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Purification of Chinese herbal extract with chitosan hydrochloride: Flocculation of single impurity and flocculation mechanism

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Abstract−Chitosan hydrochloride (CHC) was used for flocculation of impurities (tannin and protein) in Ma-xing-gan-shi solution (MSS), which is a Chinese herbal medicine solution (CHMS). To study the flocculation mechanism, simulated solutions of pure tannin and pure protein in water flocculated by CHC were investigated. For MSS flocculation by CHC, flocculation performance was assessed in terms of suspended turbidity, impurities removal rate as well as active components retention rate. CHC showed high capability of maintaining a low turbidity over a wide range of the polymer dosage. The optimum dosage of CHC was 0.6 g/L in base of different solution turbidity, and it also demonstrated the retention rate of total soluble polysaccharide (TSP) and ephedrine reached 78.4% and 92%, respectively.

Keywords: Chitosan Hydrochloride, Chinese Herbal Medicine Solution, Flocculation Mechanism, Tannin, Protein

INTRODUCTION

Traditional Chinese medicine (TCM) has evolved over thousands of years. It is also considered as one part of complementary and alternative medicine in western countries. With development of human society, the transformation of the health concept and the trend of back to nature, Chinese herbal medicine solution (CHMS) as an important type of TCM [1] has been attracting more and more attention.

Ma-xing-gan-shi solution (MSS), a famous prescription of CHMS from *Treatise on Febrile Diseases* in the Han Dynasty, is used for treatment of cough, bronchitis, pneumonia. MSS as oral pharmaceutical herbal products is mainly made by unit operations of decoction, filtration, extraction and concentration in industrial manufacture [2]. The extraction unit is mainly used to remove water-soluble macromolecules such as tannin, starch, protein, pectin, and gum. The main impurities in the MSS extract liquid mainly include tannin and protein (molecular structure is shown in Fig. 1(a) and(b)) existing as colloidal particles in solution, which can induce water-insoluble precipitate generated and high turbidity of liquid preparation, even affect the stability of oral liquid and lead to herbal liquid spoilage. So, these impurities need to be eliminated in most of the time.

Many methods have been reported for impurities removal from CHMS, including but not limited to ethanol precipitation, centrifugal separation, membrane separation, foramen-magnum absor-

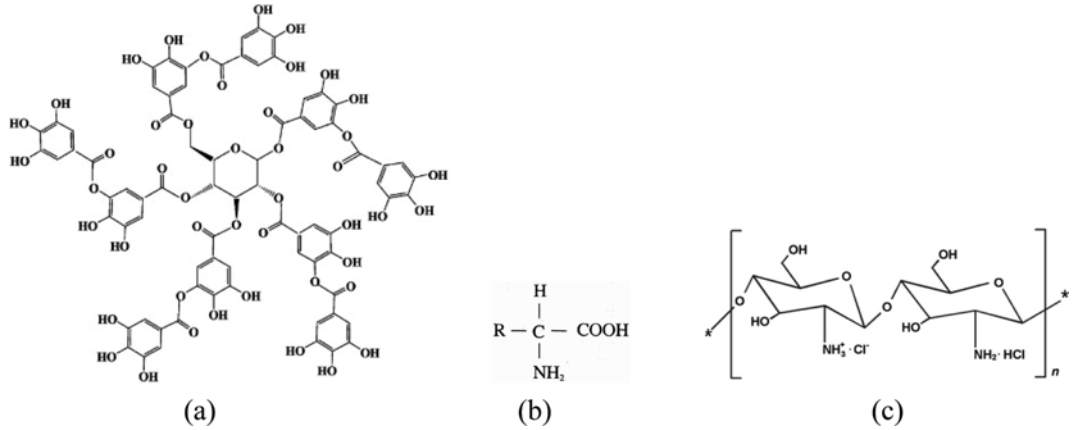

Fig. 1. Molecular structure of (a) CHC, n≥1, (b) tannin, and (c) amino acid (via peptide bond to form protein).

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bent resins and flocculation. Among these methods, traditional ethanol precipitation is still the primary way widely used in practical applications [3]. By this method, macromolecule impurities such as proteins [3,4] and polysaccharides [5,6] could be separated from the herbal aqueous extracts. However, the ethanol precipitation process is less effective for tannin removal because tannin is soluble in the ethanol solution. Furthermore, this method encompasses long operating time and a significant loss of active ingredients [5,7,8] in CHMS, such as caffeic acid, ferulic acid and some active polysaccharide. The technique of centrifugal separation still has difficulties in removing colloid particles and suffers from high energy consumption. The application of membrane separation can effectively remove impurities, retain the most effective components, and pose less pollution to the environment, but it is hampered by significant fouling of the membrane which results from macromolecules (pectin, tannins and protein) adsorbed on membrane material forming a gel layer under high pressure and some other particles or colloids as pore plugging within the internal structure of the pores [9-11]. Macroporous adsorption resin technology is mainly applied to directional separation of a kind of effective component in a single herbal medicine, and is rarely used in compound Chinese herbal medicine [12,13].

Flocculation is regarded as one of the most successful primary purification techniques for the removal organic and inorganic substances from colloidal solution or suspension in many fields [14-21] on account of its simple procedure, low cost and high efficiency, and it has been tried to apply to CHMS clarification and purification along with the development of bioflocculants. Some progress has been made about using chitosan and its derivatives as flocculants to purify CHMS [22-24], but little information concerning the individual impurities flocculated has been reported, and flocculation mechanisms are unavailable because of the complicated composition of CHMS.

In this study, we chose chitosan hydrochloride (CHC, molecular structure is shown in Fig. 1(c)) as a model positively charged biopolymers with good water solubility at neutral pH [25] as a flocculant used in CHMS. First, the simulated solution contained a single impurity only such as tannin or protein (as typical impurity in CHMS) without any other ingredients in pure water flocculated by CHC was investigated. The flocculation mechanism of single impurity flocculated by CHC was also discussed in detail by means of FTIR analysis, zeta potential, and the removal rate of impurity. Then flocculation operations for impurities removal from MSS by CHC were carried out. The influence of flocculant dosage on flocculation performance, that is, suspended turbidity, tannin and protein removal rate as well as main effective components total soluble polysaccharide (TSP) and ephedrine retention rate was investigated. In addition, the flocculation mechanism in MSS was also discussed.

EXPERIMENTAL

1. Materials

1-1. Preparation of the Impurity Solution

The two impurities solutions containing tannin 8 g/L and protein 5 g/L were prepared for flocculation process. The tannic acid was obtained from Jiangtian Chemical Technology Co. Ltd. (Tianjin, China), with an average molecular mass of 1701.23 g/mol. The isolated soy protein (>90.0% protein by weight) was purchased from Shanghai Hengdailao biological instrument Co. Ltd. Hydrochloric acid and sodium hydroxide with the concentration of 0.25 mol/L were used to adjust the pH value of tannin and protein solution. All other reagents in this work were of analytically pure grade and used as received; chromatographic grade water was used in the study.

1-2. Preparation of the Herbals Water Extract of MSS

MSS consists of ephedra (Ma Huang), bitter almond (Ku Xingren), glycyrrhiza (Gan Cao) and gypsum (Shi Gao), which were supplied by Tianjin Company of Traditional Chinese Herbs. According to the prescription of China Pharmacopoeia, the quality ratio of the four medicinal materials is 1 : 1 : 1 : 1. In this study, 400 g of each crude material referred above was added in ten times to the medicinal water, and the mixture was decocted for 1.5 hours and filtrated. Then the dregs together with 3,200 g water were decocted for 1.5 hours again. The supernatant liquid was filtered out and mixed with the former one. Thus, the herb water extraction solution of MSS was prepared at the drug concentration of 0.1 g/mL (the ratio crude drugs to the medicinal water), which turbidity was greater than 200 NTU.

1-3. Preparation of Flocculant Solution

The samples of CHC were obtained from Golden-Shell Biochemical Co., Ltd. (Zhejiang, China). Its deacetylation degree was 83%, the chloride was 16.97%, and the average molecular weight was 2.69×10^5 Da. CHC was dissolved in water under agitation to a final concentration of 1% (w/v). The stock solution was left to settle and swell for 24 hours before being used. The final pH of the chitosan hydrochloride solution was 5.5, the dynamic viscosity was 11.6×10^{-3} Pa·s, and its zeta potential was approximately +32.68 mV.

2. Methods

To evaluate the flocculation effect, the supernatant turbidity, the impurity removal rate and the active ingredient retention rate were measured in this study. Fourier transform infrared (FTIR) and zeta potential measurement were performed for investigation of flocculation mechanism.

2-1. Physico-chemical Analysis

The pH of the solution was measured with a pH meter (PHS-3C; Shanghai Leici Co. Ltd., China); Turbidity was analyzed with a WGZ-100 turbidimeter (Shanghai Optical Instrument Co. Ltd., China). Zeta potential was determined by a microelectrophoretic instrument (JS94H; Shanghai Zhongchen Co. Ltd., China), and the liquid viscosity was measured by a Brookfield Viscometer (LVDV-II, Brookfield Engineering Laboratories). The size of the impurity particles in MSS liquid was measured by a laser diffraction instrument (Malvern Mastersizer 2000, Malvern, UK).

2-2. Content of Active Ingredient

In this study, the primary active components of MSS extract are total soluble polysaccharide (TSP) and ephedrine. The TSP content was determined with the anthrone-sulfuric acid method. The content of ephedrine was measured by HPLC (UltiMate3000, Dionex Co. Ltd., U.S.) with the Agilent Zorbas Extend C18 column (250 mm×4.6 mm, 5 μm, U.S.). The mobile phase consisting of 11 : 89 (v/v) acetonitrile-0.1% orthophosphoric acid was delivered at a flow rate of 1.0 mL/min at 30 °C and detection wavelength of 207 nm.

Standard curve established with ephedrine was used for the calculation.

2-3. The Impurity Content

The tannin concentration was measured with the phosphorus molybdenum tungsten acid colorimetric method, in which the phosphorus molybdenum tungsten acid was a chromogenic agent, the casein was an adsorbent, and the gallic acid was the reference substance [3]. The gallic acid was obtained from National Institutes for Food and Drug Control of China. Protein content was analyzed by the Bradford method, and the bovine serum albumin reference substance was obtained from Shiji Aoke Bio-Chemical Co., Ltd. (Beijing, China).

After flocculation operation, the tannin and protein in the supernatant liquid were measured by a UV/VIS 7500 spectrophotometer (absorbance at 760 nm and 595 nm, respectively). The residual concentration of tannin or protein in the supernatant was obtained from the standard curve.

The removal rate and the retention rate were calculated based on Eqs. (1) and (2), respectively:

$$\text{removal rate } (\%)=(C_{i0}-C_{it})/C_{i0}\times 100\% \quad (1)$$

$$\text{retention rate } (\%)=(C_{a0}-C_{at})/C_{a0}\times 100\% \quad (2)$$

where C_{i0} and C_{a0} denote the initial concentration of each impurity and active ingredient just before flocculation, respectively, and C_{it} and C_{at} denote the impurity concentration ratio and the active ingredient, respectively, at the end of a predetermined settling time (t) after flocculation operation.

2-4. FTIR

The settled impurity flocs obtained from the jar test were collected and freeze-dried for FTIR analysis. The FTIR specimens were prepared by mixing the freeze-dried flocs with an aliquot amount of spectroscopic grade KBr, then grinding to fine powders with a mortar and pestle by hand, and final pressing to thin pellets with a pressing machine. FTIR spectra of the samples were recorded by an FTIR spectrometer (Nexus 670; Thermo Nicolet Corporation, USA).

2-5. Flocculation Procedure

Flocculation experiments were performed with a program controlled jar-test apparatus (Wuhan Meiyu Co., Ltd., China). The impurity solution or the MSS extract liquid (0.4 L) was mixed rapidly at 450 rpm for 2 min after addition of flocculant solution, followed by slow stirring at 50 rpm for 10 min. Then the flocculated solution settled for 2 hours, and the supernatant sample was collected by a syringe from about 2 cm below the supernatant layer for analysis.

RESULTS AND DISCUSSION

1. FTIR Analysis

To investigate the reaction mechanism between the flocculant and the impurities, the FTIR spectra of tannin, flocs of tannin-CHC, protein and protein-CHC were used as shown in Fig. 2 and Fig. 3.

As seen in Fig. 2, both tannin and flocs of tannin flocculated by CHC presented characteristic common bands: four strong bands,

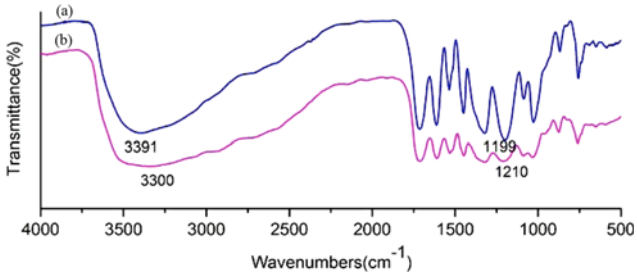

Fig. 2. FTIR spectra of tannin (a) and flocs of tannin flocculate by CHC (b).

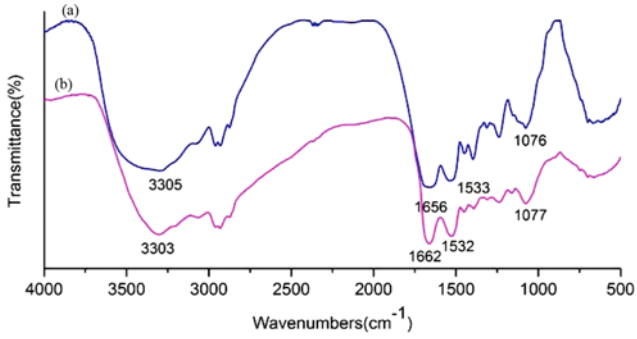

Fig. 3. FTIR spectra of protein (a) and flocs of protein flocculated by CHC (b).

1,615-1,606 cm^{-1} and 1,452-1,446 cm^{-1} assigned to aromatic ring stretch vibrations, the other two at 1,211-1,196 cm^{-1} and 1,043-1,033 cm^{-1} assigned to stretch vibrations of C-O bond [26]. Tannin-CHC flocs also presented characteristic bands of hydrolysable tannins at 1,712 cm^{-1} and 1,322 cm^{-1}. The former assigned to the stretching vibration of the carbonyl function and the later assigned to the stretching vibrations of C-O bond [27]. These results indicated that tannin presented in the tannin-CHC flocs and confirmed that the fundamental structure of tannic acid was not changed and could reflect the bridging role of flocculant. In Fig. 2(a), the band at 3,391 cm^{-1} was assigned to hydroxyl group stretching vibration of tannin, which was found to be weakened in intensity and shifted to 3,300 cm^{-1} as shown in Fig. 2(b). This change could be due to strong hydrogen bonding formed by the hydroxyl groups. The formation of hydrogen bond was induced by hydroxyl group between tannin molecules or tannin and chitosan molecules. The peaks at 1,199 cm^{-1}-1,210 cm^{-1} assigned to vibration of phenolic hydroxyl group receded in intensity after CHC was added, which suggested that the amine group of CHC interacted with negative charged sites of tannin through electrostatic attraction. At the same time, the hydrogen bonding could be formed between tannin and CHC.

As seen in Fig. 3, the spectrum of protein and protein-CHC flocs presented obvious -NH bands at 1,655-1,663 cm^{-1} and 1,530-1,540 cm^{-1}. These bands are in accordance with the reported soy protein amide I and amide II bands at 1,632 cm^{-1} and 1,536 cm^{-1}, respectively [28-30]. The similarities in FTIR peaks between CHC-protein flocs and pure protein meant that the flocs formed via hydrogen bonding between protein and the flocculant, and it also

confirmed that the primary structure of protein was not changed. Enhancement in the intensity of some bands within the range 1,500-1,700 cm^{-1} in Fig. 3(b), which were related to amino and carbonyl moieties, evidenced that these groups interacted mainly through electrostatic interactions and hydrogen bonding. It could be inferred that the minor difference was related to the slight interaction of the amino groups of the CHC with the negatively charged carboxyl groups of the protein. These results pointed out that the main interaction between protein and CHC in the flocculation process would be the electrostatic attraction and hydrogen bonds, as stated by other authors [31].

2. Flocculation of Single Impurity

2-1. Effect of the Flocculant Dosage

The effects of the dosage of CHC on tannin and protein removal rates, the supernatant turbidities and zeta potential of the two impurities flocs are presented in Fig. 4 and Fig. 5.

As shown in Fig. 4 and Fig. 5, the impurity removal rate increased first and then decreased with the rising dose of CHC, and the variation trend of supernatant turbidity was contrary to the impurity removal rate for the two impurities. In addition, the zeta potentials of both impurities were increased from negative to positive when the dosage of CHC increased.

In the solution, tannin or protein is colloidal and negatively charged, while the CHC is positively charged. When CHC was added, colloidal particles adsorbed on the flocculant by electrical neutralization. The long chains of the flocculant could have loops and tails extending some way from the adsorbed particle surface, and some sections of the macromolecular chains would attach other impurity particles. It is the bridging flocculation which can only occur if there is sufficient and effective attachment among flocculant chains adsorbed particles. When the CHC dosage was less than 0.085 g/L for tannin solution and 0.8 g/L for protein solution, the dispersion degree of CHC in solution was higher, and it was difficult for CHC chains to contact other segments adsorbed on particles. Consequently, bridging flocculation could not be formed effectively. With the flocculants dosage increased, charge neutralization was reinforced and the bridging flocculation effect improved. So, the highest impurity removal rate and the lowest supernatant turbidity were achieved at the optimum dosage. With further more flocculants added in the solution, excessive flocculants may be difficult to form charged neutralization and effective bridging flocculation with more particles. On the other hand, the presence of the excessive flocculant would make colloidal particles charged reverse, and it would cause mutual repulsion between the impurity particles. In this case, further increase of the flocculant dosage would result in the re-dispersal of flocs and the rise in turbidity.

Addition of oppositely charged polyelectrolytes to a colloidal dispersion usually leads to a significant affect in the zeta potential and

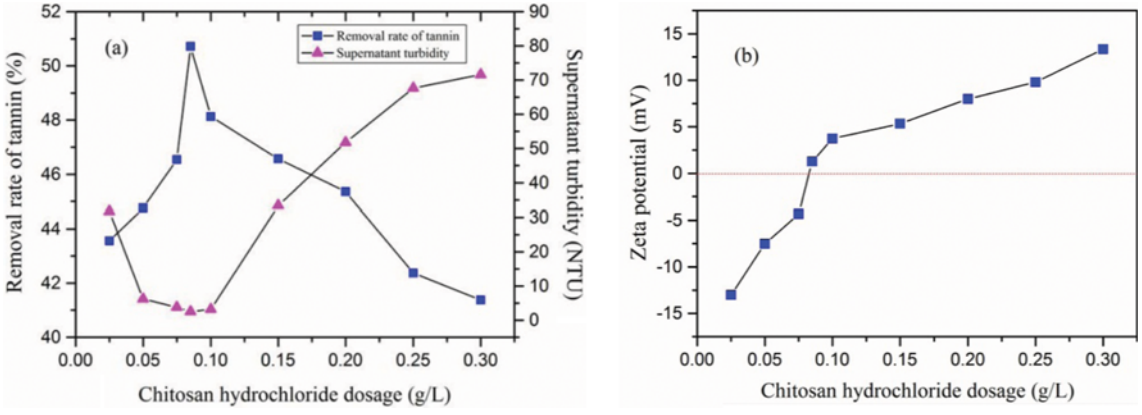

Fig. 4. Effect of CHC dosage on tannin removal rate, supernatant turbidity (a) and zeta potential of the tannin flocs (b).

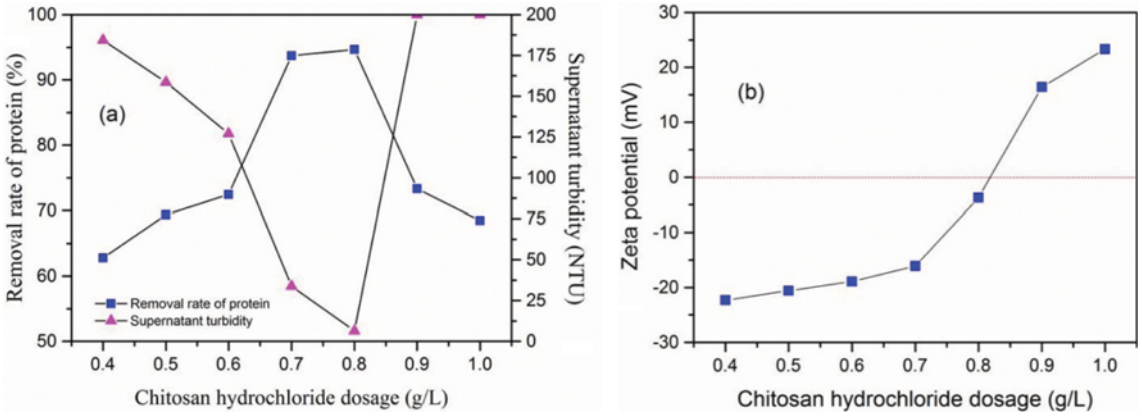

Fig. 5. Effect of CHC dosage on protein removal rate, supernatant turbidity (a) and zeta potential of protein flocs (b).

change in the polarity of particle charge, which is accompanied by destabilization of the colloidal system. As seen in Fig. 4(b) and Fig. 5(b), zeta potential of tannin and protein flocs were increasing with the raise of CHC dosage from negative value up to positive value and continued to rise to 13.3 mV and 23.3 mV, respectively. Here CHC adsorption leads to characteristic charge reversal (or overcharging) of the flocs particles after one isoelectric point. When the flocculant dosage was 0.75 g/L for tannin and 0.8 g/L for protein, respectively, the zeta potentials, which were 1.28 mV for tannin and −3.5 mV for protein, were found close to zero. At the two dosage points, the main flocculation mechanism was related to charge neutralization.

2-2. Effect of pH

In this section, the effect of pH on the removal of tannin and protein flocculated with CHC is presented. The flocculation tests of tannin solution with various pH values from 1.5 to 8.5 and protein solution from 3 to 10 were carried out. The concentration of tannin was 8 g/L and that of protein was 5 g/L, and the CHC dosages for flocculation of tannin and protein were 0.75 g/L and 0.8 g/L, respectively. The initial pH value of the tannin solution was 3.6 and that of the protein solution was 6.9.

Fig. 6 shows that the optimum pH value (the pH at which the largest removal rate of impurity was achieved) for tannin removal by CHC flocculation was 4.5, the removal rate of tannin at this pH point was 50.76%. When pH<4.5, the removal rate of tannin rose with the increase of pH value. Whereas, when pH>4.5, the removal rate of tannin decreased with the increase of pH value. This trend could be explained by that tannin was a weak organic acid and its ionization was markedly dependent on pH value. The point of zero charge of tannin is about 4.5 and the dissociation degree is about 7.05% [32], which means the surface charge of tannin is less and tannin occurs as a molecular form when pH<4.5. The flocculation of tannin at low pH could attribute to hydrogen bonding between CHC and tannin without electrostatic repulsion. With the rise of pH value, the dissociation degree and the negative surface charge of tannin increased, the charge neutralization could be enhanced. Tannin is ionized at pH>4.5, and highly dissociated when pH value was higher than 7.0 [32]. The hydrogen bonding between CHC and tannin decreased, but electrostatic attraction between tannin and CHC increased, both the electrostatic attraction together with hydrogen bonding are responsible for the flocculation of tannin. The removal rate of tannin reduction at pH>7 could be due to the two reasons: (1) some amount of CHC was consumed for neutralization of -OH in the solution; (2) the flocculation of tannin mainly arises by van der Waals interactions between tannin and the uncharged biopolymer chain in CHC [32].

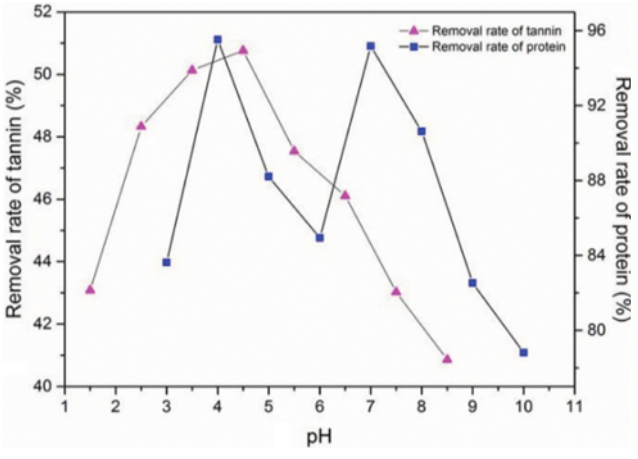

Fig. 6. Effect of pH value on tannin flocculation and protein flocculation.

The removal rate of protein rose with increase of pH value as seen in Fig. 6, the removal rate reached 95.53% at pH 4, which was close to the isoelectric point. The effective charge on colloidal particles of protein is affected strongly by pH value. Additionally, the solubility of protein is also dependent on pH value of the solution. When the pH value was near to the isoelectric point of the protein particles, the colloidal protein would begin to aggregate due to hydrophobic attraction in the absence of electrostatic repulsive forces. Protein carried patches of negative and positive charges on their surface [33]; when positively charged CHC was added in the protein solution, formation of the protein flocs could be facilitated by the presence of local patches of charge. Although CHC is positively charged and so protein is predominantly at slightly acidic pH (<6.5), there would be still a number of negatively charged groups (such as carboxyl groups) on the protein molecule [34,35]. With the rise of pH, the negative charges on the protein increased and protein solubility got higher, fluctuation of flocculation effect of protein occurred. At neutral pH value, high protein removal rate was obtained because the strong interaction between oppositely charged biopolymers induced the charge neutralization, resulting in the decrease of electrostatic repulsive force [36]. When pH> 7, the removal rate of protein decreased sharply, mainly because the declined action of charge neutralization inducing negatively charge on CHC increased with increase of pH value. It was clear that electrostatic interaction played an important role for the removal of protein.

3. Flocculation of MSS Solution

3-1. MSS Properties

The properties of the raw MSS solution are summarized in Table 1. The size of the impurity particles in MSS liquid distributed in a range of 1.2 mm to 4.8 mm with an average diameter of 2.7 mm.

Table 1. Properties of the raw MSS

Physicochemical parameters				Component (mg/ml)				Particle size (μm)			
pH	Zeta potential (mV)	Viscosity (Pa·s)	Turbidity (NTU)	Protein	Tannin	TSP	Ephedrine	D_{10}	D_{50}	D_{90}	D_{av}
5.01	−6.43	2.3×10^{-3}	>200	0.123	1.37	9.45	0.56	1.2	2.3	4.8	2.7

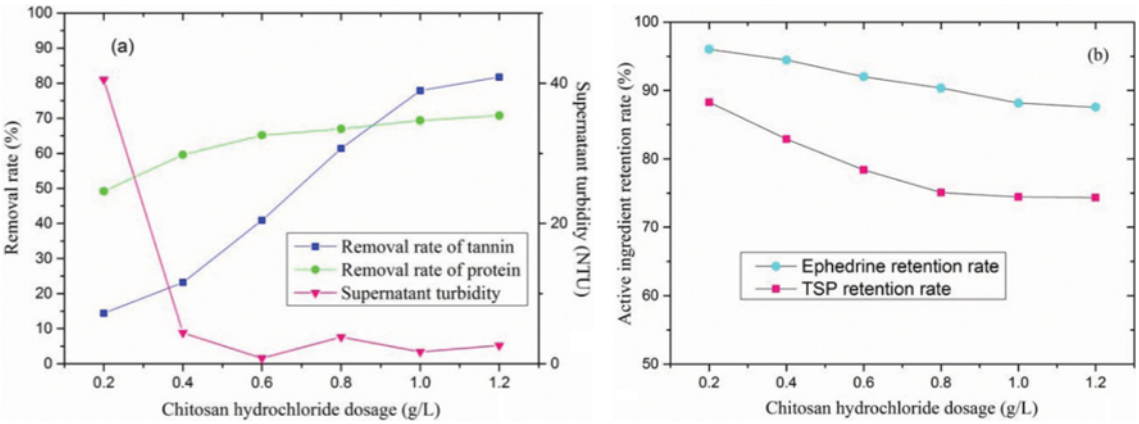

Fig. 7. Effect of CHC dosage on ineffective removal rate and supernatant turbidity (a) and active ingredient retention (b).

3-2. Effect of the Flocculant Dosage

The flocculation efficiency of the impurities in CHMS is determined mainly by the flocculant dosage and pH value of CMS. Since any variation of pH value of CHMS would change its characteristics and pharmacological function, the effect of CHC dosage on the flocculation performance of MSS was investigated here.

It is the effect of CHC dosage on the removal rate of tannin and protein, the active component retention and the supernatant turbidity shown in Fig. 7. In Fig. 7(a), the supernatant turbidity of the flocculated MSS extract initially declined markedly. With the increase of CHC dosage, the turbidity raised slightly but still less than 5 NTU. This might be attributed to repulsion of CHC adsorbed on the impurity particle surfaces. In this study, the pH value of raw MSS was 5.01. CHC was positively charged as the amino groups bonded with hydrogen ions. When CHC dosage increased to a certain value, positively charged CHC adsorbed on the surface of negatively charged colloid particles by charge neutrality flocculation, resulting in better flocculation effect. After the flocculant dosage was further increased, the presence of the excessive flocculant would make the colloidal particles charged positively, and thus cause mutual repulsion. In this case, further increase of the flocculant dosage would result in fluctuation of the supernatant turbidity. It is apparent from this result that CHC is a suitable flocculant obtaining a low turbidity at a wide range of CHC dosage from 0.4 g/L to 1.2 g/L. The TSP and ephedrine retention rate was reduced, but the removal rate of tannin and protein increased with increase of the CHC dosage. The ephedrine retention rate remained at a high level from 87.6% to 96.0%, and the loss of small molecular ephedrine may have been caused by netting or sweeping flocculation. The TSP retention rate varied between 88.3% and 74.3%, which reason was that CHC electrostatic adsorption some polysaccharides with opposite charge resulted in loss of TSP besides netting or sweeping flocculation.

As seen in Fig. 7, the optimum dosage of CHC was 0.6 g/L for the supernatant turbidity and retention rate of TSP and ephedrine, which were 0.8 NTU, 78.4% and 92%, respectively. While at this flocculant dosage, the removal rate of tannin and protein was 40.91% and 65.16%, respectively.

3-3. Flocculation Mechanism of MSS

In raw MSS, there were many suspended particles with negatively charged and average particle size 2.7 μm besides colloidal particles. Content of suspended particles was one of important factors affecting solution turbidity, which came from tiny drug residues or plant fiber which cannot be removed by simple filtration. When CHC was added, suspended particles were caught first by electrostatic interaction with flocculant, and flocs aggregated to larger ones to precipitation by bridging formation, so the turbidity descended obviously. With dosage of CHC added further, the interaction by hydrogen bonding and charge neutralization between impurity colloidal particles (tannin and protein) and flocculant improved, then removal rate of tannin and protein increased and the supernatant turbidity fluctuated around 4 NTU. But the retention rate of ephedrine retention rate was decreased within a narrow range from 87.6% to 96.0%; that was mainly due to the ephedrine being wrapped in the flocs as a small molecule. There were some polysaccharides negatively charged in solution, so the TSP loss caused by electrostatic absorbed besides netting or sweeping flocculation. Nevertheless, it was noted that netting and sweeping flocculation played the main role in the loss of small molecular active ingredient, although some active ingredient may be electrostatically absorbed by CHC.

CONCLUSIONS

The flocculation performance and mechanism between CHC and single impurity (tannin or protein) in water and raw MSS was studied, respectively. The flocculation mechanism of tannin and protein with CHC is the electrostatic adsorption and hydrogen bonding between their macromolecules. For MSS, CHC showed a good performance for achieving a low supernatant turbidity at a wide range of polymer dosage from 0.4 g/L to 1.2 g/L. At the optimum CHC dosage of 0.6 g/L, the supernatant turbidity was 0.8 NTU, the retention rate of TSP and ephedrine was 78.4% and 92%, and the removal rate of tannin and protein was 40.91% and 65.16%, respectively. In MSS flocculation process, bridging flocculation played the main role in the removal of suspended particles, and the removal of colloidal particles mainly due to a combination of electric neutralization, hydrogen bonding and bridging flocculation, while netting and sweeping flocculation was the main cause of active component losses.

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