One-step heating strategy for efficient solubilization of recombinant spider silk protein from inclusion bodies

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Research article

Keywords: heating, recombinant spidroin, inclusion body solubilization, NM IBs

Posted Date: October 31st, 2019

DOI: https://doi.org/10.21203/rs.2.16637/v1

Version of Record: A version of this preprint was published at BMC Biotechnology on July 10th, 2020. See the published version at https://doi.org/10.1186/s12896-020-00630-1.

Abstract

Background Spider silk, an ideal biomaterial with remarkable mechanical properties, is built of spider silk proteins (spidroins). Engineering spidroins have been successfully produced in a variety of heterologous hosts and the most widely used expression system is Escherichia coli (E. coli). However, recombinant expression of spidroins often results in water insoluble inclusion bodies (IBs). Target protein solubilization from IBs in a traditional way is often solved under extremely harsh conditions, e.g. 8 M urea or 6 M guanidine hydrochloride, which highly risk to poor bioactive protein recovery as well as precipitation during the following dialysis process.

Results Here we present a new mild solubilization strategy called one-step heating method to solubilize spidroins from IBs, with combining together spidroins' high thermal stability with low concentration of urea. We expressed a 430-aa recombinant protein (designated as NM) derived from the minor ampullate spidroin of Araneus ventricosus in E.coli ; the NM proteins are present in the insoluble fraction as IBs. The isolated NM IBs were solubilized parallelly by both traditional urea-denatured method and one-step heating method. The efficiency of solubilization of NM IBs in Tris-HCl (pH 8.0) containing 4 M urea by one-step heating method was comparable to that of 7 M urea by the traditional urea-denatured method. The effects of buffer, pH and temperature conditions on solubilization of NM IBs by one-step heating method were evaluated, and the optimum conditions are following: hearting temperature 70–90°C for 20 min, pH 7.0–10, urea concentration 2–4 M in normal buffers. Prepared via the one-step heating method, the recombinant NM spidroin could self-assembly into nanoparticles.

Conclusions The one-step heating method introduced here could be used to efficiently solubilize NM IBs under relatively mild conditions, which might by important for the downstream nanoparticle or fiber formation. The one-step heating method can be applied under broad buffer, pH and temperature conditions, conferring the potential to apply to not only spidroins but also other thermal stable protein IBs' solubilization.

Introduction

Spider silk is a kind of ideal biomaterial because of its extraordinary performances, such as outstanding mechanical properties and excellent biocompatibility [1, 2]. In nature silk proteins are specifically spun into fibers only, while after processed under various condition *in vitro* they can assemble into different forms with distinct morphologies, *e.g.* films, hydrogels, fibers, capsules and particles [1, 3]. The versatility of silk proteins along with their biochemical properties make silk-derived materials potentially suitable for tissue engineering and regeneration as well as controllable delivery of protein drugs and peptide vaccines [4-9]. However, due to difficulties in breeding spiders and collecting silks, native spider silk is not realistic to harvest on a large scale, which is now achieving by recombinant spider silk protein (spidroin) production [10-12]. Orb-weaving spiders can have up to seven different types of silk glands for manufacturing different types of silk with specific biological function and unique mechanical properties [13]. Most studies have focused on the dragline silk, which is displayed with high tensile strength and

extensibility [14, 15]. The dragline silk protein (major ampullate spidroin, MaSp) was shown to form nanoparticles with possibilities as drug delivery vehicles and peptide vaccines delivery system [7, 9, 16]. However, alternative biomaterials with different properties may be needed among applications of other spider silk proteins. The minor ampullate silk, distinctive from dragline silk, is used for prey wrapping and web-stabilizing auxiliary spirals [13]. Minor ampullate silk shares similar tensile strength as dragline silk but with low elasticity, and does not supercontract when hydrated [17]. Hence, minor ampullate silk could be interesting for particular biomedical applications.

Spider silk is built up of large spidroins consisting of three parts, a non-repetitive N-terminal (NT) domain, a predominant highly repetitive central domain, and a non-repetitive C-terminal (CT) domain [13, 18-21]. For heterologous expression, the repetitive gene sequence usually causes problems such as premature termination in protein synthesis, low yield and poor solubility [22-24]. Several strategies have been tried to overcome these problems, including codon optimization, growing in enriched media and use of different gene constructs [11, 22]. A variety of heterologous expression hosts have also been attempted to produce recombinant spidroins, *e.g.* bacteria, yeast, plants, mammalian cells, and transgenic animals, each with its own pros and cons in terms of cost, manipulation, expression levels and contaminations [15, 16, 25-28]. The most widely used expression system is *Escherichia coli* (*E. coli*) since the most efficient, simply manipulation and cost-efficient system suitable for large-scale production [16]. Several successful laboratory-scale *E. coli* fermentations have been reported [11, 16], although phenomena like premature termination, low yields and accumulation in inclusion bodies (IBs) existed.

To prepare pure recombinant spidroins, different strategies, *e.g.* affinity chromatography [29-31], thermal extraction [32, 33] and acidic extraction [32-34] have been pursued. Affinity chromatography is the most often used approach for purification recombinant spidroins, though an affinity tag is required. While the affinity tag might alter the protein properties, an additional step is necessary to cleave it out. Thermal and acidic extraction methods are based on spidroins' innate properties: thermal stability and solubility characteristics, during which the majority of the host proteins are precipitated at high temperature and concentrated acid, while silk proteins remain soluble. These methods circumvent the need of an affinity tag, which is favorable for biomedical applications. Unfortunately, the pure proteins from the thermal method were largely diluted and an ammonium sulfated mediated concentration step is normally following, which will induce unexpected precipitation [15, 32, 33]. To get soluble proteins, the precipitated silk proteins were dissolved in guanidine and dialyzed against non-denaturing buffer. Proteins extracted in presence of organic acids normally have to pass the affinity or ion exchange column, which is a long-lasting time and high cost [34]. Expression of recombinant spidroins has often resulted in water insoluble IBs [15]. In some case, forming of IBs sometimes is advantageous as IBs are easily isolated with high yield. Traditionally, protein solubilization from IBs is often carried out under harsh conditions, *i.e.* using high concentration of denaturant — 8 M urea or 6 M guanidine hydrochloride. Before processing the target proteins into various formats, precipitation or lyophilization steps should be required. However, solubilization of IBs with high concentration of denaturing reagents often leads to poor recovery of bioactive proteins and large amount of precipitations form during the refolding process [35, 36].

In this study, in order to make efficient preparation of recombinant minor ampullate spidroin (MiSp) and give some insights for assembly characterization, we first expressed a 430 residues construct (designated as NM) of *A.ventricosus* MiSp in *E.coli*. This construct consists of the nonrepetitive NT domain and one repetitive unit, while the full-length *Araneus ventricosus* MiSp contains 1766 amino acid, comprising a central predominant repetitive region composed of four large units and conserved nonrepetitive NT and CT domains [13]. The recombinant NM protein was expressed as IBs in *E. coli*. Then we evaluated a new relative mild strategy of solubilizing NM IBs by short-term heating in the presence of low concentration of urea, referred as one-step hearting method. By using this method, we got high concentration of recombinant NM proteins with excellent purity, and was capable of self-assembling into even nanoparticles.

Results

Silk gene construction and protein production

The detailed amino acid sequence of NM is shown in Fig 1A, while the architecture of the NM protein is shown in Fig 1B. The protein NM expression was conducted at 37°C and the final IPTG concentration was 1 mM. As shown in Fig. 1C, compared to the un-induction sample (lane U), there is an additional strong band (red arrow) appeared after induction (lane I), which is the target NM protein assessed by its apparent size of 38 kDa on SDS–PAGE. Following bacterial cell disruption, the soluble (lane S) and insoluble cell fractions (lane P) were separated, and SDS-PAGE analysis showed that the NM protein was in pellet fraction as inclusion bodies (green arrow) instead of the supernatant. The results indicate that recombinant spider silk protein NM could be efficient expressed as inclusion bodies (NM IBs) in *E. coli.*

Isolation and solubilization of NM IBs

Pure NM IBs were prepared by extensive washing with detergent containing buffer, and majority of the contaminants were removed. After the washing steps, NM IBs purity was evaluated, which is up to 70% (black arrow, Fig. 2A) and used for subsequent solubilizing testing. The purified NM IBs (10 mg/ mL wet weight concentration) were suspended in Tris-HCl at pH 8.0 containing different concentrations of urea (0-7 M) and solubilized with two strategies: one step-heating method and traditional urea-denature method. SDS–PAGE analysis of the solubilized supernatants of NM IBs were shown in Figure 2B and C, and the protein concentrations were measured by Micro BCA Protein Assay kit (Table 1), respectively. We saw that NM IBs could be largely solubilized in Tris-HCl containing 5-7 M urea by traditional urea-denatured method rather than at lower concentration of urea (0-4 M) (Fig. 2B). Differently, using the one-step heating method, Tris-HCl (pH 8.0) containing 0–7 M urea all could solubilize NM IBs, and at 4 M urea concentration the solubilization efficiency is up to 80%, which is three times higher than that of traditional urea-denature method. Interestingly, Tris-HCl (pH 8.0) alone also could solubilize NM IBs (Lane 0, Fig. 2C) to some extent. The results indicated heating improves the solubilization of NM IBs, and the capability achieved by 4 M urea via one-step heating method is comparable to that of 7 M urea with traditional urea-

denatured method (Fig. 2D). Hence, for testing of other potential factors below, the urea concentration was stuck to 4 M.

Solubilization of NM IBs at different heating temperature and pHs

In order to optimize the heating temperature that is essential for solubilization of NM IBs by one-step heating method, we tested different temperatures, range from 40 to 100°C,in 10 mM Tris-HCl at pH 8.0 containing 4 M urea. As shown in Figure 3, the solubilization capability is progressively increased when rising the heating temperature, while the plateau is around 85°C. Taking into account of the thermal stability of spidroins, the heating temperature is optimized as 85°C for following experiments. Similarly, to find out the best working pH the washed NM IBs were solubilized in 10 mM Tris-HCl at different pHs (pH 5–10) containing 4 M urea. The suspensions were mixed thoroughly and heated at 85°C for 20 min. The soluble NM protein was analyzed by SDS-PAGE and the results showed that the solubilization efficiency is also affected by the working pH, with an increasing tendency from pH 5 to 10 (Fig. 4). This observation indicates NM IBs solubilization by one-step heating method is working perfectly under neutral and alkaline conditions.

The solubilization of IBs might be influenced by different buffer conditions. To figure that out, five different buffers were tested in this study. The results showed that the buffer conditions do not really altered the solubilization of NM IBs (Fig. 5A), indicated by the bands with similar intensity in 10 mM $NaCO_3$ (pH 8.0), 10 mM K_3PO_4 (pH 8.0), 10 mM Tris–HCl (pH 8.0) and deionized water (ddH2O), even though a slight decrease in 1×PBS was observed. These results suggest that the one-step heating method is compatible to a wide range of buffers.

Self-assembly of NM protein into nanoparticles

To evaluate the NM proteins solubilized by the one-step heating method in a function point of view, we induced the NM nanoparticle assembly by salting out with potassium phosphate. Indeed, the NM proteins retain the ability to form nanoparticles. Under scanning electron microscope, well-distributed even spherical nanoparticles with diameter around 500 nm were observed (Fig. 6). The nanoparticles remained stable after dialysis against water, and showed smooth surfaces. The NM nanoparticles might have potential applications as functional biomaterials, such as controllable delivery of protein drugs /peptide vaccines.

Discussion

In the current study, we introduced a one-step heating method, which shows good compatibility to broad range of buffer conditions and pHs. Using the one-step heating method, recombinant NM proteins were successfully prepared with excellent quality from inclusion bodies. The pure NM proteins spontaneously self-assemble into structured spherical nanoparticles, which is similar to other nanoparticles formed by different silk proteins.

Over-expression of recombinant spidroins in *E.coli* has often resulted in IBs. Normally, high concentration of denaturant such as 8 M urea or 6 M guanidine or organic solvents, *e.g.* hexafluoroisopropanol (HFIP), or formic acid, are often used to dissolve them. Subsequently, extensive dialysis was performed to remove the excess denaturant before processing into various formats, which usually lead to spidroins solutions diluted and insoluble. Recently, various mild solubilization strategies for solubilizing IBs have been exploited to retain the native-like secondary structure, such as extreme pH, using detergents and freezing-thawing cycle [37-40]. And tag-free bioengineered spider silk proteins were successfully prepared by thermal extraction and acidic extraction. However, both of the two methods are usually applied to soluble recombinant spider silk protein from cell lysate, while for insoluble spider silk protein fraction, high concentration denaturants are still necessary. In the current study, we developed a new mild solubilization strategy called one-step heating method to solubilize engineered spider silk protein from IBs, with combining spidroins high thermal stability and the effect of urea. NM IBs isolated from *E. coli* with a purity about 70% by extensive washing were solubilized by one-step heating method. The purity of solubilized NM protein from IBs was more than 95% without additional purification steps. The efficiency of solubilization NM IBs in Tris-HCl (pH 8.0) containing 4 M urea by one-step heating method was comparable to that of 7 M urea by traditional urea-denatured method. Even though at 2 M urea concentration, the solubilization efficiency of NM IBs is achieved 50% by one-step heating method, there is only trace amount of soluble protein obtained by traditional urea-denatured method (less than 5%) (Fig. 2). These results indicate significantly less urea is required in the current method.

The one-step heating method holds the potential to apply to other protein preparation, because of the broad buffer choice and pHs. Our data showed just tiny effects on the solubilization efficiency of NM IBs when using different buffer conditions. On the contrary, the pHs and heating temperature have great impacts on the solubilization efficiency (Figs. 4 and 5). The higher temperature promotes IBs solubilization, however, we do not exclude the possibility that the efficiency at low temperature could be compensated by increasing incubation time. One-step heating method prefers neutral and alkaline conditions, while under acidic condition it is still working but with relatively lower efficiency. Consideration of the application of the engineered spider silk protein in bioengineering, appropriate and mild conditions to solubilize NM IBs would help. Hence, the optimum conditions of our one-step heating method are following: hearting temperature 70-90°C for 20 min, pH 7.0-8.5, urea concentration 2-4 M, and the recommended buffer is 10 mM Tris-HCl, which facilitates for the following nanoparticle formation according to the previously study [32, 33] .

Conclusion

In summary, the one-step heating method for solubilization NM IBs is much more efficient in low concentration of urea compared to the traditional urea-denatured method. Solubilization of NM IBs at such a low concentration of urea affords a mild process for downstream nanoparticle or fibers formation. The one-step heating method is not only for spider silk proteins, but also may be applicable to a board range of IBs formed with thermal stable proteins because of the flexible broad buffer condition and pHs.

Methods

Expression and purification of NM IBs in *E. coli*

A gene fragment encoding a 430-aa protein (NM) that corresponds to the 261-aa repetitive sequence and the 161-aa NT domain of *A.ventricosus* MiSp was synthesized, and an enterokinase cleavage site (DDDDK) was introduced between the NT domain and the repetitive sequence (Fig 1A). The gene fragment was inserted into pET-32a plasmid at the *NdeI* and *XhoI* restriction sites (Fig 1B) and confirmed by sequencing. The plasmid with correct sequence was transformed into *E.coli* BL21 (DE3) cells. For protein expression, *E.coli* cells were grown at 37°Cin LB medium containing ampicillin until OD600 is ~ 1, and 1 mM IPTG (final concentration) was added. The expression lasted for 4 h at 37°C for protein high expression. The cells were harvested by centrifugation and lysed by sonication for 30 min on ice. In order to obtain pure NM IBs, the insoluble pellets were washed as previously described [40].

Solubilization of NM IBs

Equal amount of purified NM IBs was resuspended in Tris buffer at pH 8.0 containing different molar concentrations of urea (0-7 M). For the traditional urea-denatured method, the suspension was stirred for 1 h at room temperature and centrifuged (12,000 g, 30 min, 4°C) to collect the supernatant. For the one-step hearting method, the suspension was heated at 85°C for 20 min and centrifuged at 12,000 g for 30 min at 4°C for collecting the supernatant. Supernatants were analyzed by SDS–PAGE. The soluble protein was quantified using Micro BCA Protein Assay Kit (Thermo, US). The protein solubilization efficiency was evaluated by the ration of soluble protein concentration to that in 7 M urea by one-step heating method.

Solubility of NM IBs in Tris buffer at different pHs and temperature

Equal amount of pure NM IBs were suspended in 1 mL 10 mM Tris-HCl at different pHs (range from 5.0 to10.0) in the presence of 4 M urea. The homogenous IBs suspensions were heated at 85°C for 20 min and centrifuged at 12,000 g for 30 min at 4°C for supernatant collections. Supernatants were analyzed by SDS–PAGE. Protein bands were quantified by densitometric analysis using ImageJ [40]. Homogenous suspension of NM IBs were suspended in 10 mM Tris-HCl at pH 8.0 containing 4 M urea. The suspensions were heated at different temperatures (40-100°C) for 20 min and centrifuged. Supernatants were analyzed by SDS–PAGE and the protein bands were quantified as above.

Solubility of NM IBs in different buffers

Five different buffers in presence of 4 M urea were employed to solubilize NM IBs: 1×PBS (pH 8.0), 10 mM sodium carbonate ($NaCO_3$, pH 8.0), 10 mM potassium phosphate (K_3PO_4, pH 8.0), 10 mM Tris–HCl (pH 8.0), and deionized water (ddH2O). Equal amount of purified NM IBs was suspended in 1 mL different buffers and mixed thoroughly to get homogenous suspensions. The IBs suspensions were heated at 85°C for 20 min and centrifuged at 12,000 g for 30 min at 4°C for clear supernatants collection,

respectively. Supernatants were analyzed parallelly by SDS–PAGE. Protein bands were quantified by densitometric analysis using Image J.

Preparation of NM nanoparticles

NM protein from IBs obtained by one-step heating method was dialyzed in 10 mM Tris-HCl at pH 8.0. One hundred microliters NM solution (2.5 mg/mL) was mixed with 1 mL 2 M potassium phosphate buffer at a pH of 8.0. The mixed solution was incubated at room temperature for 2 h and then dialyzed overnight against ultrapure water. For scanning electron microscopy (SEM), NM nanoparticles were completely washed with pure water and air-dried on a gold-coated silicon. The particles were observed under a Hitachi scanning electron microscope (Japan, S-4700).

Abbreviations

NM IBs, NM inclusion bodies; MaSp, major ampullate spidroin; MiSp, minor ampullate spidroin; *E. coli, Escherichia coli*; *A.ventricosus, Araneus ventricosus*; NT, N-terminal domain; CT, C-terminal domain; SEM, scanning electron microscopy

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and material: All data generated or analyzed during this study are included in this published article.

Competing interests: The authors declare that they have no competing interests.

Funding: This work was financially supported by National Nature Science Foundation of China (No. 31771003, No. 31400789), Jiangsu Provincial Innovative Team, Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Authors' contributions: H.C., Y.T. and H.Y. performed the experiments. G.C. designed the MiSp DNA sequence. G.C. and X.Q. analyzed the data and wrote the paper. X.Q. and S.X. conceived and supervised the study. All authors discussed the results and commented on the manuscript.

Acknowledgements: Not applicable

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Table 1

Table 1. Solubilized NM spidroins from IBs in different concentration of urea by two different methods

Urea Conc. (M)	Protein Conc. (ng/ml)	
	One-step heating method	Urea-denatured
0	483	0
1	952	58
2	1290	157
3	1866	311
4	2335	728
5	2641	1368
6	2786	2143
7	2912	2458

Solubilization of NM IBs in different buffers

Figures

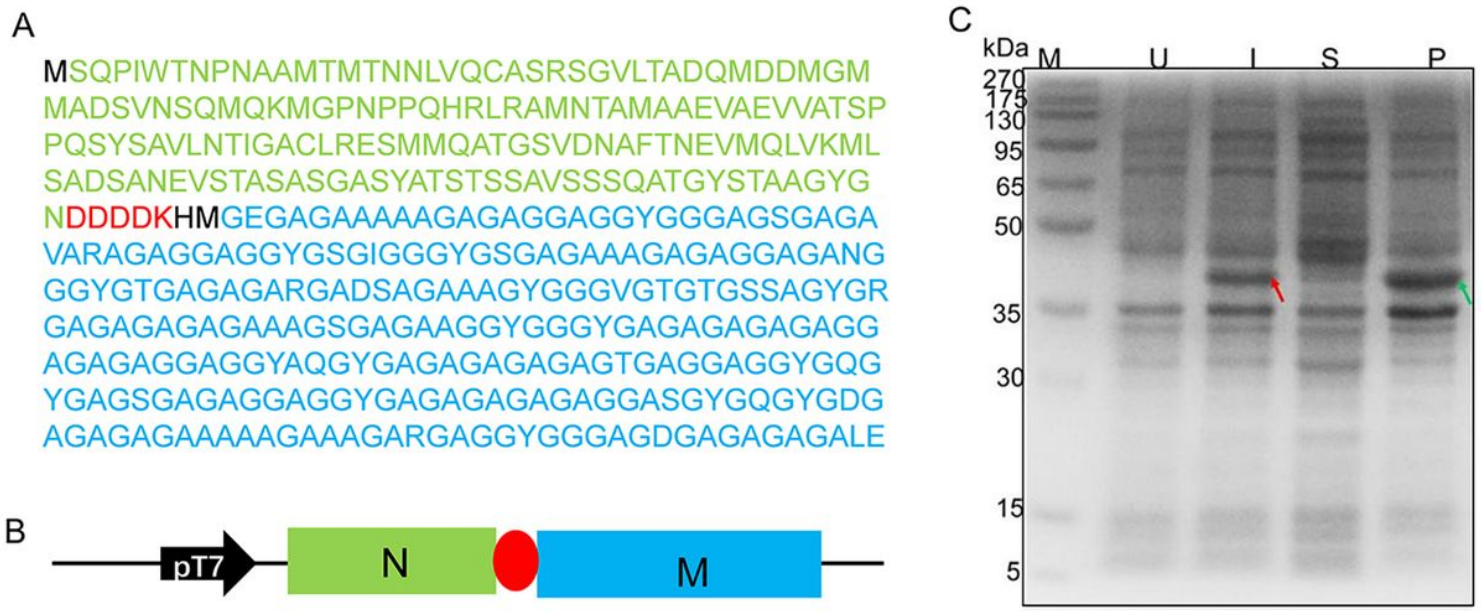

Figure 1

Amino acid sequence, plasmid construction and protein expression of NM protein. (A) Amino acid sequence of NM protein derived from the MiSp of A.ventricosus. The NM protein sequence consists of the 161-aa NT domain (green labeled) and the 261-aa one repetitive sequence (blue labeled). An enterokinase cleavage site (DDDDK) is located between the NT domain and the repetitive sequence. (B) The gene fragment NM was inserted into pET-32a plasmid at the NdeI and XhoI restriction sites to construct the recombinant protein expression vector pET-NM. (C) SDS–PAGE analysis of the recombinant protein NM expression in E. coli BL21 (DE3) cells. Lane M: protein size markers (kDa). Lanes U and I: total cellular proteins of E. coli before and after IPTG-induced protein expression, respectively. Lanes S and P: the supernatants and pellets of the bacterial cell lysate after sonication, respectively. Predicted size of NM is 38 kDa.

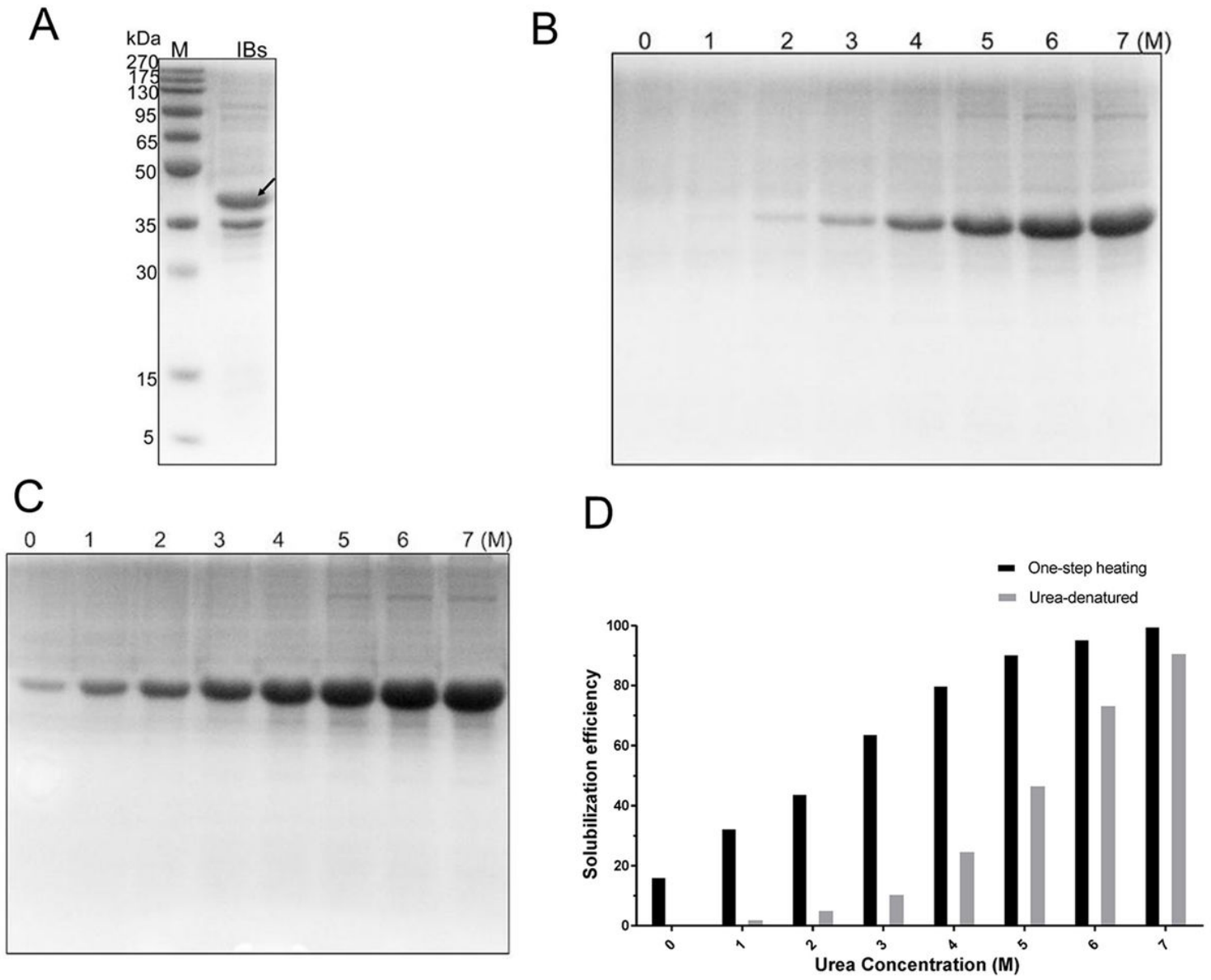

Figure 2

NM IBs isolation and solubilization by two different methods at different molar concentration of urea. (A) SDS-PAGE analysis of the purified NM IBs by extensive washing. (B) SDS-PAGE analysis of the solubilized NM protein from IBs by traditional urea-denatured method at urea concentration of 0-7 M. (C) SDS-PAGE analysis of the solubilized NM protein from IBs by one-step heating method at urea concentration of 0-7 M. (D) The protein solubilize efficiency by two different methods at 0-7 M urea. The protein solubilization efficiency was evaluated by the ration of soluble protein concentration to that in 7 M urea by one-step heating method.

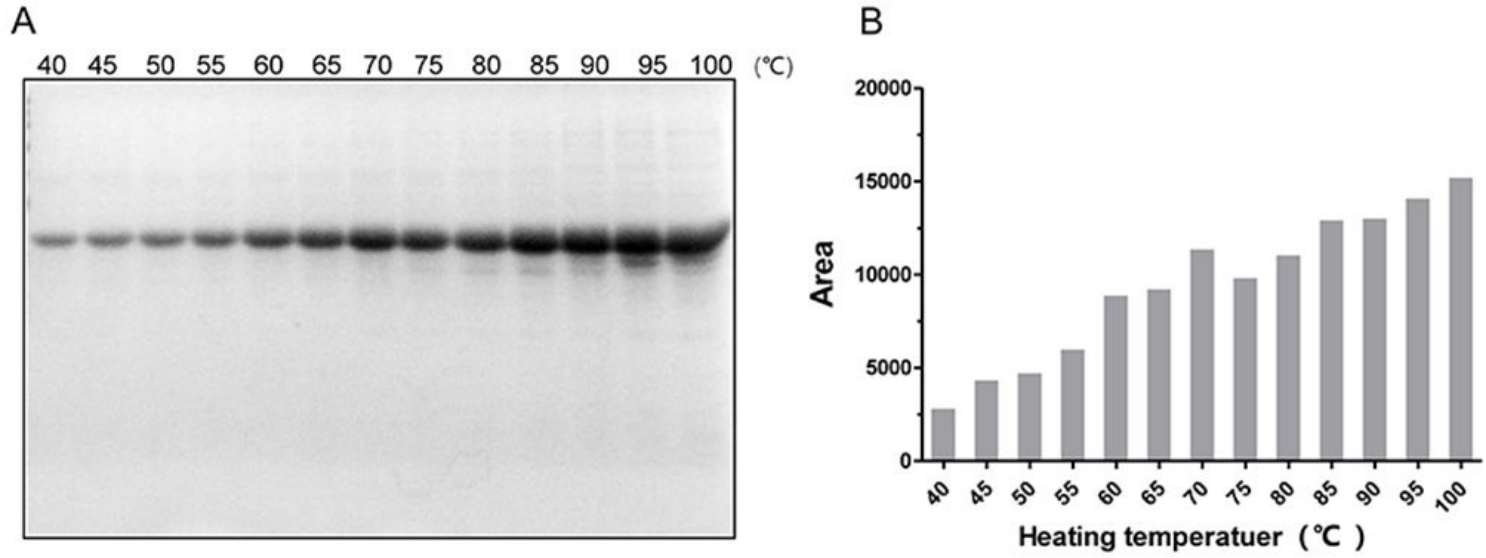

Figure 3

Effect of heating temperature on the solubility of NM IBs by one-step heating method. Equal amount of purified NM IBs was suspended in 1 mL 10 mM Tris-HCl at pH 8.0 containing 4 M urea and heating at different temperatures (40-100°C). (A) SDS–PAGE analysis of the effect of heating temperature on the solubility of NM IBs. (B) Protein bands area were quantitated by densitometric analysis using Image J Software.

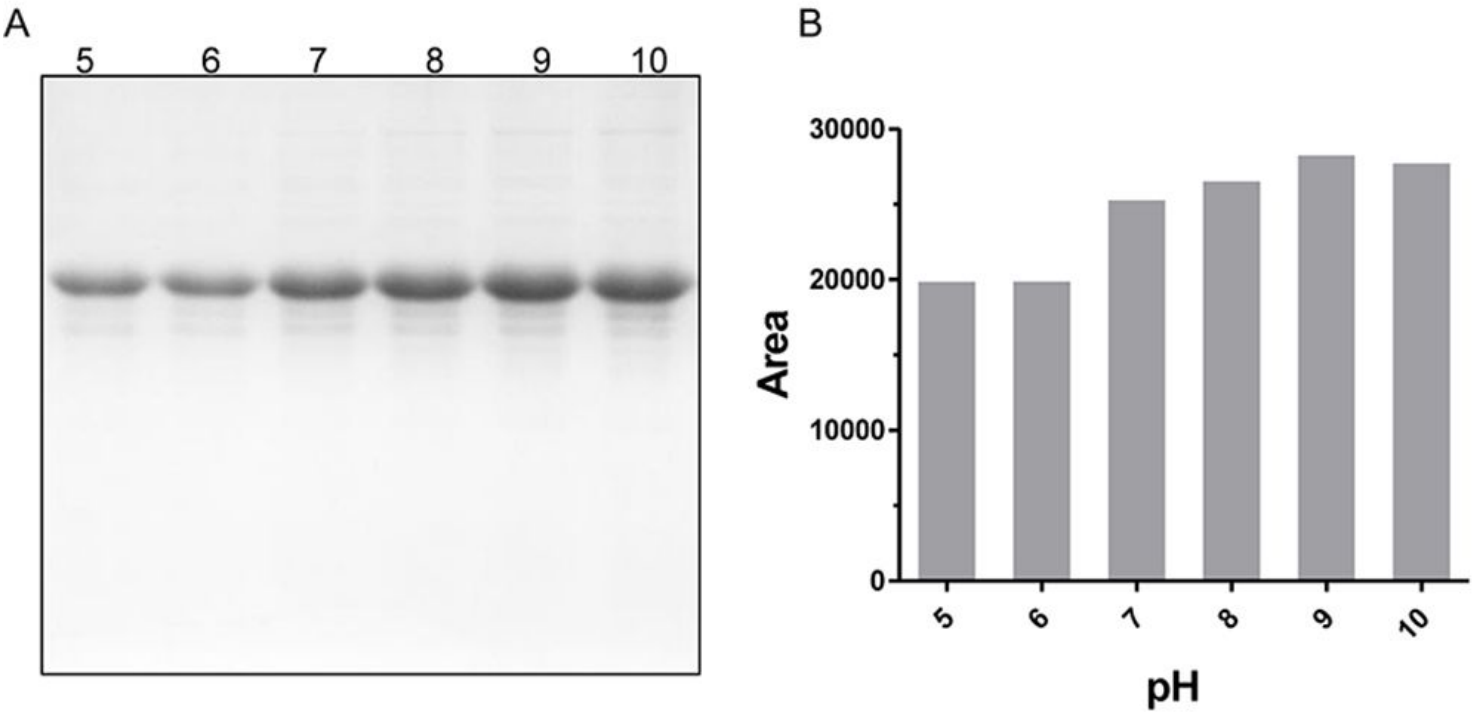

Figure 4

Effect of pH on the solubility of NM IBs by one-step heating method. The equal amount of purified NM IBs was suspended in 1 mL 10 mM Tris-HCl at different pHs (range from 5.0 to10.0) in the presence of 4

M urea. (A) SDS–PAGE analysis of the effect of pH on the solubility of NM IBs. (B) Protein bands area were quantified by densitometric analysis using ImageJ.

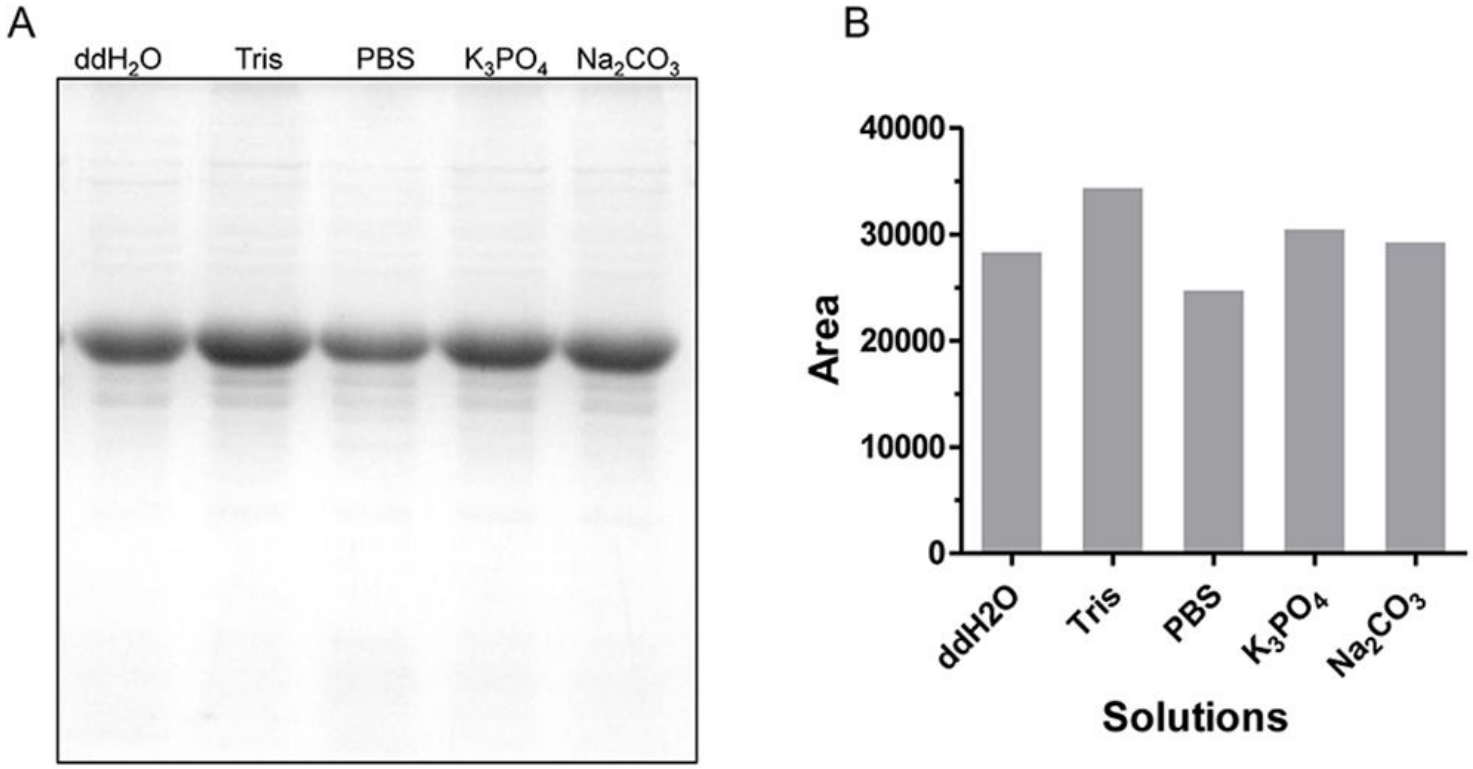

Figure 5

Effect of different buffers on the solubility of NM IBs by one-step heating method. Equal amount of purified NM IBs was suspended in 1 mL different buffers containing 4 M urea and solubilized by one-step heating method. (A) SDS–PAGE analysis of the effect of buffers on the solubility of NM IBs. ddH2O, deionized water; Tris,10 mM Tris–HCl (pH 8.0); PBS, 1×PBS (pH 8.0); K3PO4,10 mM potassium phosphate (pH 8.0); NaCO3, 10 mM sodium carbonate (pH 8.0). (B) Protein bands area were quantified by densitometric analysis using ImageJ.

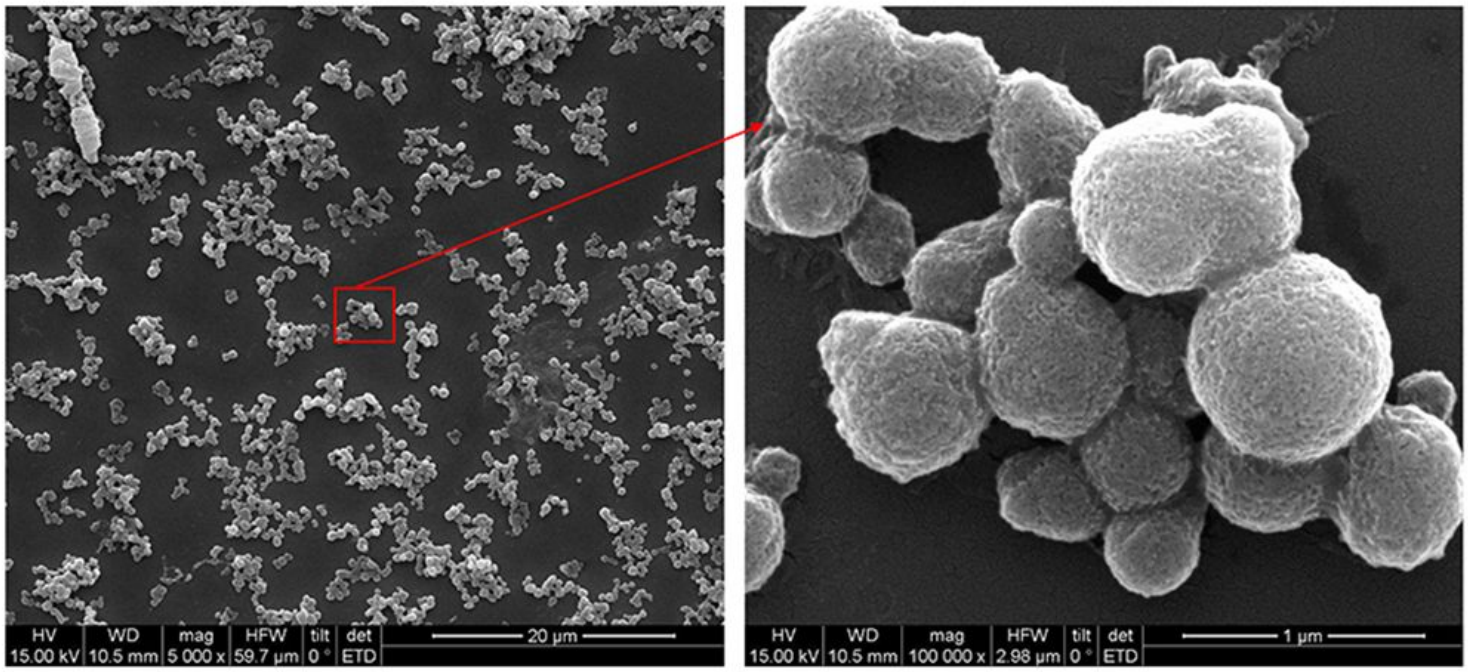

Figure 6

Scanning electron microscopy (SEM) images of nanoparticles formed by NM proteins. The particle formation was processed through salting out with potassium phosphate. One hundred microliters NM solution (2.5 mg/mL) was mixed with 1 mL 2 M potassium phosphate buffer at a pH of 8.0 and was incubated at room temperature for 2 h.