Biochimica et Biophysica Acta 1830 (2013) 5515–5525

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Human serum albumin from recombinant DNA technology: Challenges and strategies ☆

Zhen Chen, Yang He, Bo Shi, Daichang Yang *

Engineering Research Center for Plant Biotechnology and Germplasm Utilization, Ministry of Education, Wuhan University, Wuhan 430072, China
State Key Laboratory of Hybrid Rice, College of Life Sciences, Wuhan University, Wuhan 430072, China

ARTICLE INFO

Article history:
Received 16 February 2013
Received in revised form 24 April 2013
Accepted 29 April 2013
Available online 3 May 2013

Keywords:
Recombinant human serum albumin
rHSA biosafety
rHSA processing
OsrHSA
Plant bioreactor

ABSTRACT

Background: As the most abundant protein in the blood, human serum albumin (HSA) plays an important role in maintaining plasma oncotic pressure and fluid balance between the body's compartments. HSA is thus widely used in the clinic to treat diseases. However, the shortage of and safety issues arising from using plasma HSA (pHSA) underscore the importance of recombinant HSA (rHSA) as a promising substitute for pHSA.

Scope of review: Here, we review the production of rHSA, from expression to downstream processing, and highlight the scalability and cost-effectiveness of the two main expression platforms. We also discuss the biosafety of commercially available pharmaceutical rHSA with respect to impurities and contaminants, followed by an analysis of recent progress in preclinical and clinical trials. We emphasise the challenges of producing pharmaceutical-grade rHSA.

Major conclusions: rHSA can be highly expressed in various hosts and seems to be identical to pHSA. rHSA generated from yeast appears to be as efficient and safe as pHSA in a series of preclinical and clinical trials, whereas rHSA from rice seeds exhibits great potential for more cost-effective production. Cost-effective products with no adverse effects will likely play a vital role in future human therapeutics.

General significance: Our understanding of pharmaceutical-grade rHSA production has improved with respect to expression hosts, biochemical properties, downstream processing, and the detection and removal of impurities. However, due to the large dosages required for clinical applications, the production of sufficient quantities of rHSA still presents challenges. This article is part of a Special Issue entitled Serum Albumin.

1. Introduction

Human serum albumin (HSA), the most abundant protein in the human plasma (40–50 g/L), is a hydrophilic, non-glycosylated protein with a molecular mass of approximately 66.5 kDa. HSA is synthesised in the liver and mainly functions as a regulator of plasma colloid oncotic pressure and as a carrier for many exogenous and endogenous metabolites and drugs [1]. This protein is widely used in the clinic to treat hypoproteinaemia, foetal erythroblastosis, fluid loss due to burn injuries or haemorrhagic shock, and ascites caused by cirrhosis of the liver [2–4]. HSA is also utilised as an excipient, stabilizer or a supplement to cell culture for the production of recombinant pharmaceuticals [5–7].

Over the past decades, the market demand for HSA has greatly increased due to its wide applications. The need for HSA for therapeutic applications is estimated to be more than 500 t worldwide. The commercial production is mostly based on fractionating human plasma, which is limited by blood donations in such countries such as China, Brazil, and India. Moreover, plasma-derived HSA (pHSA) presents the potential risk of spreading blood-derived pathogens, even though the manufacture of pHSA has been greatly improved in recent years [8,9]. Therefore, regulatory agencies have encouraged pharmaceutical companies to use non-animal-derived sources for pharmaceutical production [10]. The production of HSA using recombinant DNA technology has thus been adopted as an alternative method for obtaining large quantities of pathogen-free HSA.

Various hosts, both prokaryotic and eukaryotic, have been used to produce recombinant human serum albumin (rHSA). However, none of these hosts has been proven to be cost-effective on an industrial scale. The production of rHSA faces many challenges, especially in large-scale manufacturing and biosafety. In this review, we discuss the rHSA from different expression systems, predominantly focusing on the protein's expression levels, downstream processing, cost-efficacy, and safety issues associated with impurities.

☆ This article is part of a Special Issue entitled Serum Albumin.

* Corresponding author at: State Key Laboratory of Hybrid Rice, College of Life Sciences, Wuhan University, Wuhan 430072, China. Tel.: +86 27 68754680.
E-mail address: dyang@whu.edu.cn (D. Yang).

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.04.037

2. rHSA expression

High dosages of HSA are required for clinical applications, typically in excess of 10 g/dose, such as for the treatment of hypoalbuminaemia or traumatic shock. Since, the unit price of HSA is very low ($3.00–$5.00 per gram) [11]. To commercially produce rHSA at an economically feasible level, a very high expression level is thus the minimum requirement [12]. A high deposition of recombinant protein could trigger unexpected toxic effects in the host, and the recombinant protein levels could also be diminished by degradation or aggregation. Additionally, HSA consists of a single polypeptide chain of 585 amino acids that is folded into three helical domains. There are 35 cysteines in HSA, and 34 of them form disulphide bonds. Such complicated disulphide-bond formation in this large recombinant protein could be a burden on the protein synthesis and folding system, potentially resulting in the low expression or incorrect folding of rHSA when rHSA highly expressed in the host cells. Therefore, it is difficult to meet the requirement if expression level is not high enough and the processing is not cost-effective. Since then, many efforts have been made to improve the expression level of rHSA in past decades, including bacteria, yeast, animals, and plant cells. However, these issues have not been solved since recent work [13].

2.1. rHSA expression in bacteria

Bacteria, such as *Escherichia coli*, were initially considered to be the most efficient platforms for rHSA production due to these organisms' well-established molecular tools, high growth rate and cultivation capacity, and significant yield of recombinant proteins [14]. *E. coli* was the earliest host to be used to produce rHSA and is currently the most common production platform for various recombinant proteins. A cDNA encoding *hsa* gene has been inserted into an expression cassette and transformed into *E. coli*, yielding an expression level of approximately 2.5 g/L. The majority of the protein is insoluble, in the form of inclusion bodies, which may be due to the protein aggregation and lack of proper folding in the cytoplasm [15,16]. Expression in *Bacillus subtilis* results in soluble secreted rHSA, but with incorrect cleavage of the signal sequence at the N-terminus [17]. As we know, incorrect folding or processing is not compatible with production of all recombinant protein in various expression systems, especially, in prokaryotes such as *E. coli*. Recently, however, *E. coli* was successfully engineered to express disulphide-rich proteins via the co-expression of the foldases and the chaperones, suggesting a novel molecular strategy for the expression of rHSA in prokaryotes [16,18].

2.2. rHSA expression in yeast

The production of rHSA in eukaryotes was initially challenging. The expression of rHSA in yeast resulted in aggregation, forming inclusion-like bodies as observed in *E. coli* [19]. Thus, collecting the secreted protein from the culture medium was adopted as the main strategy for rHSA production in yeast [19–32]. To achieve the cost-effective production of rHSA, at least several grams of the protein must be secreted into the culture medium for the starting material to be processed [12]. To enhance rHSA expression, different yeast strains, including *Saccharomyces cerevisiae* [19–23,33], *Hansenula polymorpha* [24–26], *Kluyveromyces lactis* [27,28,34] and *Pichia pastoris* [29–32], were designed to express rHSA for industrial use. Expression levels ranging from milligrams to grams per litre were obtained in yeast by employing different strategies, such as the introduction of various promoters, the insertion of introns into the *hsa* genes, and the use of signal peptides (Table 1). Additionally, many other approaches have been exploited to enhance the secretion levels, including the optimisation of the culture medium [35], the use of cyclic fed-batch [36] and repeated fed-batch cultures [29,37].

After optimising the regulatory genetic elements that control rHSA expression and the culture conditions, methylotrophic strains, including *S. cerevisiae* [22,38] and *P. pastoris* [29,30,35] seem to be the most attractive options for industrial rHSA production. These strains are preferable because the *alcohol oxidase 2* (AOX2) promoter, which controls the expression of the target gene, is very strong and strictly regulated in yeast. In *S. cerevisiae*, expression level is estimated to be higher than 3 g/L, based on Novozymes reports (http://www.biopharma.novozymes.com/en/information-centre/posters-and-presentations/Documents/ACTIP08_LesEvans_181108.pdf). The use of the AOX2 promoter and repeated fed-batch fermentation results in much higher rHSA expression, reaching up to 10 g/L in *P. pastoris* [37,39].

The proteolytic degradation of rHSA has always been observed when rHSA is secreted into the culture broth by either *S. cerevisiae* or *P. pastoris* [19,29], especially in prolonged cultures with periodic feeding [22]. This degradation has been attributed to an endogenous acidic protease that acts in an acidic pH-dependent manner [22]. A degradation rate of up to 660 mg of rHSA/L/h was observed at pH 4.3, which caused a significant decrease in rHSA production in yeast [29]. A strategy to reduce HSA degradation has been attained by disrupting the gene encoding an aspartyl protease (YAP3) [21]. The putative proteases causing such instability of rHSA in yeast remain unclear. However, to reduce the impact of proteases in existing *P. pastoris*-based protein expression systems, a new system called PichiaPink™ was recently developed. The rHSA expression level in PichiaPink™ reached 334 mg/L without any obvious degradation [40]. With the degradation issue solved, high, stable expression levels can be achieved in both *S. cerevisiae* and *P. pastoris*. These levels are suitable for rHSA production on an industrial scale, so the system is being adopted (Novozymes and Mitsubishi) for this purpose.

2.3. rHSA expression in transgenic animals

Animal cells are considered to be ideal hosts for the production of pharmaceutical proteins because the mammalian hosts can generate high-quality proteins that are identical in their biochemical features to those derived from human cells, such as solubility, structure, biological activity and nearly identical glycosylation. Thus, mammalian cell culture has been used extensively and effectively to produce many biopharmaceuticals including vaccines and antibodies. Nevertheless, the high-dosage clinical applications of and large market demand for rHSA require much higher expression levels than the levels needed for vaccines and antibodies. To obtain elevated rHSA levels, the most ideal expression platform is the blood, but it is very difficult to isolate the recombinant plasma proteins by downstream purification [41]. The mammary gland, another abundant, fluid-containing secretory tissue, is emerging as a promising alternative bioreactor for the production of pharmaceutical proteins, including rHSA [42–46]. rHSA was first expressed under the inducible promoter of β-lactoglobulin (β-LG), which highly and specifically guided rHSA expression in the mammary gland and the milk. The concentration of rHSA in the milk of transgenic mice reached 2.5 mg/mL [42]. More recently, rHSA was expressed in both virgin and lactating mice [43]. Interestingly, the rHSA expression level in mouse milk was increased to 10 g/L by combining different introns in the native *hsa* gene [44], suggesting that introns may play important roles in the maintenance of rHSA mRNA in animal cells. To further enhance rHSA expression, the promoter of whey acidic protein (WAP) was introduced to produce rHSA in mice, but this production was less efficient than using the β-LG promoter to induce rHSA expression in mouse milk. Approximately 25% of the transgenic lines produced highly secreted levels of rHSA, with levels of 0.1–2.0 mg/mL and 1–20 mg/mL in the WAP and β-LG groups, respectively. Further investigation determined that the rHSA expression level could stably reach up to 3.54 g/L when *hsa* minigene 1 was fused with the goat β-LG promoter and its 5′ upstream regulatory region [47]. However, approximately one-half (57–58%) of the transgenic lines in

Table 1
rHSA expression in various hosts.

Host	HSA coding sequence	Regulatory elements	Expression level	Remarks/company	References
E. coli	cDNA	TRP	2.5 g/L	Aggregation	[15]
B. subtilis	Fused cDNA	α-my$_{Bjp}$ or npr$_{Bnp}$	Not specific	Incorrect processing	[17]
S. cerevisiae	cDNA	PRB1	20–150 mg/L	Degradation, aggregation	[19,20]
S. cerevisiae	cDNA	GAL10	3–4 g/L	Novozymes	
S. cerevisiae	cDNA	GAL10 or GAPDH	30 mg/L	Degradation	[33]
S. cerevisiae	cDNA	AOX1	73–334 mg/L	PichiaPink™	[40]
P. pastoris	cDNA	AOX2	8–12 g/L	Repeated fed-batch culture; Mitsubishi	[29,35,37] [39]
P. pastoris	cDNA	AOX1	1.34 g/L	Cyclic fed-batch culture	[36]
P. pastoris	cDNA	AOX2	40–80 mg/L	Oleic acid addition	[35]
H. polymorpha	cDNA	GAP	0.55 g/L		[24]
H. polymorpha	cDNA	PMA1	460 mg/L		[25]
H. polymorpha	cDNA	MOX	1.3 g/L		[26]
K. lactis	cDNA		3 g/L		[27]
K. lactis	cDNA	PGK	62 mg/L		[28]
K. lactis	cDNA	PGK	>50 mg/ml		[34]
K. lactis	cDNA	KlADH4	1 g/L	Fed-batch culture	[38]
Mice	Minigene 1	Sheep β-LG	2.5 g/L		[42]
Mice	Minigene 1–2	Sheep β-LG	0.001–0.035 g/L		[42]
Mice	Minigene 1–6	Sheep β-LG	0.002–10 g/L		[44]
Mice	Whole gene	Sheep β-LG	N.D.		
Mice	cDNA	Sheep β-LG	N.D.		[42]
Mice	Whole gene	Mouse WAP	0.16 g/L		[45]
Mice	Minigene 1–6	Mouse WAP	0.001–1.6 g/L		[45]
Mice	Minigene 1	Goat β-LG	1–2 g/L		[43]
Mice	Minigene 1	Goat β-LG	0.21–3.54 g/L		[47]
Mice	cDNA	Mouse WAP	0.05–11.95 g/L		[46]
Cattle	cDNA	Goat β-LG	1-2 g/L		[48]
Silkworm	cDNA	Ser1 + BmNPV enhancer + IE1	3.0 μg/mg		[49]
Potato	cDNA	CaMV 35S	0.02% TSP	Soluble	[50]
Tobacco suspension cell	cDNA	CaMV 35S	11.88 μg/mL	Very low expression	[54]
Tobacco leaf	cDNA	16S	11.1% TSP	Aggregation	[52]
Potato tuber	cDNA	B33	0.1–0.2% TSP		[51]
Rice suspension cell	cDNA	αAmy3	11.5% TSP		[53]
Rice seed	cDNA	Gt13a	1.40–10.58% TSP	Healthgene Biotech	[55]

Notice: Minigene x: the *HSA* gene containing introns x; TSP: total soluble protein.

the WAP and β-LG groups failed to secrete detectable rHSA levels into the milk [45,47]. Recently,high expression level of rHSA (11.9 g/L) in mice has been obtained by using WAP promoter (Table 1) [46]. Despite such high secretion, rHSA from mice would hardly be able to fulfil the market demand for HSA because of the low yield of milk.

With the successful improvement of rHSA production in smaller animals, bigger animals with higher milk yields, such as cattle and sheep, have also been used to produce rHSA. Typically, a goat *β-LG* (gβ-LG) promoter and a bovine growth hormone polyadenylation (polyA) sequence are fused to the *hsa* gene, and a chicken *β-globin* insulator is used to overcome the position effects. The expression level varies from 1–2 g/L in cattle with normal lactation to 40 g/L in cattle exhibiting a shortened duration of lactation (Table 1) [48].

Beyond mammary glands, the cocoon of the silkworm (*Bombyx mori*), which contains vast quantities of silk proteins secreted by the silk glands, has also been exploited to produce rHSA. rHSA specifically accumulates in the sericin layers, with an expression level of up to 3.0 μg/mg, when driven by the *sericin-1* (*Ser1*) gene promoter, the *B. mori* nucleopolyhedrosis virus enhancer *hr3* (BmNPV), and the viral transactivator *IE1*. Approximately 83% of the rHSA expressed in transgenic cocoons can be selectively extracted in soluble form using phosphate-buffered saline [49].

2.4. rHSA expression in transgenic plants

Similar to animal cells, plant cells are eukaryotic and capable of protein synthesis and modification. Plant cells have thus been proposed as a promising alternative to microbe- and mammalian cell-based systems for pharmaceutical protein production. These cells couple eukaryotic protein processing with higher yields, lower cost, and no risk of infection with human pathogens. These advantages render plant cells an attractive platform for rHSA production.

The first attempt to produce rHSA in plants employed tobacco leaves and potato tubers. A very low expression level of 0.02% of the total soluble protein (TSP) was obtained, which was likely due to proteolytic degradation [50]. Furthermore, rHSA accumulates to up to 0.2% of the TSP by being targeted to the apoplasts of the potato tubers [51]. The expression of rHSA increases to up to 11.1% of the TSP by fusing with the untranslated regions in the tobacco-leaf chloroplasts. Unfortunately, the rHSA is incorrectly folded and degraded, even in large inclusion bodies [52].

An alternative approach to expressing rHSA in plant cells is the use of plant-cell suspension cultures. An expression level of 11.5% of TSP in the medium (15 mg/L) was achieved in a rice cell culture driven by a sugar starvation-induced promoter [53]. Very recently, rHSA expressed in tobacco Bright Yellow-2 cells reached levels of 11.88 mg/L in the culture medium [54]. However, these expression levels were much lower than the levels achieved in the yeast secretion system, suggesting that further optimisation of the plant cell cultures is necessary to improve rHSA production.

More recently, rice endosperm cells were used in our laboratory as a host for rHSA expression. The highest rHSA expression level reached 10.58% of the TSP by using an endosperm-specific promoter, vacuole targeting, and genetic codon optimisation [13]. The expression of rHSA was 20-fold higher than the minimum rHSA level that is commercially required. Because cereal crop seeds are a natural organ for protein synthesis and storage, these seeds could provide the ideal place for high-yield rHSA synthesis and processing [55].

3. Downstream rHSA processing

Because the clinical dosage of HSA often exceeds 10 g per vial, the challenge of obtaining high-purity rHSA must be addressed. For example, assuming a purity of 99.9999%, 10 μg of impurities would be administered in a single dose of rHSA (10 g/dose). Such trace impurities from the host cell could elicit an adverse response in the human body. Therefore, the potential toxic and immunologically active contaminants in rHSA must be removed. Thus, an efficient and economical manufacturing process is essential and challenging for the commercial production of rHSA.

The conventional purification of pHSA from blood uses cold ethanol fractions, leading to a purity of 95%. The purity could be improved to up to 99% via the application of the chromatographic method to the cold ethanol fractions. However, many unexpected adverse events that were attributed to contaminants in the pHSA solution have been observed [56,57]. The downstream processing of recombinant biopharmaceuticals varies depending on the properties of the product and the expression system. Many methods have been developed to isolate rHSA from different hosts, mainly in the form of pretreatments, chromatography, and formulations (Fig. 1). We will discuss these techniques in more detail, as follows:

3.1. Pretreatments in different expression platforms

Because high-dosage of rHSA is required for clinical treatments and huge market volume, rHSA production requires a large scale of high-density fermentations in *Pichia pastoris* or large-volume extractions from rice seeds. So, the pretreatment of such large amount of materials must be employed. However, tons of extracted solution or medium require clarification and stabilisation before chromatography, and the challenge is how to separate the biomass from the starting materials with a high recovery rate. Consequently, pretreatments such as cell lysis, rHSA extraction, and clarification are very important for downstream processing.

Pretreatments should increase the recovery of rHSA and reduce the impurities as much as possible. To achieve these aims, ammonium sulphate precipitation and heat treatment (65–68 °C) are used in rHSA pretreatments, based on the thermal stability of HSA. The heat treatment is mainly adopted to extract rHSA and to inactivate the proteases in yeast or plants [58,59]. Heat treatment also elevates the total protein recovery and simultaneously increases the number of impurities [54,58].

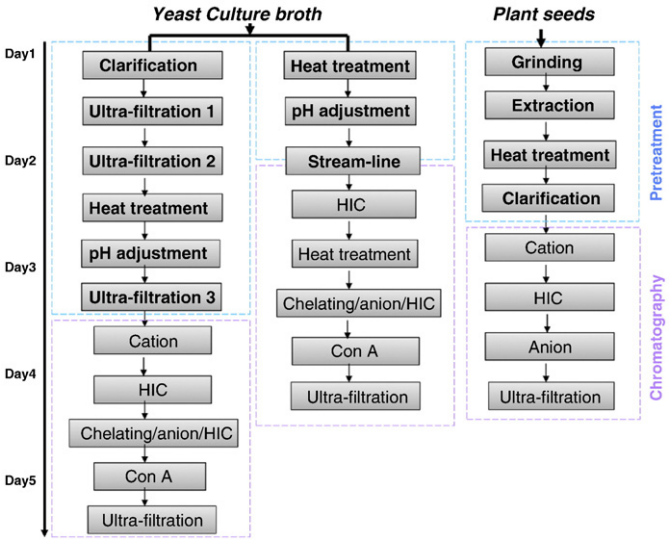

Fig. 1. Downstream processes of rHSA derived from yeast and plant. The steps involving in pretreatment and chromatography were highlighted as light blue and purple, respectively. HIC, hydrophobic interaction chromatography.

Ultrafiltration with various cut-off values (1000–100,000 Da) diminishes impurities of higher or lower molecular weights in the crude rHSA extraction solutions [58,60]. To simplify the extraction process, an ethanol/K_2HPO_4, aqueous two-phase extraction was recently developed to separate rHSA from the yeast culture broth, which dramatically decreased the proportion of host proteins in the initial recovery step [61]. Isoelectric precipitation also effectively removes the impurities of rHSA produced in rice seeds, which exhibits obviously different biochemical features. Starches and certain proteins from rice grain are unstable in an acidic environment, which is beneficial for the use of chromatography in future processing.

Centrifugation is the main method used to remove host-cell fragments from crude rHSA extracts, with the goal of clarifying the extracts [53,54]. However, it is very difficult to centrifuge tons of solution for large-scale rHSA production. A frame filter press has been used to separate yeast cells from a culture, but this process is slow and operates in an open system, increasing the risk of pyrogen contamination. STREAMLINE, which combines solid–liquid separation and cation-exchange chromatography, has been adopted to improve and simplify the purification process, greatly shortening the processing time and increasing the rHSA yield [62]. For example, membrane filtration has been used to effectively clarify the starch and crude protein extraction solutions for OsrHSA (*Oryza sativa* recombinant HSA, OsrHSA) production from rice seeds.

3.2. Chromatography-based purification

Chromatography is generally used to achieve significant levels of high-purity rHSA. As shown in Fig. 1, various chromatographic methods (ion-exchange, hydrophobic, chelating, affinity, and mixed-mode chromatography resins) followed by ultrafiltration have been successfully used to purify rHSA. During downstream processing, affinity chromatography is the most popular method for isolating pharmaceutical proteins. Affinity chromatography using Cibacron Blue can selectively and effectively separate HSA from plasma, which is widely used in the specific isolation of albumin from plasma [63]. However, rHSA is difficult to elute from the resin due to the resin's high affinity (1 M NaCl/NaSCN), and Cibacron Blue is too expensive to use in large-scale production [49,64]. There is also the potential for contamination of Cibacron Blue from the resin in the final products, which could be a risk for clinical applications. Therefore, ion exchange, hydrophobic, and chelating chromatography are the main separation strategies used to purify rHSA from yeast.

The purity of yeast-derived rHSA reaches more than 99.95% by combining ultrafiltration, ion-exchange and lipophilic affinity chromatography [60]. The purity of rHSA has largely been improved by optimising the conditions of ultrafiltration, heat treatment, and chromatography over the course of several decades [59]. The entire process for purifying rHSA from yeast contains at least 12 steps, which mainly consist of filtrations and chromatography. The final purity of the rHSA is 99.99999996%, and the concentration of yeast-derived contaminants in the purified rHSA is less than 0.1 ng/g, as determined by enzyme-linked immunosorbent assay (ELISA) [65]. In contrast, mannan polysaccharides from *P. pastoris* have not been detected in rHSA prepared by anion-exchange chromatography with pulsed amperometric detection [66]. Despite the very high purity of such rHSA, which eliminates any clinical risks, it is nearly impossible to produce in a cost-effective way, or at least as economically as pHSA, given the complicated processing that is required. Alternative purification strategies have been introduced, including STREAMLINE technology and mixed-model resin [39,62]. There has been much progress in this area, such as the improvement of rHSA production by approximately 50% in terms of processing time and 45% in terms of yield by STREAMLINE.

Several strategies have been employed to develop a cost-effective protocol for isolating OsrHSA from rice grain. These strategies include

the pretreatment of grain; extraction with heat following acidic precipitation; and several steps of cation, anion, and hydrophobic chromatography and ultrafiltration to achieve a purity of greater than 99.9%, as detected by silver staining and high-performance liquid chromatography (HPLC) [13,67]. A recovery rate of 2.75 g/kg of brown rice was achieved on both the pilot scale (110 g/lot) and the industrial scale (>1 kg/lot; unpublished data). Recently, further improvements to the downstream processing of OsrHSA increased the final purity to 99.9999%, according to the EIA's guidelines for characterising the impurities from rice (unpublished data). The process of purifying OsrHSA from rice grain seems to be more efficient and less time- and labour-intensive than the purification of rHSA from yeast. This process also has a great potential to produce rHSA in a cost-effective way, given that a safety assessment is accomplished at this purity.

3.3. Biochemical and functional fidelity

As expected, rHSA expressed in rice or yeast (*Pichia pastoris*, pprHSA) exhibits the same amino-acid sequence and molecular weight as pHSA, according to the tryptic peptides of pprHSA and mass spectrometry analysis [68,69]. Additionally, the physicochemical and immunochemical properties of OsrHSA and pprHSA in terms of viscosity, colloid osmotic pressure, and antigenicity are not significantly different from the properties of pHSA [70].

In terms of structure, OsrHSA from rice and pprHSA from yeast are identical to pHSA. As shown in Fig. 2, all of them folds into a heart shape formed by three helical domains, designated I, II, and III, with fatty acids bound to the hydrophobic cavities. The alignment of the crystal structures of OsrHSA and pprHSA to the structure of pHSA generates root-mean-square deviations of 0.605 Å and 0.374 Å, respectively (Fig. 2B). No obvious differences in the main-chain conformations are found among the structures of pprHSA, OsrHSA, and pHSA. Eight fatty acids bind to both OsrHSA and pprHSA (Fig. 2C) at the same site as in pHSA. Additionally, 17 pairs of disulphide bonds are precisely formed in plant cells and yeast cells (Fig. 2D) [13,71]. pprHSA and OsrHSA are also structural identical to pHSA in terms of thermal stability and binding to such ligands as warfarin, naproxen, and ibuprofen [69]. These conformational and functional similarities to pHSA were also observed for rHSA from transgenic silkworms and cattle [48,49]. In fact, rHSA can correctly fold in most eukaryotic cells and maintain its binding activities.

A further characterisation of rHSA indicates that rHSA retains biological activity in vivo. Supplementing culture medium with rHSA dramatically promotes the growth of several different cell lines, including CHO cells, Vero cells, hybridomas, and embryonic stem cells [13,72]. pprHSA has been used as a substitute for pHSA in the culture medium for IVF and may eliminate the risk of transmitting animal viruses and plasma-derived impurities [73,74]. More importantly, the highly purified rHSA from yeast and rice exhibits a therapeutic efficacy in liver ascites that is equivalent to the efficacy of pHSA [13,75]. Additionally, the intravenous administration of rHSA dose-dependently decreases the abdominal circumference and increases the urine volume and colloid osmotic pressure, even in combination with other chemical therapeutics [76].

However, rHSA from different hosts could have slightly dissimilar biochemical properties. For example, OsrHSA has an improved thermal stability in liposomes compared with pprHSA, possibly due to the presence of different fatty acids in OsrHSA [77]. Moreover, pprHSA has a much higher free sulphydryl content than pHSA [78]. The same result was also obtained in embryonic culture, revealing that rHSA was more reduced than pHSA, but redox variations were also noted between different OsrHSA batches and pprHSA batches as the same of pHSA [79].

3.4. Scalability and cost-effectiveness of rHSA

The cost-effective and scalability of rHSA production is very critical for commercialization of rHSA because the price of HSA is as low as 3.00–5.00 US dollars per gram and the high market demand for HSA (500 t/year) [11]. The production of rHSA in a cost-effective

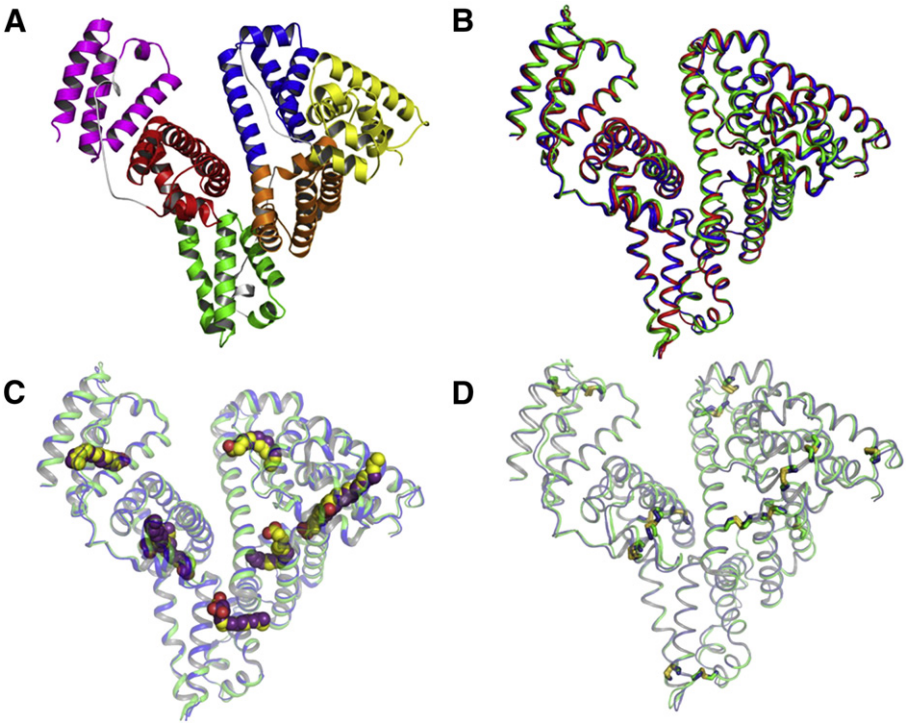

Fig. 2. Structures of rHSA from yeast and rice. A, Overall structure of recombinant human serum albumin; B, Comparison of recombinant HSA from rice (green, PDB: 3SQJ) or yeast (blue, PDB: 1E7G) to HSA from plasma (red, PDB: 2I2Z), the RMSDs of two rHSAs to pHSA were 0.605 and 0.374 Å respectively; C, Fatty acids binding in rHSAs. The fatty acids bound to rHSA from plant (green) and yeast (blue) was represented by sphere colouring as yellow and brown. D, Disulfides in rHSA from yeast and rice. Disulfide bonds were shown as yellow sticks.

way, or at least as economically as pHSA, is the most critical milestone in the development of recombinant technology for this purpose. For example, rHSA expressed in *E. coli* aggregates into inclusion bodies, so unfolding and refolding the protein upon the addition and removal, respectively, of a chemical reagent is required to produce biologically active rHSA. Although rHSA can be refolded into its native state by these treatments, low recovery has greatly increased the cost of the process, rendering such treatments unsuitable for industrial production.

For animal bioreactors, the most negative aspect of rHSA production is the timing and capital needed to transition to an industrial scale. Such production requires 3.5 months for mice, 15 months for pigs, 28 months for sheep, and 32 months for cows [80]. Additionally, the cost of the upkeep of cows using Good Agricultural Practices (GAP) is estimated to be $10,000/cow/year [81]. Therefore, it is time- and capital-consuming to produce rHSA from transgenic animals.

Yeast fermentation has been used extensively to produce recombinant pharmaceutical enzymes and cytokines. The products derived from yeast have been profitable due to the products' low dosage and high expression levels, which effectively reduce the overall cost. However, it is difficult to economically produce rHSA in yeast. The high market demand for rHSA requires investment in the instruments used for fermentation. Additionally, to eliminate potentially toxic contaminants, more complicated downstream processing is needed, which further increases the capital investment. Thus, the development of efficient and economical downstream processing could be the key to decreasing the cost of rHSA production in yeast.

In plants, the yields of rHSA have substantially improved. Recent data revealed that the expression level exceeded the estimated cost-efficacy threshold (0.1 g/kg) for the industrial production of rHSA [13,51,52]. Further evaluation, based on economic principles, suggested that rHSA from transgenic tobacco could reduce production costs and generate annual profits of millions of dollars for innovative firms [82]. More recently, the large-scale production of OsrHSA with purity >99% and a production rate of 2.75 g/kg of brown rice were achieved in our laboratory. Furthermore, OsrHSA production can be easily scaled to kilogram levels within one season once a highly expressing transgenic line is obtained. The production scale is very flexible because the seeds can retain intact recombinant protein for as long as three years [83], and the advantages over other systems include scalability, safety, and cost-effectiveness. In particular, rice endosperm cells not only provide a stable site for the deposition of recombinant proteins, preventing degradation by proteases, but also can be stored at ambient temperatures for several years. Furthermore, the entire rice genome sequence can guide the design of downstream processing, such as extraction and purification protocols, and the safety evaluation of host-cell proteins required by the FDA [84]. Thus, increasing evidence indicates that rice or cereal crop endosperm is a promising platform for producing therapeutic proteins.

However, large-scale rHSA synthesis requires the field production of genetically modified plants. Additionally, the downstream processing of rHSA should conform to GMP (Good Manufacturing Practice) and produce a sufficiently pure and homogeneous product with acceptably low levels of contaminants. GMP issues include the characterisation of the identities of contaminants and adherence to both standard operating protocols (SOPs) for genetically modified (GM) plants and regulatory guidelines for field planting regarding environmental safety [85]. For OsrHSA production from rice, many measures have been adopted to prevent the possible transfer of OsrHSA transgenic rice into the environment from the field trail, including the establishment of an isolation zone (>100 m) and a buffer zone around the field (>1.5 m); fencing; and well-established SOPs for sowing, planting, harvesting, drying, transporting, processing, and storage. In fact, there is a very low frequency (0.04–0.80%) of pollen-mediated gene flow between GM rice and adjacent non-GM plants [86], which can be easily decreased to a negligible level by short spatial isolation [87]. Because the recovery rate of OsrHSA (2.75 g/kg) is much higher than the rate estimated for tobacco (1.0 g/kg) [13,88], the production of OsrHSA for pharmaceutical applications may be cost-effective.

4. Biosafety of rHSA

There is rapidly growing public concern regarding blood-derived pathogen transmission and the potential heterogeneity and oxidation status of commercial pHSA, suggesting that rHSA is a promising substitute. In the last few decades, numerous studies have reported that rHSA is safe and efficacious in various clinical applications, with no cases of infection or allergy [89–92]. rHSA has already been approved as excipients in pharmaceutical products, such as Recombumin®, which has been used in childhood vaccines for measles, mumps and rubella following approval by the Type V Biologics Master File (BMF) of the FDA. rHSA product from Novozymes Biopharma. are also permitted as drug stabilizers and carriers [93,94]. However, no rHSA has been approved for high-dosage therapeutic use by regulatory agencies because such dosages pose a high immunogenic risk due to residual exogenous materials. For safe and efficacious therapeutic use, rHSA preparations must contain dimers and polymers at less than 1.0%, contaminations with host-cell proteins (HCPs) at less than 0.0001% and endotoxins at less than 0.05 EU/mg. As shown in Fig. 3, we designed a flowchart to develop rHSA for clinical use, according to our research. Systematic characterisations of intermediate and final products are required, including HCPs, DNA, endotoxins, and aggregation.

4.1. Residual host-cell proteins

Residual HCPs are major components of impurities in recombinant biopharmaceutical products, which are highly likely to be immunogenic. Certain levels of HCPs will elicit immune responses and possibly result in clinically relevant adverse events [95]. Because traces of any HCPs might cause severe immune reactions, the purity of rHSA is required to be greater than 99.999%, as recommended by regulatory authority [96].

To achieve acceptable HCP levels, various strategies have been employed. To date, the industrial techniques used to remove HCPs include salting out, isoelectric precipitation, aqueous two-phase extraction, and chromatography [97,98]. Strictly controlled residual HCP levels necessitate the development of rapid, accurate, and sensitive detection methods as part of rHSA production. Analytical techniques, such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and HPLC [99,100] have been typically adopted to detect and characterise HCPs, but those methods are not adequately sensitive or accurate. Thus, the more sensitive and accurate ELISA has been considered to be the golden standard for detecting HCPs. Until now, most detection technologies approved by the FDA recognised HCP levels ranging from 1–100 ppm via ELISA-based methods [101–103]. It is even more challenging to detect trace levels of HCPs without missing any components. Therefore, designing appropriate late stage-specific ELISA methods for assessing final products is essential due to the complexity of antibodies to various HCP populations and the potential variation in HCPs between the downstream rHSA purification steps. In one case, a highly sensitive ELISA was developed for the assessment of rHSA from *P. pastoris*. None of the HCPs in pprHSA was detected by ELISA at an rHSA concentration of 250 mg/mL, which is equal to less than 1.0 ng of HCP/mL [66].

Another challenge is the characterisation of individual HCPs. Because an HCP could cause potential allergies at a very low level, it is necessary to obtain as detailed information as possible on the HCPs in final rHSA products intended for high-dosage applications, even if acceptable levels of residual HCPs are achieved. A better understanding of the components of final rHSA products thus helps to avoid the perceived risks of trace HCPs. Recently, proteomic studies

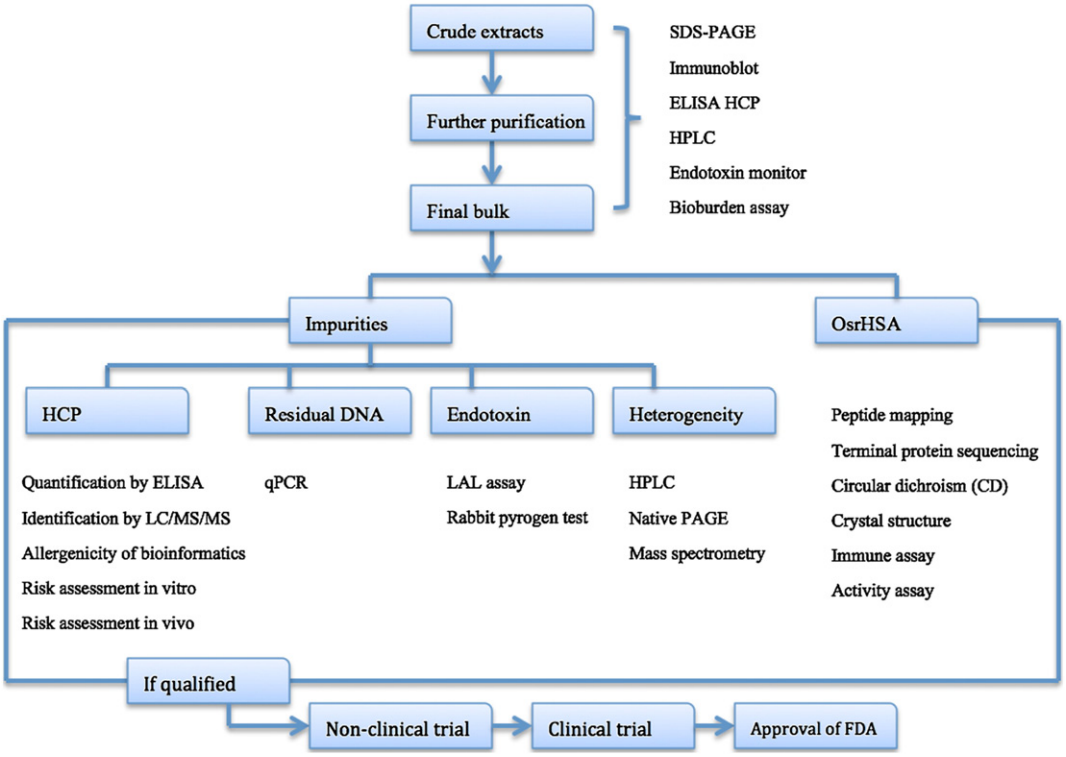

Fig. 3. Qualified control of OsrHSA for clinical application.

largely improved our capacity to identify HCPs, particularly when coupled with such techniques as two-dimensional gel electrophoresis, two-dimensional liquid chromatography and peptide library beads [104–106]. Once the identity of an individual HCP is confirmed, it is easy to further analyse the protein's properties and effects on the human body via a bioinformatics approach using an online database. According to the guidelines proposed by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), allergens can be predicted based on protein sequences [107,108]. Many potential allergens and antioxidants are present in yeast and rice, so it is essential to establish validation methods for reducing residual HCP levels and individual allergens in rHSA [66,109–112]. For example, in our laboratory, potentially adverse proteins are monitored during each processing step by Western blotting with specific antibodies against crude protein extracts from rice grain.

In all cases, the products should be sufficiently characterised prior to a well-designed preclinical safety study (ICH S6) [113]. For the high-dosage use of pharmaceutical rHSA, it is necessary to establish the safety assessment protocol for HCP before preclinical trials. In our laboratory, four-weeks toxicity of HCP from rice seed in Sprague–Dawley rats has been evaluated by intravenous injection, indicating that human body is less allergenic sensitive to plant proteins than that of yeast or bacteria (unpublished data).

4.2. Residual host-cell DNA

Residual DNA (rDNA) present in recombinant products is another safety issue in the production of rHSA. Consequently, residual DNA is often restricted to less than 100 pg per dose of a purified product, according to the authorities regulating recombinant bioproducts [114]. In 1997, the acceptable rDNA content increased to 10 ng/dose in the case of certain high-dosage clinical applications [115,116].

It is challenging to completely remove nucleic acids (DNA or RNA) when purifying a biomolecule. Certain cationic polymer adsorbents, such as chitosan beads and histidine-immobilised polymer beads [117–119], have been very useful as adsorbents for anionic, bio-related nucleic acids. However, because of the high adsorption of both nucleic acids and acidic proteins, commercially available cationic polymer adsorbents could fail to remove DNA from rHSA preparations [120]. Therefore, an alternative strategy should be employed to remove nucleic acids from rHSA.

The nucleic acids must also be quantified to detect the genetic material remaining in recombinant pharmaceuticals. Thus far, numerous quantitative methods have been developed, such as the PicoGreen assay, the hybridisation assay, the threshold immunoassay, and qPCR [103,121,122]. Certain biosensors are also used to detect residual DNA, such as monomolecular layers of alkylthiols, capacitive biosensors, surface plasmon resonance sensors, and quartz crystal microbalance [100,123]. Among these methods, qPCR is highly recommended by the regulatory authorities to quantify the DNA present in biopharmaceutical products [124–126]. Because a sensitive technique should be the key means of detecting residual nucleic acids in rHSA products, qPCR may be the best choice due to this method's ability to sensitively and quickly recognise repetitive DNA sequences. Higher sensitivity may be achieved by whole genome amplification [127]. Although there are no data regarding the rDNA in rHSA, we used a conserved fragment of the ribosome 5 s gene to conduct TaqMan qPCR to assess rHSA derived from plants.

4.3. Endotoxins

Endotoxins, also known as lipopolysaccharide, are the primary contaminants in commercially available biological products. Endotoxins can cause side effects in drug-receiving organisms, such as endotoxin shock, tissue injury, and even death if the endotoxin levels are elevated enough. The high batch rejection of biopharmaceutical products is commonly attributed to excess endotoxins. Therefore, strict control over this type of contaminant is closely related to the quality and safety of biopharmaceutical materials [128]. According to the European

Pharmacopoeia, endotoxin levels must be lower than five endotoxin units (EU) per kilogram of body weight in intravenous applications, 10 EU/mg for insulin, and 100 EU/mg for interferons [129]. Because hundreds of milligrams of HSA per kilogram of body weight are used in the clinic, the threshold endotoxin levels should be maintained at a particularly low level.

Various strategies have been employed to meet specific product requirements for endotoxin decontamination, such as ultrafiltration based on size exclusion, affinity and ion-exchange chromatography, and charged-membrane/depth filtration following electrostatic/hydrophobic interactions [130]. Generally, effective decontamination can reduce endotoxins to acceptable levels during common industrial processing according to GMP.

Karplus et al. reported failing to remove endotoxins when decontaminating bovine catalase using affinity-sorbent polymyxin B-Sepharose according to standard protocols [130]. This finding implies that potential interactions between endotoxins and biologically active substances may prevent a portion of endotoxins from being removed by typical procedures. In fact, endotoxins can bind to HSA via HSA's binding sites and compete with the fatty acids, likely interfering with endotoxin removal [131–133]. The dissociation of protein-endotoxin complexes may be an effective way of improving endotoxin reduction. An example of IgG decontamination was reported by employing the surfactant octyl-β-glucopyranoside to dissociate human IgG-endotoxin complexes and subsequently supporting endotoxin adsorption to polymyxin B-Sepharose [130]. Moreover, a method for reducing endotoxin levels in protein solutions by the use of Triton X-114 has been reported [134]; in this method, the surfactant facilitated the dissociation of endotoxins from the protein. However, all of these methods create a new problem of removing the residual surfactant. Additionally, detergents such as Triton X-114 have the potential to destroy the proper structure of rHSA.

Understanding the interactions between endotoxins and rHSA may significantly contribute to efficient endotoxin removal. For example, based on the noncompetition between albumin and endotoxins with polymyxin B, this type of filling resin may be a potentially powerful tool for removing endotoxins from rHSA solutions [135–137]. In our laboratory, endotoxins resulting from OsrHSA mainly come from rice grain, which were removed by adopting the appropriate pH, salt concentration, organic solution, and chromatographic medium at different steps of downstream processing. It is also challenging to detect endotoxins in rHSA because certain fatty acids interfere with such detection. Several assays have been established to determine endotoxin levels, such as the rabbit pyrogen test, the *Limulus* amoebocyte lysate (LAL) assay, the chicken embryo lethality assay, and the galactosamine-primed mice lethality test [138–140]. Among those tests, the LAL assay is used extensively because of the test's sensitivity, convenience, and low cost. However, many interfering factors in the coagulation cascade could result in a false-negative or false-positive result in a LAL assay. For instance, in one study, rHSA binding to endotoxins yielded a complex, leading to a false result [135]. Therefore, when assessing rHSA, other bioassays should be adopted as a complement to the LAL assay, such as the rabbit pyrogen test and whole-blood stimulation assays [141].

5. Preclinical and clinical trails

For engineered recombinant proteins with high-dosage clinical applications, it is vital to conduct a series of preclinical and clinical tests to validate the safety and efficacy of rHSA before clinical use. To assess the differences in the biological function of rHSA and pHSA, either rHSA or pHSA labelled with ^{125}I was intravenously injected into rats. No significant differences were noted in the rats' physiological symptoms, including the half-life of the HSA in the blood, urinary, and faecal excretions and the HSA's organ and tissue distributions [75]. Similarly, an animal model has been established to evaluate rHSA efficacy for the treatment of ascites during liver cirrhosis coupled with hypoalbuminaemia. Studies using this model have proven that pprHSA and OsrHSA are similar effective to pHSA for the treatment of liver cirrhosis [13,75]. Moreover, the putatively identical function of rHSA and pHSA in the treatment of liver cirrhosis induced in rats by carbon tetrachloride has been reported, resulting in decreased abdominal circumference and simultaneously improved blood colloid osmotic pressure and increased urine volume [75]. No immune reactions have been reported in these animal-based preclinical tests of rHSA. In summary, the identical efficacy of rHSA and pHSA demonstrates the feasibility of rHSA as a drug for treating liver ascites due to hypoalbuminaemia.

In clinical trials, the rHSA products from *P. pastoris* and *S. cerevisiae* are the most thoroughly studied. In particular, the safety and pharmacodynamics of pprHSA have been assessed in healthy volunteers both intravenously and intramuscularly. Nine incidences of drug-related, potentially allergic responses (four recipients of rHSA and five recipients of pHSA) were reported in the intramuscular study, indicating the similar frequency of critically adverse effects between groups [90]. As in animal tests, the dose of rHSA did not significantly influence the protein's tolerability. Moreover, rHSA performed similarly to pHSA in safety and pharmacodynamics tests, with no significant differences between rHSA and pHSA in both overall and treatment-related adverse events [90,91,142]. Furthermore, a phase III investigation by a Japanese company confirmed the high efficacy and low potential risks of pprHSA [92].

Cytokine release from leukocytes is a promising indicator of immunotoxicity in humans [143,144]. Thus, considering animal welfare and economic reasons, it becomes more important to develop an alternative assay *in vitro* based on human immune cells to precisely predict the immune reaction to rHSA in vivo.

6. Concluding remarks and perspectives

To date, rHSA has been successfully expressed using genetic engineering, and large-scale production has been achieved in yeast and rice. rHSA shows great promise as a substitute for pHSA in future clinical applications and may help resolve the shortage and safety issues associated with pHSA. However, feasible, economical commercialisation depends not only on the maintenance of biological activity but also on low-cost downstream processing and scaling. Recently, transgenic rice has been used as a novel bioreactor and has demonstrated great potential to cost-effectively produce sufficient quantities of safe OsrHSA. Further improvements in rHSA expression in various hosts and in rHSA purification will likely result in an affordable product.

Safety is the primary public concern when rHSA is proposed for clinical use. The risk of an allergenic response must be completely eliminated before clinical use. Although plant components are considered less likely to induce immune reactions than compounds derived from microbes, rHSA bioprocessing is still required to remove impurities to acceptable levels given the large dosages required for pharmaceutical applications. A well understanding of the complexities of impurities and contaminants from the host cells and processing could guide to develop the protocol for removal of HCPs, endotoxins and nucleic acids as much as possible. However, it remains very challenging to establish appropriate impurity removal and detection methods in rHSA manufacturing.

In conclusion, the limited supply and risks of viral transmission of pHSA have justified the development of rHSA. The need for an ample supply of rHSA at an affordable cost, without jeopardising quality, is now the driving force behind recombinant DNA technology, as long as this approach yields the financial returns expected by biotechnology companies. Additionally, we expect that rHSA may expand its role, either clinically or industrially, in the future.

Acknowledgements

This work was supported by National High-Tech R&D Program 863 of China No. 2011AA100604, and Major Projects of Genetically Modified Crop of China Grant 2011ZX08001-006.

References

[1] T. Peters, I. Sjöholm, Albumin: Structure, Biosynthesis, Function, Academic, San Diego, 1995.
[2] G.E. Hastings, P.G. Wolf, The therapeutic use of albumin, Arch. Fam. Med. 1 (1992) 281–287.
[3] M.R. Alexander, J.J. Ambre, B.I. Liskow, D.C. Trost, Therapeutic use of albumin, JAMA 241 (1979) 2527–2529.
[4] R.G. Romanelli, G. La Villa, G. Barletta, F. Vizzutti, F. Lanini, U. Arena, V. Boddi, R. Tarquini, P. Pantaleo, P. Gentilini, G. Laffi, Long-term albumin infusion improves survival in patients with cirrhosis and ascites: an unblinded randomized trial, World J. Gastroenterol. 12 (2006) 1403–1407.
[5] E. Marth, B. Kleinhappl, Albumin is a necessary stabilizer of TBE-vaccine to avoid fever in children after vaccination, Vaccine 20 (2001) 532–537.
[6] J. He, M. Feng, X. Zhou, S. Ma, Y. Jiang, Y. Wang, H. Zhang, Stabilization and encapsulation of recombinant human erythropoietin into PLGA microspheres using human serum albumin as a stabilizer, Int. J. Pharm. 416 (2011) 69–76.
[7] K. Kobayashi, N. Nakamura, A. Sumi, T. Ohmura, K. Yokoyama, The development of recombinant human serum albumin, Ther. Apher. Dial. 2 (1998) 257–262.
[8] M.E. Chamberland, H.J. Alter, M.P. Busch, G. Nemo, M. Ricketts, Emerging infectious disease issues in blood safety, Emerg. Infect. Dis. 7 (2001) 552–553.
[9] S. MacLennan, J.A. Barbara, Risks and side effects of therapy with plasma and plasma fractions, Best Pract. Res. Clin. Haematol. 19 (2006) 169–189.
[10] O.W. Merten, Virus contaminations of cell cultures — a biotechnological view, Cytotechnology 39 (2002) 91–116.
[11] R. Fischer, N. Emans, Molecular farming of pharmaceutical proteins, Transgenic Res. 9 (2000) 279–299.
[12] K. Kobayashi, Summary of recombinant human serum albumin development, Biologicals 34 (2006) 55–59.
[13] Y. He, T. Ning, T. Xie, Q. Qiu, L. Zhang, Y. Sun, D. Jiang, K. Fu, F. Yin, W. Zhang, Large-scale production of functional human serum albumin from transgenic rice seeds, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 19078–19083.
[14] J.L. Martinez, L. Liu, D. Petranovic, J. Nielsen, Pharmaceutical protein production by yeast: towards production of human blood proteins by microbial fermentation, Curr. Opin. Biotechnol. 23 (2012) 965–971.
[15] R.M. Lawn, J. Adelman, S.C. Bock, A.E. Franke, C.M. Houck, R. Najarian, P.H. Seeburg, K.L. Wion, The sequence of human serum albumin cDNA and its expression in *E. coli*, Nucleic Acids Res. 9 (1981) 6103–6114.
[16] J. Lobstein, C.A. Emrich, C. Jeans, M. Faulkner, P. Riggs, M. Berkmen, SHuffle, a novel *Escherichia coli* protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm, Microb. Cell Fact. 11 (2012) 56.
[17] C.W. Saunders, B.J. Schmidt, R.L. Mallonee, M.S. Guyer, Secretion of human serum albumin from *Bacillus subtilis*, J. Bacteriol. 169 (1987) 2917–2925.
[18] G. Salinas, L. Pellizza, M. Margenat, M. Flo, C. Fernandez, Tuned *Escherichia coli* as a host for the expression of disulfide-rich proteins, Biotechnol. J. 6 (2011) 686–699.
[19] A.V. Quirk, M.J. Geisow, J.R. Woodrow, S.J. Burton, P.C. Wood, A.D. Sutton, R.A. Johnson, N. Dodsworth, Production of recombinant human serum albumin from *Saccharomyces cerevisiae*, Biotechnol. Appl. Biochem. 11 (1989) 273–287.
[20] D. Sleep, G.P. Belfield, A.R. Goodey, The secretion of human serum albumin from the yeast *Saccharomyces cerevisiae* using five different leader sequences, Biotechnology (N Y) 8 (1990) 42–46.
[21] S.M. Kerry-Williams, S.C. Gilbert, L.R. Evans, D.J. Ballance, Disruption of the *Saccharomyces cerevisiae* YAP3 gene reduces the proteolytic degradation of secreted recombinant human albumin, Yeast 14 (1998) 161–169.
[22] H.A. Kang, E.S. Choi, W.K. Hong, J.Y. Kim, S.M. Ko, J.H. Sohn, S.K. Rhee, Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. 53 (2000) 575–582.
[23] S.E. Cheperegin, B.D. Efremov, D.G. Kozlov, Precipitation of human serum albumin from yeast culture liquid at pH values below 5, Protein Expr. Purif. 72 (2010) 205–208.
[24] J.H. Heo, W.K. Hong, E.Y. Cho, M.W. Kim, J.Y. Kim, C.H. Kim, S.K. Rhee, H.A. Kang, Properties of the *Hansenula polymorpha*-derived constitutive GAP promoter, assessed using an HSA reporter gene, FEMS Yeast Res. 4 (2003) 175–184.
[25] H. Cox, D. Mead, P. Sudbery, R.M. Eland, I. Mannazzu, L. Evans, Constitutive expression of recombinant proteins in the methylotrophic yeast *Hansenula polymorpha* using the PMA1 promoter, Yeast 16 (2000) 1191–1203.
[26] H.A. Kang, W. Kang, W.K. Hong, M.W. Kim, J.Y. Kim, J.H. Sohn, E.S. Choi, K.B. Choe, S.K. Rhee, Development of expression systems for the production of recombinant human serum albumin using the MOX promoter in *Hansenula polymorpha* DL-1, Biotechnol. Bioeng. 76 (2001) 175–185.
[27] R. Fleer, P. Yeh, N. Amellal, I. Maury, A. Fournier, F. Bacchetta, P. Baduel, G. Jung, H. L'Hote, J. Becquart, et al., Stable multicopy vectors for high-level secretion of recombinant human serum albumin by *Kluyveromyces* yeasts, Biotechnology (N Y) 9 (1991) 968–975.
[28] K. Blondeau, H. Boze, G. Jung, G. Moulin, P. Galzy, Physiological approach to heterologous human serum albumin production by *Kluyveromyces lactis* in chemostat culture, Yeast 10 (1994) 1297–1303.
[29] K. Kobayashi, S. Kuwae, T. Ohya, T. Ohda, M. Ohyama, H. Ohi, K. Tomomitsu, T. Ohmura, High-level expression of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation, J. Biosci. Bioeng. 89 (2000) 55–61.
[30] K. Kobayashi, S. Kuwae, T. Ohya, T. Ohda, M. Ohyama, K. Tomomitsu, High level secretion of recombinant human serum albumin by fed-batch fermentation of the methylotrophic yeast, *Pichia pastoris*, based on optimal methanol feeding strategy, J. Biosci. Bioeng. 90 (2000) 280–288.
[31] R.D. Qiu, S.Y. Li, J.G. Chen, X.F. Wu, Z.Y. Yuan, High expression and purification of recombinant human serum albumin from *Pichia pastoris*, Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai) 32 (2000) 59–62.
[32] M. Guo, Y. Zhuang, J. Chu, S. Zhang, Z. Liu, Expression of recombinant human serum albumin in genetically engineered *Pichia pastoris* in high-density fermentation, Wei Sheng Wu Xue Bao 42 (2002) 62–68.
[33] H. Kang, M. Jung, W. Hong, J. Sohn, E. Choi, R. SK, Expression and secretion of human serum albumin in the yeast *Saccharomyces cerevisiae*, J. Microbiol. Biotechnol. 8 (1998) 42–48.
[34] T. Lodi, B. Neglia, C. Donnini, Secretion of human serum albumin by *Kluyveromyces lactis* overexpressing KlPDI1 and KlERO1, Appl. Environ. Microbiol. 71 (2005) 4359–4363.
[35] K. Kobayashi, S. Kuwae, T. Ohya, T. Ohda, M. Ohyama, K. Tomomitsu, Addition of oleic acid increases expression of recombinant human serum albumin by the AOX2 promoter in *Pichia pastoris*, J. Biosci. Bioeng. 89 (2000) 479–484.
[36] M.E. Bushell, M. Rowe, C.A. Avignone-Rossa, J.N. Wardell, Cyclic fed-batch culture for production of human serum albumin in *Pichia pastoris*, Biotechnol. Bioeng. 82 (2003) 678–683.
[37] T. Ohya, M. Ohyama, K. Kobayashi, Optimization of human serum albumin production in methylotrophic yeast *Pichia pastoris* by repeated fed-batch fermentation, Biotechnol. Bioeng. 90 (2005) 876–887.
[38] M. Saliola, C. Mazzoni, N. Solimando, A. Crisa, C. Falcone, G. Jung, R. Fleer, Use of the KlADH4 promoter for ethanol-dependent production of recombinant human serum albumin in *Kluyveromyces lactis*, Appl. Environ. Microbiol. 65 (1999) 53–60.
[39] M. Belew, M. Li, W. Zhang, K. Caldwel, Purification of recombinant human serum albumin (rHSA) produced by genetically modified *Pichia Pastoris*, Sep. Sci. Technol. 43 (2008) 3134–3153.
[40] B. Li, Y. Cao, L. Zhou, C. Liang, F. Sun, A novel protein expression system-PichiaPink ™- and a protocol for fast and efficient recombinant protein expression, Afr. J. Biotechnol. 10 (2011) 19464–19471.
[41] L.M. Houdebine, Transgenic animal bioreactors, Transgenic Res. 9 (2000) 305–320.
[42] M. Shani, I. Barash, M. Nathan, G. Ricca, G.H. Searfoss, I. Dekel, A. Faerman, D. Givol, D.R. Hurwitz, Expression of human serum albumin in the milk of transgenic mice, Transgenic Res. 1 (1992) 195–208.
[43] I. Barash, A. Faerman, A. Baruch, M. Nathan, D.R. Hurwitz, M. Shani, Synthesis and secretion of human serum albumin by mammary gland explants of virgin and lactating transgenic mice, Transgenic Res. 2 (1993) 266–276.
[44] D.R. Hurwitz, M. Nathan, I. Barash, N. Ilan, M. Shani, Specific combinations of human serum albumin introns direct high level expression of albumin in transfected COS cells and in the milk of transgenic mice, Transgenic Res. 3 (1994) 365–375.
[45] I. Barash, A. Faerman, M. Richenstein, R. Kari, G.M. Damary, M. Shani, M.J. Bissell, In vivo and *in vitro* expression of human serum albumin genomic sequences in mammary epithelial cells with beta-lactoglobulin and whey acidic protein promoters, Mol. Reprod. Dev. 52 (1999) 241–252.
[46] X. Wu, Y. Lin, F. Xiong, Y. Zhou, F. Yu, J. Deng, P. Huang, H. Chen, The extremely high level expression of human serum albumin in the milk of transgenic mice, Transgenic Res. 21 (2012) 1359–1366.
[47] Y. Huang, Z. Huang, J. Yan, Z. Ma, M. Sheng, Z. Ren, Y. Zeng, S. Huang, High expression of human serum albumin in milk of transgenic mice directed by the goat β-casein gene promoter region, Chin. Sci. Bull. 46 (2001) 582–585.
[48] Y. Echelard, J.L. Williams, M.M. Destrempes, J.A. Koster, S.A. Overton, D.P. Pollock, K.T. Rapiejko, E. Behboodi, N.C. Masiello, W.G. Gavin, J. Pommer, S.M. Van Patten, D.C. Faber, J.B. Cibelli, H.M. Meade, Production of recombinant albumin by a herd of cloned transgenic cattle, Transgenic Res. 18 (2009) 361–376.
[49] S. Ogawa, M. Tomita, K. Shimizu, K. Yoshizato, Generation of a transgenic silkworm that secretes recombinant proteins in the sericin layer of cocoon: production of recombinant human serum albumin, J. Biotechnol. 128 (2007) 531–544.
[50] P.C. Sijmons, B.M. Dekker, B. Schrammeijer, T.C. Verwoerd, P.J. van den Elzen, A. Hoekema, Production of correctly processed human serum albumin in transgenic plants, Biotechnology (N Y) 8 (1990) 217–221.
[51] I. Farran, J.J. Sánchez-Serrano, J.F. Medina, J. Prieto, A.M. Mingo-Castel, Targeted expression of human serum albumin to potato tubers, Transgenic Res. 11 (2002) 337–346.
[52] F.S. Millán, A. Mingo-Castel, M. Miller, H. Daniell, A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation, Plant Biotechnol. J. 1 (2003) 71–79.
[53] L.F. Huang, Y.K. Liu, C.A. Lu, S.L. Hsieh, S.M. Yu, Production of human serum albumin by sugar starvation induced promoter and rice cell culture, Transgenic Res. 14 (2005) 569–581.
[54] Q.Y. Sun, L.W. Ding, G.P. Lomonossoff, Y.B. Sun, M. Luo, C.Q. Li, L. Jiang, Z.F. Xu, Improved expression and purification of recombinant human serum albumin from transgenic tobacco suspension culture, J. Biotechnol. 155 (2011) 164–172.
[55] O.S. Lau, S.S. Sun, Plant seeds as bioreactors for recombinant protein production, Biotechnol. Adv. 27 (2009) 1015–1022.

[56] P. Matejtschuk, C.H. Dash, E.W. Gascoigne, Production of human albumin solution: a continually developing colloid, Br. J. Anaesth. 85 (2000) 887–895.
[57] N. Coker, P. Amoroso, Production of human albumin solution: a continually developing colloid, Br. J. Anaesth. 86 (2001) 898.
[58] D. Yang, C. He, G. Li, Method for extracting recombinant human serum albumin from transgenic rice grain, United States Patent (2012).
[59] T. Ohmura, A. Sumi, W. Ohtani, N. Furuhata, K. Takeshima, K. Kamide, M. Noda, M. Kondo, S. Ishikawa, K. Oohara, Yokoyama, N. Fujiwara, Recombinant human serum albumin, process for producing the same and pharmaceutical preparation containing the same, United States Patent (1999).
[60] L. Van Der, M. Piet, Process for the purification of serum albumin, European Patent, EP0524681 (1992).
[61] Y. Dong, F. Zhang, Z. Wang, L. Du, A. Hao, B. Jiang, M. Tian, Q. Li, Q. Jia, S. Wang, Z. Xiu, Extraction and purification of recombinant human serum albumin from *Pichia pastoris* broths using aqueous two-phase system combined with hydrophobic interaction chromatography, J. Chromatogr. A 1245 (2012) 143–149.
[62] A. Sumi, K. Okuyama, K. Kobayashi, W. Ohtani, T. Ohmura, K. Yokoyama, Purification of recombinant human serum albumin efficient purification using STREAMLINE, Bioseparation 8 (1999) 195–200.
[63] J. Travis, R. Pannell, Selective removal of albumin from plasma by affinity chromatography, Clin. Chim. Acta 49 (1973) 49–52.
[64] I. Farran, J.J. Sanchez-Serrano, J.F. Medina, J. Prieto, A.M. Mingo-Castel, Targeted expression of human serum albumin to potato tubers, Transgenic Res. 11 (2002) 337–346.
[65] T. Ohmura, A. Sumi, W. Ohtani, N. Fuluhata, K. Takeshima, K. Kamide, M. Noda, M. Kondo, S. Ishikawa, K. Oohara, K. Yokoyama, Recombinant human serum albumin process for producing the same and pharmaceutical perparation containing the same, United States Patent (1995).
[66] W. Ohtani, T. Ohda, A. Sumi, K. Kobayashi, T. Ohmura, Analysis of *Pichia pastoris* components in recombinant human serum albumin by immunological assays and by HPLC with pulsed amperometric detection, Anal. Chem. 70 (1998) 425–429.
[67] Y.H. D. Yang, G. Li, J. Liu, Method for isolating and purifying recombinant human serum albumin from transgenic rice grain, in, United States Patent, 2012.
[68] J. Flensburg, M. Belew, Characterization of recombinant human serum albumin using matrix-assisted laser desorption ionization time-of-flight mass spectrometry, J. Chromatogr. A 1009 (2003) 111–117.
[69] H. Watanabe, K. Yamasaki, U. Kragh-Hansen, S. Tanase, K. Harada, A. Suenaga, M. Otagiri, *In vitro* and in vivo properties of recombinant human serum albumin from *Pichia pastoris* purified by a method of short processing time, Pharm. Res. 18 (2001) 1775–1781.
[70] W. Ohtani, Y. Nawa, K. Takeshima, H. Kamuro, K. Kobayashi, T. Ohmura, Physicochemical and immunochemical properties of recombinant human serum albumin from *Pichia pastoris*, Anal. Biochem. 256 (1998) 56–62.
[71] K. Ikegaya, M. Hirose, T. Ohmura, K. Nokihara, Complete determination of disulfide forms of purified recombinant human serum albumin, secreted by the yeast *Pichia pastoris*, Anal. Chem. 69 (1997) 1986–1991.
[72] E.S. Ng, R. Davis, E.G. Stanley, A.G. Elefanty, A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies, Nat. Protoc. 3 (2008) 768–776.
[73] S. Friedler, M. Schachter, D. Strassburger, K. Esther, R. Ron El, A. Raziel, A randomized clinical trial comparing recombinant hyaluronan/recombinant albumin versus human tubal fluid for cleavage stage embryo transfer in patients with multiple IVF-embryo transfer failure, Hum. Reprod. 22 (2007) 2444–2448.
[74] M. Bungum, P. Humaidan, L. Bungum, Recombinant human albumin as protein source in culture media used for IVF: a prospective randomized study, Reprod. Biomed. Online 4 (2002) 233–236.
[75] S. Horie, H. Nagai, T. Yuuki, S. Hanada, N. Nakamura, Effectiveness of recombinant human serum albumin in the treatment of ascites in liver cirrhosis: evidence from animal model, Gen. Pharmacol. 31 (1998) 811–815.
[76] M. Tsukada, M. Shintome, T. Matsui, H. Tsuchiyama, T. Maruyama, T. Yuki, S. Hanada, N. Nakamura, Effects of recombinant human serum albumin on ascites in rats with puromycin aminonucleoside-induced nephropathy, Gen. Pharmacol. 31 (1998) 209–214.
[77] G.E. Frahm, T.D. Cyr, D.G. Smith, L.D. Walrond, M.J. Johnston, Investigation of the differences in thermal stability of two recombinant human serum albumins with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine liposomes by UV circular dichroism spectropolarimetry, J. Phys. Chem. B 116 (2012) 4661–4670.
[78] K. Ikegaya, K. Nokihara, T. Yasuhara, Characterization of sulfhydryl heterogeneity in human serum albumin and recombinant human serum albumin for clinical use, Biosci. Biotechnol. Biochem. 74 (2010) 2232–2236.
[79] J. Otsuki, Y. Nagai, Y. Matsuyama, T. Terada, S. Era, The redox state of recombinant human serum albumin and its optimal concentration for mouse embryo culture, Syst. Biol. Reprod. Med. 59 (2012) 48–52.
[80] W. Soetaert, E.J. Vandamme, Industrial BiotechnologyWiley-VCH, 2010, p. 64.
[81] A.L. Demain, P. Vaishnav, Production of recombinant proteins by microbes and higher organisms, Biotechnol. Adv. 27 (2009) 297–306.
[82] K. Genti, F.M. Bradford, W.N. George, Potential impacts of pharmaceutical uses of transgenic tobacco: the case of human serum albumin (Has), The Annual Meeting of the American Agricultural Economics Association, Denver, Colorado (August 1-4, 2004).
[83] E. Stöger, C. Vaquero, E. Torres, M. Sack, L. Nicholson, J. Drossard, S. Williams, D. Keen, Y. Perrin, P. Christou, Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies, Plant Mol. Biol. 42 (2000) 583–590.
[84] S.A. Goff, D. Ricke, T.-H. Lan, G. Presting, R. Wang, M. Dunn, J. Glazebrook, A. Sessions, P. Oeller, H. Varma, A draft sequence of the rice genome (*Oryza sativa L. ssp. japonica*), Science 296 (2002) 92–100.
[85] R. Fischer, S. Schillberg, S. Hellwig, R.M. Twyman, J. Drossard, GMP issues for recombinant plant-derived pharmaceutical proteins, Biotechnol. Adv. 30 (2012) 434–439.
[86] J. Rong, Z. Song, J. Su, H. Xia, B.R. Lu, F. Wang, Low frequency of transgene flow from Bt/CpTI rice to its non-transgenic counterparts planted at close spacing, New Phytol. 168 (2005) 559–566.
[87] J. Rong, B.R. Lu, Z. Song, J. Su, A.A. Snow, X. Zhang, S. Sun, R. Chen, F. Wang, Dramatic reduction of crop-to-crop gene flow within a short distance from transgenic rice fields, New Phytol. 173 (2007) 346–353.
[88] G. Kostandini, B.F. Mills, G.W. Norton, The potential impact of tobacco biopharming: the case of human serum albumin, Am. J. Agric. Econ. 88 (2006) 671–679.
[89] V. Chuang, M. Otagiri, Recombinant human serum albumin, Drugs Today (Barc.) 43 (2007) 547–561.
[90] D. Bosse, M. Praus, P. Kiessling, L. Nyman, C. Andresen, J. Waters, F. Schindel, Phase I comparability of recombinant human albumin and human serum albumin, J. Clin. Pharmacol. 45 (2005) 57–67.
[91] K. Ohnishi, A. Kawaguchi, S. Nakajima, H. Mori, T. Ueshima, A comparative pharmacokinetic study of recombinant human serum albumin with plasma-derived human serum albumin in patients with liver cirrhosis, J. Clin. Pharmacol. 48 (2008) 203–208.
[92] A. Kasahara, K. Kita, E. Tomita, J. Toyota, Y. Imai, H. Kumada, Repeated administration of recombinant human serum albumin caused no serious allergic reactions in patients with liver cirrhosis: a multicenter clinical study, J. Gastroenterol. 43 (2008) 464–472.
[93] E. Tarelli, A. Mire-Sluis, H.A. Tivnann, B. Bolgiano, D.T. Crane, C. Gee, X. Lemercinier, M.L. Athayde, N. Sutcliffe, P.H. Corran, Recombinant human albumin as a stabilizer for biological materials and for the preparation of international reference reagents, Biologicals 26 (1998) 331–346.
[94] B. Elsadek, F. Kratz, Impact of albumin on drug delivery—new applications on the horizon, J. Control. Release 157 (2012) 4–28.
[95] J. Lupker, Residual host cell protein from continuous cell lines. Effect on the safety of protein pharmaceuticals, Dev. Biol. Stand. 93 (1998) 61–64.
[96] USP, the United States Pharmacopoeia — National Formulary (USP-NF), Albumin human, the United States Pharmacopoeia Convention, , 2009.
[97] S. Gräslund, P. Nordlund, J. Weigelt, J. Bray, O. Gileadi, S. Knapp, U. Oppermann, C. Arrowsmith, R. Hui, J. Ming, Protein production and purification, Nat. Methods 5 (2008) 135–146.
[98] F. Fortis, L. Guerrier, P.G. Righetti, P. Antonioli, E. Boschetti, A new approach for the removal of protein impurities from purified biologicals using combinatorial solid-phase ligand libraries, Electrophoresis 27 (2006) 3018–3027.
[99] K. Hoffman, Strategies for host cell protein analysis, Biopharm-Eugene 13 (2000) 38–47.
[100] B. Mattiasson, K. Teeparuksapun, M. Hedström, Immunochemical binding assays for detection and quantification of trace impurities in biotechnological production, Trends Biotechnol. 28 (2010) 20–27.
[101] L.C. Eaton, Host cell contaminant protein assay development for recombinant biopharmaceuticals, J. Chromatogr. A 705 (1995) 105–114.
[102] A.S. Rathore, S. Sobacke, T. Kocot, D. Morgan, R. Dufield, N. Mozier, Analysis for residual host cell proteins and DNA in process streams of a recombinant protein product expressed in *Escherichia coli* cells, J. Pharm. Biomed. Anal. 32 (2003) 1199–1211.
[103] W. Tanja, R. Andreas, Assays for controlling host-cell impurities in biopharmaceuticals, BioProcess Int. 3 (2005) 40–46.
[104] E.J. Hayduk, L.H. Choe, K.H. Lee, A two-dimensional electrophoresis map of Chinese hamster ovary cell proteins based on fluorescence staining, Electrophoresis 25 (2004) 2545–2556.
[105] M.R. Schenauer, G.C. Flynn, A.M. Goetze, Identification and quantification of host cell protein impurities in biotherapeutics using mass spectrometry, Anal. Biochem. 428 (2012) 150–157.
[106] P. Antonioli, F. Fortis, L. Guerrier, S. Rinalducci, L. Zolla, P.G. Righetti, E. Boschetti, Capturing and amplifying impurities from purified recombinant monoclonal antibodies via peptide library beads: a proteomic study, Proteomics 7 (2007) 1624–1633.
[107] R.E. Hileman, A. Silvanovich, R.E. Goodman, E.A. Rice, G. Holleschak, J.D. Astwood, S.L. Hefle, Bioinformatic methods for allergenicity assessment using a comprehensive allergen database, Int. Arch. Allergy Immunol. 128 (2002) 280–291.
[108] M.B. Stadler, B.M. Stadler, Allergenicity prediction by protein sequence, FASEB J. 17 (2003) 1141–1143.
[109] A. Zargari, B. Härfast, S. Johansson, S. Johansson, A. Scheynius, Identification of allergen components of the opportunistic yeast *Pityrosporum orbiculare* by monoclonal antibodies, Allergy 49 (2007) 50–56.
[110] S. Suzuki, Immunochemical study on mannan, the antigenic polysaccharide of pathogenic yeasts in man of genus Candida, Yakugaku Zasshi 115 (1995) 280.
[111] R. Nakamura, T. Matsuda, Rice allergenic protein and molecular-genetic approach for hypoallergenic rice, Biosci. Biotechnol. Biochem. 60 (1996) 1215.
[112] Y. Usui, M. Nakase, H. Hotta, A. Urisu, N. Aoki, K. Kitajima, T. Matsuda, A 33-kDa allergen from rice (*Oryza sativa* L. Japonica) cDNA cloning, expression, and identification as a novel glyoxlase I, J. Biol. Chem. 276 (2001) 11376–11381.
[113] J.A. Cavagnaro, The principles of ICH S6 and the case-by-case approach, Preclinical Safety Evaluation of Biopharmaceuticals: A Science-Based Approach to Facilitating Clinical Trials, 2007. 43–65.
[114] K. Zoon, Points to consider in the manufacture and testing of monoclonal antibody products for human use, FDA, 1997.

[115] Acceptability of cell substrates for production of biologicals. Report of a WHO Study Group on Biologicals, World Health Organ. Tech. Rep. Ser. (1987) 1–29.
[116] V. Grachev, D. Magrath, E. Griffiths, WHO requirements for the use of animal cells as *in vitro* substrates for the production of biologicals (Requirements for biological susbstances no. 50), Biologicals 26 (1998) 93–175.
[117] C. Hirayama, H. Ihara, Method for preparing granular porous chitosan, in, EP Patent 0,345,017, 1994.
[118] M. Sakata, M. Nakayama, T. Fujisaki, S. Morimura, M. Kunitake, C. Hirayama, Chromatographic removal of host cell DNA from cellular products using columns packed with cationic copolymer beads, Chromatographia 62 (2005) 465–470.
[119] H. Matsumae, S. Minobe, K. Kindan, T. Watanabe, T. Sato, T. Tosa, Specific removal of endotoxin from protein solutions by immobilized histidine, Biotechnol. Appl. Biochem. 12 (1990) 129–140.
[120] C. Hirayama, M. Sakata, K. Moriguchi, H. Mizokami, H. Ihara, K. Ohkuma, Pore-size controlled and aminated poly (GAMMA.-methyl L-glutamate) particles for selective removal of nucleic acids, Polym. J. 30 (1998) 616–621.
[121] S.J. Ahn, J. Costa, J.R. Emanuel, PicoGreen quantitation of DNA: effective evaluation of samples pre-or post-PCR, Nucleic Acids Res. 24 (1996) 2623–2625.
[122] X. Ji, K. Lee, B. DiPaolo, Short technical report high-sensitivity hybridization assay for quantitation of residual *E. coli* DNA, Biotechniques 32 (2002) 1162–1167.
[123] A. Numnuam, P. Kanatharana, B. Mattiasson, P. Asawatreratanakul, B. Wongkittisuksa, C. Limsakul, P. Thavarungkul, Capacitive biosensor for quantification of trace amounts of DNA, Biosens. Bioelectron. 24 (2009) 2559–2565.
[124] K.Y. Wang, Y.J. Guo, S.H. Sun, K. Shi, S. Zhang, K.H. Wang, Z.H. Chen, 16S rRNA gene probe quantitates residual host cell DNA in pharmaceutical-grade plasmid DNA, Vaccine 24 (2006) 2656–2661.
[125] H. Cai, X. Gu, M.S. Scanlan, C.R. Lively, Development of a quantitative PCR assay for residual mouse DNA and comparison of four sample purification methods for DNA isolation, J. Pharm. Biomed. Anal. 55 (2011) 71–77.
[126] P.M. Nissom, Specific detection of residual CHO host cell DNA by real-time PCR, Biologicals 35 (2007) 211–215.
[127] M.J. Kang, H. Yu, S.K. Kim, S.R. Park, I. Yang, Quantification of trace-level DNA by real-time whole genome amplification, PLoS One 6 (2011) e28661.
[128] V. Salema, L. Saxena, P. Pattnaik, Removing endotoxin from biopharmaceutical solutions, Pharm. Technol. Eur. 21 (2009).
[129] European Pharmacopoeia, 3rd ed., 1997.
[130] T.E. Karplus, R.J. Ulevitch, C.B. Wilson, A new method for reduction of endotoxin contamination from protein solutions, J. Immunol. Methods 105 (1987) 211–220.
[131] G.J. van der Vusse, Albumin as fatty acid transporter, Drug Metab. Pharmacokinet. 24 (2009) 300–307.
[132] S. David, P. Balaram, V. Mathan, Characterization of the interaction of lipid A and lipopolysaccharide with human serum albumin: implications for an endotoxin carrier function for albumin, J. Endotoxin Res. 2 (1995) 99–106.
[133] T.L. Gioannini, D.S. Zhang, A. Teghanemt, J.P. Weiss, An essential role for albumin in the interaction of endotoxin with lipopolysaccharide-binding protein and sCD14 and resultant cell activation, J. Biol. Chem. 277 (2002) 47818–47825.
[134] Y. Aida, M.J. Pabst, Removal of endotoxin from protein solutions by phase separation using Triton X-114, J. Immunol. Methods 132 (1990) 191–195.
[135] G. Jürgens, M. Müller, P. Garidel, M.H.J. Koch, H. Nakakubo, A. Blume, K. Brandenburg, Investigation into the interaction of recombinant human serum albumin with Re-lipopolysaccharide and lipid A, J. Endotoxin Res. 8 (2002) 115–126.
[136] S. Tang, L. Kong, J. Ou, H. Zou, Adsorption of endotoxin on polymyxin B immobilized affinity matrices, Chin. J. Anal. Chem. 34 (2006) 455–459.
[137] K. Teramoto, Y. Nakamoto, T. Kunitomo, H. Shoji, T. Tani, K. Hanazawa, M. Kodama, Removal of endotoxin in blood by polymyxin B immobilized polystyrene-derivative fiber, Ther. Apher. 6 (2002) 103–108.
[138] C. Galanos, M.A. Freudenberg, Mechanisms of endotoxin shock and endotoxin hypersensitivity, Immunobiology 187 (1993) 346–356.
[139] C. Galanos, E.T. Rietschel, O. LÜDERITZ, O. Westphal, Interaction of lipopolysaccharides and lipid A with complement, Eur. J. Biochem. 19 (1971) 143–152.
[140] F. Pearson, J. Dubczak, M. Weary, G. Bruszer, G. Donohue, Detection of endotoxin in the plasma of patients with gram-negative bacterial sepsis by the Limulus amoebocyte lysate assay, J. Clin. Microbiol. 21 (1985) 865–868.
[141] T. Hartung, A. Wendel, Detection of pyrogens using human whole blood, In Vitro Toxicol. 9 (1996) 353–359.
[142] F. Schindel, C. Andersen, D. Bosse, Comparison of recombinant human albumin with human serum albumin: safety, tolerability and pharmacodynamics, J. Clin. Pharmacol. 43 (2003) 1032.
[143] R.V. House, Cytokine measurement techniques for assessing hypersensitivity, Toxicology 158 (2001) 51–58.
[144] D. Lankveld, H. Van Loveren, K. Baken, R. Vandebriel, *In vitro* testing for direct immunotoxicity: state of the art, Methods Mol. Biol. 598 (2009) 401–423.