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Purification and characterization of the major protein isolated from *Semen Armeniacae Amarum* and the properties of its thermally induced nanoparticles

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1 **Abstract:**

2 From the aqueous extract of *Semen Armeniacae Amarum*, a major protein isolate
3 was purified and characterized as a novel member of the 11S globulin family, which is
4 composed of three polypeptides linked by disulfide bond. Furthermore, the feasibility
5 of using the isolated protein for fabricating nanocarriers was investigated. The results
6 indicate that thermal treatment of the globulin induced the rearrangement of the
7 disulfide bond to form homodimers of acid polypeptides during the formation of
8 nanoparticles. The harvested nanoparticles produced by heat-induced assembly are
9 spherical in shape, with an average size of 92 nm and exhibited low cytotoxicity to L-
10 02 and MDCK cell lines. These nanoparticles are capable to encapsulate paclitaxel,
11 estimated the maximum encapsulation efficiency of paclitaxel loaded to the
12 nanoparticles was 92.6% and the maximum release of paclitaxel was 57.4%. This
13 research suggests that the screening of traditional herbal extracts could provide a novel
14 source of protein nanocarriers.

15

16 **KEYWORDS:** protein nanoparticles; thermal assembly; disulfide bond.

1. Introduction

The plant kingdom contains many substances which may have therapeutic potential for the prevention or treatment of human diseases. However, these bioactive ingredients (e.g. vitamins and alkaloids) often exhibit low bioavailability and chemical or biological instability [1]. Recently, various technics of improve drug delivery were developed to address these weakness in bioavailability and stability [2]. One concept of controlled drug delivery involves the association of active molecules with a carrier system [3]. Nanoparticles (NPs) are the most promising drugs carrier that may allow efficient delivery of bioactive compounds and achieve an increase in bioavailability [4]. Among NPs, protein-based NPs have special interests because of their advantage in being biodegradable through metabolic processes [5]. Protein NPs have unique properties compared to other NPs since they are derived from natural sources, are easy to manipulate, and most importantly, are usually nontoxic [6]. The properties of protein NPs can be manipulated by adjusting pH, temperature, protein concentration, etc., which are suitable for scale production. Natural food proteins, i.e., whey protein, casein and legumin, have been studied for their tendency to encapsulate either hydrophobic or hydrophilic molecules by heat-induced, calcium ion-induced or pH-induced approaches [7-9]. Besides these food-derived proteins, herbal proteins remain as an untapped resource of raw materials for protein nanoparticles. It is well known that “decocting” which refers to boiling water extracts of herbs, often results in generation of micro- or nano-scale colloidal particles [10, 11]. The investigations on these herbal colloidal particles has revealed that herbal proteins are the main contributors for the formation of nanoparticles among the macromolecules [12]. Up to now, herbal proteins in herbal decoctions were scarcely studied, since neither their bioactivities nor their structures were believed to be conserved after boiling.

Semen Armeniacae Amarum (SA), commonly known as bitter apricot kernel, is the dry ripe seed obtained from several plants of the Rosaceae family, including *Prunus armeniaca* L. var. *ansu* Maxim., *P. sibirica* L., *P. mandshurica* (Maxim.) Koehne and *P. armeniaca* L [13]. Bitter apricot kernel is a popular food ingredient and a widely-used ingredient for traditional Chinese medicine (TCM) formulas. Its annual production is about 193,000 metric tons in China [14], which are mainly used for oil extraction and in protein drinks after detoxification [15]. A by-product of the kernel oil processing,

bitter apricot kernel meal, which contains approximately 34% crude protein [16], but has rarely been investigated for use as a functional food ingredient.

Most plant seeds contain 11S globulins as major storage proteins for nutrients [17]. Globulins are used in a wide range of practical applications not only because of their biological activity but also because of their inherent properties. These protein molecules would unfold and interact to form covalent NPs at sufficiently high temperatures [18]. As a nano-carrier needs to be stable in the circulatory system, the interchain disulfide linkage between subunits is the ideal stabilization strategy [19]. Hence, the conformational features protein NPs exhibit potential to be used as functional NPs to deliver bioactive components, especially for hydrophobic drugs.

Paclitaxel (PTX) is one of the most widely used antineoplastic drugs found from nature in the past decades [20], has demonstrated significant activity against a variety of tumors, such as metastatic breast cancer [21]. However, PTX is a hydrophobic drug with poor aqueous solubility. To increase its solubility for clinical applications, nanotechnology has been used to design the PTX-loading carrier, such as nanoencapsulation [22], nanoemulsions [23] and nanocrystals [24]. The use of chemical cross-linking agents to stabilize nano-carrier is challenging due to its potential toxicity. For this reason, a natural material with appropriate properties needs to be developed.

In this study, the major proteins/subunits of *Semen Armeniacae Amarum* (SAP) were isolated and purified by chromatography; the characterization of proteins was measured by electrophoresis, mass spectrometry and N-terminal amino acid sequencing. The SAP nanoparticles (SAP-NPs) were assembled by thermal treatment, and the effect of medium pH value, heating temperature, heating time and SAP concentration were systematically evaluated. The SAP-NPs features were characterized by morphology, size and surface charge. The mechanism of assembly was analyzed based on an investigation of structure dissociation in the presence of urea, dithiothreitol (DTT) and sodium dodecyl sulphate (SDS). The PTX loading and release tests were carried out, and cytotoxicity of SAP-NPs was evaluated by the MTT test *in vitro* using human normal liver cells (L-02) and Madin-Darby canine kidney (MDCK) cells.

2. Materials and methods

2.1 Materials

Semen Armeniacae Amarum (SA) was purchased from Beijing Yanjing Chinese Medicinal Herbs Co. Ltd. (China). BCA kit was purchased from Nanjing Jiancheng Bioengineering Institute (China). High Q ion exchange gel was purchased from Bio-Rad Laboratories (USA). The TSK-GEL G6000 PW column was purchased from TOSOH (Japan). β -Mercaptoethanol was purchased from Amresco Co. (USA). Paclitaxel (99.5% purity), sodium dodecyl sulphate, glycine and Trizma base were purchased from Sigma Chemical Co. (USA). Ethanol, acetone, carbamide, hydrochloric acid, glacial acetic acid, sodium chloride, *N,N,N',N'*-Tetramethyl ethylene diamine, tween 20, bromophenol blue, ammonium persulphate and EDTA were purchased from Sinopharm Chemical Reagent Co., Ltd (China).

2.2 Purification of SAP

After the *Semen Armeniacae Amarum* was ground and defatted with acetone (1:10 w/v) for 4h, the residual acetone in the slurry was then evaporated overnight at 4°C. The defatted powder was homogenized in 10 mM phosphate buffer (pH7.4, 1:50 w/v) for 1 h. After centrifugation at 6000 g for 10 min, the crude protein solution was then purified by a two-step chromatographic method using High Q anion exchange chromatography and TSK-GEL G6000 PW gel filtration chromatography. 5 mL of defatted protein solution was loaded onto a High Q anion exchange column equilibrated with Tris-HCl buffer (20 mM, pH 8.1) and then eluted with a linear gradient of NaCl (0-0.3M) contained in Tris-HCl (20 mM, pH 8.1) buffer in a total volume of 150 mL at 1.0mL/min. Fraction SP1 was collected and then dialyzed against 50 mM phosphate buffer (pH 8.1 containing 0.02% NaN₃). Then 50 μ L of dialyzed SP1 was subsequently purified by gel filtration chromatography with phosphate buffer in a total volume of 30 mL at 0.5 mL/min. The eluate was monitored at UV 280 nm. Fraction SP2 was obtained and collected for further study. The protein concentration was determined using a BCA kit. Bovine serum albumin (BSA) was used as the protein standard.

Three polypeptides were collected by preparative SDS-PAGE using the AE-6760 preparation system (ATTO, Japan) connected to a micro collector (ATTO, Japan). After concentrating gel (T=5%) and separating gel (T=10%) were cast in the tapered tube assembly, the concentrated protein sample (~30 mg) was loaded onto the top of the gel

avoiding bubbles. The power supply was set at a constant rate of 30 mA, and the Tris/glycine/SDS running buffer was eluted at 1 mL/min. The micro collector was started running after eluting for 6 h and the fractions were collected for 1 mL per tube.

2.3 Characterizations of SAP

Total proteins and chromatography resulting fractions were analyzed by SDS-PAGE. It was performed by using concentrating gel (T=4%) and separating gel (T=12.5%) in an electrophoretic system (ATTO, Japan). Reducing conditions were obtained by adding DTT (20 mmol/L) in sample buffer. A 30 μ L sample was loaded and the gels were run in Tris/glycine/SDS buffer. The gels were stained with Coomassie Brilliant Blue R 250 and were examined using a scanning instrument (PeiQing, China).

The isoelectric focusing (IEF)-PAGE was performed using a 2-dimensional electrophoresis system (ATTO, Japan). The gels were stained with Coomassie blue R250. The molecular weights of polypeptides were determined by a Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectrometer (Applied Biosystem, USA). The amino acid sequence of three purified polypeptides was sequenced by Edman degradation using a protein sequencer (Shimadzu, Japan).

2.4 Preparation and characterizations of SAP-NPs

After dialyzing with distilled water, the purified SAP was lyophilized and used to prepare the SAP-NPs. The NPs formation was examined by varying pH (2-10) with 1mg/mL SAP solution at 95 $^{\circ}$ C for 10 min; varying protein concentration (0.25, 0.5, 1, 2, 4 and 8 mg/mL) at pH 9.0, 95 $^{\circ}$ C for 10 min; varying temperature (85, 90, 95 and 100 $^{\circ}$ C) with 1mg/mL SAP solution heating for 10 min; varying heating time (10-60 min) with 1mg/mL SAP solution at pH 9.0, 95 $^{\circ}$ C. All samples were cooled down immediately in ice after the heating process to terminate the assembly formation.

The Z-average diameter (ZD), Zeta-potential, polydispersity index (PDI), derived count rate (DCR), Zeta potential (Zeta P) and distribution of light scattering spectrum intensity of SAP-NPs were measured using a Zetasizer Nano ZS (Malvern, UK).

The microstructure of NPs was observed by a JEM-1230TEM (JEOL, Japan). 20 μ L sample was dispensed onto a carbon-coated copper grid and then stained with 2% (w/v) uranyl acetate for 5 min.

2.5 Protein thermal analysis

Protein conformational changes before and after NPs formation were measured by Far-UV CD spectra. CD was acquired in the range of 190-250 nm using a J-710 spectropolarimeter (JASCO, Japan) with Quartz cuvettes path length of 1 cm. The spectral resolution was 0.5 nm, time per point 1 s, bandwidth 1 nm. The data were calculated by Spectra Manager software. The thermal properties of SAP were examined using a VP-DSC thermal analyzer (Malvern, UK). Approximately 1 mg of protein were heated in aluminum pans from 15 to 100°C at a rate of 10 °C/min.

2.6 Nanoparticles dissociation testing

β-Mercaptoethanol (β-ME), urea and SDS were chosen to investigate the molecular interactions to form SAP-NPs such as β-ME for breaking disulfide bonds, urea for attenuating hydrogen bonds and SDS for attenuating hydrophobic interactions. The reagents were respectively added to SAP solutions at final concentrations as following, 2% (w/w) for β-mercaptoethanol, 6 M for urea and 10 mM for SDS, the concentrations of these denaturing reagents was determined according to the previous studies [25, 26]. The mixtures were heated and kept at 95°C for 10 min, and then subjected to diameter distribution proportion analyses by dynamic light scattering. The reagents were also added to SAP-NPs to investigate their influences on the structures.

2.7 PTX loading and release

A predetermined amount of PTX were dissolved in methanol and added to SAP solution with the PTX/SAP weight ratios (w/w) of 2.5%, 5%, 10%, 20% and 50%, respectively. The mixtures were heated in a water bath at 95 °C for 10 min and cooled to 25°C immediately. Non-entrapped PTX was removed by ultrafiltration (with MW cut-off of 100 kDa, 5000 g, 30 min). The SAP-NPs on the ultrafiltration membrane were washed and resuspended in an aqueous dispersion. The amount of PTX was analyzed by high performance liquid chromatography (Bio-Red Laboratories, USA) equipped with a Phenomonex C18 (5 μm, 150×4.6 mm) column (USA) according to the method of Ahmed Abu-Fayyad et al. [27]. Encapsulation efficiency (EE%) and PTX loading content were calculated by the following equation:

$$\text{Encapsulation efficiency} = (\text{Mass of PTX in NPs} / \text{Mass of the initial PTX}) \times 100\% \quad (1)$$

$$PTX \text{ loading content} = (\text{Mass of PTX in NPs} / \text{Mass of total PTX and NPs}) \times 100\% \quad (2)$$

The *in vitro* release testing of paclitaxel from SAP-NPs was carried out by a dialysis tube method described by Federica Novelli *et al.* [28]. A 0.5 mL sample of PTX loaded SAP-NPs in PBS buffer (pH 7.4, 10 mM) was introduced into a dialysis tube with MW cut-off of 8-14 kDa. The dialysis tube then was dialyzed against PBS buffer containing 5% Tween 20 (w/v) in a beaker. The beaker was maintained at 37 °C and gently stirred (100 rpm) for 48 h. The release media were replaced with fresh release media of equal volume at regular intervals. Quantification of PTX in release medium was performed by HPLC as described above.

2.8 Cytotoxicity of SAP-NPs *in vitro*

L-02 and MDCK cells were seeded into 96-well plates (Costar, Corning, NY) at a density of 5×10^4 cells/well in the MEM medium. Prior to incubation, SAP and SAP-NPs was added in varied SAP concentrations of 0.25, 0.5, 1.0 and 2.0 mg/mL with 4 duplicates for each concentration. Control group were treated with PBS buffer (20 mM, pH 9.0). The 96-well plates then were incubated at 37°C with 5% CO₂ for 24 h. The cellular viability was then assessed by cell proliferation (MTT assay) (Loosdrecht *et al.*, 1994), and presented as the percentage of viable cells (Means $\pm$ SD, n=4) given by:

$$Viability = (\text{mean } OD_{570 \text{ nm sample}} / \text{mean } OD_{570 \text{ nm control}}) \times 100\% \quad (3)$$

2.9 Statistical analysis

All experiments were performed at least three times. Data were analyzed by Origin imaging software (Origin pro 2017, Inc., USA). Results were expressed in tables as mean $\pm$ standard deviation (SD). Error bars represent SD on figures. The significance levels were examined with Student's t-test and ranked as $P < 0.05$.

3. Results and discussion

3.1 Purification of SAP

The SDS-PAGE result of aqueous extract is presented in Figure 1 (a), the majority of proteins was observed at an approximate molecular weight (Mw) of 65 kDa, appeared within band A (named SAP) compared to several other weak bands. The protein electrophoresis pattern was obviously changed by boiling the aqueous extract,

shown in lane 2. The appearance of new protein bands in four regions, marked by B-E, was coincident with the diminution of band A, suggesting that heating process changed the protein structures (i.e., quaternary structures). The proteins within region D and E have relatively smaller Mw compared to SAP, which may represent the polypeptides composed of SAP. The proteins within region B have larger Mw, which implied the recombination or re-arrangement among the polypeptides of SAP. This resulted in forming huge protein complexes or aggregates and their sizes are too large to pass through the separating gel (as shown in region C). The purification and characterization of SAP were carried out to understand the transformation among the subunits of SAP by heating and their heating-induced assembly.

The crude protein solution was subjected to anion exchange and gel filtration chromatography for further purification (Figure 1 b-c), fraction SP2 containing SAP was obtained. The results of SDS-PAGE (Figure 1 d) showed that SAP contains two proteins (non-reducing conditions) with Mw of 65.9 and 64.2 kDa, respectively. Furthermore, these two proteins consisted of three polypeptides with Mws of 45.3, 43.6 and 20.6 kDa under reducing conditions, suggesting that they are heterodimers. Each of them contains two polypeptides linked by an interchain disulfide bond, one of the polypeptides with Mw of 20.6 kDa shared by these two proteins. It also revealed that the interchain disulfide bonds were broken by heating, shown in lane 2 of Figure 1 (a). Subsequently, three polypeptides were purified by preparative SDS-PAGE under reducing conditions, and were subjected to IEF, MALDI-TOF and N-terminal amino acid sequencing (Shown in Table 1). Each N-terminal amino acid sequence was compared to sequences in NCBI databases using the BLAST suite of programs. They were detected and possessed a high degree of sequence similarity with a sequence deduced from a cDNA clone, which encoded the almond 11S globulin precursor Pru1. The AP-1 and AP-2 owned the same 10 N-terminal amino acid sequence of ARQSQLSPQN, which was totally identical to a region within the Pru1 N-terminal sequence. The BP-1 displayed the highest homology with a motif starting from residue A248 of Pru1 containing a NGxEET motif of 11S globulins [29]. 11S globulins typically are hexamer consisting of acidic and basic polypeptide chains, which are covalently linked by disulfide bond. The masses of the acidic chains are typically ~40 kDa, while the basic chains usually have masses of ~20 kDa [30, 31]. In our case, the hexamer of 11S globulin is composed of two types of subunits. The SAP fraction is

the mixture of these two subunits, whose acidic polypeptide chains are AP-1 and AP-2, while both of their basic polypeptide chains are BP-1.

3.2 The influences on protein structures by heating

Heat treatment led to a thermal denaturation of protein and induced the exposure of initially embedded hydrophobic amino acids and sulfhydryl groups that are responsible for NP formation. To study the relationship between unfolding of thermally induced protein and particle formation, circular dichroism (CD) spectra were obtained at the far UV range to trace the secondary structure of SAP as affected by heating (shown in Figure 2 a). The unheated sample exhibited a strong positive peak at 190-200 nm and a broad negative peak in the region of 205-215 nm. The secondary structure of the unheated SAP composed of 6.2% α -helix, 73% β -sheet and 20.8% random structures. When the SAP was heated for 10 min, the peak at 195 nm was vanished and the negative peak was shifted towards lower wavelength with slight increase. This may be caused by the release of monomers from the protein during heat treatment [32]. There was about 4.1% of α -helix converted to random structures, while β -sheet remained unchanged, which was probably because the SAP retained much of the intramolecular β -sheet structure when heated at 95°C, pH 9.0 for 10 min. The thermal behavior of SAP was studied by differential scanning calorimetry (DSC) that is illustrated in Figure 2 (b). A single peak of thermal transition midpoint (T_m) was observed, indicating a denaturation of SAP at 76.31°C. SAP was denatured and participated in the formation of large protein complex at a temperature above the T_m value. Therefore, these conformational features of thermal treatment could be used in the development of custom-made carrier.

On the other hand, the disulfide bonds among polypeptides were also influenced by thermal treatment. As seen in Figure 2 (c), the result of SDS-PAGE showed that the interchain disulfide bonds of SAP were broken down after heating at 95 °C for 10 min, indicated by the occurrence of the polypeptides with Mw 45 and 43 kDa under non-reducing conditions. Meanwhile, either of these two polypeptides was able to form homodimer via an interchain disulfide bond, revealed by the occurrence of two new protein fractions which exhibited two bands with Mw ~90 and ~86 kDa in non-reducing

SDS-PAGE and 45 and 43 kDa bands in reducing SDS-PAGE. The results suggested that the subunits with Mw of 45 and 43 kDa were released from SAPs and able to form homodimers upon heating, respectively. Notably, after heat treatment, while the band intensity of 66 kDa reduced and the band of 45 kDa occurred, which indicated the breakdown of disulfide bridges, the band of BP-1 was not observed in SDS-PAGE, which suggested that BP-1 were precipitated by heat treatment. The thermal instability of the basic polypeptides was also reported in Amandin [26].

3.3 Preparation of SAP-NPs by heating-induced assembly

Due to their sizes, nanoparticles have large surfaces per unit volume to improve the bioavailability, delivery properties and stability for drug and thus their biological activity [33]. The scale of nanoparticles can be modulated by controlling the various factors, such as protein concentration, pH, heating time and temperature. In addition, pH conditions also significantly influence the protein interactions. At pHs far away from its pI, protein molecules repel to each other due to electrostatic repulsion.

As discussed earlier, the *pIs* of SAP polypeptides were around pH 5-6. When SAP solution was heated in the pH range 2-6, an obvious precipitate was noticed and the supernatant showed very low light scattering spectrum intensity (data not shown). As can be seen in Figure 3 (a), the Z-average diameter (ZD) at pH 7.0 was 42.3 nm, the DCR was about 4000, indicating that no obvious colloids were produced by heating at pH 7.0. When increasing the pH value to around pH 8.0-9.0, the formation of protein NPs with size ranged from 90-92 nm was observed. The PDI value was less than 0.2 at pH 9.0, whereas the PDI was near to 0.34 at pH 8.0, implying that the formation of NPs at pH 9.0 were more homogeneously dispersed than those of pH 8.0. When the pH was above 10.0, the ZD of the particle became larger with an obvious decrease in DCR and less NPs were formed. The results suggest that the proteins were unfolded and its content of ordered structures, e.g. α -helix, were decreased, while heating above pH 7.0, conformational changes could influence on their aggregation [34]. It is worth mentioning that the protein molecules have a great charge density, which increases intermolecular repulsion at extreme pH values. Therefore, the protein NPs can be formed by assembly with a balance between electrostatic repulsion and intermolecular

hydrophobic attraction [35].

The size and PDI of particles at different protein concentration were determined and shown in Figure 3 (b). The particle sizes increased with the increase of the protein concentration, and aggregates or even precipitation were observed when the protein concentration exceeded 4.0 mg/mL accompanied with the significant decrease of DCR values. The PDI pattern was narrower than those at other concentrations when the ZD of NPs was about 92 nm at protein concentration of 1.0 mg/mL, and the DCR was also the highest among the protein concentrations. Hence, the SAP protein concentration for heat-induced assembly of NPs was optimized as 1.0 mg/mL.

The influence of temperature and heating time on assembly of SAP-NPs was investigated. According to T_m of 76.3 °C determined by DSC, the SAP solutions (pH 9.0, C = 1.0 mg/mL) were heated at the temperature of 85 °C, 90 °C, 95 °C and 100 °C. As shown in Figure 3 (c), the particle size increased with the increase of heating temperature in the range from 85 to 100 °C. The DCR values increased dramatically when the temperature rose from 90 to 95 °C. A further increase of temperature to 100 °C did not affect the DCR values. The PDI at 95 °C reached to the minimum among the tested temperatures. As demonstrated in Figure 3 (d), heating time showed little influence on the formation of SAP-NPs, while the ZDs and PDIs were slightly increased with the extension of heating time, the DCRs were slightly decreased. Considering the preference of smaller scale, higher intensity of light scattered and narrower dispersion, the optimal heating conditions for fabricating SAP-NPs were then selected at 95 °C for 10 min. In summary, 1 mg/mL of SAP heated at 95 °C for 10 min at pH of 9.0 formed the SAP-NPs with well monodispersed particle distributions and optimum diameter of approximately 92 nm under the optimum conditions.

3.4 Morphology and Stability of Nanoparticles

Figure 4 (a) showed the morphology of the SAP-NPs by TEM. SAP-NPs are spherical in shape with uniform size around 80 nm. It was observed in Figure 4 (b) that the size distribution for DLS analysis exhibited a unimodal and narrow pattern, which implied that heating could be an effective monodisperse approach to produce uniform

SAP-NPs. The result showed that SAP-NPs have a Zeta-potential of -26 mV and a Z-average diameter (ZD) of 92 nm. It is notable that the NPs size observed by TEM was apparently smaller compared to that by DLS (correspond to hydrodynamic diameter). It is probably due to extension of attached hydrophilic groups in aqueous medium. Similar observations were reported by Vrushti Kansara [36].

3.5 Nanoparticle formation and dissociation testing

Internal bonds which are responsible for the formation of SAP-NPs have been investigated on dispersion in varied denaturing reagents [37]. The denaturing reagents were mixed with SAP solution and heated to form NPs at 95 °C for 10 min, respectively. The effects of various reagents on the dissociation of SAP-NPs was presented in Table 2. After incubation with β -ME, SAP was precipitated instead of forming soluble NPs. By mixing with either SDS or urea, SAP was assembled into nanostructures with size distribution of 1-5 nm and 10-30 nm, which were smaller than NPs with Z-average diameter of 92 nm. The results suggested that disulfide bonds, hydrogen bonding and hydrophobic interactions are all important for heat-induced assembly of SAP-NPs. Among these three intermolecular forces, disulfide bonds are essential for formation of SAP nanostructures. Besides, the breakage of disulfide bonds cleaved by β -ME also prevented the formation of homodimers of the basic chains with molecular weight of 86 and 90 kDa, which implied that SAP-NPs are assembled by the homodimers via hydrogen bonding and hydrophobic.

The diameter distribution proportion analyses of SAP-NPs were carried out after incubation with the denaturing reagents to investigate the internal bonds within SAP-NPs. As shown in Table 2, nearly 60% of SAP-NPs were broken down into smaller pieces after treatment with SDS, implying that smaller nanostructures with size of 1-5 nm and 10-30 nm were assembled via hydrophobic interaction. In contrast, neither β -ME nor urea influenced the size distribution of SAP-NPs. The hydrogen and disulfide bonds are less important to maintain the intact structural integrity of SAP-NPs compared to hydrophobic bonds. It might be due to the conformation of SAP-NPs, which embed their disulfide bonds and hydrogen bonds into their internal structures so that the SAP-NPs were able to resist to the presence of β -ME and urea.

Based on the results above, an idealized schematic representation of thermally induced self-assembly of SAP is given as Figure 5. During thermal treatment, the

disulfide bonds of both subunits were broken, resulting in releasing of a basic polypeptide with Mw of 20 kDa and two acidic polypeptides with Mw of 45 and 43 kDa, respectively. Both the released acidic polypeptides tend to subsequently form new homodimers with Mw of 86 and 90 kDa (also as shown in Figure 2 c) via disulfide bonds. Upon thermal treatment, the homodimers assemble into nanoparticles. In addition, the CD spectra indicated that the secondary structure of SAP was changed by heat (as shown in Figure 2 a), the unfolding of secondary structure (the decrease of α -helix) led to the exposure of hydrophobic groups, which can enhance the hydrophobic interactions during the formation of the SAP-NPs.

3.6 Cytotoxicity evaluation

The toxicities of SAP and SAP-NPs towards L-02 cell and MDCK cell was evaluated by MTT assay, shown in Figure 6 (a). The results suggested that the cellular viability of SAP-NPs exhibited little cytotoxicity on L-02 cell and MDCK cell at the concentration as high as 1 mg/mL. The non-toxic concentrations of purified SAP for L-02 cell and MDCK cell both were 1 mg/mL for comparison. Therefore, the self-assembled nanostructures of the SAP proteins did not significantly change their cytotoxicities. The results of cytotoxicity also indicated that the SAP-NPs were safe nano-vehicle for active ingredients delivery.

3.7 Drug loading and in vitro releasing

Paclitaxel (PTX) was employed to evaluate the potential of SAP-NPs as carrier for water-insoluble drugs in this study. PTX-loaded SAP-NPs were prepared with varied PTX/SAP weight ratio (w/w) by heat-induced assembly at 95 °C for 10 min, the loading content of PTX was subsequently determined by a HPLC-based method and the encapsulation efficiency was calculated. Table 3 summarized the encapsulation characteristics of PTX-SAP-NPs. It was seen that the PTX to SAP mixtures in different PTX/SAP ratios were able to assemble into micro/nanoparticles by the heat-induced method, and the particles sizes increased from 213 nm to 945 nm with the increase of PTX/SAP ratio. In contrast, the encapsulation efficiency was reduced with the increase of PTX/SAP ratio, and the maximum encapsulation efficiency of $92.6 \pm 2.0\%$ was obtained while the PTX/SAP ratio was 0.03. The maximum loading content of PTX was 9.38 wt.%, whereas the PTX percent and EE% were 10% and 84.5%, respectively.

When PTX/SAP ratio rose beyond 0.1, the EE% decreased significantly and the loading content of PTX also decreased. The surface charge appeared to be important in determining whether the NPs adhered to other molecules with opposite charge. The absolute values of zeta-potential slightly increased with the increase of the PTX content, which suggested that more PTX was entrapped into SAP-NPs to form the stable PTX-SAP-NPs complexes.

The PTX release test for PTX-SAP-NPs, which was assembled with PTX/SAP ratio of 1/20, was carried out at 37 °C for 50 hours *in vitro* in 10 mM phosphate buffer (pH 7.4) containing 5% w/v Tween 20 to prevent the sink condition [38]. As shown in Figure 6 (b), the release pattern composed of a fast growth phase in the first 20 hours, followed by a stabilizing phase in the last 30 hours. There was about 12.6% loaded PTX released from PTX/SAP-NPs during the first 1 h. The release was then gradually increased until 20 h and remained constant even after 30 h. The largest release percentage was observed for SAP-NPs loaded with free PTX was 57.4% for the drug. Chitosan is often used to fabricate nanocarrier with a high paracellular permeability, it was reported the PTX encapsulation efficiency was as high as 88.3% to the optimized chitosan nanoparticle, and the released amount of PTX was over 50% within 24 h [39]. Compared with chitosan nanocarrier, SAP-NPs exhibited a higher encapsulation efficiency and more promising sustained release potential. The next work will focus on the delivery and release *in vivo*.

4. Conclusion

In summary, the major protein fraction SAP from the aqueous extract of *Semen Armeniacae Amarum* was purified and identified as a new member of 11S globulin family, which poses a heterohexamer composed of two types of subunits. Like most 11s globulins, both subunits are heterodimers, each consisting of a basic polypeptide chain and an acid polypeptide chain, which are covalently linked by a single disulfide bond. The SAP was assembled into NPs by heating at 95 °C for 10 min at pH 9.0, to give SAP-NPs with a Z-average diameter in the range of 90-92 nm Thermal treatment disrupted the disulfide bonds of heterodimers and formed new disulfide bonds between same subunits to produce homodimers. The results of formation testing suggested that disulfide bonds are essential for the formation of SAP-NPs. SAP-NPs are capable of

encapsulating bioactive components with low aqueous solubility, such as paclitaxel. It was estimated that the maximum encapsulation efficiency of PTX loaded to SAP-NPs was 92.6% and the maximum release of PTX was 57.4%.

It is speculated that this study may provide a new insight into “decocting”, which is the earliest and most popular method of preparation practice in traditional Chinese medicine. It is conceivable that major proteins formed NPs during the boiling process could encapsulated insoluble compounds and prevented their precipitation. From this perspective, “decocting” could serendipitously be a method for producing natural protein NPs and the screening of herbal extracts could provide new protein nanocarriers.

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Figure legends

Figure 1. Purification and characterization of SAP. **(a)** SDS-PAGE of extraction. Lane: 1, molecular weight marker (non-reducing); 2, heated protein solution; 3, natural protein extraction. **(b)** Anion exchange chromatography on High Q. The unloaded fraction was washed with 30 mL and loaded protein was eluted with 120 mL linear NaCl gradient (0-0.3 M). **(c)** Fraction peak SP1 was collected and loaded onto a TSK-GEL G6000 PW gel filtration column, eluted by phosphate buffer (pH 8.1, 20 mM). The protein content of the eluate was monitored at 280 nm. **(d)** SDS-PAGE of SAP with non-reducing and reducing. An enlarged view of SAP was diluted 20 times by PBS buffer.

Figure 2. Thermal analysis of SAP. **(a)** Circular dichroism spectra of unheated and heated SAP. **(b)** DSC thermograms of SAP. C_p : heat capacity. **(c)** SDS-PAGE of unheated and heated SAP with non-reducing and reducing. Lane: 1, molecular weight marker (non-reducing); 2, unheated SAP solution; 3, heated SAP for 10 min at 95 °C.

Figure 3. Influence of experimental conditions on fabrication of SAP-NPs. **(a)** pH. **(b)** Protein concentration. **(c)** Temperature. **(d)** Heating time.

Figure 4. **(a)** TEM micrographs of SAP-NPs dispersions, the scale bar represents 100 nm. **(b)** Size distribution of SAP-NPs. The NPs prepared with 1mg/mL SAP solution (pH 9.0) heated at 95 °C for 10 min.

Figure 5. Idealized schematic representation for self-assembly of SAP-NPs.

Figure 6. **(a)** Percentage of cell viability evaluated by MTT assay on L-02 and MDCK. Control group were treated with PBS buffer. $*P < 0.05$ compared with the control group. **(b)** *In vitro* release profile of PTX from the loaded SAP-NPs.

582 Table 1. N-terminal sequence, IEF and Molecular weight of SAP polypeptides.

Polypeptides	N-terminal amino acid sequence	IEF	Molecular weight (kDa)
AP-1	<u>ARQSQLSPQNQ</u>	5.44	45.31
AP-2	<u>ARQSQLSPQNAYQLVQQQAR</u>	5.32	43.57
BP-1	<u>GVEETFCSARLSQNIGDPSR</u>	6.54	20.62

583 Sequence alignment of three polypeptides and almond 11S protein precursor Pru1 chain A without
584 signal peptide (GenBank: AND 39440.1, NCBI database). The underlined amino acids are homologous
585 to the N-terminal sequences of Pru1.

586

587 Table 2. Effects of various reagents on the dissociation of SAP-NPs.

	Denaturing reagents	Distribution proportion (%)		
		1-5 nm	10-30 nm	90-100 nm
Heating with SAP solutions	2% β -ME	P ^a		
	6 M urea	45.5	54.5	-- ^b
	10 mM SDS	33.2	66.8	--
Incubation with SAP-NPs	2% β -ME	--	--	100
	6 M urea	--	--	100
	10 mM SDS	35.4	24.5	40.1

588 ^a Precipitation; ^b Not detected.

589

590 Table 3. The encapsulation characteristics of PTX-SAP-NPs.

Sample	Encapsulation Efficiency (%)	PTX loading content (%)	Z- Average diameter (nm)	Zeta-potential (mV)
SAP-NPs	-	-	91.54±2.01	-26.1±0.3
PTX (2.5%) SAP-NPs	92.6±2.0	2.26±0.09	213.07±2.60	-28.2±0.3
PTX (5%) SAP-NPs	90.3±3.1	4.30±0.34	305.45±4.05	-29.3±0.6
PTX (10%) SAP-NPs	84.5±2.4	9.38±0.68	416.92±6.72	-30.6±0.4
PTX (20%) SAP-NPs	34.2±3.5	8.08±1.02	421.77±5.98	-31.2±0.6
PTX (50%) SAP-NPs	16.7±1.1	7.05±1.73	945.23± 8.81	-31.0±0.9

591

592

Figure 1. Purification and characterization of SAP. **(a)** SDS-PAGE of extraction. Lane: 1, molecular weight marker (non-reducing); 2, heated protein solution; 3, natural protein extraction. **(b)** Anion exchange chromatography on High Q. The unloaded fraction was washed with 30 mL and loaded protein was eluted with 120 mL linear NaCl gradient (0-0.3 M). **(c)** Fraction peak SP1 was collected and loaded onto a TSK-GEL G6000 PW gel filtration column, eluted by phosphate buffer (pH 8.1, 20 mM). The protein content of the eluate was monitored at 280 nm. **(d)** SDS-PAGE of SAP with non-reducing and reducing. An enlarged view of SAP was diluted 20 times by PBS buffer.

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614

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 617 (pH 9.0) heated at 95 °C for 10 min.

618

● Acidic polypeptides
 ● Basic polypeptides
 ○ Surface negative charge

Figure 5. Idealized schematic representation for self-assembly of SAP-NPs.

Figure 6. **(a)** Percentage of cell viability evaluated by MTT assay on L-02 and MDCK. Control group were treated with PBS buffer. * $P < 0.05$ compared with the control group. **(b)** *In vitro* release profile of PTX from the loaded SAP-NPs.