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Access to gram scale amounts of functional globular adiponectin from *E. coli* inclusion bodies by alkaline-shock solubilization

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ABSTRACT

The adipose tissue derived protein adiponectin exerts anti-diabetic, anti-inflammatory and anti-atherosclerotic effects. Adiponectin serum concentrations are in the microgram per milliliter range in healthy humans and inversely correlate with obesity and metabolic disorders. Accordingly, raising circulating adiponectin levels by direct administration may be an intriguing strategy in the treatment of obesity-related metabolic disorders. However production of large amounts of recombinant adiponectin protein is a primary obstacle so far.

Here, we report a novel method for large amount production of globular adiponectin from *E. coli* inclusion bodies utilizing an alkaline-shock solubilization method without chaotropic agents followed by precipitation of the readily renaturing protein. Precipitation of the mildly solubilized protein capitalizes on advantages of inclusion body formation. This approach of inclusion body protein recovery provides access to gram scale amounts of globular adiponectin with standard laboratory equipment avoiding vast dilution or dialysis steps to neutralize the pH and renature the protein, thus saving chemicals and time. The precipitated protein is readily renaturing in buffer, is of adequate purity without a chromatography step and shows biological activity in cultured MCF7 cells and significantly lowered blood glucose levels in mice with streptozotocin induced type 1 diabetes.

1. Introduction

Adipose tissue as an endocrine organ secretes a wide variety of bioactive proteins and lipids (adipokines). Adiponectin is one of the most abundant adipokines. Since its identification by four independent research groups in the mid 1990s [1–4] adiponectin has emerged as a key player in the regulation of insulin sensitivity, inflammation and energy homeostasis [5–7]. Adiponectin plasma concentrations are inversely correlated to obesity and its related disorders including type 2 diabetes, steatosis hepatis, atherosclerosis and cardiovascular disease [8–10], suggesting adiponectin as a promising candidate for drug development and treatment of obesity-related metabolic disorders.

Adiponectin circulates in microgram levels per milliliter in the circulation and full-length adiponectin (fAd) forms a wide range of complexes from low, medium to high molecular weight oligomers (LMW, MMW and HMW) and appears to be involved in different and tissue specific signaling pathways [11]. Serum concentrations of the proteolytic globular adiponectin fragment (gAd) are significantly lower, but gAd seems to be the more potent signaling molecule compared to the full-length protein in animal studies [5,12].

Two receptors have been described as adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). AdipoR1 shows high affinity for gAd and a reduced affinity for fAd whereas AdipoR2 exerts medium affinity for both variants [13]. The receptors contain seven transmembrane domains but are structurally and functionally distinct from G-protein coupled receptors (GPCRs). We recently confirmed the inverse membrane orientation of AdipoRs in the plasma membrane compared to GPCRs with an intracellular N-terminus and an extracellular C-terminus [14] and reported protein kinase CK 2 as interaction partner of the AdipoR1 [15].

Despite the ongoing discussion which form of recombinant adiponectin (HMW, MMW or LMW) should be used for therapeutic administration, bacterially expressed recombinant gAd exerts a great therapeutic potential. Fruebis et al. [6] demonstrated increased β-oxidation in muscle tissue after acute gAd treatment which resulted in sustained weight loss in high fat/sucrose fed mice. In addition, gAd expressed in yeast significantly lowered blood glucose levels in mice with STZ-induced type 1 diabetes (T1D) and promoted free fatty acid clearance in mice with dyslipidemia [16].

Abbreviations: ACC, acetyl-CoA carboxylase; AdipoR, adiponectin receptor; fAd, full-length adiponectin; gAd, globular adiponectin; HMW, MMW, LMW, high, medium, low molecular weight.

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Recently, chronic treatment with the trimeric fraction of recombinant gAd was shown to improve glucose tolerance and completely reversed insulin resistance in mice with high-fat diet induced diabetes [17].

However the major drawback for a therapeutic application of adiponectin remains the lack of an easy, cost efficient production of functional protein. This could be overcome by a novel strategy for adiponectin production. Here, we report the alkaline-shock method to solubilize inclusion body derived gAd without chaotropic agents which yields gram amounts of pure, folded and biologically active protein.

2. Materials and methods

2.1. Plasmids, antibodies and chemicals

Globular adiponectin was cloned into pET15b bacterial expression vector (Novagen). Protease inhibitor cocktail was from Sigma–Aldrich. ACC and phospho-ACC specific antibodies were from Cell Signalling Technologies (Danver, MA).

2.2. Bacterial strains

Escherichia coli strain DH5α was used as a host for plasmid constructions and *E. coli* BL21(DE3)pLysS_RARE was used for bacterial expression of His_6-tagged protein.

2.3. Protein expression

Escherichia coli Bl21(DE3)plysS_RARE were transformed with the plasmid gAd_pET15b and grown on solid LB/ampicillin (100 μg/mL) plates at 37 °C overnight. A single colony was selected to grow a 50 mL starter culture overnight at 37 °C. The starter culture was centrifuged at 1500*g* for 5 min and the cell pellet was resuspended in 10 mL LB. The starter culture was inoculated in 1.5 L LB/ampicillin (100 μg/mL) and incubated at 37 °C with shaking until the OD_{600} reached 0.6. Expression of recombinant gAd was induced by addition of IPTG to a final concentration of 1 mM and expression was continued for 6 h at 37 °C. Finally cells were harvested and collected by centrifugation. The cell pellet was resuspended in lysis buffer (25 mM Tris, 500 mM NaCl, 5 mM $MgCl_2$, pH 8, containing lyophilized DnaseI) and lysis was performed in five freeze and thaw cycles using liquid nitrogen and a 30 °C water bath. Bacterial lysates were centrifuged at 18,000*g* at 4 °C for 45 min and the pellet was stored at −70 °C.

2.4. Inclusion body treatment

The pellet (~3 g wet pellet) was resuspended in ~5 volumes of buffer containing 25 mM Tris, 500 mM NaCl, 2 M urea, 0.25% Triton X-100, pH 8.0, incubated for 1 h at RT and centrifuged at 18,000*g* for 45 min at 4 °C. This was repeated twice and a third and fourth time without Triton X-100 to remove detergent. The washed pellet was subjected to three solubilization steps. Inclusion bodies were resuspended in buffer (20 mM Tris, 500 mM NaCl, 8 M urea, 5 mM β-mercaptoethanol, pH 8), incubated for 1 h at RT and centrifuged at 18,000*g* for 45 min at 4 °C.

2.5. Alkaline-shock solubilization and subsequent protein precipitation of pure recombinant gAd

The remaining pellet (~1 g wet pellet) was resuspended in 10 mL of buffer (20 mM Tris, pH 8) and the pH was increased to 12.5 using 1 M NaOH resulting in complete solubilization of the inclusion bodies within seconds. The clear solution was centrifuged for 15 min at 18,000*g* and the supernatant was filtered through a 0.45 μm sterile filter. Protein was precipitated by addition of ~5 volumes of acetone and precipitated protein was collected by centrifugation. The pellet was washed once with acetone, spun down and air dried for storage at −70 °C. Precipitated protein readily dissolved in distillated water or buffer (10 mM Tris, 150 mM NaCl, pH 7.5) up to concentrations of 100 mg/mL. Endotoxin removal for recombinant gAd used in *in vivo* experiments was performed using an ActiClean Etox column (Sterogene) following the manufacturers' instructions.

2.6. SDS–PAGE and Western blot

Samples were run on 15% SDS gels and stained with Coomassie Brilliant Blue R250 (Sigma–Aldrich) or silver stained (Fermentas). SDS gels (7%) of cell lysates (30 μg/lane) were transferred onto PVDF membrane (HyPond, GE Healthcare), blocked with protein free blocking solution (Pierce). After respective antibody incubation blots were developed using ECL-substrate (Pierce) and G:BOX ChemieXL camera (Syngene). Bands were quantified using GeneSnap software (Syngene) and values are presented as means ± SEM of three independent experiments performed. Statistical evaluation of the data was done using one-way analysis of variance (ANOVA) with the Graph-Pad Prism program (Graph-Pad, Inc.). Statistical significance is indicated as follows: $^{*}P < 0.01$.

2.7. Protein concentration determination

Protein concentrations were determined with Bio-Rad Protein Assay (Bio-Rad) using BSA as a standard.

2.8. MALDI-TOF/TOF mass spectrometry

Recombinant protein was analyzed by MALDI-TOF mass spectrometry using a MALDI-TOF/TOF UltraflexIII (Bruker Daltonics) in linear mode. Spectra were calibrated using a mixture of recombinant cytochrome *c* and myoglobin from horse heart (both Fluka) as standard. Tryptic in solution digests were analyzed by MALDI-TOF MS and MSMS. Peak lists of the tryptic peptide masses were generated and searched against the National Center for Biotechnology Information non-redundant database using the Mascot search engine (Matrix Science, London, UK; http://www.matrixscience.com) in order to identify the proteins. For database searches the following parameters were used – species: *Homo sapiens*, tryptic digestion with a maximum of one missed cleavage site, monoisotopic masses; variable modification: methionine residues oxidized; mass tolerance MS: 50 ppm; mass tolerance MSMS: 0.25 Da.

2.9. Circular dichroism spectroscopy

CD spectra were recorded using a JASCO model J720 spectropolarimeter over 250–185 nm at 20 °C in a N_2 atmosphere. Protein solution was measured at a concentration of 5 μM in water. Each measurement was repeated three times using a thermo stable sample cell with a path of 0.2 cm and following parameters: response time of 4 s, scan speed of 20 nm/min, sensitivity of 10 mdeg, step resolution of 0.2 nm, and bandwidth of 2 nm. The CD spectrum of the solvent was subtracted from the CD spectra of the protein solutions to eliminate the interference from solvent and optical equipment.

High-frequency noise was reduced by means of a low-path Fourier transform filter. The ellipticity was expressed as the mean residue weight ellipticity $[\theta]$NRW in deg cm^2 $dmol^{-1}$.

2.10. Cell culture and activity assay

MCF7 cells were grown in DMEM Ham's F12 medium (PAA) supplemented with 10% FCS. Cells were serum starved for 5 h before stimulation experiments. After treatment with 5 μg/mL gAd in DMEM Ham's F12 for 5 min cells were scraped into ice-cold lysis buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail, pH 7.5) and sonicated on ice (15 pulses, 20% power, 0.5 amplitude). Centrifuged lysates were subjected to SDS–PAGE and Western blot analysis.

2.11. In Vivo experiments

Pathogen free 12-week-old male C57BL/6J mice were maintained under controlled animal care conditions with free access to standard chow and water. Mice were given two i.p. injections of streptozotocin (STZ) (150 mg/kg BW) after fasting for 14 h. Two weeks later, glucose levels were measured by Hitado Analyzer after an overnight fast. Those mice with glucose levels >17 mmol/l were defined as STZ-induced T1D and randomized into two groups, which either received 5 mg/kg body weight (BW) recombinant gAd ($n = 4$) or pyrogen-free saline ($n = 4$) by intra-peritoneal injections. Four hours after injection, blood glucose levels were analyzed. All experiments were performed in accordance with the rules for animal care of the local government authorities (Landesdirektion Leipzig, Germany) and were approved by the Institutional Animal Care Committee.

3. Results

3.1. Recombinant gAd is expressed as inclusion bodies and prone to urea solubilization

SDS–PAGE analysis after cell lysis revealed expression of gAd as inclusion bodies. After inclusion body washing steps and three solubilization steps using 8 M urea about one-third of the pellet remained unsolubilized. SDS–PAGE analysis showed inefficient solubilization of further protein. The urea-solubilized fraction contained major impurities and less than 50% gAd (Fig. 1). The major host impurity was identified as *E. coli* 16 kDa heat shock protein A according to Mascot peptide mass fingerprint analysis following in gel tryptic digestion and MALDI-TOF MS (data not shown). On column refolding and purification of urea-solubilized His-tag gAd using the ÄKTA-purifier chromatography system yielded low mg-amounts of recombinant protein per liter of expression culture (data not shown). SDS–PAGE analysis of the residual pellet revealed the vast majority of the recombinant gAd with high purity in the urea insoluble pellet (Fig. 1A).

3.2. Alkaline-shock solubilization of gAd inclusion bodies yields pure, non-aggregated and properly folded gAd in gram scale

After first tests using urea and alkaline pH (>12), a simple Tris buffer adjusted to pH 12.5 as an alkaline-shock was identified to

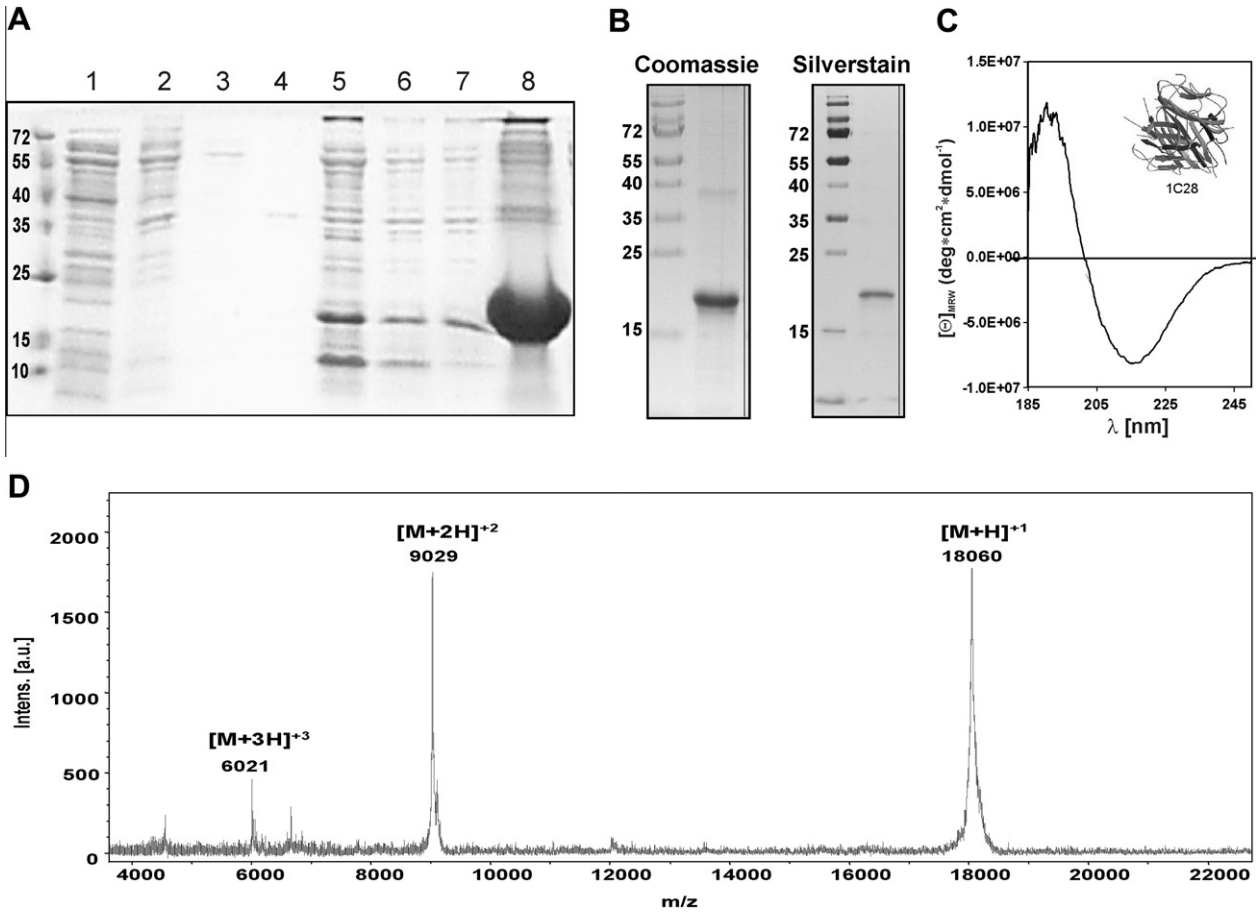

Fig. 1. Alkaline-shock solubilizes gAd from inclusion bodies and yields recombinant protein of great purity. (A) SDS–PAGE analytic at different stages of gAd inclusion body preparation and solubilization in a Coomassie stained gel of the lysis supernatant (lane 1), inclusion body washings (lanes 2–4), urea (lanes 5–7) and alkaline-shock solubilizations (lane 8). (B) Coomassie and silver stain of acetone precipitated gAd dissolved in water (1 μg per lane and 200 ng per lane, respectively). (C) Circular dichroism spectrum of alkaline-shock solubilized gAd at 5 μM in water. CD data are presented as mean residue weight ellipticity. Insertion shows the rendering of murine gAd homotrimer based on PDB ID: 1C28. (D) MALDI-TOF spectrum of gAd in linear mode ($[M+H]^{+1}$ theoretical = 18,069. Together with the singly charged ions doubly and triply charged ions are observed ($[M+2H]^{+2}$ theoretical = 9035, $[M+3H]^{+3}$ theoretical = 6024).

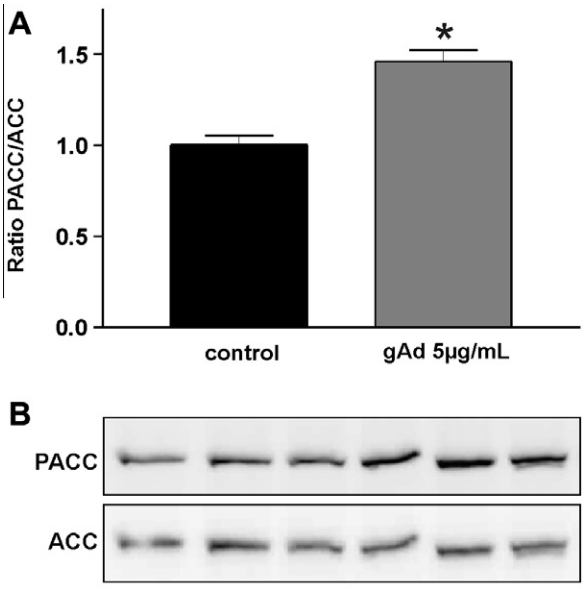

Fig. 2. Induction of ACC phosphorylation (Ser79) in MCF7 cells by alkaline-shock solubilized gAd. MCF7 cells were serum starved (5 h) and treated with gAd (5 µg/mL) for 5 min. Cell lysates were separated by SDS–PAGE, subjected to immunoblotting using anti-ACC and anti-PACC antibodies. The graphic representation of ACC phosphorylation is shown in (A), respective Western blot bands in (B). Data shown represent means ± SEM of three experiments performed. *Statistically different from control with $P < 0.01$.

be sufficient for the solubilization of the urea insoluble pellet within seconds. To avoid irreversible modification of the protein, the soluble protein was immediately precipitated by the addition of five volumes of ice-cold acetone. After centrifugation and washing with acetone, the air dried protein pellet (~1 g) was found to be readily soluble in water or Tris buffer at concentrations up to 100 mg/mL. SDS–PAGE followed by Coomassie or silver stain revealed >95% purity of the recovered protein (Fig. 1B). MALDI-TOF MS (Fig. 1D) and Mascot peptide mass fingerprint analysis after in solution tryptic digestion proved protein identity as His-tag gAd (data not shown). Size exclusion chromatography showed only very little aggregated protein (<5%, data not shown). Circular dichroism studies confirmed this, resulting in spectra consistent with a negative Cotton effect at 218 nm and a positive Cotton effect at 192 nm. This is in agreement with the 10-β-strand jellyroll folding topology revealed by X-ray structure determination of murine gAd [18] (Fig. 1C).

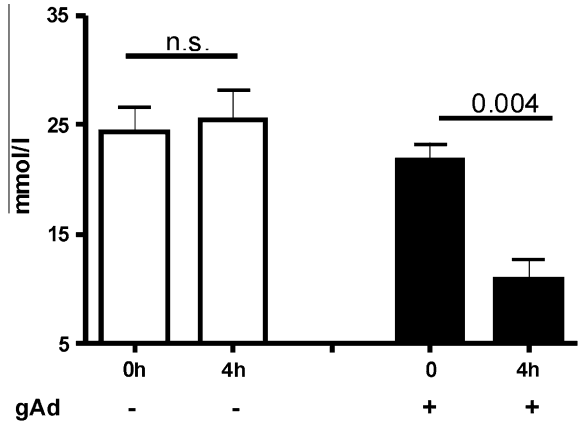

Fig. 3. Blood glucose lowering in STZ-induced T1D mice by alkaline-shock solubilized gAd. T1D mice with blood glucose levels >17 mmol/L were injected with gAd (5 mg/kg BW, i.p.) or pyrogen-free saline and blood glucose levels were determined 4 h after treatment.

Table 1
Effect of gAd on blood glucose levels in STZ-induced type 1 diabetic mice.

	Blood glucose (mmol/l)	
	0 h	4 h
Control (saline)	24.4 ± 4.5	25.5 ± 5.4
gAd 5 mg/kg BW	21.7 ± 3.1	10.9 ± 3.7

3.3. Recombinant gAd induces ACC phosphorylation in MCF7 cells

Biological activity was tested *in vitro* by treating serum starved MCF7 cells with 5 µg/mL gAd in DMEM for 5 min. Western blot analysis using phospho-ACC and ACC antibodies revealed that alkaline-shock solubilized gAd significantly increased ACC phosphorylation about 1.5-fold over basal level (Fig. 2).

3.4. Recombinant gAd lowers blood glucose in STZ-induced type 1 diabetic mice

The biological activity of gAd as a blood glucose lowering agent *in vivo* was investigated in C57Bl6 mice after STZ-induced diabetes (Fig. 3). Animals showed a 50% decrease in blood glucose levels ($P = 0.004$) 4 h after gAd treatment compared to unchanged glucose levels in the saline treated control group (Table 1).

4. Discussion

Adiponectin has emerged as a potent insulin sensitizing protein as well as a protector against cardiovascular disease with beneficial effects on many parameters associated with the metabolic syndrome (reviewed in [19–21]). *In vivo* and *in vitro* data established adiponectin as a reliable biomarker for these disorders and an important element of metabolic improvement thus representing an intriguing therapeutic agent, but recombinant expression of large amounts of active adiponectin remains the bottleneck for therapeutic application [19].

We managed to express globular adiponectin from *E. coli* in large amounts to overcome this problem.

Recombinant expression of adiponectin was previously performed by others mainly in *E. coli* or in the eukaryotic system of HEK 293 cells [12,22–25]. Bacterial recombinant adiponectin was obtained as inclusion bodies or as soluble protein [22,23]. For inclusion body derived adiponectin, yields of 100 mg/L culture medium have been reported [25]. Liu and Liu [16] published the expression gAd in *Pichia pastoris* with yields of about 50 mg/L culture medium. Recently, high-cell density *E. coli* expression protocols have been reported based on auto-induction or high-cell density IPTG induction consistently yielding 280–680 mg/L cell culture for several proteins tested [26,27].

Our results in *E. coli* provide access to much higher levels of bacterially expressed gAd which is in agreement with data obtained for other proteins with nine out of 31 therapeutic proteins approved from 2003 to 2006 are being produced in *E. coli* with yields up to 5 g/L [28].

The recovered gAd was of good purity without a chromatography step and biological activity was demonstrated in a cell culture model and *in vivo*. Recombinant gAd was expressed as inclusion bodies in *E. coli* as His-tag fusion protein. The formation of inclusion bodies due to overexpression in *E. coli* may present a disadvantage at first sight yielding in principle inactive protein with the need for solubilization and subsequent refolding to recover the native protein [29]. An advantage is a lower degradation risk

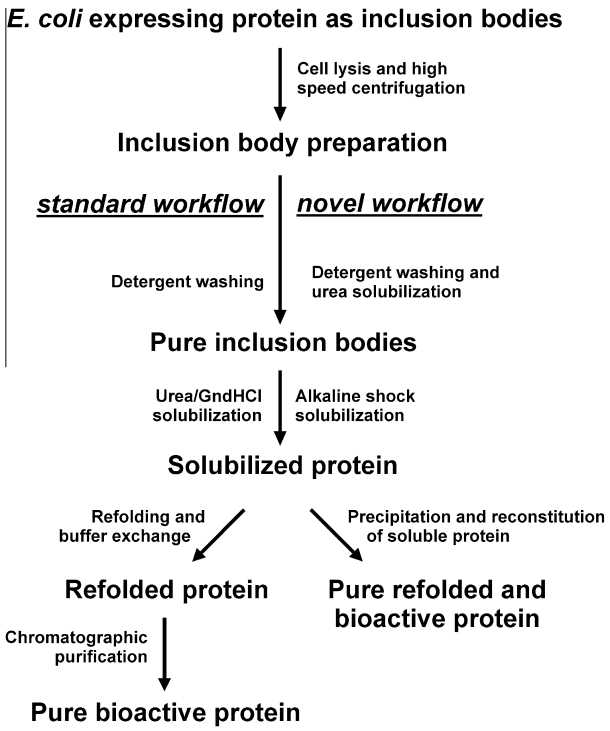

Fig. 4. Novel solubilization and purification strategy for inclusion bodies. Workflow comparison of the alkaline-shock protocol for improved recovery of recombinant bioactive gAd and a standard recovery protocol.

with the inclusion body state protecting against proteolysis by host proteases. By applying a standard solubilization procedure, only a minor fraction of gAd, but a major fraction of host cell impurities are solubilized. Thus, we incorporated the 8 M urea step into the inclusion body washing procedure to achieve near homogeneity before appropriate solubilization.

To capitalize on the presence of extensive native-like secondary structures in inclusion body proteins, we recovered gAd by a chaotropeless alkaline-shock solubilization of the rigorously washed inclusion bodies followed by immediate acetone precipitation to prevent protein modification due to the extreme pH. Our protocol moreover facilitates the renaturation of the recombinant gAd. The mild solubilization method preserves native-like secondary structure of the protein, which after precipitation readily renatures in water or Tris buffer (pH 7.5). The complete solubilization of inclusion bodies in least amounts of buffer compared to insolubility at neutral pH suggests that the charge distribution at alkaline pH across the polypeptide facilitates the solubilization of gAd. Hydrophobic interactions seem to be neglectable in the process resulting in complete solubilization without the addition of chaotropes. This suggests ionic interactions as the main contribution to gAd aggregation. The lone cysteine in the gAd sequence is not involved in disulfide bond formation, as demonstrated in the crystal structure of murine gAd [18].

In the 1990s several groups reported pH assisted solubilization of inclusion bodies. While some alkaline pH assisted procedures still demanded high chaotropic agent concentrations (8 M urea) [30,31] mild solubilization strategies employing only 2 M guanidine hydrochloride or urea at pH $\geqslant$ 12 have been applied for the recovery of human growth hormone (HGH) and zona pellucida protein [32,33]. This mild solubilization process without altering the native-like secondary structure significantly increased the recovery of bioactive HGH via pulsed renaturation process, simple dilution or dialysis. In contrast to our protocol, all procedures required urea or guanidine hydrochloride. [34].

5. Conclusion

Our combination of mild alkaline-shock solubilization and immediate precipitation of the protein bypasses the need for large volumes of cost-prohibitive refolding buffers and expensive chemicals and additives required for most widely used dilution-based refolding strategies [35], thus saving time and money (Fig. 4). Furthermore the precipitated protein can be recovered at very high concentrations (100 mg/mL) making protein sample preparation for follow up chromatography steps very convenient. If protein purity after acetone precipitation is sufficient as with gAd, this strategy gives convenient access to tagless recombinant protein.

Taken together our strategy already gives access to gram scale amount of active gAd out of 1 L expression culture without any optimization regarding expression time, temperature, culture medium, etc. The use of fed-batch fermentation could increase the yield to the magnitude of kilograms. This production scheme could clear the hurdle of limited access to active recombinant adiponectin by supplying sufficiently large amounts for the therapeutical application of this potent adipokine and promising pharmaceutical agent.

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