Article

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Comparative Analysis of Post-Translational Modifications of Human Serum Albumin Derived from Human Plasma and Recombinant Sources in China

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Background: The variations in sequence, three-dimensional structure, and post-translational modifications (PTMs) of human serum albumin (HSA) are crucial for its physiological functions. This study aims to analyze and compare the disparities in PTMs between HSA derived from human plasma and genetically recombinant sources for clinical treatments in China.
Methods: Six distinct PTMs, namely acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, and lactylation, were identified using pan-specific antibodies via Western blot analysis. The samples, comprising human plasma-derived HSA (pHSA) from six different manufacturers and recombinant HSA (rHSA) expressed in yeast and *Oryza sativa*, underwent detection for various types of PTMs. Additionally, a 4D label-free quantitative proteomic analysis was performed to identify N-glycosylation and the aforementioned PTMs in both pHSA and rHSA samples. This analysis aimed to discern disparities in modification sites and levels.
Results: Through Western blot analysis, all six pHSA and two rHSA samples displayed positive bands for albumin (66.5 kDa) across the six PTMs. Subsequent analysis using 4D label-free quantitative proteomics revealed 25 (29) acetylated, 30 (32) succinylated, 41 (50) malonylated, 15 (23) phosphorylated, 36 (30) beta-hydroxybutyrylated, and 27 (34) lactylated modification sites in pHSA and rHSA samples, with no N-glycosylation modification sites detected. The analysis identified 1 acetylation (ALB_K160), 2 beta-hydroxybutyrylation (ALB_K569, ALB_K426), and 3 crotonylation (ALB_K264, ALB_K581, ALB_K560) specific modification sites in pHSA, as well as 3 crotonylation (ALB_K560, ALB_K562, ALB_K75), 1 succinylation (ALB_K490), and 23 phosphorylation specific modification sites in rHSA. In pHSA (rHSA), 2 (6) acetylation, 10 (12) succinylation, 0 (9) crotonylation, 1 (9) phosphorylation, 6 (0) beta-hydroxybutyrylation, and 0 (7) lactylation specific modification sites were found. Moreover, in the shared modification sites between pHSA and rHSA, pHSA exhibited up-regulation of amberylation (16:1) and beta-hydroxybutyrylation (12:2) in more sites, and up-regulation of acetylation (7:11), crotonylation (2:11), phosphorylation (1:8), and lactylation (1:14) in fewer sites compared to rHSA.
Conclusion: In clinical practice, both pHSA and rHSA utilized in China commonly display acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, and lactylation. Notably, there exist distinctions in the site characteristics and modification levels of these alterations between pHSA and rHSA. Further experimental inquiries are imperative to delve into the implications of these disparities in PTMs on the biological functionality, effectiveness, and safety of pHSA and rHSA.

Keywords: human serum albumin; post-translational modification; human plasma-derived; recombinant

Introduction

Serum albumin (SA) is the most prevalent protein in the plasma of all vertebrates [1]. Human serum albumin (HSA) further claims its position as the primary plasma protein in the human body, constituting over 60% of the total protein mass within human plasma. Given its abundance in the circulatory system and its role as a carrier for exogenous and endogenous substances, HSA reigns as the most prolific carrier protein in human serum [2]. HSA fulfills a multitude of biological functions, chiefly encompassing the maintenance of plasma colloid osmotic pressure and the regulation of fluid exchange [3], as well as substance binding and transportation [4], antioxidant properties [5], and enzymatic catalysis [6].

HSA preparations, derived from isolating, purifying, and mass-producing blood products from human plasma, are currently prevalent in clinical practice. They are widely employed in the treatment of fluid resuscitation and hypoalbuminemia associated with various critical ill-

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nesses, including sepsis, septic shock, hemorrhagic shock, acute respiratory distress syndrome, burns, malignant tumors, renal edema, and decompensated liver cirrhosis [7]. Presently, the HSA preparations utilized in clinical therapy are sourced exclusively from healthy human plasma. However, due to constraints in the availability of raw plasma, the supply of plasma-derived HSA (pHSA) falls short of demand in China. Recombinant HSA (rHSA) produced via gene recombination technology emerges as a viable solution. This approach not only mitigates the challenges posed by raw plasma shortages but also reduces the risk of blood-borne pathogen contamination.

Indeed, the international community has been pursuing the production of rHSA through genetic engineering technology since 1981, marking over 30 years of concerted efforts without a significant breakthrough. Despite this extensive duration, the safety, efficacy, and stability of rHSA in practical clinical applications remain within the realm of clinical trials. Presently, two domestic production entities are actively engaged in clinical trials associated with the therapeutic use of rHSA in liver cirrhosis ascites.

Prior investigations have highlighted the substantial consistency between rHSA and pHSA across various dimensions, including pharmacokinetics [8], crystal structure, and three-dimensional (3D) configuration of albumin molecules [9], as well as immunogenicity profiles [10,11]. In 2014, Frahm *et al.* [12] shed light on notable distinctions between rHSA expressed in *Oryza sativa* (*Os*rHSA) and pHSA. Specifically, they observed a higher prevalence of hexose-glycated arginine and lysine residues in *Os*rHSA compared to pHSA. Additionally, they identified variability among suppliers and batches in the degree of glycation at specific lysine and arginine residues for *Os*rHSA. The extensive glycation observed across multiple suppliers may potentially impact the therapeutic utility of *Os*rHSA.

Jean-Luc Plantier *et al.* [13], in 2016, corroborated that distinct HSA preparations exhibit varying antioxidant properties, linked to the redox status of Cys34 residues on HSA molecules. Moreover, one study has elucidated that non-enzymatic glycation modifications can accelerate albumin degradation through the lysosomal degradation pathway, consequently increasing its degradation rate [14]. This phenomenon correlates with the shortened half-life of albumin observed in diabetic patients. Additionally, disparities in the post-translational modifications (PTMs) of HSA sourced from different origins can impact its interactions with drugs [15,16]. These PTM variances may influence the binding affinity and release kinetics of HSA-bound drugs, thereby modulating drug transport and release mechanisms. Consequently, disparities in HSA PTMs may exert a significant influence on the pharmacokinetics and clinical efficacy of HSA-associated medications. These research findings underscore the imperative of investigating HSA modifications in the context of drug interactions and treatment.

In recent years, the liver disease community has introduced the concept of the "effective albumin concentration theory" [17]. This theory pertains to the concentration of albumin required to maintain normal physiological functions primarily through non-colloidal mechanisms. Professor Caraceni highlighted that effective albumin denotes a detectable subtype of albumin possessing complete functional attributes. In patients with decompensated cirrhosis, functional impairment leads to a more pronounced reduction in effective albumin compared to total albumin [18]. PTMs serve as crucial origins of albumin subtypes [19]. As previously mentioned, PTMs can modulate the properties and functions of HSA. These modifications may arise from enzymatic or non-enzymatic reactions, influenced by the pathophysiological state of the body and the biochemical milieu surrounding the protein. Due to variations in source materials and purification technologies, PTMs occurring in pHSA and rHSA preparations may diverge. Alterations in albumin subtypes induced by different PTMs in pHSA and rHSA preparations could potentially impact their biological functions, consequently affecting the efficacy of HSA preparations.

In summary, we posit that the identification and comparative analysis of PTMs in pHSA and rHSA hold paramount importance in elucidating the heterogeneity, efficacy, and safety profiles of HSA preparations sourced from different origins. To address this, our study employed a sequential approach integrating pan-specific antibody Western blot and 4D label-free PTMs quantitative proteomic techniques. This comprehensive methodology enabled us to detect and analyze differences in acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, lactylation, as well as N-glycosylation modifications present in pHSA and rHSA formulations.

Materials and Methods

HSA

In order to provide a comprehensive overview of the PTMs landscape of albumin in pHSA and rHSA formulations utilized in current clinical therapy within China, we embarked on a concerted effort to procure samples from nearly all domestic manufacturers of pHSA formulations. Additionally, we obtained samples of rHSA formulations sourced from both yeast and rice origins, which are currently in the initial stages of clinical trials. It is noteworthy that all of these HSA preparations conform rigorously to the quality standards outlined in the Chinese Pharmacopoeia, exhibiting remarkable stability and consistency across various batches [20].

Human Plasma-Derived HSA (pHSA)

pHSA samples were procured from six domestic blood product companies, each with the following specifications:

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10 g per bottle (20% concentration, 50 mL volume). The details of the samples, including batch numbers, are provided below: (1) pHSA-1, batch number: A052202207; (2) pHSA-2, batch number: 20220205A1; (3) pHSA-3, batch number: 20210737; (4) pHSA-4, batch number: 202209092; (5) pHSA-5, batch number: 202203007; (6) pHSA-6, batch number: 202204022.

Recombinant HSA (rHSA)

rHSA samples were acquired from two biopharmaceutical companies, each with the following specifications: 10 g per bottle (20% concentration, 50 mL volume). The details of the samples, including batch numbers and expression sources, are provided below: (1) rHSA-1 (expressed in yeast), batch number: ART102L-230712-01; (2) rHSA-2 (expressed in *Oryza sativa*), batch number: C202208003.

Reagents and Instruments

Reagents

Acrylamide (A2792-1L, Sigma-Aldrich, Saint Louis, MO, USA); Trometamol (A610195-0005, Sangon Biotech, Shanghai, China); Glycine (A502065-0005, Sangon Biotech, Shanghai, China); Sodium chloride (S9888, Sigma-Aldrich, Saint Louis, MO, USA); Sodium lauryl sulfate (S8010-500g, Solarbio, Beijing, China); Ammonium persulfate (A600072-0025, Sangon Biotech, Shanghai, China); Skim milk powder (M203-10G-10PK, Amresco, Solon, OH, USA); Serum albumin (A8020-500, Solarbio, Beijing, China); β-mercaptoethanol (M6250-250ML, Sigma-Aldrich, Saint Louis, MO, USA); Bromophenol blue (114391-25G, Sigma-Aldrich, Saint Louis, MO, USA); Tetramethylethylenediamine (T9281-100mL, Sigma-Aldrich, Saint Louis, MO, USA); Absolute alcohol (23221151, Hangzhou Gaojing Jingxi Chemical Co., Ltd., Hangzhou, China); PONCEAU S (P3504, Sigma-Aldrich, Saint Louis, MO, USA); PageRuler™ Plus Prestained Protein Ladder (26619, ThermoFisher scientific, Waltham, MA, USA); Chemiluminescent HRP substrate (WBKLS0500, Millipore, Bedford, MA, USA); anti-mouse secondary antibody (31430, Pierce, Rockford, IL, USA); anti-rabbit secondary antibody (31460, Pierce, Rockford, IL, USA); TWEEN 20 (T104863, Aladdin, Shanghai, China); Nitrocellulose filter membrane (66485, PALL, Ann Arbor, MI, USA); anti-acetyllysine, anti-succinyllysine, anti-crotonyllysine, anti-phosphotyrosine, anti-beta-Hydroxybutyryllysine, and anti-lactyllysine primary antibodies (PTM-105RM, PTM-419, PTM-502, PTM-702RM, PTM-1201RM, PTM-1401RM, PTM Bio, Hangzhou, China); modified enriched antibody resin: acetylation, succinylation, crotonylation, beta-hydroxybutyrylation, and lactylation (PTM104, PTM402, PTM503, PTM1204, PTM1404, PTM Bio, Hangzhou, China); formic acid (A117-50, Fluka, Neu-Ulm, Germany); acetonitrile (204433, ThermoFisher Scientific, Waltham, MA, USA); Glycerol (A100854, Sangon Biotech, Shanghai, China); Coomassie brilliant blue dye (B802270-5g, Hangzhou Mick Chemical Instrument Co., Ltd., Hangzhou, China); acetic acid (11236005, Hangzhou Gaojing Jingxi Chemical Co., Ltd., Hangzhou, China); Ethanol (211065165, Hangzhou Gaojing Jingxi Chemical Co., Ltd., Hangzhou, China); Protein marker (26620, ThermoFisher Scientific, Waltham, MA, USA); Ultrapure water (10977015, ThermoFisher Scientific, Waltham, MA, USA); bicinchoninic acid assay (BCA) kit (P0011, Beyotime, Shanghai, China); Trypsin (V5117, Promega, Beijing, China); Triethylammonium bicarbonate buffer (T7408-500mL, Sigma-Aldrich, Saint Louis, MO, USA); acetone (23200501, Hangzhou Hannuo Chemical Co., Ltd., Hangzhou, China); iodoacetamide (V900335-5G, Sigma-Aldrich, Saint Louis, MO, USA); DL-dithiothreitol (D9163-25G, Sigma-Aldrich, Saint Louis, MO, USA); trifluoroacetic acid (302031-1L, Sigma-Aldrich, Saint Louis, MO, USA); Methanol (A452-4, ThermoFisher Scientific, Waltham, MA, USA); C18 ZipTips (ZTC18M096, Millipore, Bedford, MA, USA).

Instruments

Mini-PROTEAN® Tetra electrophoresis chamber (1658004, Bio-rad, Hercules, CA, USA); PowerPac™ Basic, Electrophoresis Power Supply (1645050, Bio-rad, Hercules, CA, USA); horizontal rotating shaker (TS-200, Kylin-bell, Haimen, China); automatic chemiluminescence imaging analysis system (5200, Tanon, Shanghai, China); nanoElute UHPLC system (nanoElute, Bruker, Billerica, MA, USA); timsTOF Pro mass spectrometry (timsTOF Pro, Bruker, Billerica, MA, USA); Solid phase extraction column (8B-S100-AAK, Phenomenex, Torrance, CA, USA); Refrigerated centrifuge (centrifuge 5427R, Eppendorf, Hamburg, Germany); Centrifuge Concentrator (concentrator plus, Eppendorf, Hamburg, Germany); Thermostat water bath (DK-8D, Shanghai Boxun Medical Biological Instrument Co., Ltd., Shanghai, China); Thermostatic blast drying oven (DHG-9240A, Shanghai Yiheng Instrument Co., Ltd., Shanghai, China); Ultra-Low Temperature Freezers (906-ULTS, ThermoFisher Scientific, Waltham, MA, USA); Biosafety cabinet (1200 A2, Hfsafe, Shanghai, China); Microplate reader (IMARK, Bio-rad, Hercules, CA, USA); Water purification instrument (RD0GP1000, Direct-Pure Genie, Molsheim, France); Ice machine (XB130-FZ, GRANT, Cambridge, UK); NanoPhotometer (NP80Touch, IMPLEN, Munich, Germany); Electrophoresis apparatus (1645050, Bio-rad, Hercules, CA, USA); Automated solid-phase Extractor (Resolvex A100, TECAN, Crailsheim, Germany); Scanner (V600, Epson, Beijing, China).

Analysis Software

MaxQuant (max planck institute of biochemistry, Martinsried, Germany; https://www.maxquant.org/maxquant/).

Experimental Methods

Pan-Antibody Western Blot Detection for Various PTMs

Electrophoresis. A 5 µg protein sample was mixed with 4× sample buffer and diluted to 1× concentration. Subsequently, an appropriate volume of protein lysis buffer was added to adjust the sample concentration to 1~2 mg/mL, followed by heating for 10 minutes at 95 °C. Sequentially, 20% pre-stained protein markers and 5 µg protein sample, each at the same volume, were loaded onto the gel. An equal volume of 1× loading buffer was added for blocking. Electrophoresis was conducted under the following conditions: a constant pressure of 80 V for 30 minutes, followed by a constant pressure of 120 V until bromophenol blue migrated out from the separation gel.

Membrane Transference. The electrotransfer buffer was pre-cooled in a refrigerator at 4 °C for 1 hour. Subsequently, the nitrocellulose membrane was immersed in the electrotransfer buffer and allowed to equilibrate for approximately 30 minutes. Carefully, the gel was placed flat on the membrane to prevent the formation of bubbles between the gel and the membrane. Following this, the transfer clip, oriented in the correct electrode direction, was placed into the electric Trans-blot tank. The tank was then positioned in a 4 °C environment, and electrotransfer was conducted at a constant current of 200 mA for 1 hour.

Membrane Blocking. The membrane was blocked with 5% skim milk powder diluted in 1× Tris-buffered saline containing Tween 20 (TBST) at room temperature for 1 hour.

Antibody Dilution. Dilute the antibodies in TBST containing 2.5% bovine serum albumin (BSA). The primary antibody dilutions are as follows: anti-acetyllysine at 1:1000, anti-succinyllysine at 1:6000, anti-crotonyllysine at 1:1000, anti-phosphotyrosine at 1:1000, anti-beta-Hydroxybutyryllysine at 1:300, and anti-lactyllysine at 1:1000. The secondary antibodies should be diluted at 1:10000.

Incubation with Primary and Secondary Antibodies. After blocking, the membrane was washed with TBST three times (10 minutes each time). The primary antibodies, diluted in TBST containing 2.5% BSA, were then incubated with the membrane overnight at 4 °C with mild shaking. Subsequently, the membrane was rinsed with TBST three times (10 minutes each time) and then incubated with secondary antibodies at room temperature. After 1 hour, the membrane was washed again with TBST three times (10 minutes each time).

Exposure. The membrane was incubated with a chemiluminescent HRP substrate for 2 minutes, and the signals were captured following the operating instructions of the chemiluminescent imaging system. The exposure time was adjusted based on the strength of the signal to achieve the optimal exposure signal.

4D Label-Free PTMs Quantitative Proteomic Analysis for Various PTMs

Protein Extraction. The samples stored at 4 °C were retrieved and subjected to protein concentration determination using a BCA kit (P0011, Beyotime, Shanghai, China).

Trypsin Digestion. Equal amounts of protein samples were digested with trypsin and adjusted to a consistent volume with the lysis buffer. Subsequently, a final concentration of 20% Trichloro acetic acid (TCA) was slowly added, and the mixture was thoroughly vortexed and precipitated at 4 °C for 2 hours. After centrifugation at 4500 ×g for 5 minutes, the supernatant was removed, and the precipitate was washed 2–3 times with pre-cooled acetone. Following drying, the precipitate was reconstituted with 200 mM TEAB, sonicated, and then incubated with trypsin at a ratio of 1:50 (protease: protein, m/m) overnight. Subsequently, dithiothreitol (DTT) was added to the treated samples to achieve a final concentration of 5 mM and reduced at 56 °C for 30 minutes. Upon reaching a final concentration of 11 mM with the addition of iodoacetamide (IAA), the samples were further incubated for 15 minutes at room temperature in the dark.

Modification Enrichment. Acetylation, succinylation, crotonylation, beta-hydroxybutyrylation, and lactylation were enriched using modified antibody resin. Specifically, the peptide segments were dissolved in IP buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0). The supernatant was then transferred to pre-washed resin (Kac, PTM-104; Ksucc, PTM-402; Kcr, PTM-503; Kbhb, PTM-1204; Kla, PTM-1404; PTM Bio, Hangzhou, China), placed on a horizontal rotating shaker at 4 °C, gently shaken, and incubated overnight. The resin was subsequently washed four times with IP buffer and two times with deionized water. Elution of the peptide segments bound to the resin was achieved by applying 0.1% trifluoroacetic acid eluent thrice. The eluent was collected and drained under freezing and vacuum. Salt removal was carried out according to the instructions of C18 ZipTips. Following draining under freezing and vacuum, liquid chromatography-mass spectrometry analysis was performed. For phosphorylation modification enrichment, the peptide segments were dissolved in an enrichment buffer (50% acetonitrile/0.5% acetic acid). The supernatant was transferred to pre-washed IMAC (Immobilized Metal Affinity Chromatography) materials and incubated with mild shaking on a rotating shaker. Subsequently, the materials were washed three times using 50% acetonitrile/0.5% acetic acid and 30% acetonitrile/0.1% trifluoroacetic acid buffer. A wash with 10% aqueous ammonia was performed

Table 1. Modification sites in pHSA and rHSA for various PTMs using 4D label-free PTMs quantitative proteomic analysis.

Modification types \ Sample classification	pHSA	rHSA
Acetylated modification sites	ALB_K588, ALB_K597, ALB_K298, ALB_K236, ALB_K229, ALB_K214, ALB_K36, ALB_K249, ALB_K549, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K499, ALB_K186, ALB_K460, ALB_K437, ALB_K286, ALB_K130, ALB_K548, ALB_K305, ALB_K438, ALB_K219, ALB_K456, ALB_K160	ALB_K588, ALB_K597, ALB_K298, ALB_K229, ALB_K214, ALB_K219, ALB_K36, ALB_K558, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K456, ALB_K499, ALB_K186, ALB_K286, ALB_K236, ALB_K249, ALB_K549, ALB_K460, ALB_K97, ALB_K305, ALB_K438, ALB_K383, ALB_K130, ALB_K257, ALB_K598, ALB_K264, ALB_K437
Succinylated modification sites	ALB_K588, ALB_K597, ALB_K298, ALB_K236, ALB_K229, ALB_K214, ALB_K36, ALB_K249, ALB_K549, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K499, ALB_K186, ALB_K565, ALB_K341, ALB_K558, ALB_K383, ALB_K460, ALB_K437, ALB_K305, ALB_K438, ALB_K205, ALB_K463, ALB_K468, ALB_K28, ALB_K97, ALB_K117, ALB_K598	ALB_K588, ALB_K597, ALB_K298, ALB_K229, ALB_K214, ALB_K219, ALB_K36, ALB_K558, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K456, ALB_K499, ALB_K186, ALB_K565, ALB_K305, ALB_K438, ALB_K383, ALB_K130, ALB_K468, ALB_K28, ALB_K584, ALB_K160, ALB_K198, ALB_K569, ALB_K581, ALB_K463, ALB_K543, ALB_K183, ALB_K402, ALB_K490
Malonylated modification sites	ALB_K588, ALB_K597, ALB_K298, ALB_K236, ALB_K229, ALB_K214, ALB_K36, ALB_K249, ALB_K549, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K499, ALB_K186, ALB_K565, ALB_K341, ALB_K558, ALB_K383, ALB_K460, ALB_K437, ALB_K286, ALB_K130, ALB_K548, ALB_K305, ALB_K438, ALB_K205, ALB_K463, ALB_K468, ALB_K28, ALB_K97, ALB_K219, ALB_K456, ALB_K117, ALB_K257, ALB_K562, ALB_K88, ALB_K584, ALB_K264, ALB_K581, ALB_K560	ALB_K588, ALB_K597, ALB_K298, ALB_K229, ALB_K214, ALB_K219, ALB_K36, ALB_K558, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K456, ALB_K499, ALB_K186, ALB_K565, ALB_K286, ALB_K236, ALB_K249, ALB_K549, ALB_K460, ALB_K97, ALB_K305, ALB_K438, ALB_K383, ALB_K130, ALB_K468, ALB_K28, ALB_K205, ALB_K341, ALB_K548, ALB_K88, ALB_K257, ALB_K584, ALB_K160, ALB_K598, ALB_K426, ALB_K198, ALB_K569, ALB_K581, ALB_K463, ALB_K543, ALB_K183, ALB_K402, ALB_K117, ALB_K264, ALB_K437, ALB_K560, ALB_K562, ALB_K75
Phosphorylated modification sites	ALB_S256, ALB_S504, ALB_S443, ALB_S89, ALB_S82, ALB_S294, ALB_S297, ALB_T190, ALB_T590, ALB_T502, ALB_T107, ALB_T551, ALB_T444, ALB_T71, ALB_T92	ALB_S504, ALB_S443, ALB_S216, ALB_S226, ALB_S459, ALB_S89, ALB_S82, ALB_S294, ALB_S297, ALB_T190, ALB_T590, ALB_T502, ALB_T107, ALB_T551, ALB_T444, ALB_T446, ALB_T376, ALB_T103, ALB_T71, ALB_T92, ALB_T498, ALB_T267, ALB_Y162
Beta-hydroxybutyrylated modification sites	ALB_K588, ALB_K597, ALB_K298, ALB_K236, ALB_K229, ALB_K214, ALB_K36, ALB_K249, ALB_K549, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K499, ALB_K186, ALB_K565, ALB_K341, ALB_K558, ALB_K383, ALB_K460, ALB_K437, ALB_K286, ALB_K130, ALB_K548, ALB_K205, ALB_K463, ALB_K468, ALB_K28, ALB_K97, ALB_K219, ALB_K456, ALB_K257, ALB_K562, ALB_K88, ALB_K569, ALB_K426	ALB_K588, ALB_K597, ALB_K298, ALB_K229, ALB_K214, ALB_K219, ALB_K36, ALB_K558, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K456, ALB_K499, ALB_K186, ALB_K565, ALB_K286, ALB_K236, ALB_K249, ALB_K549, ALB_K460, ALB_K97, ALB_K468, ALB_K28, ALB_K205, ALB_K341, ALB_K548, ALB_K88, ALB_K257, ALB_K426

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Table 1. Continued.

Modification types \ Sample classification	pHSA	rHSA
Lactylated modification sites	ALB_K588, ALB_K597, ALB_K298, ALB_K236, ALB_K229, ALB_K214, ALB_K36, ALB_K249, ALB_K549, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K499, ALB_K186, ALB_K565, ALB_K341, ALB_K558, ALB_K383, ALB_K286, ALB_K130, ALB_K548, ALB_K305, ALB_K438, ALB_K117, ALB_K584, ALB_K598	ALB_K588, ALB_K597, ALB_K298, ALB_K229, ALB_K214, ALB_K219, ALB_K36, ALB_K558, ALB_K161, ALB_K375, ALB_K223, ALB_K300, ALB_K456, ALB_K499, ALB_K186, ALB_K565, ALB_K286, ALB_K236, ALB_K249, ALB_K549, ALB_K460, ALB_K97, ALB_K305, ALB_K438, ALB_K383, ALB_K130, ALB_K205, ALB_K341, ALB_K548, ALB_K88, ALB_K584, ALB_K160, ALB_K598, ALB_K117

Note: no *N*-glycosylation modification sites were detected in pHSA and rHSA for various PTMs using 4D label-free PTMs quantitative proteomic analysis.

to wash the phosphopeptides, and the eluent was collected and drained under vacuum and freezing. Salt removal was conducted following the instructions of C18 ZipTips. After draining under freezing and vacuum, liquid chromatography-mass spectrometry analysis was performed.

For N-glycosylation modification enrichment, the peptide segments were dissolved in 200 µL of enrichment buffer (80% acetonitrile/5% trifluoroacetic acid). The supernatant was then transferred to a hydrophilic (HILIC) microcolumn and centrifuged at 1000 g for approximately 15 minutes to complete the enrichment process. Subsequently, the HILIC microcolumns were washed three times with enrichment buffer. Elution of glycopeptides was achieved using 0.1% trifluoroacetic acid, 50 mM ammonium bicarbonate solution, and 50% acetonitrile. The eluent was collected, mixed, and drained under vacuum and freezing. Following this, the glycopeptides were re-dissolved in 50 mM ammonium bicarbonate buffer dissolved in 50 µL of heavy-oxygen water. The samples were then subjected to reaction with 2 µL of PNGase F glycosidase and digestion overnight at 37 °C. Desalting was carried out according to the instructions provided with C18 ZipTips, and liquid chromatography-mass spectrometry analysis was performed after vacuum-freeze drying. For phosphorylation modification enrichment, peptide mixtures were initially incubated with IMAC microspheres suspension in a loading buffer (50% acetonitrile/0.5% acetic acid) with vibration. To remove non-specifically adsorbed peptides, the IMAC microspheres were washed sequentially with 50% acetonitrile/0.5% acetic acid and 30% acetonitrile/0.1% trifluoroacetic acid. Subsequently, enriched phosphopeptides were eluted by adding elution buffer containing 10% NH_4OH with vibration. The supernatant containing phosphopeptides was collected and lyophilized for LC-MS/MS analysis.

Liquid Chromatography-Mass Spectrometry Analysis. The peptide segments were dissolved in liquid chromatography mobile phase A and separated using the NanoElute ultra-high performance liquid chromatography system. Mobile phase A consisted of an aqueous solution containing 0.1% formic acid and 2% acetonitrile, while mobile phase B was an acetonitrile-aqueous solution containing 0.1% formic acid. For acetylation, succinylation, crotonylation, beta-hydroxybutyrylation, and lactylation, the gradient liquid phase was set as follows: 0–40 minutes, 6%–24% B; 40–52 minutes, 24%–35% B; 52–56 minutes, 35%–80% B; 56–60 minutes, 80% B; with a flow rate of 450 nL/min. For *N*-glycosylation, the gradient liquid phase was set as follows: 0–48 minutes, 2%–22% B; 48–52 minutes, 22%–35% B; 52–56 minutes, 35%–90% B; 56–60 minutes, 90% B; also with a flow rate of 450 nL/minute. For phosphorylation, the gradient liquid phase was set as follows: 0–76 minutes, 2%–22% B; 76–82 minutes, 22%–35% B; 82–86 minutes, 35%–90% B; 86–90 minutes, 90% B; with a flow rate of 450 nL/minutes. The peptide segments were separated by an ultra-high performance liquid chromatography system and injected into a Capillary ion source for ionization before being analyzed by timsTOF Pro mass spectrometry, with the ion source voltage set to 1.7 kV. The peptide parent ions and their secondary fragments were detected and analyzed using high-resolution TOF. The scanning range of the secondary mass spectrometry was set to 100–1700. Parallel accumulation-serial fragmentation (PASEF) mode was utilized for data collection. A first-order mass spectrum was followed by 10 times collection of second-order mass spectrum at PASEF modes with charge numbers of parent ions ranging from 0 to 5. The dynamic exclusion time for tandem mass spectrometry scanning was set to 24 seconds to avoid repeated scanning of parent ions.

Data Analysis

The second-order mass spectrum data were processed using MaxQuant (v1.6.15.0, Max Planck Institute of Biochemistry, Martinsried, Germany) with the following parameters:

- Database: Homo_sapiens_9606_SP_20230103.fasta (20389 sequences), supplemented with a reverse library to calculate the false positive rate (FDR) caused by random matching.

- Digestion method: Trypsin/P.

- Maximum missed cleavages: 4 (phosphorylation set as 2 and *N*-glycosylation).

- Minimum peptide length: 7 amino acid residues.

- Maximum number of peptide modifications: 5.

- Mass tolerance of primary parent ions in First search and Main search: 20 ppm.

- Mass tolerance for secondary fragment ions: 20 ppm.

- Fixed modification: Carbamidomethyl (C) for cysteine alkylation.

- Variable modifications: Oxidation of methionine, *N*-terminal acetylation of protein, acetylation of lysine, succinylation of lysine, crotonylation of lysine, phosphorylation of serine, threonine, and tyrosine, Deamidation of asparagine (18O), beta-hydroxybutyrylation of lysine, and lactylation of lysine.

- False discovery rate (FDR) for protein identification and peptide-spectrum match (PSM) identification: 1%.

Results

Pan-Antibody Western Blot Results for Various PTMs in Albumin Samples

The Western blot analysis conducted with pan-specific antibodies targeting six PTMs—acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, and lactylation—revealed positive bands for both pHSA and rHSA derived from yeast and *Oryza sativa* obtained

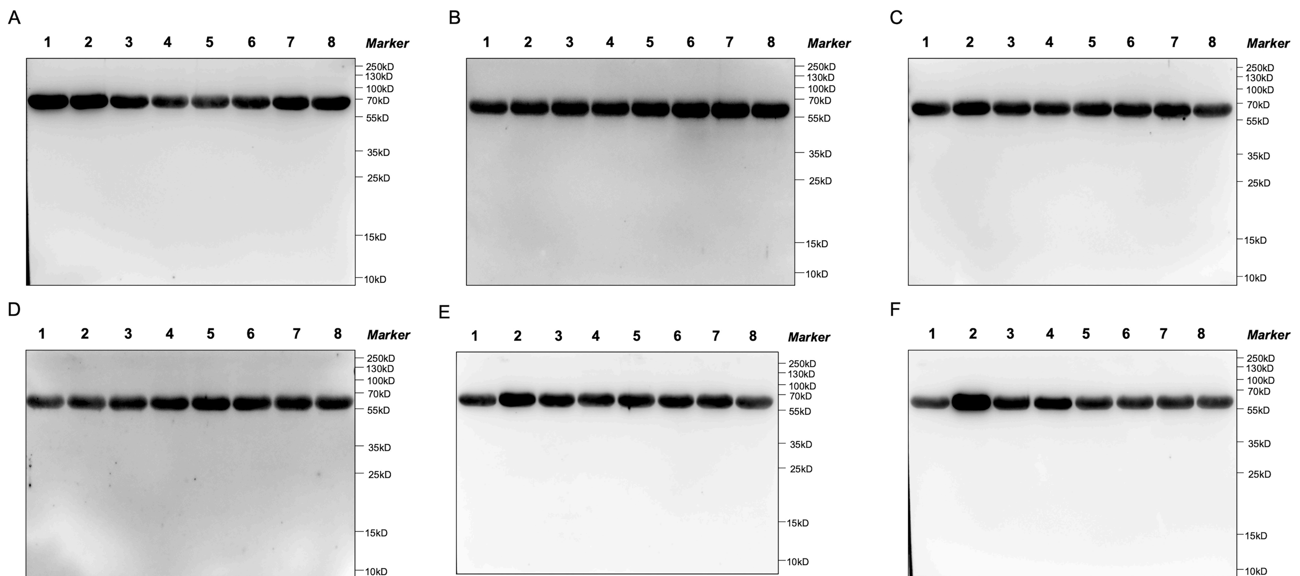

Fig. 1. Western blot results of pan-specific antibodies for various post-translational modifications (PTMs) in various albumin samples. (A–F) Western blot results of pan-specific antibodies for PTMs, acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, and lactylation (Pan-specific antibody gel without *N*-glycosylation modification); 1 referred to rHSA expressed in yeast; 2 referred to rHSA expressed in *Oryza sativa*; 3–8 referred to pHSA from 6 different companies. HSA, human serum albumin; pHSA, plasma-derived HSA; rHSA, recombinant HSA.

from six different companies. These bands corresponded to the expected molecular weight of albumin (66.5 kDa) (Fig. 1).

4D Label-Free PTMs Quantitative Proteomic Analysis Results of pHSA and rHSA for Various PTMs

According to the results of 4D label-free PTMs quantitative proteomic analysis, amino acid sites that underwent the aforementioned six modifications were detected in both pHSA and rHSA. Specifically, 25 (29) acetylated, 30 (32) succinylated, 41 (50) malonylated, 15 (23) phosphorylated, 36 (30) beta-hydroxybutyrylated, and 27 (34) lactylated modification sites were identified in both pHSA and rHSA (Table 1).

Further analysis of the six PTMs in pHSA and rHSA revealed specific modification sites. In pHSA, one acetylation site (ALB_K160), two beta-hydroxybutyrylation sites (ALB_K569, ALB_K426), three crotonylation sites (ALB_K264, ALB_K581, ALB_K560), and 15 phosphorylation sites were identified. Each of these sites exhibited only one type of modification. In contrast, in rHSA, three crotonylation sites (ALB_K560, ALB_K562, ALB_K75), one succinylation site (ALB_K490), and 23 phosphorylation sites were detected. All fifteen (twenty-three) phosphorylation sites identified in pHSA and rHSA were specific modification sites (Fig. 2).

Difference Analysis Results between pHSA and rHSA for Various PTMs

Through analysis of the differences in PTM sites between pHSA and rHSA, it was found that in pHSA and rHSA, there were 2 (6) acetylation, 10 (12) succinylation, 0 (9) crotonylation, 1 (9) phosphorylation, 6 (0) beta-hydroxybutyrylation, and 0 (7) lactylation specific modification sites. These specific modification sites were only detected in either pHSA or rHSA. However, the above-mentioned six PTMs were also found at common sites in both pHSA and rHSA, indicating that the corresponding site modifications occurred in both pHSA and rHSA (Fig. 3).

Further analysis of common sites for the six PTMs in pHSA and rHSA revealed differences in the prevalence of modifications between the two. Compared to rHSA, pHSA exhibited a higher occurrence of succinylation (16:1) and beta-hydroxybutyrylation (12:2) modifications at common sites. Conversely, relatively fewer common sites in pHSA showed up-regulation of acetylation (7:11), crotonylation (2:11), phosphorylation (1:8), and lactylation (1:14) modifications (Fig. 4).

To provide a clearer visualization and analysis of the location of the six PTMs and modification sites within the amino acid residue sequences and 3D spatial structure of pHSA and rHSA, we generated distribution sequence diagrams and 3D structural diagrams of PTM residue sites in albumin. These diagrams depict the specific sites as well as common sites with or without differences that were analyzed previously in pHSA and rHSA (Figs. 5,6).

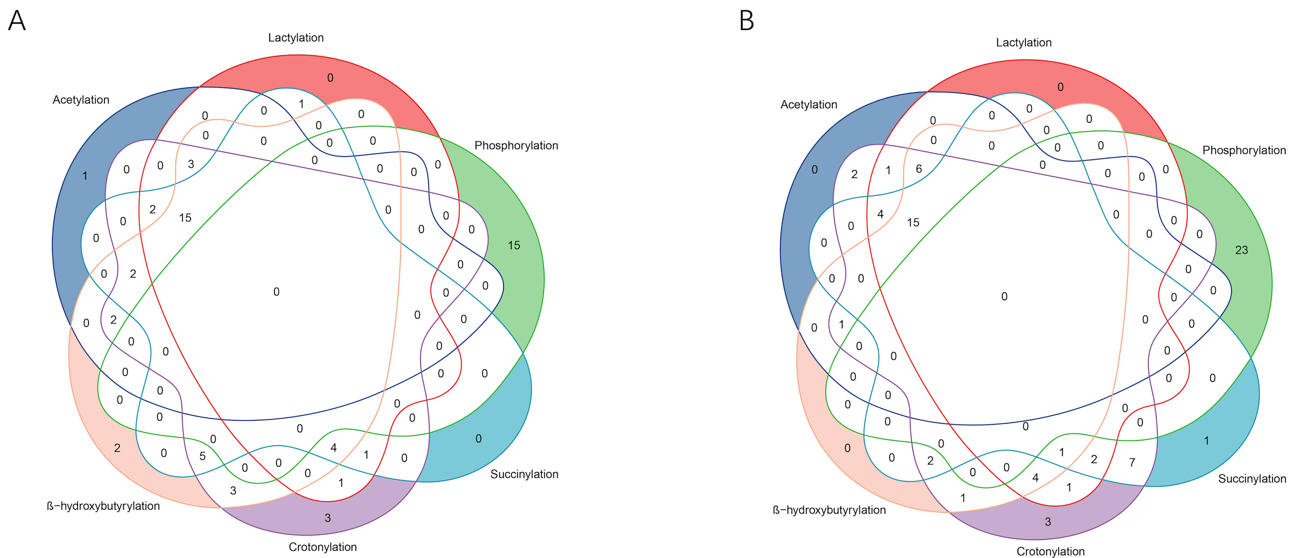

Fig. 2. Petal image of site analysis results regarding 6 PTMs in pHSA and rHSA. (A) Relationship of modification sites in pHSA. (B) Relationship of modification sites in rHSA.

Fig. 3. Venn diagram of difference analysis of modification sites between pHSA and rHSA for various PTMs. (A–F) Difference analysis of acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, and lactylation modification sites in pHSA and rHSA (RY: pHSA; CZ: rHSA).

Discussion

PTMs of proteins denote the alterations that occur to proteins after translation. These modifications can be categorized into enzymatic and non-enzymatic types depending on whether enzymatic catalysis is involved, and they may occur either intracellularly or extracellularly. PTMs encompass various processes, including protease cleavage, enzymatic addition or removal of chemical groups (such as phosphate, methyl, acetyl, and even macromolecules like ubiquitin), as well as non-enzymatic chemical reactions involving organic and inorganic compounds. Albumin, a protein present throughout the human body, interfaces with nearly all cells and tissues. Consequently, PTMs may result

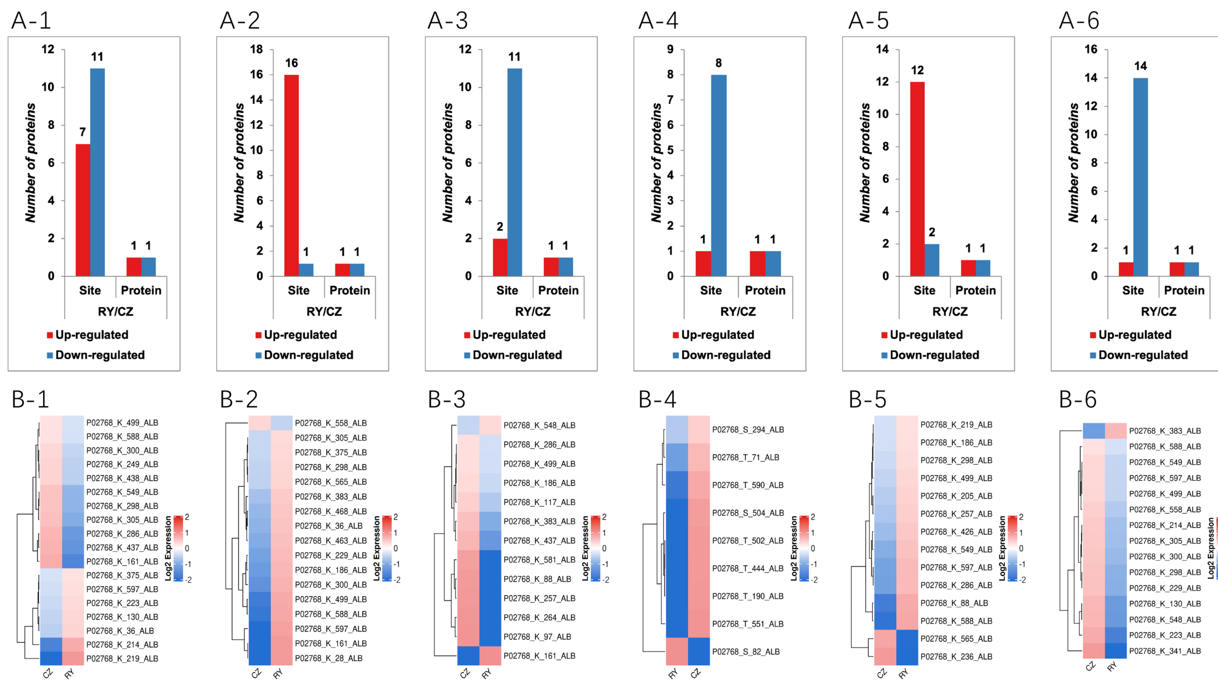

Fig. 4. Differential analysis statistical graph (A) and heatmap (B) of the commonly modified sites shared between pHSA and rHSA for 6 PTMs. 1–6 were the analysis results of acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, and lactylation, respectively.

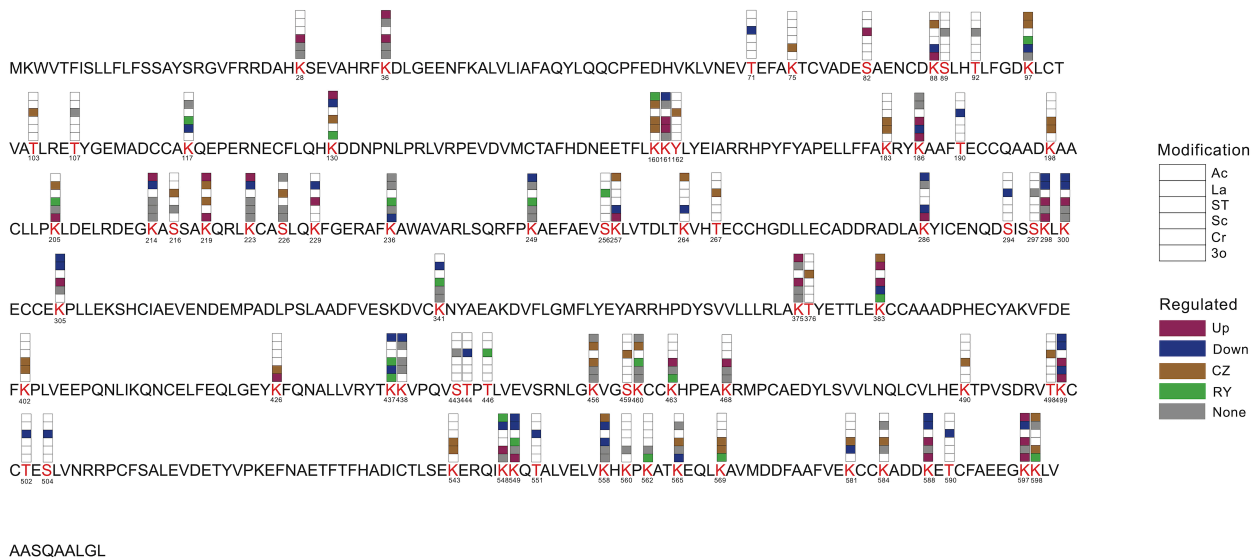

Fig. 5. Distribution sequence diagram of 6 PTM residue sites in pHSA and rHSA. The total distribution sequence diagrams of acetylation (Ac), succinylation (Sc), crotonylation (Cr), phosphorylation (St), beta-hydroxybutyrylation (3o), and lactylation (La) modification residue sites in pHSA and rHSA (Up: relatively high expression in pHSA; Down: relatively low expression in pHSA; CZ: only occurring in rHSA; RY: only occurring in pHSA).

from enzymatic catalysis by cell-associated enzymes. Additionally, diverse chemical reactions occurring in plasma or interstitial fluid can induce PTMs, such as glycosylation [21].

Moreover, phosphorylation, glycosylation, acetylation, carbonylation, and methylation modifications of HSA have been documented. As of January 2022, over 210 types of PTMs of HSA have been identified [22]. Given the prolonged circulating half-life of HSA in the body and its exposure to various stressors, preparations of pHSA derived from a blend of healthy human plasma often exhibit diverse PTMs [23]. These modifications can also occur during the

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Fig. 6. 3D structural diagram of the distribution of 6 PTM residue sites in pHSA and rHSA. (A–F) 3D structural diagram of the distribution of acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, and lactylation modification residue sites in pHSA and rHSA (Up: relatively high expression in pHSA; Down: relatively low expression in pHSA; CZ: only occurring in rHSA; RY: only occurring in pHSA).

production, processing, and storage of pHSA preparations. A study has suggested that alterations in the PTMs of albumin molecules in HSA preparations may impact functions beyond osmotic pressure regulation [24]. Due to the substantial distinction in the source materials between pHSA and rHSA preparations, rHSA formulations produced and purified via genetic engineering techniques may contain non-human PTMs. These modifications could influence their biochemical and biophysical characteristics, as well as their pharmacokinetic and immunological properties.

Protein acetylation modifications play a central and vital role in reshaping protein structure and function across numerous biological processes, encompassing cell metabolism, proliferation, differentiation, and apoptosis [25]. Research indicates that the positioning of many phosphorylation sites in albumin may disrupt the binding of lipids to albumin and affect its stability [26]. Succinylation on lysine residues of proteins can induce various biological changes, contributing to cellular processes such as proliferation, growth, differentiation, metabolism,

and even tumorigenesis [27]. Lysine crotonylation has been identified in both histone and non-histone proteins and has been implicated in various diseases and biological processes, including neurodegenerative diseases, carcinogenesis, spermatogenesis, tissue damage, and inflammation [28]. Recent studies have demonstrated that beta-hydroxybutyrylation modification can enhance the stability of antibodies against proteases and heat treatment. Moreover, beta-hydroxybutyrylation has been shown to stabilize antibodies in plasma [29].

This is significant because HSA stands as the most abundant protein in human plasma, thus emphasizing the potential importance of beta-hydroxybutyrylation modification for its stability. Ongoing research has substantiated the widespread presence of lactylation modification, which plays a role in coordinating various biological processes such as transcription, metabolism, and inflammatory responses [30]. Building upon these insights, to comprehensively understand the PTMs of albumin in HSA preparation and to explore new types of modifications, this study utilized 4D label-free PTMs quantitative proteomic analysis. It aimed to detect seven modification types—acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, lactylation, and *N*-glycosylation—in both pHSA and rHSA preparations. Furthermore, the study aimed to analyze the differences between pHSA and rHSA preparations.

In a prior study, the acetylation and phosphorylation modifications of HSA have been reported in a previous study [26]. Compared to the previously reported 25 acetylation modification sites in HSA [31], our analysis revealed a total of 31 distinct acetylation modification sites in the albumin of pHSA and rHSA preparations. Notably, Notably, all acetylation modification sites we detected were novel compared to previous findings. Illustrated in Fig. 3A, 23 sites represented common acetylation modification sites, with 6 (ALB_K558, ALB_K97, ALB_K383, ALB_K257, ALB_K598, ALB_K264) and 2 (ALB_K548, ALB_K160) unique acetylation modification sites of pHSA and rHSA, respectively. Prior research results have demonstrated that acetylation modification can alter the binding affinity of albumin to drugs and other molecules. Specifically, following after acetylation modification, albumin exhibits increased affinity for binding to phenylbutyric acid but decreased affinity for binding to flufenamic acid [31].

Additionally, acetylation inhibits the binding of albumin to bilirubin and reduces its affinity for prostaglandins [32]. Therefore, the location of acetylation sites may bind to lipids, while acetylation might also decrease the alkaline charge on the surface and increase its isoelectric point. However, further comprehensive structural and functional investigations are needed to elucidate the significance of acetylation modification differences between pHSA and rHSA. According to the Phosphosite Plus database (https://www.phosphosite.org/), 53 phosphorylation sites of HSA have been documented, involving three amino acid residues of serine, threonine, and tyrosine. We found a total of 24 different phosphorylation modification sites, of which 4 (ALB_S226, ALB_S459, ALB_T103, ALB_T267) were newly discovered phosphorylation modification sites in rHSA. Notably, as depicted in Fig. 2A,B, 15 [23] phosphorylation modification sites found in pHSA (rHSA) were exclusive modification sites, with no other modifications detected. Prior studies have utilized cAMP-dependent protein kinase and casein kinase 1 to phosphorylate BSA, thus verifying the phosphorylation process of albumin *in vitro* [33]. It has been documented that this phosphorylation process relies on the sulfydryl-disulfide bond state of BSA, suggesting that the albumin phosphorylation *in vivo* may be influenced by the redox state of its sulfydryl group [34]. These findings demonstrate that albumin phosphorylization can be regulated by the intracellular redox, particularly the balance between reducing sulfydryl groups and oxidative disulfide bonds. Phosphorylation may significantly alter the structural characteristics of molecules. Therefore, understanding and controlling the phosphorylation modification of HSA are crucial for better understanding the function of albumin and for enhancing the quality control of the HSA production and preparation.

In addition to the previously reported modification types of acetylation and phosphorylation in HSA, our investigation for the first time delved into new modification types including succinylation, crotonylation, beta-hydroxybutyrylation, and lactylation in HSA. Corresponding modification sites were identified in both pHSA and rHSA preparations (Table 1). Through techniques such as Stokes radius, friction ratio, UV spectroscopy, solvent perturbation, solubility, and immune cross-reactivity, S. Tayyab *et al.* [35] demonstrated that the native conformation of BSA gradually alters with increasing degrees of succinylation. The alteration becomes significant when the degree of modification exceeds 50%, whereas it remains less apparent below that threshold. Subsequent research by the same group revealed that progressive succinylation leads to a steady decrease in bilirubin-induced fluorescence quenching intensity, accompanied by diminished visible spectral changes of bilirubin-albumin complexes at 480 nm [36]. Furthermore, Murphy MC *et al.* [37] confirmed that succinylation results in increased electrostatic repulsion among BSA molecules, decreased relative hydrophobicity, and reduced α-helix content, consequently impairing foam stability, gel formation, and emulsifying properties. Protein crotonylation was initially discovered by a research team led by Professor Yingming Zhao from the University of Chicago in 2011, earning recognition as one of the "Five Breakthroughs of the Year" by Cell due to its significant research implications [38].

Lysine residues, with their positive charge, play pivotal roles in protein-protein interactions and catalytic activity. However, crotonylation neutralizes this positive

charge, thereby influencing protein function. Crotonylation of various proteins can impact a range of biological functions, including gene transcription, DNA repair, metabolic pathways, protein activity regulation, cell cycle, heterochromatin localization, and MTORC1 regulation [39]. Among the array of novel protein acylation modifications, beta-hydroxybutyrylation modification is facilitated by beta-hydroxybutyric acid, a critical metabolite in ketone body metabolism, which is closely linked to fatty acid oxidation metabolism and the regulation of energy metabolism [40]. Extensive investigations into lactylation have underscored its significant role in physiological and pathological processes. However, early research on lactylation primarily focused on its epigenetic effects on histones [41].

The results from modification-specific proteomics have revealed that, besides histones, numerous non-histone proteins undergo lactylation, playing significant roles in key cellular processes associated with both physiology and disease. Subsequent studies have reinforced the notion that protein lactylation serves as a crucial mechanism for lactate to exert its functions, participating in essential activities such as glycolysis-related cellular functions [42], macrophage polarization [43], nervous system regulation [44], and rice grain development [45]. However, as of now, there are no available reports detailing the impacts of succinylation, crotonylation, beta-hydroxybutyrylation, and lactylation on HSA-related functions. Consequently, extensive studies are required to provide further elucidation on this matter.

It's noteworthy that 36 different N-glycosylation sites in HSA have been reported [21,46]. Additionally, non-enzymatic glycosylation can trigger lysosomal degradation of albumin, leading to an increase in its decomposition rate. This phenomenon may be linked to the reduced half-life of albumin observed in diabetic patients [14]. Glycosylation can induce structural alterations in numerous drug and fatty acid binding sites on albumin. Such changes in drug binding site activity resulting from this PTM have the potential to disrupt albumin's normal function as a drug carrier [47]. However, in the present study, no *N*-glycosylation sites were detected in the albumin of pHSA and rHSA using 4D label-free PTMs quantitative proteomic analysis.

We hypothesized that the *N*-glycosylation sites in HSA samples could be linked to the pathophysiological condition of the study subjects. Previous research has identified 18 different glycosylation sites in HSA samples from patients with type 2 diabetes [48], and notably, the level of glycosylated albumin is significantly elevated in patients with Alzheimer's disease [49]. The reason why we did not detect any *N*-glycosylation in the HSA samples could be attributed to the fact that the pHSA samples were derived from the plasma of healthy donors, while the rHSA samples were purified from HSA expressed using genetic engineering technology. Further research is necessary to determine whether glycosylation of albumin in HSA preparations can impact its functional activity. Additionally, exploring the scientific significance of using glycosylation levels as a quality evaluation indicator for related products is crucial.

In light of Fig. 2, both pHSA and rHSA preparations exhibited partial specific modification sites where only one type of modification was detected. The occurrence of specific PTMs at these sites may be associated with the characteristics of their amino acid residues. Based on Fig. 3, specific sites among the detected modification types in this study were found exclusively in the albumin of either pHSA or rHSA preparation. These differences may result from variations in the raw materials used for pHSA and rHSA preparations. The impacts of non-human specific sites resulting from gene recombination expression on the safety and efficacy of rHSA deserve attention.

As depicted in Fig. 4, differences in modification levels were observed for some common modification sites in the albumin of pHSA and rHSA. These disparities in PTMs between pHSA and rHSA may be intricately linked to factors such as their genetic origins, physicochemical conditions, production methodologies, and storage environments. For example, due to HSA's broad affinity for impurities, it can effectively bind to various contaminants that may arise during fermentation and purification processes. Therefore, in the purification process, it is imperative not only to address impurities inherent in the fermentation broth but also to eliminate endogenous or exogenous impurities bound to rHSA. Thus, compared to pHSA, the separation and purification procedures for rHSA are more refined and complex.

Figs. 5,6 reveal that we comprehensively displayed and analyzed the location information of six PTMs and modification sites in the amino acid residue sequences and 3D spatial structure of albumin in pHSA and rHSA. This information reveals the diversity in the type, site, source, and spatial distribution of PTMs in HSA. Since these diverse modifications do not occur simultaneously on the same albumin molecule, different albumin molecules may have varied modifications, resulting in the diversity of albumin subtypes in HSA preparation.

From the perspective of the "effective albumin concentration theory", it can be concluded that research on PTMs of albumin in HSA preparation is of great significance for its clinical safety and efficacy evaluation. It also provides a theoretical basis for studying the heterogeneity of products from different suppliers, especially between pHSA and rHSA preparations, thereby further enhancing the product quality of HSA preparation in China.

The catalog of PTMs in human serum albumin serves as a valuable resource for understanding the diverse roles of albumin in both normal physiological and disease conditions [50,51]. PTMs have the ability to modify the surface characteristics of albumin, potentially altering its structure and, consequently, its functionality. Mass spectrometry has emerged as an unbiased method for analyzing PTMs, ca-

pable of detecting over 300 known PTMs by ionization. Moreover, MALDI offers the potential for multiple ionizations from the same sample through adjustments in laser energy and ionization conditions. The mass accuracy and sensitivity of modern mass spectrometers surpass the necessary requirements for the secure and precise determination of PTMs, supported by advanced tools and databases for their identification. The development of mass spectrometry databases and tools for exploring PTMs within peptide sequences has significantly improved the efficiency and reliability of PTM recognition [52,53].

Additionally, three-dimensional structural analysis serves as a powerful tool for predicting the functional impacts of PTMs [54,55]. With existing three-dimensional structures of HSA, we can pinpoint identified PTMs and assess their potential implications on albumin function

Conclusion

This study, employing advanced 4D label-free PTMs quantitative proteomic analysis, unveiled the prevalence of acetylation, succinylation, crotonylation, phosphorylation, beta-hydroxybutyrylation, and lactylation modifications in clinical therapeutic pHSA and rHSA formulations in China. Interestingly, *N*-glycosylation modifications were not detected. Additionally, distinct variances in site characteristics and modification levels were observed between pHSA and rHSA. Further experimental investigations are warranted to explore the implications of these PTMs differences on the biological functions, efficacy, and safety of pHSA and rHSA formulations.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available in the [PRIDE] repository, https://www.ebi.ac.uk/pride/archive/projects/PXD050752.

Author Contributions

QL: Data curation, experiment, methods, validation, writing-original draft. ZFL, CQL: acquisition of data, visualization. ZKW, JX, LC: analysis and interpretation of data. LM, ZHW: conception and design, analysis and interpretation of data, writing-review, and project administration. All authors contributed significantly to editorial changes of important content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

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Conflict of Interest

JX and LC are consultants of Shanghai RAAS Blood Products Co., Ltd. and declare no conflicts of interest. All other authors have reported no conflicts relevant to the contents of this paper to disclose.

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