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TITLE:

Limited proteolysis and gel electrophoresis in the presence of metal cations: Au(III)-binding luminescent domain in serum albumins

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KEYWORDS:
Synthesis, bovine serum albumin, BSA, gold, Au, proteolysis, limited proteolysis, luminescence, electrophoresis

SUMMARY:
We present a protocol for studying the binding domain of Au(III) in bovine serum albumin (BSA).

ABSTRACT:
The purpose of the presented protocols is to determine the domain of Au(III) binding in BSA. The BSA-Au(III) compound exhibits ultraviolet (UV)-excitable red luminescence (λ_{em} = 640 nm), with unusual Stokes shifts compared to the innate UV/blue fluorescence arising from the aromatic residues. Red-luminescent complexes are formed in highly alkaline conditions above pH 10 and require a conformation change within the protein to occur. In addition, preservation of Cys-Cys disulfide bonds in BSA is necessary to obtain this red luminescence. In order to understand the mechanism of this luminescence, elucidation of the luminophore-forming Au(III) binding site is essential. A facile way to assess the luminophore-forming site would be to (1) predictably fragment the protein by enzymatic digestion, (2) react the obtained fragments with Au(III), then (3) perform gel electrophoresis to observe the well-separated fragment bands and analyze the in-gel red luminescence. However, due to the alkaline conditions and the reaction with metal cations, new limited proteolysis techniques and gel electrophoresis conditions must be applied.

Particularly, the presence of metal cations in gel electrophoresis can make the band separations technically difficult. We describe this new protocol in steps to identify the red-luminophore-forming metal binding domain in BSA. This protocol can thus be applied for analyzing protein fragments that must remain in a non-denatured or a partially denatured state, in the presence of metal cations. Because the majority of proteins need metal cations to function, analyses of metal-bound proteins are often desired, which have relied on x-ray crystallography in the literature. This method, on the other hand, could be used in supplement to study the interactions of proteins with metal cations without requiring the protein crystallization and at a desired pH condition.

INTRODUCTION:

Bovine serum albumin[1-3] (BSA)–gold (Au) complexes, obtained by reactions in highly alkaline conditions (pH > 10), are known to exhibit UV-excitable red luminescence (λ_{em} = 640 nm)[4-7]. Numerous applications of this compound has been proposed and investigated, including sensing,[8-10] imaging[11-13], and nanomedicine[14-16]. However, the mechanism of the luminescence is not fully understood. Identifying the location of Au(III) binding and the luminophore formation in BSA is an important step.

It has been recently elucidated that pH-controlled dynamic conformation change of BSA, followed by a Au(III) binding to a Cys-Cys disulfide bond, is necessary for yielding the red luminescence[4]. In order to gain further insights into the mechanism of this luminescence, elucidation of the luminophore-forming Au(III) binding site is essential. A facile way to assess the luminophore-forming site is to fragment the BSA-Au compound by enzymatic digestion, and to analyze each fragment for the luminescence. However, due to the alkaline conditions and the presence of metal cations, new proteolysis and gel electrophoresis protocols are needed.

We employed limited enzymatic proteolysis as the method of protein fragment preparations, while preserving the Cys-Cys disulfide bonds. In the conventional proteolysis, cleaving of all disulfide bonds and linearization of a protein (by denaturing agents such as dithiothreitol and urea, as well as heat) is necessary. Herein, we demonstrate a Cys-Cys bond-preserving proteolysis and evaluate the obtained fragments and their luminescence after the reaction with Au(III). We use trypsin for the digestive enzyme, as a concrete example.

The protocol generally describes the gel electrophoresis of proteins and fragments in the presence of metal cations. Because the majority of proteins need metal cations to function[17,18], analyses of metal-bound proteins are often desired, which have relied on x-ray crystallography in the literature. Structures of BSA, and their fragments, are not known for non-neutral pH conformations including at pH > 10. Therefore, the structural details of the Au(III) coordination cannot be analyzed by gel electrophoresis alone. This method, on the other hand, could be used in supplement to study the interactions of proteins with metal cations without requiring the protein crystallization, which may not be possible at a desired functional pH condition. The presence of metal cations can cause significant “smearing” of the gel bands. The focus of this paper is to overcome this technical difficulty and to present a protocol to minimize the metal-induced band smearing.

PROTOCOL:

1. Synthesis of BSA-Au complex fragments

1.1. Dissolve 5 mg of BSA in 1 mL of HPLC water containing 50 mM Tris-HCl and 50 mM NaCl with a pH of 8.0 in a 5 mL vial.

1.2. Dissolve 2 mg of trypsin in 1 mL of a freshly prepared solution of HPLC water containing 50 mM Tris-HCl and 50 mM NaCl with a pH of 8.0.

1.3. Place the reaction vial of BSA in a 37 °C water bath and stir vigorously at 750 rpm using a magnetic stirrer.

1.4. Immediately after stirring begins, add 50 µL of the freshly prepared trypsin to the solution.

NOTE: No sodium dodecyl sulfate (SDS), dithiothreitol (DDT), or urea should be added to the solution, as opposed to the conventional enzyme digestion reactions. Also, no temperature annealing should be performed. Due to this limited proteolysis, Cys-Cys disulfide bonds will be kept intact and only surface accessible random coil segments will be cleaved by the enzyme.

1.5. Dissolve Au(III) chloride (chloroauric acid) in 1 mL of HPLC grade water to a concentration of 750 µM.

1.6. Into the reaction vial, add the chloroauric acid solution for a resulting BSA:Au molar ratio of 1:10.

1.7. Stir the mixture for 2 minutes at 37 °C and at 750 rpm using a magnetic stirrer.

1.8. Add 100 µL of 1 M NaOH to the reaction vial to achieve a pH of 12.5.

NOTE: The high alkaline conditions of the reaction should induce the formation of the red luminescent complex and quench the enzymatic activity of trypsin.

1.9. Stir the mixture vigorously at 750 rpm for 2 hours at 37 °C.

NOTE: The final product was used immediately without further purification.

2. Gel electrophoresis of BSA-Au complex fragments by limited proteolysis

2.1. Rinse a pre-cast 4-12% gradient Bis-Tris gel using deionized water and place in a gel electrophoresis tank.

2.2. Prepare 500 mL of MES running buffer solution from a concentrated stock solution, diluting with deionized water.

2.3. Prepare for each well lane by diluting samples to 1 μg of protein/μL in a 20% glycerol solution. This dilution brings the pH from 12.5 to ~8.

NOTE: No SDS, DTT, or urea is used in the sample buffer. Additionally, temperature annealing of samples should not be performed.

2.4. Add 10 μL of each sample solution to each lane of the gel.

2.5. Run the gel for 1 hour at a constant voltage of 150 V.

2.6. After running the gel, remove the gel from the cast and rinse 3 times for 1 minute each using deionized water to remove running buffer.

2.7. Store the gel in 200 mL of deionized water and immediately measure the in-gel fluorescence, using a gel imaging system.

2.8. Prepare a fresh staining solution containing 200 mg of Coomassie Brilliant Blue in 200 mL of the following solution: methanol, acetic acid, and water at a volume ratio of 50:10:40.

2.9. Wash the gel in 200 mL of staining solution for 30 minutes using gentle rocking.

2.10. Prepare a fresh de-staining solution by mixing methanol, acetic acid, and water at a volume ratio of methanol:acetic acid:water = 50:10:40.

2.11. Wash the gel in 100 mL of de-staining solution for 1 hour using gentle stirring.

2.12. Repeat the above procedure 4 times and finally store the fixed gel deionized water at room temperature.

3. Analysis of BSA-Au complex fragments by limited proteolysis

3.1. Examine the amino acid sequence of BSA and prepare a table of expected fragments that can be obtained by enzymatic digestion, assuming Cys-Cys bond preservation (limited proteolysis). In the case of trypsin digestion (**Table 1**), cut locations are C-terminus of Lys and Arg, except followed by Pro. Account for the small errors in fragment molecular weights, arising from the ambiguity in tryptic cut locations.

NOTE: Analyzing the amino acid sequence of BSA, the expected limited tryptic fragments obtained from this step are: [*A*] (7.3 kDa, residues 1 – 64); [*B*] (5.9 kDa, residues 65 – 114); [*C*] (20.1 ~ 22.4 kDa, residues 115/117 – 294/312); [*D*] (21.3 ~ 23.4 kDa, residues 295/313 – 499); and [*E*] (9.5 kDa, residues 500 – 583). Ambiguity in tryptic cut locations result from segments

outside the Cys-connected units. For BSA, the residues 107 – 114 (0.9 kDa) and residues 295 – 312 (2.1 kDa) can appear as the N-or C-terminus part of a Cys-Cys bond-connected fragment.

3.2. Identify location(s) of surface-exposed Cys in these expected fragments. For trypsin-digested BSA, the only surface-exposed Cys34 is in fragment [*A*].

3.3. Prepare the list of molecular weights observed as gel electrophoresis bands, below ~66 kDa (molecular weight of BSA).

NOTE: For trypsin digestion, the observed gel electrophoresis bands are: Band(1) = undigested BSA; Band(2) ~50 kDa; Band(3) ~44 kDa; Band(4) ~42 kDa; Band(5) ~36 kDa; Band(6) ~32 kDa; Band(7) ~26 kDa; Band(8) ~21 kDa; Band(9) ~15 kDa; Band(10) ~12 kDa; Band(11) ~10 kDa; and Band(12) ~8 kDa.

3.4. Reconstruct the list of the observed molecular weights in the gel, by the sequential additions of the expected BSA fragments. For trypsin, fragment [*A*] can form [*A*]-[*A*] dimer through the surface-exposed Cys residue.

REPRESENTATIVE RESULTS:

The observed twelve gel bands were uniquely reconstructed from the five expected BSA fragments [*A*] – [*E*] (**Figure 1**). The results were consistent with the literature, in which the secondary structures including α-helices and β-strands are preserved[19-23]. Band(1) = [*ABCDE*] (undigested); Band(2) = [*ABCD*] (connected fragments); Band(3) = [*BCD*]; Band(4) = [*CD*]; Band(5) = [*A*]+[*ABC*]; Band(6) = [*ABC*]; Band(7) = [*BC*]; Band(8) = [*D*]; Band(9) = [*A*]+[*A*]; Band(10) = [*AB*]; Band(11) = [*E*]; Band(12) = [*A*] (**Figure 2a**). Band(5) and Band(9) contain dimerized fragment [*A*], which are justified by the surface-exposed Cys34[2]. Bands above the molecular weight of BSA ~66 kDa can be excluded from further analyses of BSA fragments.

Smearing of the gel bands was observed in the presence of Au(III). We attribute this smearing to the interaction of the surface-bound Au(III)[5] with the gel matrix[24,25]. This smearing can be minimized, when the protocol is followed. Out of the twelve gel bands of BSA-trypsin, nine bands were observed as [BSA-trypsin]-Au (**Figure 2b**). Multimer bands (above ~66 kDa) were pronounced, due to aggregation caused by surface-bound Au(III)[12].

Five bands exhibited the in-gel red luminescence (**Figure 2c**). These bands were Band(1) = [*ABCDE*] (undigested BSA); Band(2) = [*ABCD*]; Band(3) = [*BCD*]; Band(6) = [*ABC*]; Band(7) = [*BC*]. Luminescence was absent in Band(8) = [*D*], Band(9) = [*A*]+ [*A*], Band(10) = [*AB*], and Band(12) = [*A*] (**Figure 2d**). This in-gel luminescence band pattern can be explained by the presence of a red luminophore in the tryptic fragment [*C*] (residues 115/117 – 294/312).

The gel band smearing was exacerbated with extended digestion times, in the presence of Au(III). The smearing was minimized when the protocol described above was used. Examples of non-optimized gel electrophoresis are described in **Figures 3-6**, to demonstrate the factors affecting the limited tryptic proteolysis and the gel electrophoresis in the presence of metal cations.

FIGURE AND TABLE LEGENDS:

Figure 1. List of Cys-Cys bond-preserving bands of BSA by limited tryptic proteolysis. Tryptic cleaving in the random coil regions can result in 15 (#i–xv) fragments, but Cys-Cys bonds connect the sequences as indicated. This table has been modified from Dixon et al.[6].

Figure 2. Gel-electrophoresis of the tryptic fragments of BSA obtained by the limited proteolysis. (**a**) A total of twelve bands were observed in gel-electrophoresis of BSA by the limited tryptic proteolysis. (**b**) Tryptic BSA fragments reacted with Au(III). (**c**) In-gel luminescence of (**b**). (**d**) Drawing of the observed in-gel bands. In-gel red luminescence was observed with Band(1) = undigested BSA, Band(2) = [*ABCD*], Band(3) = [*BCD*], Band(6) = [*ABC*], and Band(7) = [*BC*] (indicated by †).

Figure 3. Optimizing the time of reaction with Au. (A) BSA; (B) BSA-Au (reacted for 2 h); (C) BSA-Au (reacted for 1 h); (D) BSA-Au (reacted for 30 min); (E) BSA-Au (reacted for 5 min). The red luminescence of BSA-Au complexes required at least 30 min at 37 °C, and luminescence was most intense in the gel after reaction for 2 hours. However, the longer reaction time led to more smearing in the gel electrophoresis bands. In order to maximize the luminescence intensity and to minimize the smearing, the optimum reaction time was chosen to be 2 hours.

Figure 4. Optimizing the timings of the addition of trypsin for digestion, and Au for reaction. The reaction time with Au was explored in the range of 2 to 24 h. (A) BSA; (B) [BSA-trypsin]-Au (after 2 h); (C) [BSA-trypsin]-Au (after 4 h); (D) [BSA-trypsin]-Au (after 8 h); (E) [BSA-trypsin]-Au (after 24 h). Longer reaction time resulted in more smearing of the gel bands.

Figure 5. Effects of metal cations and the gel band smearing. The smearing of the gel bands by the additions of and reactions with Au(III) were similarly observed with Cu(II) and Ni(II). (A) BSA; (B) BSA-Au; (C) [BSA-trypsin]-Au (2-hour digestion); (D) [BSA-trypsin]-Au (overnight digestion); (E) [BSA-trypsin]-Cu (2-hour digestion); (F) [BSA-trypsin]-Cu (overnight digestion); (G) [BSA-trypsin]-Ni (2-hour digestion); (H) [BSA-trypsin]-Ni (overnight digestion). The red luminescence was not observed upon reactions with Cu(III) or Ni(II).

Figure 6. Effects of pH and the order of the additions of trypsin and Au. The fragmented compounds were prepared by reacting Au with digested BSA ([BSA-trypsin]-Au), or by digesting the pre-formed BSA-Au ([BSA-Au]-trypsin), and then run in gel as is or after adjusting the pH to 7 in sample buffer. (A) BSA; (B) BSA-Au (as is); (C) BSA-Au (pH=7); (D) BSA-trypsin; (E) [BSA-trypsin]-Au (as is); (F) [BSA-Au]-trypsin (as is); (G) [BSA-trypsin]-Au (pH=7); (H) [BSA-Au]-trypsin (pH=7). The presence of the red luminescence was not affected by the order of the additions of trypsin and Au, however the smearing of the gel bands was more pronounced for [BSA-Au]-trypsin. The adjustment of pH to neutral in the sample buffer did not significantly improve the gel band smearing, however increased the aggregation of fragments as indicated by the gel bands above ~66 kDa.

DISCUSSION:

The purpose of the present protocol was to identify the red-luminophore-forming domain in BSA-Au complexes. We employed limited tryptic proteolysis to obtain the BSA fragments, while preserving the Cys-Cys bonds that are necessary to produce the red luminescence. We optimized the conditions for proteolysis and electrophoresis in the presence of Au(III). The same principles can be broadly applied to the gel analyses of fragmented proteins in the presence of metal cations.

We performed multiple optimizations to enable the analysis of BSA-Au fragments and their in-gel luminescence. The red luminescence of BSA-Au complexes and [BSA-trypsin]-Au fragments required at least 30 min of reaction time at 37 °C to be observable in gels, and luminescence was most intense in the gel after reaction for 2 hours (**Figure 3, Figure 4**). However, the longer reaction time led to more smearing in the gel electrophoresis bands. In order to maximize the luminescence intensity and to minimize the smearing, the optimum reaction time was chosen to be 2 hours.

The presence of metal cations appears to be the major cause of the gel band smearing. The smearing of the gel bands were observed by the additions of and reactions with Au(III), as well as with Cu(II)[24,25] and Ni(II) (**Figure 5**). We also assessed the effects of the order of the addition of trypsin and Au, and the sample loading buffer pH (**Figure 6**). We observed the in-gel red luminescence regardless of the order of the additions of trypsin and Au. However, smearing of the gel bands was suppressed for [BSA-trypsin]-Au, namely by digesting BSA first and then reacting the obtained fragments with Au. The adjustment of pH to neutral in the sample loading buffer for the gel electrophoresis did not significantly improve the gel band smearing, rather, it increased the aggregation of fragments as indicated the gel bands above the molecular weight of 66.4 kDa. Other important factors that we note to optimize the gel-band analysis are the ratio of BSA:Au (maximum intensity was obtained for the ratio of 1:25[5], but we optimized it to 1:10 to minimize the metal cation-induced gel smearing, while maximizing the red luminescence intensity) and the gel loading (10 μg of protein per lane, to maximize the in-gel band luminescence intensity while avoiding the overloading). For gel-based proteomics, a further improvement in the smearing may be necessary.

The majority of proteins need metal cations to function, and at a particular physiological pH. For a complete understanding of these proteins, x-ray crystallography is ultimately required for the structural analyses and the identification of metal coordination. However, protein crystallization is often difficult and is not always possible at desired (physiologically functional) pH. Taking the experiments presented herein as an example, the structures of BSA, and their fragments, are not known for the non-neutral pH conformation that is required to produce the red luminescence. Yet we were able to obtain critical functional information of the metal-bound BSA fragments at the desired pH. This method could therefore be used in supplement to study the interactions of proteins with metal cations in a facile manner without requiring the protein crystallization.

Molecular cloning can be technically difficult in some cases, due to low solubility of the product fragment or low expression in *E. coli*, and is usually time-consuming[6]. The facile method based on the limited proteolysis presented here can complement the molecular cloning approach.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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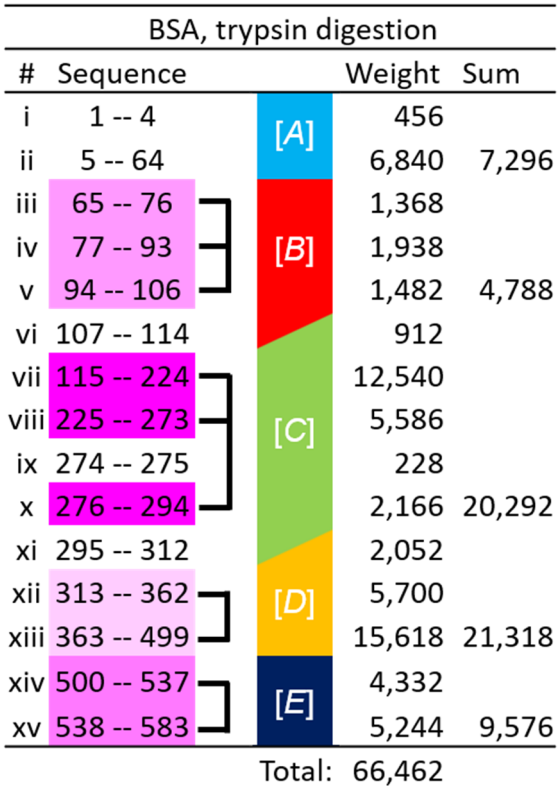

BSA, trypsin digestion				
#	Sequence		Weight	Sum
i	1 -- 4	[A]	456	
ii	5 -- 64		6,840	7,296
iii	65 -- 76	[B]	1,368	
iv	77 -- 93		1,938	
v	94 -- 106		1,482	4,788
vi	107 -- 114	[C]	912	
vii	115 -- 224		12,540	
viii	225 -- 273		5,586	
ix	274 -- 275		228	
x	276 -- 294		2,166	20,292
xi	295 -- 312		2,052	
xii	313 -- 362	[D]	5,700	
xiii	363 -- 499		15,618	21,318
xiv	500 -- 537	[E]	4,332	
xv	538 -- 583		5,244	9,576
		Total:	66,462	

Figure 2

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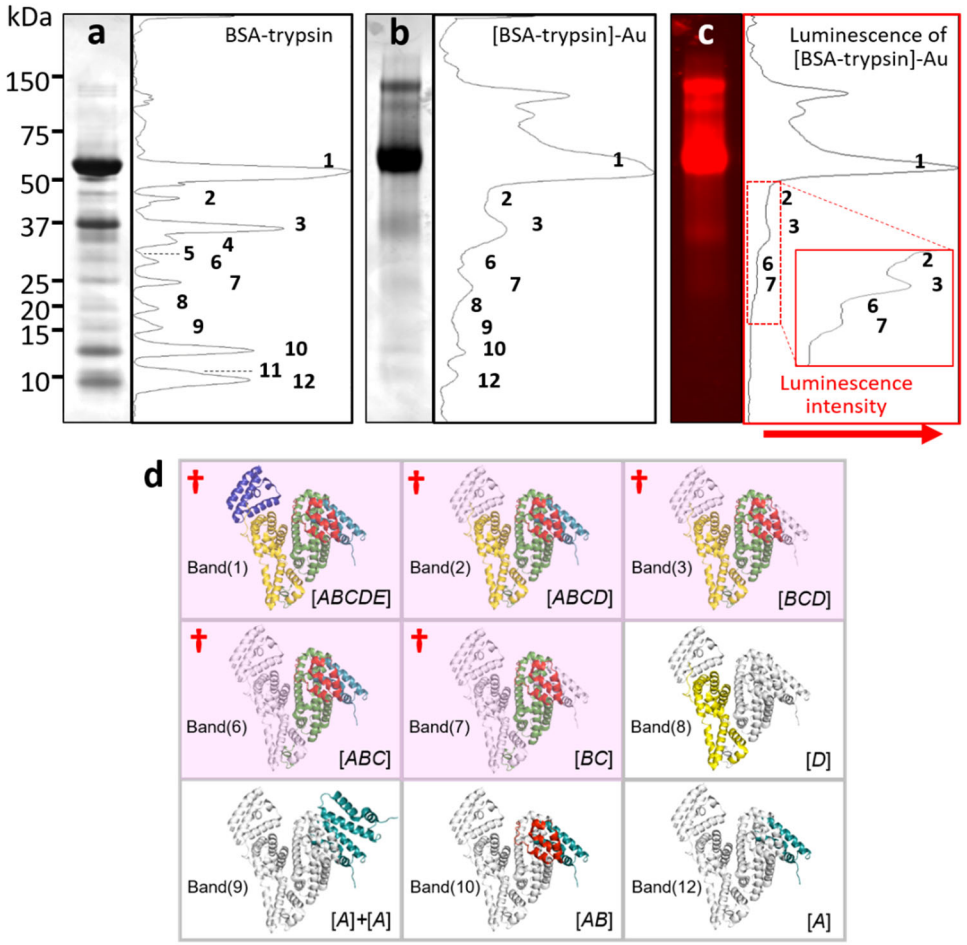

Figure 3

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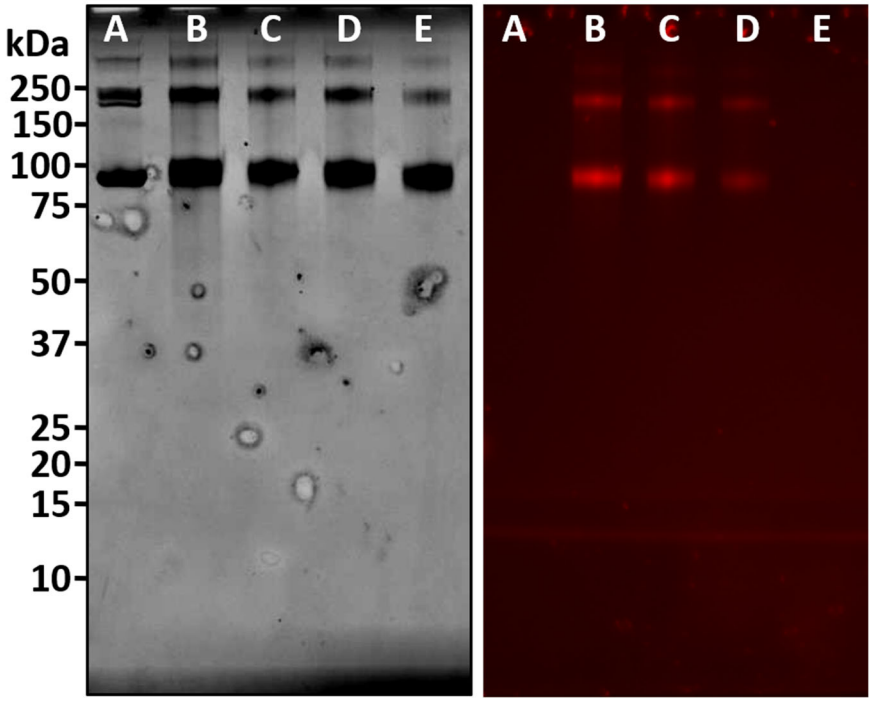

Figure 4

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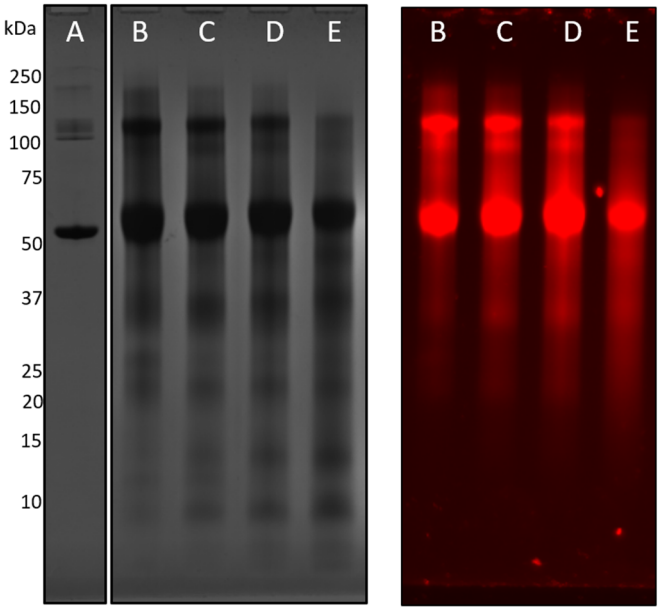

Figure 5

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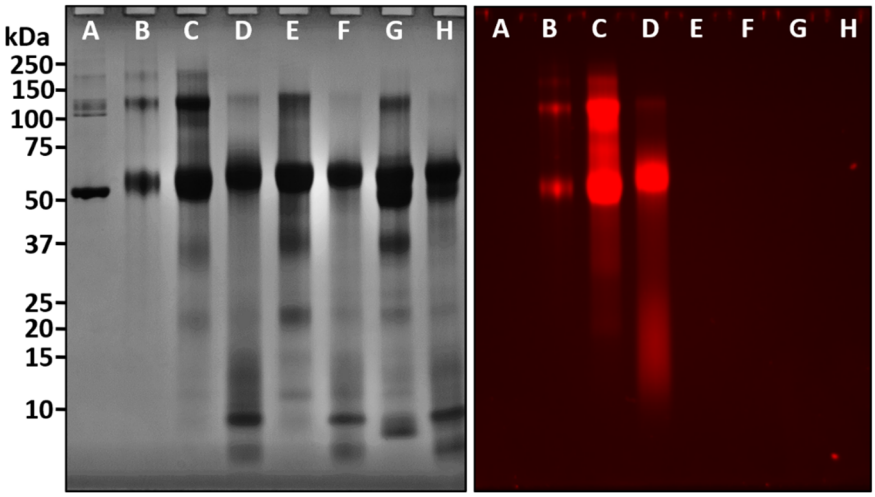

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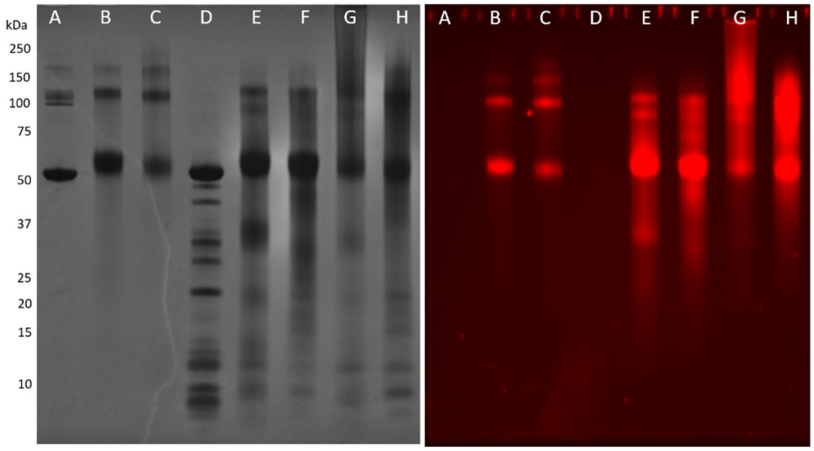

Name of Material/ Equipment	Company	Catalog Number	Comments
Ammonium bicarbonate, 99.5%	Sigma-Aldrich	9830	
Azure Biosystems C400 gel imaging system	Azure Biosystems	C400	
Bovine Serum Albumin (BSA), 96%	Sigma-Aldrich	A5611	
Glycerol, >99.0%	Sigma-Aldrich	G5516	
gold (III) chloride trihydrate, 99.9%	Sigma-Aldrich	520918	
NuPAGE 4-12% Bis-Tris Mini Protein Gel	Thermo Fisher	NP0321BOX	
NuPAGE MES Running Buffer (20X)	Thermo Fisher	NP0002	
Sodium Chloride (NaCl), >99.5%	Sigma-Aldrich	S7653	
Sodium hydroxide, >98.0%	Sigma-Aldrich	S8045	
Tris Hydrochloride (Tris-HCl)	Sigma-Aldrich	10812846001	
Trypsin from Bovine Pancreas (>10,000 BAEE units/mg)	Sigma-Aldrich	T1426	

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October 15, 2020

Dr. Nam Nguyen, Manager of Review
Journal of Visualized Experiments

Dear Dr. Nguyen:

We thank you and the reviewers for the critical reading and comments. With the revisions below, I hope the manuscript is ready for publication.

Response to Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We checked for typos.

2. Please rename Short Abstract as Summary, and rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

We used Summary as the heading, and followed your instructions.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

We corrected the locations of reference numbers in text.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: NuPAGE, Azure Biosystems C400,

We removed the product names from the text, and modified the Materials Table accordingly.

5. 3.1: as the disulfide bonds are still intact, do you use any specific method to list expected fragments by taking into account 3D conformation?

We added a description: “Examine the amino acid sequence of BSA and…”

6. Lines 201-203: as smearing of the gel bands was observed even with your protocol, please comment (as you have done in lines 213-214) what might have happened and how to resolve this smearing.

We modified the description: “The gel band smearing was exacerbated with extended digestion times, in the presence of Au(III). The smearing was minimized when the protocol described above was used.”

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We attached the copyright permission from ACS. The figure legend was corrected.

8. Representative Results: lines 273-275: did you try an intermediate duration, e.g. 1 hour and lines 283-284: did you try adjusting to higher pH just before gel electrophoresis?

We believe the data presented are sufficient to describe the optimized conditions.

9. As we are a methods journal, please add limitations of the technique to the Discussion.

Thank you for this comment. We added the limitations of this technique: “For gel-based proteomics, a further improvement in the smearing may be necessary.”

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal names.

We corrected and used the specified reference style.

11. Figure 1 looks like a table. Please rename it as Table 1 and refer to it that way.

We renamed and referred to it as Table 1.

12. Please sort the Materials Table alphabetically by the name of the material.

We corrected the Materials Table.

Reviewer #1:

In this video protocol, Egusa et al. reported the method to identify the Au(III) binding domain in

serum albumins. Based on their recent papers (JPC Lett. 2020 etc.), they describe the detailed protocol on how to determine the red luminophore-forming domain in serum albumin-gold complexes using proteolysis. This red luminophore is the origin of metal clusters, although the mechanisms of the luminescence of the cluster are not fully understood. Therefore, this study is one of the important studies to clarify the origin of cluster formation as well as the basics of metal binding to protein. However, the current introduction is relatively narrow and specialized, and the protocol is of interest to the limited scientists. This reviewer recommends revising the introduction to strengthen the importance of the study, such as metal binding to proteins and cluster formation.

We thank the reviewer for the careful reading and positive evaluation. Following the reviewer's suggestion, we added in introduction to strengthen the rationale of this protocol: "Identifying the location of Au(III) binding and the luminophore formation in BSA is an important step."

Reviewer #2:

Manuscript Summary:

The manuscript describes an experimental protocol suitable for the controlled digestions of of proteins while preserving disulfide bonds as well as a protocol to minimize band smearing during gel electrophoresis in the presence of protein bound metal ions. These protocols facilitate the identification of the metal binding domains of the protein, which can be the interest in the case of many metal ion containing enzymes. The methods are demonstrated on the BSA/Au(III) system, which have a peculiar red fluorescence. The protocols are described clearly in details, which allows the readers to easily reproduce the presented methods. I recommend the publication of the manuscript as it.

Major Concerns:

-

Minor Concerns:

-

We thank the reviewer for the positive comments.

The annotated version is attached for your reference. We look forward to hearing from you soon.

Sincerely,

Shunji Egusa, Ph.D.
Assistant Professor

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Identifying the Red-Luminophore-Forming Domain in Serum Albumin–Gold Complexes

Author: Jacob M. Dixon, Junya Tomida, Shunji Egusa

Publication: Journal of Physical Chemistry Letters

Publisher: American Chemical Society

Date: May 1, 2020

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Author(s): Jacob M. Dixon and Shunji Egusa

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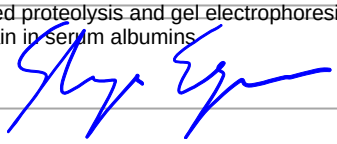
Signature:	
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