 200 $\mu$M $MnCl_2$ inhibited 79% of the activity (Table III). $Na_2Cr_2O_7$, $MgCl_2$,

Table III: Effect of Divalent Cations on Rabbit Pulmonary BLM Hydrolase Activity

| | relative BLM hydrolase activity (% of control)[a] | | | |
|---|---|---|---|---|
| divalent cation | 2 mM | 0.2 mM | 0.02 mM | 0.002 mM |
| none | 100 | 100 | 100 | 100 |
| $CuSO_4$ | 0 | 0 | 0 | 2 |
| $CdSO_4$ | 0 | 0 | 13 | ND[b] |
| $ZnSO_4$ | 0 | 0 | 40 | ND |
| $CoCl_2$ | 0 | 0 | 48 | ND |
| $FeSO_4$ | 0 | 36 | 53 | 67 |
| $MnCl_2$ | 2 | 21 | 86 | ND |
| $Na_2Cr_2O_7$ | 82 | 99 | 100 | ND |
| $MgCl_2$ | 94 | 111 | 109 | ND |
| $CaCl_2$ | 108 | 126 | 119 | ND |

[a] Each data point is an average of two or more experiments. [b] Not determined.

Table IV: Inhibitors of Rabbit Pulmonary BLM Hydrolase

| | relative BLM hydrolase activity (% of control)[a] | | |
|---|---|---|---|
| inhibitor | 2 mM | 0.2 mM | 0.02 mM |
| none | 100 | 100 | 100 |
| leupeptin | 0 | 15 | 75 |
| *N*-ethylmaleimide | 5 | 40 | 77 |
| puromycin | 7 | 35 | 81 |
| [Met]enkephalin | 5 | 71 | 89 |
| arginine-$\beta$-naphthylamide | 13 | 57 | 81 |
| [Lys]bradykinin | 26 | 67 | 95 |
| glutathione | 34 | 91 | 100 |

[a] Each data point is an average of two or more experiments.

and $CaCl_2$ did not affect BLM hydrolase activity (Table III). Thus, divalent cations inhibited BLM hydrolase in the following order $Cu^{2+} > Cd^{2+} > Zn^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Cr^{2+} > Mg^{2+} > Ca^{2+}$. Furthermore, concentrations of NaCl up to 0.4 M did not affect BLM hydrolase activity whereas comparable concentration of KCl increased BLM hydrolase by 2-fold (data not shown).

(*F*) *Other Affectors of BLM Hydrolase.* The BLM hydrolase fractions from the phenyl-Sepharose column were pooled, and aliquots were preincubated with potential inhibitors as described under Materials and Methods. The control samples that were preincubated with Tris buffer in the absence of affectors had an average BLM hydrolase specific activity of 120 nmol of BLM $dA_2$ per absorbance unit (280 nm) per hour. The most potent inhibitor tested was leupeptin, which inhibited BLM hydrolase by 85% at a concentration of 200 $\mu$M (Table IV). NEM and puromycin were also potent inhibitors of BLM hydrolase, and each had an $IC_{50}$ of less than 200 $\mu$M (Table IV). [Met]enkephalin, arginine-$\beta$-naphthylamide, [Lys]bradykinin, and glutathione also inhibited BLM hydrolase but to a lesser extent; each $IC_{50}$ was less than 2 mM (Table IV). Cysteine, EGTA, and EDTA (2 mM) did not affect BLM hydrolase to a great extent (data not shown). Finally, mercaptoethanol, bestatin, sucrose, and glycerol had no effect on BLM hydrolase (data not shown).

## Discussion

The metabolic inactivation of BLM by BLM hydrolase is believed to play an important role in controlling the cellular responsiveness to BLM in both normal and malignant tissues (Umezawa, 1980, 1979; Lazo & Humphreys, 1983; Akiyama et al., 1981; Lazo et al., 1984). Therefore, the purification and characterization of this biologically relevant enzyme are essential to our understanding of the biochemical basis of malignant and nonmalignant tissue responsiveness to BLM. Two major obstacles to the purification of BLM hydrolase have been the lack of a rapid and sensitive enzyme assay and the highly fragile nature of this enzyme activity. Umezawa et al. (1974) used a bioassay to measure BLM hydrolase activity and showed that this enzyme was cytosolic and had a molecular mass greater than 25 000 daltons. The authors also suggested that this enzyme may be an aminopeptidase B like enzyme. Their preparation, however, was only partially (27-fold) purified, and attempts to isolate this enzyme further by conventional bioseparation techniques resulted in loss of activity (Umezawa et al., 1974). Sebti and Lazo (1987) recently developed an HSLC assay for BLM hydrolase coupled with FPLC and demonstrated that BLM hydrolase had no aminopeptidase B activity associated with it and that BLM hydrolase was clearly distinct from all aminopeptidases in rabbit lungs. We have now utilized HSLC, FPLC, and affinity chromatograhy to purify this enzyme to homogeneity. Our best preparation was purified over 6000-fold comprising a single protein as judged by PAGE. In the native state, BLM hydrolase had a molecular mass of 250 000 daltons and appeared to contain five subunits of 50 000 daltons each. When BLM $A_2$ was used as a substrate for BLM hydrolase, $K_m$ and $V_{max}$ values of 1.3 mM and 5.9 $\mu$mol mg$^{-1}$ h$^{-1}$ were obtained. These kinetic values agree favorably with previous results from our laboratory (Lazo et al., 1984) for crude homogenates from pulmonary cells.

Although BLM hydrolase is a cytosolic enzyme, the phenyl-Sepharose column elution profile suggested that this enzyme may contain several hydrophobic domains, since it eluted last from this column. This is in agreement with the amino acid composition analysis, which showed that 48% of the residues from the 50 000-dalton subunit (Table II) were hydrophobic amino acids. The hydrophobic regions may have some functional role in recognizing either cellular membranes or the bithiazole domain of BLM. Chromatofocusing studies revealed three isoforms (BH1, BH2, and BH3) of BLM hydrolase with apparent p*I*'s of 5.3, 4.5, and 4.3, respectively. In rabbit lungs, BH1 and BH3 were the major forms whereas BH2 was present in a much smaller amount. We do not know, at this time, whether the ratio of these three isoforms of BLM hydrolase is different in other organs or whether this ratio is critical in determining the susceptibility of an organ or a tumor to BLM-induced toxicity. Furthermore, the fact that all isoforms of BLM hydrolase had low p*I*'s suggested that this enzyme may be acidic. Our amino acid composition analysis of the 50 000-dalton subunit of BLM hydrolase (Table II) showed that 37% of the residues were acidic amino acids.

Cellular BLM hydrolase may be regulated by several factors. For example, NEM inhibited the enzyme activity, suggesting that the active site may contain a sulfhydryl moiety, which could provide an ionic site of interaction with the positive charges of the BLM molecule. Divalent cations, in particular $Cu^{2+}$, $Cd^{2+}$, $Zn^{2+}$, and $Co^{2+}$, were very potent inhibitors. It is worth noting that the divalent cation concentrations in these studies are well below the substrate concentration and, therefore, the inhibition appears to reflect an action on the enzyme rather than the substrate. $Cu^{2+}$ and $Zn^{2+}$ could modulate BLM hydrolase activity by chelating SH groups. This supports the idea of the presence of an SH group in the active site as suggested by the NEM study. $Co^{2+}$ and $Cd^{2+}$ are not likely to have any regulatory effect in vivo since neither one is likely to be present in cells. In contrast, glutathione, while not the most potent inhibitor, produced more than a 60% decrease in enzyme activity at concentrations found in cells. BLM hydrolase does not appear to require metals for its activity, since chelating agents such as EDTA and EGTA do not affect activity. Peptidase-specific inhibitors, such as leupeptin,

strongly inhibited BLM hydrolase, suggesting that BLM hydrolases may have some similarities to other known peptidases. In agreement with our previous findings (Sebti & Lazo, 1987), however, the purified BLM hydrolase does not appear to act as an aminopeptidase.

Thus, rabbit pulmonary BLM hydrolase can now be purified to homogeneity. The described purification scheme for this biologically relevant enzyme should be useful for subsequent attempts to further characterize BLM hydrolase and to determine its amino acid sequence. The availability of pure BLM hydrolase will permit investigation of the potential endogenous substrates for this deamidating enzyme. In addition, substrate-specificity studies of the pure enzyme may result in the discovery of BLM analogues with better antitumor activity.

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# 8-Hydroxy-5-deazaflavin-Reducing Hydrogenase from *Methanobacterium thermoautotrophicum*: 1. Purification and Characterization†


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ABSTRACT: The 8-hydroxy-5-deazaflavin (coenzyme $F_{420}$) reducing hydrogenase from the obligate anaerobe *Methanobacterium thermoautotrophicum* ΔH has been purified 41-fold to apparent homogeneity. The major active enzyme form is a high molecular weight aggregate of $M_r$ ca. 800 000, composed of three subunits, $\alpha$ ($M_r$ 47K), $\beta$ ($M_r$ 31K), and $\gamma$ ($M_r$ 26K). The hydrogenase is purified aerobically in reversibly inhibited form, and conditions for anaerobic reductive activation with $H_2$, high salt, thiols, and electron acceptors have been defined. The minimal species transferring electrons from $H_2$ to coenzyme $F_{420}$ appears to be an $\alpha\beta\delta$ ($M_r$ 115K) complex. The tightly associated redox cofactors per 115K species are 0.6–0.7 nickel atom, 0.8–0.9 flavin adenine dinucleotide (FAD), and 13–14 iron atoms in iron–sulfur centers. The subunits have been separated by denaturing gel electrophoresis, which has permitted determination of amino acid composition, subunit N-terminal sequencing, and preparation of subunit-directed antibodies. There is iron associated with the $\alpha$-subunit, but placement of the nickel and FAD has not been established.


The thermophilic methanogenic bacterium *Methanobacterium thermoautotrophicum* ΔH produces large fluxes of methane during growth on $CO_2$, $H_2$, and inorganic salts (including $Ni^{2+}$ ions). The eight-electron reduction of $CO_2$ to $CH_4$ consumes four molecules of $H_2$, oxidized by hydrogenase action in the cell. As $H_2$ is the only source of reducing power,

$$4H_2 \rightarrow 8H^+ + 8e^-$$

$$CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2H_2O$$

$$\text{sum:}\quad 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

the hydrogenases play a critical role in biogenesis of methane. *M. thermoautotrophicum* ΔH contains two separable hydrogenase enzymes, distinguishable initially by assay with the methanogen 8-hydroxy-5-deazaflavin redox coenzyme, coenzyme $F_{420}$[1] (see Scheme I) (Jacobson et al., 1982; Walsh,

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[1] Abbreviations: $F_{420}$, 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphoryllactylglutamylglutamate; $F_0$, 8-hydroxy-5-deazariboflavin; MV, methylviologen; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; $H_2$ase, hydrogenase; DEAE, diethylaminoethyl; NAD, nicotinamide adenine dinucleotide; NADH, dihydronicotinamide adenine dinucleotide; QAE, quaternary aminoethyl; PMSF, phenylmethanesulfonyl fluoride; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; $KP_i$, potassium phosphate buffer; $Me_2SO$, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EXAFS, extended X-ray absorption fine structure spectroscopy; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)-aminomethane; TBS, Tris-buffered saline; Bis-Tris propane, 1,3-bis-[[tris(hydroxymethyl)methyl]amino]propane; IgG, immunoglobulin G; DTT, dithiothreitol; TLC, thin-layer chromatography; EPR, electron paramagnetic resonance; GdmCl, guanidinium chloride; 2D, two dimensional; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; CAPS, 3-(cyclohexylamino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

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