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Ammonium sulfate precipitation combined with liquid chromatography is sufficient for purification of bovine serum albumin that is suitable for most routine laboratory applications

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Ammonium sulfate precipitation combined with liquid chromatography is sufficient for purification of bovine serum albumin that is suitable for most routine laboratory applications

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Abstract

The use of bovine serum albumin (BSA) in routine biochemical assays such as restriction enzyme digestion and immunodetection is plagued largely by two common contaminants, DNase and immunoglobulins G (IgGs). Acetylation of BSA to inactivate DNase limits its use as a protein standard due to interference with color development in assays such as Lowry's. In spite of the availability of inexpensive BSA, its purification involves several cumbersome and time-consuming steps. In this work, we employed a modified strategy of ammonium sulfate precipitation coupled with liquid chromatography to purify BSA that is free of DNase and IgGs. Purified BSA tested negative for the presence of DNase and IgGs using DNase and immunodetection assays respectively. We conclude that carefully controlled ammonium sulfate precipitation and liquid chromatography techniques are sufficient to purify BSA suitable for most routine laboratory applications. This purification strategy can yield more than 40 g BSA per liter of serum.

Keywords: Bovine serum albumin, ammonium sulfate, nuclease, purification, immunoglobulin G, chromatography

Introduction

Bovine serum albumin (BSA) is widely used in various biochemical and immunological applications due to its high abundance in bovine plasma, structural stability, and its non-invasive stabilizing interaction with most proteins [1-5]. Albumin is used as a carrier for several molecules in drug delivery systems, a stabilizing protein to dilute antibodies as well as a blocking agent in various immunoassays and detection protocols [1-5]. Most current approaches to purification of BSA combine the original or modified version of the Cohn method [6,7] with several additional steps such as ion exchange chromatography [8-14]. The relative inexpensiveness of certain brands of commercial BSA is due to abundance of the protein in bovine serum rather the simplicity, optimization or robustness of the purification strategies. There are several grades of commercially prepared BSA depending on the intended laboratory application. The most common contaminants of commercially prepared BSA are nucleases, immunoglobulins G (IgGs) and endotoxins. Due to its relative thermostability, heat inactivation at a temperature between 55°C and 70°C for 1 hr is commonly employed during BSA purification to denature contaminating proteins [13,15]. Thermocoagulation removes contaminating proteins such as nucleases and proteases; however, prolonged exposure to heat can cause BSA molecules to form hydrophobic aggregates which may not revert to monomers upon cooling [16-20]. In some preparations, nucleases are inactivated by acetylation using chemical agents such as p-nitrophenyl acetate [21,22]. Acetylation of tyrosine residues in BSA limits its laboratory use by interfering with color development in assays such as the Lowry's. Several reports on the purification of BSA claim purity level greater than 90% without indicating specific contaminants or testing for suitability of the purified protein for immunological applications [23-25]. Purification of albumin by salt precipitation is rarely reported in literature [26,27]. In this work, we employed a modified strategy of ammonium sulfate precipitation coupled with liquid chromatography to purify BSA that is free of DNase and IgGs, and which is suitable for most laboratory applications.

Materials and methods

Ammonium sulfate precipitation

Clotted cow blood was obtained from the Texas Veterinary Center in Nacogdoches, Texas. The blood was centrifuged for 15 min at 2500xg, room temperature. Supernatant obtained was centrifuged at 5000xg for 15 min, room temperature to obtain clean serum. Proteins were sequentially precipitated from 100 mL of serum (varying volumes ranging from 3 to 100 mL were used) by stepwise addition of solid ammonium sulfate with stirring, according to the protocol in Table 1 followed by incubation on ice for at least 2 hr and centrifugation at 10,000xg, 4°C for 15 min. The pellet obtained after each centrifugation was resuspended in 100 mL of buffer containing 20 mM Tris, pH 9, and 100 mM NaCl. Aliquots of precipitation fractions were analyzed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Acetone precipitation

Following ammonium sulfate precipitation, all fractions

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Table 1. Scheme for ammonium sulfate precipitation of albumin from 100 mL of cow serum.

% $(NH_4)_2SO_4$ saturation	Start volume (mL)	Grams of $(NH_4)_2SO_4$ added	End volume (mL)
40	100.00	23.000	83.30
50	83.30	4.960	76.50
60	76.50	4.720	66.40
70	66.40	4.250	60.00

containing BSA, except the 40% fraction, were pooled and used in acetone precipitation step. Ice-cold acetone was added to the BSA solution up to four times the volume before incubation at -20°C for 1 hr. The mixture was centrifuged at 15,000xg, 4°C for 15 min. Precipitate obtained was incubated at room temperature for 1 hr to allow residual acetone to evaporate, and then resuspended in 20 mM Tris, pH 9.

Anion exchange and size exclusion chromatography

The concentration of acetone-precipitated BSA was diluted to 0.1 mg/mL in 20 mM Tris, pH 9. The protein solution was loaded onto a pre-equilibrated Q Sepharose column at a flow-rate of 0.5 mL/min. In each anion exchange purification step, the column was loaded to half the binding capacity of the bed volume and washed extensively with the binding buffer, 20 mM Tris, pH 9. Elution of protein was carried out in a Tris-buffered 1 M NaCl solution by 50 mM stepwise increment of salt concentration. At each salt increment step, the column was eluted with five times bed volume of buffer and 5 mL fractions collected. BSA fractions were pooled together and concentrated to 15 mg/mL. 200 µL of the concentrated protein was loaded onto a pre-equilibrated Superose 12 10/300 GL column and fractionation carried out in buffer containing 20 mM Tris, pH 9 and 100 mM NaCl, at a flow rate of 0.5 mL/min. Aliquots of chromatographic fractions were resolved on 10% SDS-PAGE.

DNase I assay

Assay was performed in a buffer containing 10 mM Tris-HCl, pH 7.6, 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$. In separate 25-µL assay reactions, 20 µg of purified BSA, 20 µg of acetylated BSA, 2 µg of commercial bovine gamma globulin (BgG) (Pierce Biotechnology, Inc., Rockford, IL, USA), and 1 unit of DNase I were used to digest 1 µg of p202 plasmid DNA for 10 min at 37°C. Assay was stopped by addition of 5 µl of 6 X DNA gel loading buffer and 20 µl of reaction mixture was resolved on 1% agarose gel.

Western blotting and chemiluminescence

100 µg each of bovine serum and purified BSA, 50 µg of commercial BSA (Pierce Biotechnology, Inc., Rockford, IL, USA), and 10 µg of BgG were resolved on 10% SDS-PAGE and the proteins blotted onto nitrocellulose membrane. The membrane was incubated with HRP-conjugated goat anti bovine IgG (Southern Biotech, Birmingham, AL, USA) diluted in fat-free milk to a ratio of 1:5000. Antigen-antibody interaction was detected by chemiluminescence.

Results and Discussion

BSA is widely used as a blocking agent in several immunological assays or a stabilizing protein in biochemical reactions. Conventional purification of BSA is based on the original Cohn method [6,7] which involved pH and ethanol precipitations. Recent modifications include anion exchange and size exclusion chromatography [8-14]. Despite these improvements, BSA preparations are still plagued with contaminants such as DNase I, RNase, proteases, immunoglobulins and endotoxins [23-25]. For its use as a stabilizer and blocking agent, the main drawback is contamination with DNase and IgGs. More stringent purification steps are required to make BSA preparations suitable for use in blood transfusion and other clinical applications [4,12]. Even though BSA is in high abundance in cow blood, commercial purification of molecular grade BSA involves several cumbersome and time-consuming steps [14]. One way companies avoid high cost of production is to purify BSA of varying research grades depending on the intended use [23]. Purification of BSA by salt precipitation is not a common practice, even though it is a relatively easy and inexpensive step [26]. We therefore attempted to purify BSA by a combination of ammonium sulfate precipitation, anion exchange chromatography and gel filtration.

Similar to the Cohn pH and ethanol fractionation [6,7], we found that BSA did not precipitate out of serum in a discrete fraction during ammonium sulfate precipitation; rather the protein was found in almost all the fractions collected (**Figure 1**). However, the amount of BSA precipitated increased as the percent saturation of ammonium sulfate increased and appeared to peak between 50% and 60% (**Figure 1**). The major contaminant that was removed by ammonium sulfate was IgG and perhaps other globulins (**Figure 1**, lane 3, arrowed bands). It appeared that most of the globulins were precipitated at 50% ammonium sulfate saturation (**Figure 1**). At 70% ammonium sulfate saturation, almost all contaminating proteins were removed except for a 20-kDa protein (**Figure 1**, lanes 6 and 7, arrowed band). This observation suggests that carefully designed fractionation of serum using ammonium sulfate is effective to purify albumin to a significant purity level.

The partially purified BSA at 70% ammonium sulfate saturation and the final supernatant could be pooled and further purified by size exclusion chromatography; however, the recovery of original BSA would be extremely low since a significant amount of the protein has been lost in the lower fractions. Therefore, we pooled BSA from 50% fraction and upward for further purification. The pooled BSA solution was precipitated using ice-cold acetone to remove lipids. However, it appeared that this step did not contribute significantly to the purity of the protein and therefore might not be necessary. The partially purified BSA was subjected to anion

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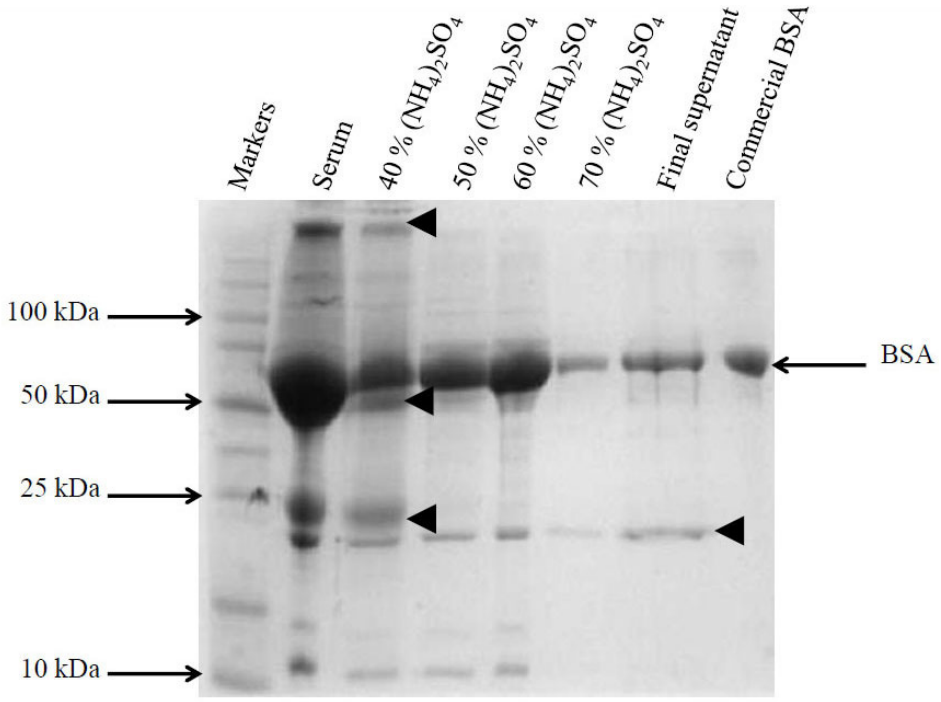

Figure 1. SDS-PAGE analysis of BSA purification from serum by ammonium sulfate precipitation. Proteins were sequentially precipitated from serum by stepwise addition of solid ammonium sulfate and the precipitate reconstituted in buffer containing 20 mM Tris, pH 9, and 100 mM NaCl. Amount of BSA precipitated increased as the percent saturation of ammonium sulfate increased and appeared to peak between 50% and 60%. The major contaminant that was removed by this step was IgG and perhaps other globulins (arrowed bands in lane containing 40% $(NH_4)_2SO_4$). It appeared that most of the globulins were precipitated at 50% ammonium sulfate saturation. At 70% ammonium sulfate saturation, almost all contaminating proteins were removed except for a 20-kDa protein (arrowed bands in lanes containing 70% $(NH_4)_2SO_4$ and final supernatant).

exchange chromatography on a Q Sepharose column. It has been demonstrated that parameters such as sample concentration, sample size and flow rate significantly influence chromatographic separation of BSA and IgG [**28**]. We therefore diluted the partially purified BSA solution to a low concentration of 0.1 mg/mL and loaded the ion exchange column to half its binding capacity at a flow rate of 0.5 mL/min. BSA started eluting from the column at a NaCl concentration of 150 mM but not significantly until 250 mM (**Figure 2**). Transferrin appeared to be the major contaminant that was removed from the BSA preparation by anion exchange (**Figure 2**, lane 2, arrowed band) and to some extent light chains of immunoglobulins. The smaller bands observed in the 250 mM NaCl elution fractions were degradation products of albumin as suggested by western blotting (result not shown). At this stage of the purification, most of the major contaminating bands were absent from the BSA preparation (**Figure 2**).

Finally, we subjected the BSA solution to gel filtration on a Superose 12 10/300 GL column in Tris buffer. At a loading sample concentration of 15 mg/mL, the chromatogram revealed a single major peak corresponding to a molecular weight of 67 kDa based on extrapolation from a calibration curve (**Figure 3A**). No peaks corresponding to contaminating proteins were detected on the chromatogram. SDS-PAGE analysis of the purified BSA (**Figure 3B**) showed the presence of a major band corresponding to the size of BSA. The bands with lower mobilities are clearly oligomers formed during heat denaturation of the protein for SDS-PAGE (**Figure 3B**). The slightly lower mobility of the commercial BSA (**Figure 3B**) may be due to its acetylation. Purified BSA was concentrated to 10 mg/mL and gave a yield of 28 g/L. We estimated that the total BSA yield would be higher than 40 g/L if the protein lost in the lower ammonium sulfate precipitation fractions (≤ 40%) were recovered.

The purified BSA was assayed for the presence of contaminating immunoglobulins by western blotting and chemiluminescence. The blotted protein samples were probed with a HRP-conjugated secondary antibody that reacts with the complete bovine IgG protein as well as the light chains of other bovine immunoglobulins. Despite loading twice the amount of commercial BSA, autoradiograph

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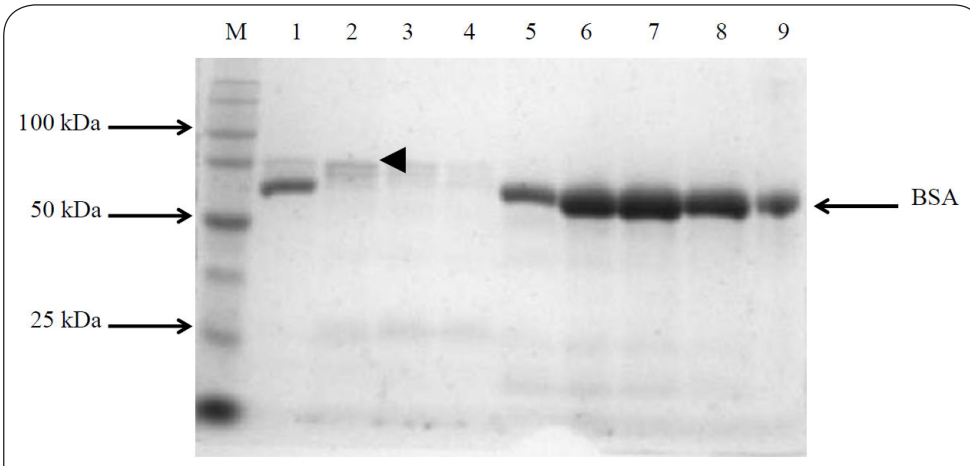

Figure 2. SDS-PAGE analysis of BSA purification by ion exchange chromatography. Fractions (from 50% and above) of BSA partially purified by ammonium sulfate precipitation were pooled and diluted to 0.1 mg/mL (lane 1). The protein solution was then loaded onto a pre-equilibrated Q Sepharose column at a flow rate of 0.5 mL/min, to half its binding capacity. After washing, proteins were eluted from the column by 50 mM stepwise increment of a Tris-buffered 1 M NaCl solution. Transferrin (arrowed band in lane 2), one of the major contaminants of BSA, was removed by this purification step at a NaCl concentration of 150 mM (lanes 2-4). BSA started eluting from the column at a NaCl concentration of 250 mM (lanes 5-8). Light chains of immunoglobulin were also removed to some extent. The smaller bands observed in the 250 mM NaCl elution fractions were probably degradation products of albumin. At this stage of the purification, most of the major contaminating bands were absent from the BSA preparation. Lane 9 is commercial BSA. The non-appearance of the prominent 20-kDa protein seen in figure 1 was due to dilution of the pooled partially purified BSA solution to low concentration of 0.1 mg/mL.

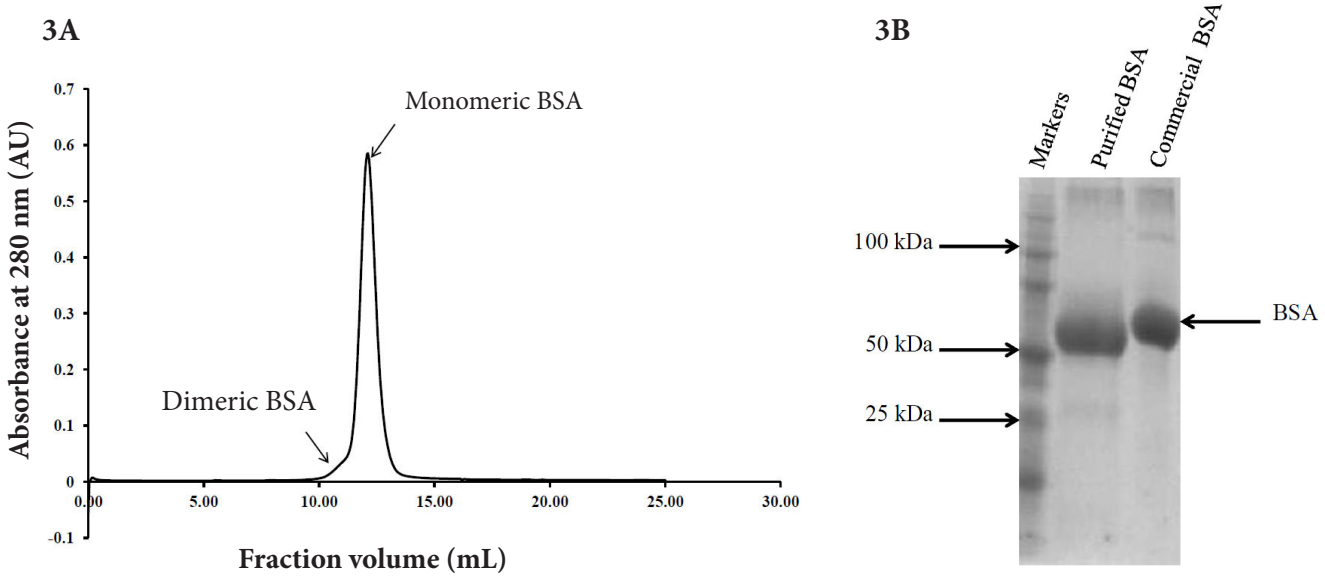

Figure 3. Analysis of purified BSA by gel filtration.
200 µL of 15 mg/mL BSA was fractionated on a pre-equilibrated Superose 12 10/300 GL column at a flow rate of 0.5 mL/min, in buffer 20 mM Tris, pH 9 and 100 mM NaCl,. (A) Chromatogram showed a prominent band corresponding to 67-kDa protein with no contaminating proteins detected. (B) SDS-PAGE analysis showed the presence of a major band corresponding to the size of BSA. The bands running above 100 kDa are oligomers formed during heat denaturation of the protein for SDS-PAGE. The slightly lower mobility of the commercial BSA may be due to its acetylation.

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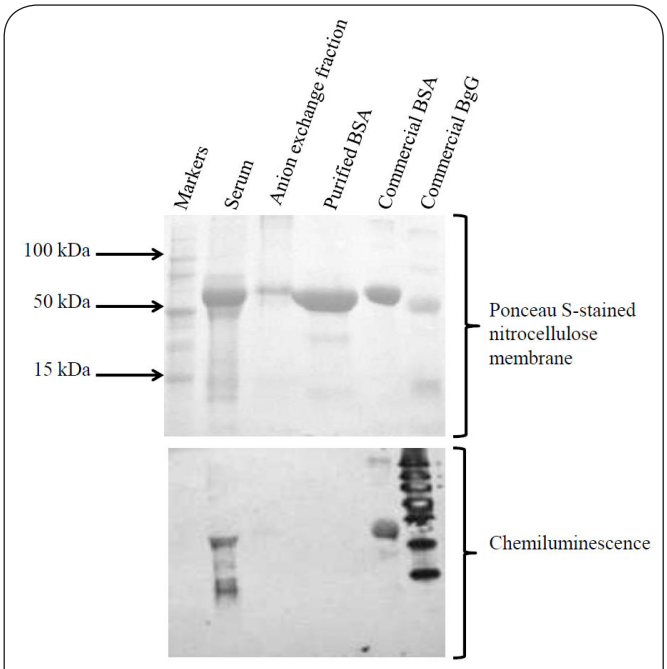

Figure 4. Analysis of purified BSA for the presence of IgGs.
Protein samples were blotted onto nitrocellulose membrane and probed with a HRP-conjugated antibody that reacts with the complete bovine IgG protein as well as the light chains of other bovine immunoglobulins. Chemiluminescence revealed no detectable immunoglobulin band in the purified BSA preparation, despite loading twice the amount of commercial BSA. Several bands corresponding to immunoglobulin heavy and light chains and their oligomers were detected in the commercial BSA sample as well as in the original bovine serum. Sample from one of the elution fractions from anion exchange was loaded instead of the pooled concentrated solution. However, this did not affect the outcome and conclusions of this experiment.

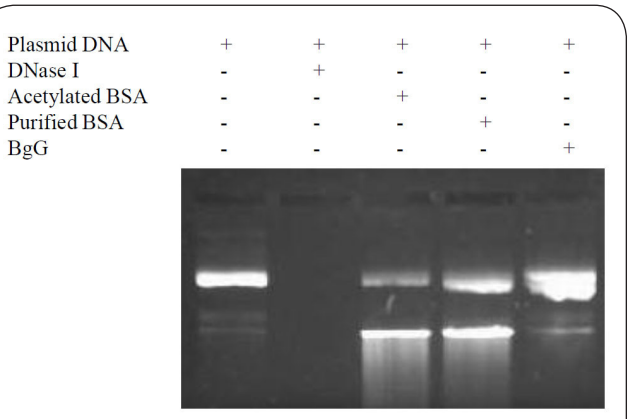

Figure 5. Testing of purified BSA sample for nuclease contamination by DNase I assay.
In separate 25-µL assay reactions, 20 µg of purified BSA, 20 µg of acetylated BSA, 2 µg of commercial BgG, and 1 unit of DNase I were used to digest 1 µg of p202 plasmid DNA. To ensure that any trace amount of DNase would be detected, 20 µg each of purified BSA and acetylated BSA were used in the reaction volume, which is ten times the amount of BSA used in routine restriction digestions. Analysis by agarose gel electrophoresis revealed no detectable amount of nucleases in both the purified and acetylated BSA samples, whereas DNase I completely digested the double-stranded plasmid DNA used as substrate. The streaking effect observed in the BSA assays was due to the high amount of protein in the samples and not a result of nuclease activity.

revealed no detectable immunoglobulin band in the BSA preparation (**Figure 4**). On the other hand, several bands corresponding to immunoglobulin heavy and light chains and their oligomers were detected in the commercial BSA sample as well as in the original bovine serum. These data clearly showed that the purified BSA was free of IgG and possibly other immunoglobulins. Nuclease contamination in the purified BSA sample was tested by assaying for DNase I, a common contaminant. Acetylated BSA, commonly used as stabilizing agent in restriction digestions, was used as a control. To ensure that any trace amount of DNase would be detected, we used 20 µg each of purified BSA and acetylated BSA in the reaction volume. This is ten times the amount of BSA used in routine 20-25 µL reaction volume. Analysis by agarose gel electrophoresis revealed no detectable amount of nucleases in both the purified and acetylated BSA samples (**Figure 5**), whereas DNase I completely digested the double-stranded plasmid DNA used as substrate. The streaking effect observed in the BSA assays was due to the high amount of protein in the samples and not a result of nuclease activity. These data confirmed that the purified BSA was nuclease-free and suitable for routine restriction enzyme digestions. Finally, we tested the purified BSA for the presence of endotoxin using the ToxinSensor Chromogenic LAL assay kit (GenScript, Piscataway, NJ, USA) and found that it contained 4 EU/mL endotoxin (result not shown).

In conclusion, we have used a combination of ammonium sulfate precipitation and liquid chromatography to purify BSA that is free of IgG and DNase, and suitable for most routine biochemical applications. Our approach presents a simple, inexpensive, scalable strategy for purification of BSA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Odutayo O. Odunuga was involved in the design of hypothesis and experiments, collection, analysis and interpretation of data, and all stages of writing of manuscript. Alina Shazhko was involved in collection and analysis of data, and writing of the first draft of manuscript.

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References

1. Peters T. **Serum Albumin**. In Putnam FW **(Ed.), The plasma proteins: structure, function and genetic control**, 1975, **1**: 141, 147.
2. Peters T Jr. **Serum albumin**. *Adv Protein Chem.* 1985; **37**:161-245. | PubMed
3. Peters T. **Albumin in Medicine**. In Peters T (**Ed.**), **All about albumin: biochemistry, genetics, and medical applications**, 1995, 251-284. | Book
4. Tanaka K, Sawatani E, Shigueoka EM, Dias GA, Nakao HC and Arashiro F. **Isolation of bovine plasma albumin by liquid chromatography and its polymerization for use in immunohematology**. *Braz J Med Biol Res.* 2001; **34**:977-83. | Article | PubMed
5. Bertucci C and Domenici E. **Reversible and covalent binding of drugs to human serum albumin: methodological approaches and physiological relevance**. *Curr Med Chem* 2002; **9**:1463-81. | Article | PubMed
6. Cohn EJ, Strong LE and et al. **Preparation and properties of serum and plasma proteins; a system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids**. *J Am Chem Soc.* 1946; **68**:459-75. | Article | PubMed
7. Cohn EJ, Gurd FRN, Surgenor DM, Barnes BA, Brown RK, Derouaux G, Gillespie JM, Kahnt FW, Lever WF, Liu CH, Mittelman D, Mouton RF, Schmid K and Uroma E. **A system for the separation of the components of human blood**. *J. Am. Chem. Soc*. 1950; **72**:465-474. | Article
8. Curling JM, Berglof J, Lindquist LO and Eriksson S. **A chromatographic procedure for the purification of human plasma albumin**. *Vox Sang.* 1977; **33**:97-107. | Article | PubMed
9. Hao Y. **A simple method for the preparation of human serum albumin**. *Vox Sang.* 1979; **36**:313-20. | Article | PubMed
10. Harvey MJ. **The application of affinity chromatography and hydrophobic chromatography to the purification of serum albumin.** In Curling J (**Ed.**), **Methods of plasma protein fractionation.** 1980; pp189-200.
11. Berglof JH, Eriksson S and Curling JM. **Chromatographic preparation and in vitro properties of albumin from human plasma**. *J Appl Biochem* 1983; **5**:282-92. | Article | PubMed
12. Denizli A. **Plasma fractionation: conventional and chromatographic methods for albumin purification.** Hacettepe J. Biol. & Chem. 2011; **39:** 315–341. | Pdf
13. Burnouf T. **Chromatography in plasma fractionation: benefits and future trends**. *J Chromatogr B Biomed Appl.* 1995; **664**:3-15. | Article | PubMed
14. Tanaka K, Shigueoka EM, Sawatani E, Dias GA, Arashiro F, Campos TC and Nakao HC. **Purification of human albumin by the combination of the method of Cohn with liquid chromatography**. *Braz J Med Biol Res.* 1998; **31**:1383-8. | Article | PubMed
15. Rothschild MA, Oratz M and Schreiber SS. **Serum albumin**. *Hepatology.* 1988; **8**:385-401. | Article | PubMed
16. Aoki K, Sato K, Nagaoka S, Kamada M and Hiramatsu K. **Heat denaturation of bovine serum albumin in alkaline pH region**. *Biochim Biophys Acta.* 1973; **328**:323-33. | Article | PubMed
17. Takeda K, Wada A, Yamamoto K, Moriyama Y and Aoki K. **Conformational change of bovine serum albumin by heat treatment**. *J Protein Chem.* 1989; **8**:653-9. | Article | PubMed
18. Fiocchi A, Restani P, Riva E, Mirri GP, Santini I, Bernardo L and Galli CL. **Heat treatment modifies the allergenicity of beef and bovine serum albumin**. *Allergy.* 1998; **53**:798-802. | Article | PubMed
19. Arakawa T and Kita Y. **Protection of bovine serum albumin from aggregation by Tween 80**. *J Pharm Sci.* 2000; **89**:646-51. | Article | PubMed
20. Moriyama Y, Watanabe E, Kobayashi K, Harano H, Inui E and Takeda K. **Secondary structural change of bovine serum albumin in thermal denaturation up to 130 degrees C and protective effect of sodium dodecyl sulfate on the change**. *J Phys Chem B.* 2008; **112**:16585-9. | Article | PubMed
21. Means GE and Bender ML. **Acetylation of human serum albumin by p-nitrophenyl acetate**. *Biochemistry.* 1975; **14**:4989-94. | Article | PubMed
22. Howard JC. **The acylation of bovine serum albumin with diacetylcycloserine**. *Int J Pept Protein Res.* 1977; **9**:175-81. | Article | PubMed
23. Slaaby R, Andersen AS and Brandt J. **IGF-I binding to the IGF-I receptor is affected by contaminants in commercial BSA: the contaminants are proteins with IGF-I binding properties**. *Growth Horm IGF Res.* 2008; **18**:267-74. | Article | PubMed
24. Trujillo LE, Castellanos L, Garcia O and Herrera L. **A protocol for the purification of bovine serum albumin free of deoxyribonuclease activity**. *Biotechniques.* 1990; **9**:620-2. | Article | PubMed
25. Poteser M and Wakabayashi I. **Serum albumin induces iNOS expression and NO production in RAW 267.4 macrophages**. *Br J Pharmacol.* 2004; **143**:143-51. | Article | PubMed Abstract | PubMed Full Text
26. Svensson H. **Fractionation of serum with ammonium sulfate and water dialysis, studied by electrophoresis**. *J. Biol. Chem*. 1941; **139**: 805-825. | Article
27. Tayyab S and Qasim MA. **Purification and properties of buffalo serum albumin**. *Biochem Int.* 1990; **20**:405-15. | PubMed
28. Issaq HJ, Fox SD, Mahadevan M, Conrads TP and Veenstra TD. **Effect of experimental parameters on the HPLC separation of peptides and proteins**. *J. Liq. Chromatogr. R. T.* 2003; **26**: 2255-2283. | Article

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