Inhibitory Effect of Recombinant Tyrosine-sulfated Madanin-1, a Thrombin Inhibitor, on the behavior of MDA-MB-231 and SKOV3 Cells in vitro

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Article

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Abstract

Thrombin, which plays a crucial role in hemostasis, is also implicated in cancer progression. We investigated the effects of the thrombin-targeting recombinant tyrosine-sulfated madanin-1 on cancer cell behavior and signaling pathways compared with wild-type (WT) madanin-1. We generated recombinant madanin-1 2 sulfation (2S) and madanin-1 WT proteins using E. coli. SKOV3 and MDA-MB-231 cells were treated with purified recombinant proteins with or without thrombin stimulation. Migration and invasion of cells were analyzed by a wound healing assay and transwell assay, respectively. Thrombin markedly increased cell migration and invasion in both SKOV3 and MDA-MB-231 cells, which were significantly suppressed by madanin-1 2S ($p < 0.05$). Madanin-1 2S also significantly suppressed thrombin-induced expression of phosphorylated Akt and extracellular signal-regulated kinase (ERK) in both cell lines ($p < 0.05$), but not by madanin-1 WT in MDA-MB-231 cells. Furthermore, madanin-1 2S significantly reversed the expression of E/N-cadherin and vimentin in thrombin-treated MDA-MB-231 cells ($p < 0.05$), whereas madanin-1 WT did not show any effect. In conclusion, madanin-1 2S suppressed migration and invasion of cancer cells more effectively than madanin-1 WT. We postulate that inhibiting thrombin via the sulfated form of madanin-1 may be a potential candidate for enhanced cancer therapy, albeit further in vivo validation is required.

Introduction

Thrombin, a serine protease derived from the plasma protein prothrombin, is generally widely recognized for its role in hemostasis$^1$. Previous studies have reported that thrombin plays a diverse role as a growth-regulatory protein in addition to its role in hemostasis; thrombin enhances mitogenesis$^2$, the proliferation of vascular smooth muscle cells$^3$ and fibroblasts$^4$, cellular adhesion$^5,6$, and angiogenesis$^7$. Moreover, accumulating evidence suggests that hemostatic system components, such as thrombin, are involved in cancer progression through a variety of mechanisms$^8,9$. Protease-activated receptor-1 (PAR-1), which is activated by proteases including thrombin, is a G-protein coupled receptor (GPCR)$^{10}$; PAR-1 signaling is constitutively activated in cancer cells in contrast to normal cells and is involved in carcinogenesis, metastasis, and angiogenesis$^{11}$. PAR-1 has also been observed to be overexpressed in many cancers, including breast and ovarian cancer, and may be utilized as a therapeutic target$^{10,11}$.

Several anticoagulant substances have been isolated and characterized to date from blood-feeding invertebrates$^{12–14}$. Madanin-1 (MEROPS inhibitor family I53) is a small cysteine-free protein isolated from the salivary glands of ticks (Haemaphysalis longicornis), and it acts as a competitive thrombin inhibitor$^{15}$. According to previous studies, madanin-1 also competes with physiological substrates for binding to the exosite I of α-thrombin, a secondary recognition site that interacts with fibrinogen and PARs$^{16}$. An assay using human plasma showed that madanin-1 prolonged the prothrombin time (PT) and activated partial thromboplastin time (aPTT) in a dose-dependent manner$^{16}$, as well as doubled the thrombin time (TT) at a concentration of 5 µM$^{15}$. It has recently been shown that post-translational
sulfation of tyrosine residues within acidic stretches substantially enhanced the thrombin inhibitory and anticoagulant potency of madanin-1 by binding the molecule to the active site and the exosite II.

Although anticoagulants are widely used for treating and preventing cancer-associated venous thromboembolism, they have not yet been used as a direct anti-cancer therapy in clinical settings. However, in the context of the comprehensive role of thrombin in cancer biology, several studies have reported the potential therapeutic effects of targeting thrombin and PAR-1. In this study, we investigated the effect of recombinant tyrosine-sulfated madanin-1 on the migration and invasion of cancer cells and the intracellular signaling pathways compared with wild-type (WT) madanin-1. We performed an in vitro assay using SKOV3, an ovarian cancer cell line, and MDA-MB-231, a highly aggressive breast cancer cell line.

**Results**

**Inhibition of cancer cell migration by madanin-1 2 sulfation (2S) protein**

We examined the effect of sulfated madanin-1 compared to wild-type madanin-1 on the migration of SKOV3 and MDA-MB-231 cells through the wound healing assay (Fig. 1). Cells were pretreated with 10 µg/ml of madanin-1 WT or madanin-1 2S for 30 minutes before treatment with 2 units/ml of thrombin for 24 hours. Compared with the serum-free condition, cells treated with thrombin (2 units/ml) presented more cell migration in both SKOV3 and MDA-MB-231 cells by +18.5% and +33.3% compared with untreated cells, respectively. Thrombin-induced migration was significantly inhibited by madanin-1, and the inhibitory effect was more effective when cells were treated with madanin-1 2S (93.3% in SKOV3 and 75% in MDA-MB-231 cells) compared with madanin-1 WT (38.2% in SKOV3 and 47.1% in MDA-MB-231 cells).

**Inhibition of cancer cell invasion by madanin-1 2S protein**

The invasive capacity of cancer cells was analyzed using a transwell cell invasion assay (Fig. 2). Cells were treated with thrombin (2 units/ml) with or without 10 µg/ml of madanin-1 WT or madanin-1 2S for 24 hours. The results showed that thrombin treatment significantly enhanced cell invasion in both SKOV3 and MDA-MB-231 cells (by +800% and +385% compared to untreated cells, respectively). Madanin-1 2S significantly suppressed thrombin-induced cell invasion in both cell lines; the inhibitory effect was higher by 476% in SKOV3 cells and by 300% in MDA-MB-231 cells compared with madanin-1 WT.

**Effect of madanin-1 WT and madanin-1 2S on thrombin-associated extracellular signal-regulated kinase (ERK) and the Akt signaling pathway**
Western blot analysis was performed to investigate the potential mechanism of madanin-1 WT and madanin-1 2S on thrombin-associated signaling pathways (Fig. 3). Cells were untreated or pretreated with 10 µg/ml of madanin-1 WT or madanin-1 2S for 30 minutes, followed by treatment with thrombin (2 units/ml for 15 minutes). The expression of phosphorylated (p)-ERK and p-Akt was induced by thrombin; madanin-1 2S significantly inhibited the thrombin-induced expression of p-ERK and p-Akt in both SKOV3 and MDA-MB-231 cells, whereas madanin-1 WT did not significantly inhibit p-ERK and p-Akt expression in MDA-MB-231 cells.

**Effects of madanin-1 WT and madanin-1 2S on thrombin-related E-cadherin, N-cadherin, and vimentin protein expression**

We analyzed protein expression, including cadherin and vimentin expression, using western blotting in MDA-MB-231 cells (Fig. 4). Cells were untreated or pretreated with 10 µg/ml of madanin-1 WT or madanin-1 2S for 30 minutes, followed by treatment with thrombin (2 units/ml for 24 hours). The results showed that thrombin suppressed E-cadherin expression, and the expression was significantly increased by madanin-1 2S but not by madanin-1 WT. Furthermore, thrombin-induced N-cadherin and vimentin expression was significantly suppressed by madanin-1 2S but not by madanin-1 WT.

**Discussion**

In this study, we investigated whether tyrosine-sulfated madanin-1, a thrombin inhibitor, affected malignant tumor cell behavior compared with wild-type madanin-1, using SKOV3 and MDA-MB-231 cells. Using a wound healing assay and transwell cell invasion assay, we showed that sulfated madanin-1 significantly attenuated thrombin-induced cancer cell migration and invasion. To the best of our knowledge, this is the first study to investigate the effects of madanin-1 on thrombin-related signaling pathways as well as cancer cell behavior.

Our knowledge of the association between hypercoagulability and the poor prognosis of cancer has grown considerably. Cancer has many mechanisms that even induce thrombin production, e.g., fibroblast and epithelial expression of thrombin are significantly increased in invasive breast cancer. Thrombin activates signaling pathways, including the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascade via the PAR-1 receptor; and stimulation of PAR-1 receptors on tumor cells and stromal cells mediates diverse effects, including cancer progression, inflammation, and immunosuppression. Moreover, Zhong et al. reported that thrombin treatment of SKOV3 cells not only increased invasion but also upregulated PAR-1. In inflammatory breast cancer and epithelial ovarian cancer, the therapeutic effect of inhibiting thrombin action via PAR-1 inhibitors, such as vorapaxar, has been reported in cell experiments. Furthermore, in animal experiments, the group injected with PAR-1-deficient lung cancer cells showed a higher survival rate and lower tumor volume, and the promotion of invasion and metastasis by thrombin was suppressed in PAR-1-deficient cells. Likewise,
our data showed that thrombin treatment increased p-Akt and p-ERK expression as well as cancer migration and invasion in SKOV3 and MDA-MB-231 cells. We suggest that thrombin inhibition by sulfated madanin-1 could effectively block PAR-1 signaling in the tumor microenvironment.

The process of tyrosine sulfation, which is a post-translational modification, is involved in various biological processes, including hemostasis; sulfation of a tyrosine residue within the acidic tale of anticoagulant hirudin increased the affinity of hirudin for thrombin by more than 10-fold. Two tyrosine residues (Y32 and Y35) of madanin-1 are found within acidic stretches and provide suitable sites for sulfation by tyrosylprotein sulfotransferase enzymes that catalyze the transfer of the sulfuryl group. Thompson et al. demonstrated that disulfated variants of madanin-1 at Y32 and Y35 enhanced the magnitude of inhibiting thrombin relative to unsulfated madanin-1; disulfated madanin-1 prolonged TT to a similar degree at 500-fold lower concentrations than unsulfated madanin-1 (28.7 ± 0.9 seconds at 0.01 µM and 5 µM, respectively). We manufactured recombinant tyrosine-sulfated madanin-1 protein using pET41a and pSUPAR6-L3-3SY plasmid in an E. coli cell system, and this purified madanin-1 2S protein was used for the experiments. The present study showed that migration and invasion of SKOV3 and MDA-MB-231 cells induced by thrombin were more effectively suppressed by madanin-1 2S treatment than by madanin-1 WT.

Epithelial-mesenchymal transition (EMT) is a reversible cellular process that allows epithelial cells to acquire mesenchymal features, which increases the metastatic potential of cancer cells and confer resistance to several therapies. EMT activation triggers the downregulation of E-cadherin and upregulation of vimentin and N-cadherin, which are thought to play vital roles in causing invasion and metastasis. E-cadherin is known to be a potent tumor suppressor, and the aberrant expression of N-cadherin negatively affects the overall survival and progression of cancer patients. There is mounting evidence that targeting E-cadherin and N-cadherin could be a promising approach for cancer treatment. In the context of thrombin, several studies have reported that thrombin induces EMT in various cells, including gastric and ovarian cancer cells. Our data were consistent with these studies; thrombin significantly reduced E-cadherin expression in MDA-MB-231 cells, dramatically increased with statistical significance by madanin-1 2S treatment but not by madanin-1 WT. Moreover, madanin-1 2S significantly reduced thrombin-induced expression of vimentin and N-cadherin, while madanin-1 WT showed no effect. The switch in expression from E-cadherin to N-cadherin by thrombin indicates activation of the EMT process, and these findings suggest that madanin-1 2S may have a therapeutic effect by inhibiting metastasis.

The PI3K/Akt/mTOR (mammalian target of rapamycin) and MAPK/ERK signaling pathways, which regulate fundamental cellular functions, are activated downstream from cell surface receptors, such as receptor tyrosine kinases (RTKs) and GPCRs. Mutations in the molecules involved in these pathways have been found in various types of cancer, and dysregulated signaling transduction plays an important role in mediating oncogenic signals, enhancing cancer cell growth, survival, and metabolism. Moreover, activated Akt and ERK are also implicated in orchestrating EMT by regulating EMT-inducing transcription...
Accordingly, research and clinical trials for anti-cancer agents targeting molecules in these pathways are being actively conducted. Several agents are commonly used in clinical practice; alpelisib (PI3K p110α inhibitor) and everolimus (mTOR complex 1 inhibitor) have been approved by the US Food and Drug Administration (FDA) for the treatment of hormone receptor-positive human epidermal growth factor receptor 2 (HER2)-negative breast cancer. In our study, thrombin-induced expression of p-Akt and p-ERK was significantly inhibited by madanin-1 2S in both SKOV3 and MDA-MB-231 cells. Taken together, thrombin may promote ovarian and breast cancer development via the PI3K/Akt/mTOR and MAPK/ERK signaling pathway, and madanin-1 2S may have a therapeutic effect by inhibiting this process.

The exact mechanism of madanin-1 in the pathogenesis of cancer is not elucidated, and further studies on its mode of action are needed. While other cysteine-less thrombin inhibitors like variegin and anophelin occupy the active site of thrombin with strong affinity, madanin-1 competitively binds to thrombin with low affinity. Madanin-1 is cleaved by clotting factor Xa as well as thrombin, and thrombin-cleaved madanin-1 consequently loses its inhibitory function, limiting its effectiveness as an inhibitor. Thrombin rapidly cleaved both unsulfated and sulfated madanin-1 at specific sites; however, unlike cleaved fragments from unmodified madanin-1, synthesized disulfated madanin-1 fragments (1–54, 22–54) showed similar inhibitory activity to the full-length molecule. These findings indicate that the inhibitory activity of disulfated madanin-1 is not substantially affected by thrombin processing, and madanin-1 2S might be a more effective therapeutic agent than madanin-1 WT. We showed the therapeutic potential of madanin-1 2S for cancer for the first time in vitro. However, because a tumor microenvironment that was closely related to cancer development was not sufficiently reflected in this study, further in vivo and clinical trials are necessary to verify the actual effects and biological mechanisms of sulfated madanin-1.

In conclusion, post-translational sulfation of madanin-1, a small cysteine-free anticoagulant, showed enhanced inhibitory activity of thrombin on cancer cell behavior in vitro. The strengthened inhibitory effect of madanin-1 2S on thrombin may have resulted in the restoration of E-cadherin, N-cadherin, and vimentin protein expression, and inhibition of Akt and ERK phosphorylation, leading to suppression of breast and ovarian cancer cell migration and invasion. Taken together, we suggest the possibility of tyrosine-sulfated madanin-1 as a promising therapeutic candidate in the field of cancer therapy.

**Methods**

**Expression and purification of madanin-1 wild-type and 2 sulfation proteins**

The wild-type madanin-1 was purchased from CosmoGenetech (Seoul, Korea). The two TAC of madanin-1 WT sequences were changed to two TAG for making madanin-1 2 sulfation protein (Table 1). The nucleotides were added to make EcoRI and HindIII restriction sites in parenthesis. The synthesized
double-stranded oligonucleotide was inserted into the EcoRI and HindIII digested pET-41a (Novagen) vector, which has an N-terminal GST, polyhistidine (6×His) tag, and a C-terminal polyhistidine for purification.

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence (5’-3’)</th>
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<tr>
<td>Madanin-1 WT</td>
<td>AAA GAA TTC TAC CCA GAG CGT GAT TCC GCA AAA GAA GGT AAC CAG GAA CAA GAG CGG GCG TTG CAT GTA AAG GTG CAA AAG CGC ACT GAC GGC GAT GCT GAC TAC GAT GAA TAC GAA GAG GAT GGC ACA ACT CCA ACG CCC GAT CCG ACT GCA CCT ACG GCC AAG CCC AGA CTG CGT GGT AAC AAG CCT TAA AAG CCT TAA AAG CTG TAA</td>
</tr>
<tr>
<td>Madanin-1 2S</td>
<td>AAA GAA TTC TAC CCA GAG CGT GAT TCC GCA AAA GAA GGT AAC CAG GAA CAA GAG CGG GCG TTG CAT GTA AAG GTG CAA AAG CGC ACT GAC GGC GAT GCT GAC TAG GAT GAA TAG GAA GAG GAT GGC ACA ACT CCA ACG CCC GAT CCG ACT GCA CCT ACG GCC AAG CCC AGA CTG CGT GGT AAC AAG CCT TAA AAG CTG TAA</td>
</tr>
</tbody>
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Madanin-1 WT pET-41a (50 µg/ml kanamycin), madanin-1 2S pET-41a, and pSUPAR6-L3-3SY (50 µg/ml kanamycin and 50 µg/ml chloramphenicol) were transformed into *E. coli* BL21(DE3) (Fig. 5a, b). Then, the selected BL21 colony was cultured in 3 ml of Luria-Bertani (LB) medium overnight. The next day, cultured madanin-1 WT transformants were inoculated into 250 ml of LB medium until OD₆₀₀ = 0.5, then 0.1 mM of IPTG was added and lasted for 5 hours at 37°C to overexpress the fusion proteins. Cultured madanin-1 2S transformants were also inoculated into 250 ml of LB medium with 10 mM of sulfotyrosine (Bachem) and cultured until OD₆₀₀ = 1, then 1 mM of IPTG was added and lasted for 20 hours at 25°C to overexpress the madanin-1 2S proteins.

After centrifuging the medium at 10000 g for 10 minutes, the bacterial pellets containing proteins suspended in 10 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) were sonicated. Again, the disrupted cells were centrifuged at 14000 g for 30 minutes, and the supernatant was then used for further purification. The supernatant was applied to pre-equilibrated Ni-NTA resin (Novagen) with distilled water and binding buffer and allowed to flow by gravity. The column was washed with 10 volumes of binding buffer and elution buffer (100 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Next, dialysis (20 mM Tris-HCl, 50 mM NaCl, 0.5 mM β-mercaptoethanol, pH 7.5) was accomplished to remove imidazole for properly storing purified proteins. Finally, madanin-1 WT and madanin-1 2S proteins were resolved in 10% SDS-PAGE. After electrophoresis, the gels were stained with Coomassie Blue R-250 and then destained (Fig. 5c).

**Wound healing assay**

SKOV3 (2x10⁶ cells/well) and MDA-MB-231 cells (3x10⁶ cells/well) (Korea Cell Line Bank, Seoul, Korea) were seeded into 24-well plates. After reaching 100% confluence, the cell monolayers were scratched
with a 200 µl sterile pipette tip to create a vertical wound\textsuperscript{47}. To avoid any influence from the cell growth rate, the cell culture medium was changed from RPMI-1640 with 10% FBS to serum-free RPMI-1640. Phase-contrast images were acquired after scratching and after 24 hours of incubation at 37°C. Cell migration rates were calculated with ImageJ software 6.0, and each experiment was repeated three times. To quantify the effect of the reagents on the scratch wound area, the migration distance (mm) between the edges of the gap was measured in each image.

**Transwell cell invasion assay**

The invasion assay was carried out using an 8 µM pore, 24-well Transwell (Costar) coated with PBS buffer containing 25 µg Matrigel (sigma) and 0.1% gelatin (sigma). Cells were grown to 80% confluence in growth medium, followed by starvation in serum-free RPMI-1640 for 24 hours, and then seeded (1 x 10\(^6\) cells/ml) on top of the chamber\textsuperscript{47}. The bottom chamber contained serum-free medium within the presence or absence of madanin-1 WT or madanin-1 2S (10 µg/ml) in the presence of thrombin (2 units/ml). The cells were fixed in 100% methanol for 10 minutes and stained with 0.1% crystal violet (fisher) for 10 minutes. A cotton swab removed cells on the top side of the filter. Cells that migrated to the underside of the filter were photographed with an Olympus DP70 microscope; three fields per sample were captured at 10× magnification. Quantitation of cells was determined by measuring the pixel density of crystal violet-stained cells using a DP controller.

**Western blotting assay**

Cells were extracted in cold 1x Cell Lysis Buffer (Cell Signaling) containing a 1 mM PMSF and 50 mM NaF mixture. The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein were resolved by 6 ~ 10% SDS-PAGE. The proteins were transferred to nitrocellulose membranes (Millipore) and then probed with primary antibodies (1:1000) to p-Akt, Akt, p-ERK, ERK, β-actin, E-cadherin (Cell Signaling), N-cadherin, and Vimentin (Santa Cruz). The specific immunoreaction was detected with mouse or rabbit secondary antibodies conjugated to chemiluminescence reagent (Santa Cruz).

**Statistical analysis**

Statistical analysis data are expressed as the mean of at least three independent experiments ± standard deviation (SD). To identify statistically significant differences in the experimental data, a one-way analysis of variance test was used. Tukey post-hoc analysis was performed for pairwise comparisons between conditions. IBM SPSS Statistics 20.0 (IBM, Armonk, NY, USA) was used to analyze the data. A \(p\)-value less than 0.05 was considered to indicate statistical significance.

**Declarations**

**Data Availability**

The data in this study are available from the corresponding author.
Acknowledgements

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Author contributions

J.H.L. and J.S.Y. conceived and designed the study; G.H.J. and S.A.J. performed the experiments; J.S.Y. and T.H.R. drafted and revised the paper; all authors reviewed and approved the final version of the manuscript.

Additional Information

Competing interests: The authors declare no competing interests.

References


**Figures**

**Figure 1**

**Effects of madanin-1 wild-type (WT) and madanin-1 2 sulfation (2S) on cancer cell migration.** A monolayer of confluent cells was scraped with a sterile pipette tip after being preincubated with 10 μg/ml of madanin-1 WT or madanin-1 2S for 30 minutes. SKOV3 (a) and MDA-MB-231 (b) cells were treated with or without stimulation of thrombin (2 units/ml) for 24 hours, and wound closure observed by phase contrast microscopy was photographed. The average distance between the edges of the gap was...
measured in three independent experiments. Madanin-1 2S inhibited thrombin-induced migration with statistical significance more effectively than madanin-1 WT. Results are presented as mean ± standard deviation of three independent experiments (*$p < 0.05$).

**Figure 2**

**Effect of madanin-1 wild-type (WT) and madanin-1 2 sulfation (2S) on transwell invasion of cancer cells.** Invasion of SKOV3 (a) and MDA-MB-231 (b) cells were investigated by a transwell invasion assay with Matrigel for 24 hours. The bottom chamber contained serum-free medium with or without madanin-1 WT or madanin-1 2S (10 μg/ml) in the presence of thrombin (2 units/ml). Representative images and the statistical graph indicating the density of invaded cells per field 24 hours after seeding are shown. Only madanin-1 2S treatment significantly inhibited thrombin-induced cell invasion. Results are presented as mean ± standard deviation of three independent experiments completed in triplicate (*$p < 0.05$).
Figure 3

Effects of madanin-1 wild-type (WT) and madanin-1 2 sulfation (2S) on signaling pathways. SKOV3 and MDA-MB-231 cells were pretreated with madanin-1 WT or madanin-1 2S (10 μg/ml for 30 minutes) prior to treatment with thrombin (2 units/ml for 15 minutes). The expression levels of phosphorylated (p)-Akt, Akt, p-extracellular signal-regulated kinase (ERK), and ERK protein in SKOV3 and MDA-MB-231 cells were analyzed by western blot. (a) Representative gel images are shown. (b) In SKOV3 cells, thrombin-induced p-Akt and p-ERK expression was significantly attenuated by both madanin-1 WT and madanin-1 2S treatment. In MDA-MB-231 cells, thrombin-induced p-Akt and p-ERK expression was significantly inhibited only by madanin-1 2S treatment. The protein levels were normalized to the level of β-actin in the same sample. Results are presented as the mean relative density ratio ± standard deviation of three independent experiments completed in triplicate (*p< 0.05). Original blots are presented in Supplementary Figure 1.
Effects of madanin-1 wild-type (WT) and madanin-1 2 sulfation (2S) on the expression of E-cadherin, N-cadherin, and vimentin in MDA-MB-231 cells. MDA-MB-231 cells were pretreated with madanin-1 WT or madanin-1 2S (10 μg/ml for 30 minutes) prior to treatment with thrombin (2 units/ml for 24 hours). The expression levels of E-cadherin, N-cadherin, and vimentin protein were analyzed in MDA-MB-231 cells by western blot. (a) Representative gel images are shown. (b) Madanin-1 2S significantly increased E-cadherin expression, which was suppressed by thrombin treatment. The expression of N-cadherin and vimentin induced by thrombin was decreased with statistical significance by madanin-1 2S. The protein levels were normalized to the level of β-actin in the same sample. Results are presented as the mean relative density ratio ± standard deviation of three independent experiments completed in triplicate (*p<0.05). Original blots are presented in Supplementary Figure 2.
Figure 5

Cloning and purification of madanin-1 wild-type (WT) and madanin-1 2 sulfation (2S) proteins. (a) Diagram of the expressed control, madanin-1 WT, and madanin-1 2S proteins. Madanin-1 was cloned into the EcoRI and HindIII restriction sites of the pET-41a vector. (b) Along with pET41a plasmid encoding madanin-1 WT and 2S protein, pSUPAR6-L3-3SY plasmid encoding the components necessary for translational incorporation of sulfotyrosine in response to the TAG codon was used. (c) Purified fusion proteins were detected by Coomassie brilliant blue staining after 10% SDS-PAGE gel.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SRmadaninFigure3original.tif
• SRmadaninFigure4original.tif