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Developments in purification methods for obtaining and evaluation of collagen derived endogenous angioinhibitors

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Abstract

Collagen constitutes one of the vital components of the basement membrane scaffolds. Non-collagenous domains (NC1) derived from collagens exhibit potent anti-angiogenic properties, thus attaining significance in regulation of angiogenesis promoted diseases. Individual NC1 domains essential for anti-angiogenic evaluations are generally obtained through purification of individual non-collagenous domains, which have undergone steady developments for enhancing the yields, purpose of biological evaluations and solubility based on the nature of different NC1 domains. This review focuses on the method developments in obtaining biologically active NC1 domains and for specific evaluations in different scenarios.

Keywords

Extra cellular matrix (ECM); Type IV collagen; non-collagenous domains (NC1); anti-angiogenesis; different purification methods and different expression systems

Introduction

Purification of individual proteins of interest to homogeneity for functional and structural characterization forms the basis for development of protein purification methodologies. Purification of individual proteins is affected by different factors such as occurrence of protein of interest as individual moiety or subunit, quantity of proteins present in biological systems and physico-chemical properties of the proteins including primary sequence,

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isoelectric values (pI), solubility, size or relative molecular mass (Mr), charge and hydrophobicity. Each of these factors influence the functions of the target purified protein and are considered critical during optimization of purification methods, which are developed based on the factors effecting purification. Finally, the efficacy of purification methodologies applied for obtaining proteins of interest are indicated through the quantity and also the quality of purified proteins; evaluated through functional, biophysical and biochemical characterizations. Thus, protein purification methods are optimized for enhancement of quality and quantities of purified proteins of interest, which can be exemplified by considering the developments in the purification strategies applied for therapeutically important proteins such as anti-angiogenic non- collagenous domains obtained from collagens.

Purification methods and significance of NC1 domains

Collagens constitute vital components of the extracellular matrix (ECM) that forms the scaffold for tissue organization, along with other ECM components. Different collagen types (29 types) differing primarily in their compositions have been identified to date [1]. A common feature of collagens includes the typical triple helical conformation, arising from the glycine-proline-hydroxyproline (Gly-X-Y) repeats occurring in the characteristic filamentous domains of collagens [2]. Based on the type, collagens also contain non-collagenous (NC1) sequences or domains with sequences differing from repeated Gly-X-Y, that generally occur at the amino and carboxy-termini of the monomeric collagen subunits [3]. The common triple helical filamentous supra-molecular structure was considered to be the major functional moiety of collagens, until novel functional roles of NC1 domains were deciphered [4, 5]. Non-collagenous domains of collagens were identified to possess structural, pathological and therapeutic properties; which were characterized through the isolation and purification of individual NC1 domains. Both the collagenous and non-collagenous sequences constitute different domains of the same monomeric chains of collagens (Figure 1); warranting the need for isolation and purification of the individual non-collagenous domains for respective evaluations. Purification of individual non-collagenous (NC1) domains of collagens thus gained significance for evaluation of their structural, pathological and anti-angiogenic activities, which was achieved through the gradual developments in purification methodologies applied for enhancing the quality and quantity of these domains, which are highlighted in this review.

Purification of non-collagenous domains for biophysical and biochemical evaluations

Purification methods for isolation of non-collagenous (NC1) domains were initially applied for obtaining these domains to decipher their composition and structural significance in collagens. The methodologies used for such identification and characterization of non-collagenous (NC1) domains of collagen and for identification of role of NC1 domains in collagen structural assembly were based on the digestion of collagen obtained from tissues and subjecting them to either size exclusion or ion exchange chromatography. These methods enabled the identification and purification of NC domains for biochemical and biophysical characterizations. NC1 domains of type IV collagen were initially identified from the collagenase and protease resistant fragments of type IV collagen [6, 7]. NC1 domains were prepared from the collagenase and peptidase treated samples of lens tissues and Engelbreth-Holm-Swern (EHS) tumors for the identification and elucidation of role of NC1 domains in self-assembly of type IV collagens, respectively. The purification steps included size exclusion and ion exchange chromatographies for separation of NC1 domains of type IV collagen from collagenase digested samples [6–8]. Dimers of NC1 domains were obtained through the digestion of tumor derived type IV collagen with bacterial collagenases, followed by dialysis and Sephacryl-S-300 column chromatography. These

isolated dimers of NC1 domains were shown to play a role in lateral assembly of type IV collagen, through turbidometric and rotary shadow analyses [8].

The NC1 domain's structural assembly into hexamers from dimeric and monomeric forms were deciphered by purification of globular NC1 domains from placental, ligament and tumoral tissues by collagenase digestion, followed by chromatography on HPLC [9]. Hexameric, dimeric and monomeric forms of the NC1 domains were also subsequently isolated from human placenta by collagenase and size exclusion methods, which lead to characterization of associations arising between NC1 domains [10]. Thus, purification of NC1 domains to their homogeneity was achieved through the protease and size exclusion or ion exchange chromatography methodologies, for enumerating the role of NC1 domains in collagen structural maintenance. However, novel functional roles of NC1 domains other than the role played by these domains in maintaining of collagen structure were also deciphered, which required purification of NC1 domains exclusively for functional characterization.

Purification of NC1 domains for antigenicity evaluations

Specific isoforms of NC1 domains of type IV collagen were identified as antigens for autoimmune disorders viz., Good-pasture and Alport-syndromes [11]. Purification of NC1 domains from glomerular basement membrane (GBM) and those expressed using recombinant expression facilitated the characterization of antigen features of these domains. Goodpasture antigen (GA) was initially detected by purification from collagenase digested GBM extracts, indicating the origin of this antigen from collagen [12]. In the initial study, gel filtration chromatography was applied for enhancing the GA from collagenase treated GBM extracts, and characterized as GA through antibody inhibition radioimmunoassay. Concomitantly, another study isolated GA, following similar method of collagenase digestion of GBM, which reported identification of both non-reduced (dimeric) and reduced (monomeric) forms of GA, that were characterized as GA, through the reactivity against anti-GBM antibodies obtained from patient sera [13].

Subsequent studies applied purification strategies for characterization of GA as the type IV collagen $\alpha 3$ chain. The source for such characterization was the GA obtained by purification of collagenase digested GBM of sheep using autoantibodies of GS patients' sera. Amino acid sequence of purified GA was utilized for identifying GA's corresponding gene as, type IV collagen $\alpha 3$ chain [14]. The antigenic nature of alpha 3 chain of type IV collagen was further localized to the NC1 domain of this collagen by characterizing the $\alpha 3(\text{IV})\text{NC1}$ (tumstatin) as GA; through cloning and purification of this antigen. Individual recombinant $\alpha 3(\text{IV})\text{NC1}$ was obtained as soluble FLAG tagged fusion protein from transformed human embryonic kidney 293 cell culture media and purified by affinity chromatography for GA characterization [15]. Antigenic epitopes of $\alpha 3(\text{IV})\text{NC1}$ characteristic for Alport and Goodpasture antigenicity were also characterized by purification of recombinant human $\alpha 1$ - $\alpha 5\text{NC1}$ domains and chimeric $\alpha 1/\alpha 3\text{NC1}$ domains expressed in human kidney 293 cells by affinity chromatography on immobilized anti-FLAG monoclonal antibodies specific for the antigens [16]. Thus, antigenic roles played by NC1 domains were deciphered by the purification of individual domains.

Purification of NC1 domains for anti-angiogenic evaluations

Purification methodologies for obtaining non-collagenous domains underwent gradual modifications with identification of anti-angiogenic properties of NC1 domains. Pathological angiogenesis leads to the progression of tumoral growth and metastasis in cancers, neovascularization in ocular disorders and arthritis [17]. Regulating pathological angiogenesis became a complementary approach in treatment of these neovascular diseases.

In this scenario, identification of anti-angiogenic properties of NC1 domains suggested therapeutic significance to NC1 domains for treatment of neovascular disorders [18–21]. Thus, obtaining NC1 domains for anti-angiogenic evaluations formed the novel criteria for NC1 domain purification.

Purification of NC1 domains for initial anti-angiogenic characterization

Non-collagenous domain of type XVIII collagen or endostatin was the first one identified with anti-angiogenic properties, which was purified from conditioned media of mouse hemangioendothelioma (EOMA) cells. Endostatin was also expressed as recombinant protein using baculovirus and bacterial recombinant cloning systems [22]. In this initial study, affinity chromatography methods were applied for the purification of endostatin, by utilizing the heparin binding property of NC1 domains of collagen. The native form of endostatin released into the conditioned media of EOMA cell cultures and recombinant protein secreted by Sf-21 cells into culture media were purified by using heparin affinity matrix. Size exclusion and HPLC methods were also employed in this purification methodology to obtain the anti-angiogenic endostatin.

In the same purification study, recombinant endostatin was expressed as a fusion protein with His-tags and purified by using Ni^{2+} affinity chromatography, which yielded higher quantities of recombinant endostatin (30–40 mg/L culture). The soluble form of His-tagged endostatin, and the endostatin obtained from EMOA cell conditioned or transformed Sf-21 cell media exhibited anti-angiogenic activities *in vitro*. However, major quantities of recombinant endostatin, purified from *E. coli* did not exhibit *in vivo* anti-angiogenic and tumor growth inhibitory potential due to precipitation [22]. This study indicated the significance of recombinant methods of expression, in enhancing the yields of NC1 domains and the solubility of NC1 domains as a critical factor in determining the efficacy of purification strategy applied for obtaining cloned for angioinhibitory molecules.

Characterization of angioinhibitory properties of endostatin opened new arena in NC1 domain purification. Rather than purifying NC1 domains from native collagens of tissue origin, methods of purification were optimized for obtaining NC1 domains that were cloned and expressed by recombinant methods. The NC1 domain of type XV collagen termed as restin, was identified to possess anti-angiogenic properties, based on homology search with endostatin gene and obtained by cloning and purification from *E. coli* [23].

Cloning and expression of individual NC1 domains using heterologous expression systems continued as the basic approach along with affinity based purification methods for obtaining the NC1 domains of interest. Type IV collagen contains six different isoforms of monomeric alpha chains denoted as $\alpha 1$ – $\alpha 6$ [21, 24]. Bacterial expressed soluble, recombinant carboxy-terminal NC1 domain of type IV collagen alpha-2 chain ($\alpha 2(\text{IV})\text{NC1}$ /canstatin) was purified by Ni^{2+} chromatography. This protein exhibited partial insolubility during dialysis; however, the solubilized protein exhibited potent anti-angiogenic and anti-tumor properties [25]. The carboxy-terminal NC1 domains of all six isoform chains [$\alpha 1(\text{IV})\text{NC1}$, $\alpha 2(\text{IV})\text{NC1}$, $\alpha 3(\text{IV})\text{NC1}$, $\alpha 4(\text{IV})\text{NC1}$, $\alpha 5(\text{IV})\text{NC1}$ and $\alpha 6(\text{IV})\text{NC1}$; termed as arresten- $\alpha 1(\text{IV})\text{NC1}$, canstatin- $\alpha 2(\text{IV})\text{NC1}$, tumstatin- $\alpha 3(\text{IV})\text{NC1}$ and hexastatin- $\alpha 6(\text{IV})\text{NC1}$ respectively] were also characterized as anti-angiogenic molecules, through recombinant cloning of individual cDNA's into expression vectors containing FLAG sequences. FLAG containing NC1 domains were purified by affinity chromatography from culture media supernatants of transformed human embryonic kidney 293 cells and evaluated for anti-angiogenic potencies using different *in vitro* and *in vivo* studies along with characterization of the ligand specificity of purified NC1 domains [5].

Challenges in purification of NC1 domains for therapeutic evaluations—

As evident from the above studies, recombinant methods of cloning facilitated expression of individual NC1 domains, which can be purified as individual moieties, rather than mixed with other collagen components. However, maintenance of native conformations of NC1 domains was identified as a major challenge in purification of these domains expressed through different recombinant systems. As an evident from the early purification methods of endostatin, which showed that solubility of recombinant endostatin expressed in *E. coli* was lower compared to native endostatin [22]. Similar to this observation another study also showed the precipitation of endostatin purified by Ni^{2+} affinity chromatography after expression in *E. coli* [26]. Nevertheless, purification of NC1 domains using recombinant methods of expression enabled in obtaining higher yields, which was essential for therapeutic evaluations. Thus, NC1 domains purification methodologies were confronted with further, qualitative and quantitative challenges, which were circumvented in subsequent studies through optimization of purification methods.

Anti-angiogenic NC1 domains modified purification methods—Initial purification strategies of NC1 domains aimed at the characterization of their anti-angiogenic properties. Based on these methods, subsequent purification methodologies were optimized for obtaining similar NC1 domains for enhancing the solubility and yields of NC1 domains along with identification of mechanisms through which these molecules exhibit anti-angiogenic properties.

Recombinant mouse endostatin and respective mutant forms were purified from both bacterial and yeast expression systems using affinity chromatography methods, within the same study [26]. This study reported higher yields of soluble endostatin obtained through purification of recombinant endostatin expressed in yeast, *Pichia pastoris*. In this study, recombinant endostatin was also purified from bacterial inclusion bodies by Ni^{2+} affinity chromatography purification, under denaturing conditions by applying urea, which formed precipitates during dialysis. However, Ni^{2+} affinity chromatography purification applied for purification of His-tagged endostatin expressed as soluble form in cultures of transformed *P. pastoris*, resulted in higher yields of functionally renatured endostatin, which exhibited anti-angiogenic properties. This study reported yeast expression system as a new means for obtaining recombinant NC1 domains and also reported higher yields of renatured NC1 domains for anti-angiogenic evaluations. Considering the low cost effectiveness of this yeast expression system, attempt was also made by applying expanded bed absorption method for purification of soluble endostatin from the culture media of *P. pastoris*. In this method, soluble recombinant endostatin released into the culture supernatants of transformed *P. pastoris* were purified on expanded cation exchange bed rather than heparin columns for decreasing the time of purification [27]. However, similar yields of endostatin were obtained from both the studies.

Affinity chromatography purification on Ni^{2+} columns was also applied for fractionation of non-collagenous domains expressed in culture media of transformed insect cell lines. Recombinant tumstatin, canstatin and arresten expressed in transformed insect cell lines of *Drosophila melanogaster* S2 cells, *Trichoplusia ni* BTI Tn 5B1-4 cells and *Bombyx mori* Bm5 (Bm5) cells, were purified through fractionation on Ni^{2+} affinity chromatography, with higher yields of respective functional anti-angiogenic molecules [28–32]. These Ni^{2+} purification methodologies were facilitated by through the expression of NC1 domains with His tags. In another study, recombinant arresten was expressed with fused His tag using baculovirus expression system in Sf9 cells, and purified using Ni^{2+} affinity chromatography, which exhibited anti-angiogenic activities by binding to $\alpha\text{I}\beta\text{1}$ integrins, after the cleavage of His-tags from purified protein [33–35]. Similar strategy of His-tags purification was later applied for purification of recombinant arresten from bacterial expression system.

Interestingly, retention of His-tags in the recombinant human His-tagged arresten, expressed in *E. coli* did not affect the anti-angiogenic properties of arresten, which was predominantly isolated as soluble form [36, 37].

Recombinant human hexastatin was expressed with His-tags and purified through Ni^{2+} affinity chromatography with lower yields of soluble protein compared to insoluble fraction [38]. The soluble hexastatin purified in this study inhibited the proliferation of cultured human umbilical vein endothelial cells (HUVECs) and also reduced the neovascularization and tumor growth in Rip1Tag2 mice [38, 39]. Human recombinant tetrastatin [$\alpha 4(\text{IV})\text{NC1}$] was also purified, recently by utilizing the His tags on Ni^{2+} affinity columns, which inhibited melanoma cellular migration and *in vivo* tumor growth [39]. Fusion tag other than His tag also facilitated the purification of NC1 domains such as the maltose binding protein fusion tag was used for purification of soluble, anti-angiogenic, recombinant tumstatin from bacterial culture systems [40].

Renaturation strategies in purification methods of NC1 domains—Though the purification of recombinant NC1 domains expressed in different systems enabled the functional anti-angiogenic characterization of these domains, purification method improvements were still warranted to isolate higher yields of NC1 domains in soluble forms from cost effective systems such as bacterial expression systems, for therapeutic evaluations. Solubility and yields of the NC1 domains were addressed in the subsequent studies by considering the purification from bacterial expressed systems, which required solubilization of recombinant NC1 domains through renaturation or refolding.

Considering the cost effective nature of bacterial expression systems compared to those of yeast, recombinant soluble endostatin was purified from bacterial expression system, by modification of inclusion body preparation and refolding methods in the subsequent study [41]. In this study, recombinant mouse endostatin was cloned into pTB01#4 and expressed in competent BL21(DE3)pLysS bacterial cells to obtain both insoluble and soluble recombinant endostatin purified by two different methods. This study demonstrated that purification of recombinant endostatin using Ni^{2+} chromatography under denaturing conditions by applying 8.0 M urea resulted in mostly insoluble form of endostatin. However, another method of purification including repeated washing and centrifugation of endostatin containing bacterial lysates resulted in soluble endostatin, with anti-angiogenic and anti-tumor properties. This method included lysis of bacterial pellet containing recombinant proteins using lysozyme, sonication and detergent; followed by repeated washing and centrifugation of 'inclusion bodies' in buffers containing detergents and reducing agent, dithiothreitol (DTT). The subsequent steps of purification included dialysis against buffers containing oxidized and reduced glutathiones, enabling the renaturation or refolding of the recombinant proteins. This gradual refolding method enabled purification of soluble endostatin at higher yields, utilizing bacterial expression systems [41].

Applying Ni^{2+} affinity chromatography and subsequent refolding methods also purified recombinant mouse restin expressed in *E. coli*, which yielded soluble restin with anti-proliferative and pro-apoptotic properties. In this study, recombinant restin expressed in *E. coli* was purified under denaturing conditions on Ni^{2+} -NTA columns and the purified protein was subjected to renaturation in refolding buffer containing arginine and glutathione as renaturing agents, followed by dialysis to remove the renaturing agents [42]. Thus, inclusion of renaturation method for restin purification also yielded functional NC1 domain. Refolding of recombinant NC1 domain was also applied in upstream of purification to recombinant canstatin expressed in *E. coli* BL21 (DE3) and purified by gel filtration on Sephadex-G-75, which inhibited angiogenesis in chick embryo micro-capillary vessel [43]. Refolding was also applied for Ni^{2+} affinity matrix purified, insoluble His tagged

recombinant tumstatin expressed in *E. coli*, which exhibited anti-tumor and anti-angiogenic activities [44].

Simultaneous renaturation and purification strategy in purification of NC1 domains

Refolding strategies applied in purification of NC1 domains facilitated the purification of anti-angiogenic domains effectively from bacterial expression systems. However, purified NC1 domains were subjected to refolding or renaturation either prior to or after or column purification as evident from the studies mentioned at renaturation strategies in purification methods of NC1 domains section. Simultaneous renaturation and purification methods provide another alternative strategy for obtaining refolded protein during purification process [45]. In a recent study, L-arginine mediated on-column renaturation coupled with size exclusion chromatography was applied for obtaining higher yields of soluble hexastatin from bacterial inclusion bodies [46, 47]. In this study recombinant human hexastatin was expressed in transformed *E. coli* as inclusion bodies. Applying size exclusion chromatography methods using Sephadex-G100 and Superdex-200, without utilizing His tags, purified the expressed hexastatin protein. The purified hexastatin exhibited anti-angiogenic and anti-tumor activities by inhibiting proliferation and migration of proliferating endothelial cells.

Conclusions

Purification strategies for obtaining non-collagenous domains (NC1) underwent gradual modifications based on the purpose of purification of NC1 domains. The purification methods applied initially were aimed at isolation of NC1 domains for their structural characterization, without major emphasis on the quantities of the purified NC1 domains. Subsequent identification of antigenic roles played by the NC1 domains still did not warrant the purification of higher quantities of NC1 domains. However, purification methodologies enabled the characterization and localization of antigenic epitopes from the respective NC1 domain. Enhancement of solubility and quantities of NC1 domains attained significance with the identification of anti-angiogenic activities of NC1 domains. These criteria affected subsequent purification methodologies, which resulted in 'effective' qualitative and quantitative purification methods that enabled enhancement of soluble NC1 domains in higher quantities. An overview of developments in purification strategies applied for NC1 domain purification is presented in the following schematic diagram (Figure 2 and Table 1), which shows that purification methods underwent developments based on the criteria of enhancing solubility and yields of NC1 domains, according to the needs of evaluation. Developments can be further expected in this area of anti-angiogenic and anti-tumor specific collagen derived NC1 domain purification methodologies, which would result in purification of maximum yields of specific NC1 domains for clinical evaluations.

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Highlights

- Developments in methods for obtaining collagen derived endogenous angioinhibitors.
- A comprehensive review discussed different purification methods.
- Sequential developmental methodology from history.
- Various modified methods to improve higher yields of biologically active collagen proteins.
- This review would be of broad interest to many protein biologists for purification methods.

Figure 1. Schematic illustration showing structures of type XVIII, XV and IV collagens
 Structure of three collagens is presented with focus on the domains (highlighted in different colors for distinction). NC domains of three collagens play role in structural stability of collagens. Type IV collagen NC1 domains possess antigenic properties. Type XVIII and XV collagen contain homologous carboxy-terminal NC1 domains termed as endostatin and restin (endostatin-like domain), respectively. Six isoforms of type IV collagen NC1 domains are identified. Carboxy-terminal NC domains of three collagens exhibit anti-angiogenic and anti-tumor properties.

Overview of NC domain purification methods

Figure 2. Schematic illustration showing developments in NC domain purification methods
 Source for purification of NC domains altered with the purpose of NC domain purification. Collagen obtained from tissues formed the source for purifying NC domain for biochemical and biophysical evaluations, which was subjected to purification after collagenase and protease digestion. Single head arrows indicate multiple steps in purification, where as double head arrow indicates single step in purification.

Table 1

Developments in Non-collagenous domain purification strategies

Source	Purification strategies	Characterization	Ref.
Purification of NC domains for structural evaluations			
Lens tissues	Protease, collagenase digestion and gel filtration	Identification of non-collagenous domains	7
EHS tumors	Protease, collagenase digestion, gel and ion exchange chromatography	Role of NC domains in collagen structure and self assembly	6,8
Tumors, placenta and ligaments	Protease, collagenase digestion, exclusion chromatography and HPLC for sub unit complex analyses	Hexameric, dimeric and monomeric subunit characterization of NC domains	9
Human placenta	Collagenase digestion and exclusion chromatography	Hexameric, dimeric and monomeric subunit role identified collagen assembly	10
Purification of NC domains for antigenicity evaluations			
Glomerular basement membrane	Collagenase digestion and gel filtration	Origin of Goodpasture antigen from collagen	12
Glomerular basement membrane	Collagenase digestion	Identification of dimeric and monomeric forms of Goodpasture antigen	13
Glomerular basement membrane	Collagenase digestion	Demonstrates Goodpasture antigen to be the alpha 3 chain of type IV collagen	14
Transformed human embryonic kidney 293 cell culture media	FLAG Affinity chromatography	Individual recombinant $\alpha 3(IV)NC1$ was obtained as soluble FLAG tagged fusion protein for Goodpasture antigen characterization	15
Recombinant $\alpha 1-\alpha 3NC1$ domains and chimeric $\alpha 1/\alpha 3NC1$ domains of Human kidney 293 cells	FLAG Affinity chromatography	Antigenic epitopes of $\alpha 3(IV)NC1$ characterized for Alport and Goodpasture antigenicity	16
Purification of NC domains for anti-angiogenic evaluations			
Recombinant His-tagged endostatin in EOMA cells, Sf-21 cells, <i>E. coli</i>	Ni^{2+} affinity chromatography, Heparin affinity matrix, size exclusion and HPLC methods	Anti-angiogenic activity <i>in vitro</i> and tumor growth inhibitor	22
Bacterial expressed recombinant canstatin	Ni^{2+} chromatography followed by solubilization.	Anti-angiogenic and anti-tumoral properties	25
Recombinant endostatin human embryonic kidney 293 cells	FLAG affinity chromatography	Six NC1 isoforms exhibited anti-angiogenic activity	26
Recombinant His-tags mouse endostatin & mutant forms in <i>P. pastoris</i>	Ni^{2+} affinity chromatography	Anti-angiogenic activity	26
Recombinant His-tags endostatin in <i>P. pastoris</i>	Expanded bed cation exchange chromatography	Anti-angiogenic activity	27
Recombinant tumstatin, in <i>D. melanogaster</i> S2 cells, Sf9 cells and <i>Trichoplusia ni</i> BTI Tn 5B1-4 cells	Ni^{2+} affinity chromatography	Anti-angiogenic activity	28 29 32
Recombinant canstatin in <i>D. melanogaster</i> S2 cells and <i>Bombyx mori</i> Bm5 (Bm5) cells.	Ni^{2+} affinity chromatography	Anti-angiogenic activity	30 31
Recombinant arresten with fused His tag using baculo virus expression system in Sf9 cells,	Ni^{2+} affinity chromatography.	Anti-angiogenic activity	34
Recombinant arrestin in <i>E. coli</i>	Simultaneous renaturation and size exclusion chromatography	Inhibition of endothelial cells proliferation and promotes apoptosis	37

Source	Purification strategies	Characterization	Ref.
Recombinant His-tags human hexastatin in <i>E. coli</i>	Ni ²⁺ affinity chromatography	Inhibition of HUVEC proliferation, neovascularization and tumor growth	38
Recombinant human His tags tetrastatin from <i>E. coli</i>	Ni ²⁺ affinity chromatography columns	Inhibition of melanoma cell migration and tumor growth	40
Recombinant human tumstatin- MBP in <i>E. coli</i>	Affinity chromatography	Inhibition of chick embryo angiogenesis	40
Recombinant mouse endostatin in BL21(DE3) pLysS	Ni ²⁺ chromatography under denaturing conditions followed by solubilization.	Inhibition of angiogenesis and tumor growth	41
Recombinant mouse restin in <i>E. coli</i> .	Ni ²⁺ affinity chromatography, refolding in arginine	Anti-proliferative and apoptotic properties	42
Recombinant canstatin in <i>E. coli</i> BL21 (DE3)	Gel filtration on Sephadex G-75	Inhibition of angiogenesis in chick embryo micro- capillary vessel	43
His tagged, tumstatin in <i>E. coli</i>	Refolding and Ni matrix purification ²⁺ affinity	Anti-tumoral and anti- angiogenic activities	45
Recombinant hexastatin in <i>E. coli</i>	Simultaneous renaturation and size exclusion chromatography	Anti angiogenic evaluations on endothelial cells	46 47