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New approaches for high-yield purification of Müllerian inhibiting substance improve its bioactivity

Hans K. Lorenzo, Jose Teixeira, Nima Pahlavan, V. Matt Laurich, Patricia K. Donahoe, David T. MacLaughlin*

Pediatric Surgical Research Laboratories, Massachusetts General Hospital and the Department of Surgery, Harvard Medical School, Boston, MA 02114, USA

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Abstract

We have established a new method to purify Müllerian inhibiting substance (MIS) with higher purity and recovery over existing procedures. Recombinant human MIS was expressed in Chinese hamster ovary cells and secreted into chemically defined serum-free media. The secreted products were concentrated by either precipitation with ammonium sulfate or lectin-affinity chromatography, each of which was followed by anion-exchange chromatography. Further separation of the active carboxy-terminal domain of MIS was achieved after cleavage by plasmin followed by lectin-affinity chromatography. This method may be applicable to other members of the transforming growth factor β family with which MIS shares sequence homology.

Keywords: Protein purification; Müllerian inhibiting substance; Transforming growth factor β

1. Introduction

The Müllerian ducts give rise to the internal female reproductive tract organs during fetal mammalian development. Regression of the Müllerian ducts, which is required for normal male reproductive tract development, is an active process controlled by a testicular factor, Müllerian inhibiting substance (MIS), also known as anti-Müllerian hormone [1]. Other functions for fetal MIS have been described, such as inhibition of fetal lung surfactant production [2] and estradiol production by fetal ovaries [3]. Holo-MIS is also produced in adult gonads [4,5], where in males it regulates testosterone homeostasis by inhibiting P450 Cyp17 transcription [6–8], while in females MIS can cause inhibition of granulosa cell proliferation and steroidogenesis [9,10] and inhibition of oocyte meiosis [11,12]. MIS has also been shown to inhibit ovarian cancer cell growth in vitro [13] and, therefore, may have utility as a new naturally occurring cancer therapeutic. The bioactivity of the purified MIS carboxy-terminal domain in vivo has yet to be determined.

MIS is a member of the transforming growth factor β (TGFβ) superfamily of growth and differentiation factors. It is a 140 kDa dimer of identical 70 kDa disulfide-linked monomers each composed of a

*Corresponding author. Pediatric Surgical Research Laboratories, Warren 10, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114, USA. Tel.: +1-617-7241-617; fax: +1-617-7265-057.

E-mail address: maclaugh@helix.mgh.harvard.edu (D.T. MacLaughlin).

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57 kDa amino-terminal and a 12.5 kDa carboxy-terminal domain [14]. It is the carboxy-terminal active domain which shares amino acid homology with other TGFβ family members, such as β 1, 2, and 3, inhibin, activin, and bone morphogenetic proteins [15]. The structure of the MIS carboxy-terminal domain is supported by seven cysteines involved both in intra- and intermolecular disulfides bridges that lead to its structural stability, as revealed by homology to the three dimensional structure of TGFβ [16] using molecular modeling (Lorenzo et al., unpublished data).

Like other TGFβ family members, MIS can be cleaved by plasmin [17,18] which generates its amino- and carboxy-terminal domains. This proteolytic process is required for its physiological activity [19] and occurs at a site in a position similar to the dibasic cleavage site found in the sequence of TGFβ. The resultant products are tightly associated in a non-covalent complex that dissociates at low pH; therefore, plasmin treatment and molecular size-exclusion chromatography have been previously used to separate the carboxy terminus from the amino terminus [20]. The physiological function of the amino terminus is unclear but may be to direct protein folding and to deliver the carboxy-terminal to its receptor [21].

The procedures described for MIS purification, whether from testis or recombinant sources, have employed some combination of ion-exchange [22], carbohydrate [14], or immunoaffinity chromatography [20]. Attempts to produce bioactive MIS in non-mammalian sources such as bacteria, yeast, and insect cells have been unsuccessful or suboptimal because of aggregation, incomplete processing, or increased degradation (MacLaughlin and Donahoe, unpublished). In the present report, we describe MIS purification after expression in Chinese hamster ovary (CHO) cells and secretion into serum-free media. A number of purification protocols of MIS containing serum-free media were compared for increased yield and specific activity; these included immunoaffinity chromatography, ammonium sulfate precipitation followed by anion-exchange chromatography, or lectin-affinity chromatography followed by anion-exchange chromatography (see Fig. 1). The non-immunoaffinity methods have greatly improved the yield and specific activity of MIS over existing procedures and avoided possible bovine serum and mouse immunoglobulin contaminants and immunogens from the preparation, thus making these methods useful if the purified bioactivated MIS is to be used in a clinical setting. Furthermore, this purification process can be applicable to other members of the TGFβ family because of the similarities in their structure.

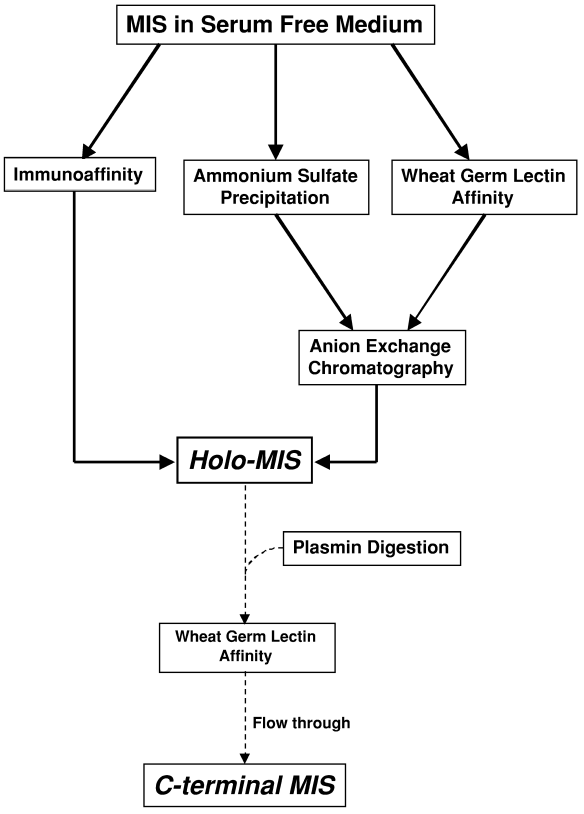

Fig. 1. Strategy for MIS purification. Three different pathways to purify holo-MIS are represented by solid arrows (immunoaffinity, ammonium sulfate precipitation and lectin affinity). The dashed arrows represent the common pathway to purify the carboxy-terminal domain of MIS.

2. Experimental

2.1. Cell cultures

Recombinant human MIS was expressed in dihydrofolate reductase-deficient CHO cells [23]

grown at 37°C in an atmosphere of 10% CO_2, 2% O_2, and 88% N_2 in roller bottles containing modified Eagle's medium minus (Invitrogen Life Technologies) supplemented with 60 μ*M* methotrexate (Immunex), penicillin/streptomycin, 1% L-glutamine, and 5% female fetal bovine serum (FBS). Cultures were subjected to progressively lower concentrations of fetal serum and increasing proportions of CD CHO media (Invitrogen Life Technologies) until serum-free conditions were reached. Cells were maintained in 5% female FBS (Aires/Biologos) for 3 days, 2.5% FBS for 2 days, and then serum-free media only for 2 days. Benzamidine (4 m*M*, Sigma) was added to media and methotrexate was omitted. This concentration of benzamidine is not toxic [14] in the Müllerian duct organ culture regression assay [24]. The serum-free medium was collected, passed through a 0.45-μm filter before being stored at −20°C. The purification protocols compared in the present study are shown schematically in Fig. 1 and described below.

2.2. Immunoaffinity purification

Mouse monoclonal anti-human MIS antibody, purified as described previously [20], was coupled to 5 ml of protein A Sepharose beads and equilibrated in 20 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4. The mouse monoclonal antibody used for this column is highly specific for human MIS and does not interact with other members of the TGFβ family of proteins. This same monoclonal antibody is used as the primary reagent in the human MIS enzyme-linked immunosorbent assay (ELISA) [26]. MIS conditioned media was loaded at one column volume per hour at 4°C and the column was washed with a minimum of 10 column volumes of 20 m*M* HEPES, pH 7.4, then equilibrated in 20 m*M* HEPES, 0.5 *M* NaCl, pH 7.4, and the bound MIS eluted with 1 *M* acetic acid, pH 3.0. The acid eluted fractions were neutralized with NaOH to pH 7.0–7.4, concentrated and desalted via centrifugal filtration (Centricon 10, Amicon). The MIS concentration was determined by the Bradford method [25] and by an MIS-specific ELISA [26]. Purity was assessed by silver staining after polyacrylamide gel electrophoresis (PAGE). The fractions containing MIS were tested in the organ culture bioassay for regression [24] of the Müllerian duct as described below.

2.3. Ammonium sulfate precipitation

MIS containing serum-free media was precipitated with 40% $(NH_4)_2SO_4$ [20]. The precipitated protein was then harvested by centrifugation (10 000 *g*, 30 min at 4°C), resuspended in 25 m*M* glycine, pH 10.5 with protease inhibitors (COMPLETE, Roche), and then dialyzed (approximately 30 000 fold) against 25 m*M* glycine, pH 10.5, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), and 2 m*M* benzamidine. The dialyzed product was filtered through a 0.45-μm filter prior to anion-exchange chromatography.

2.4. Wheat-germ lectin affinity

Serum-free media collected after 48 h of incubation of the CHO cells producing MIS was loaded onto a 5 ml wheat-germ lectin-Sepharose column (Amersham Pharmacia BioTech) previously equilibrated in 20 m*M* Tris, pH 7.4, 0.5 *M* NaCl. Media was passed over the column at a flow-rate 1 ml/min. The column was extensively washed with 20 m*M* Tris, pH 7.4, 0.5 *M* NaCl and equilibrated in 20 m*M* Tris, pH 7.4. Lectin bound proteins were eluted with 20 m*M* Tris, pH 7.4, 50 m*M* NaCl, and 0.5 *M* *N*-acetylglucosamine, and dialyzed against 25 m*M* glycine, pH 10.5 (approximately 30 000 fold).

2.5. Anion-exchange chromatography

The protein recovered after either ammonium sulfate precipitation or wheat-germ lectin affinity chromatography was applied to an anion-exchange column (1 ml MonoQ 5/5 HR in an ÄKTA-FPLC system, Amersham Pharmacia BioTech) previously equilibrated in the starting buffer (25 m*M* glycine, pH 10.5) at a flow-rate 1 ml/min. Flow-through was recovered and the column washed with several hundred ml of starting solvent. A linear salt gradient (0–300 m*M* NaCl in 10 column volumes at 1 ml/min) was developed with 25 m*M* glycine, 1*M* NaCl, pH 10.5. The column was stripped with 1 *M* NaCl for reuse. Elution fractions were subjected to PAGE, then pooled, dialyzed against phosphate-buffered saline (PBS), and the MIS concentration quantified

by the Bradford method [25] and ELISA. The activity of this product was assessed by bioassays as described below.

2.6. Purification of the active carboxy-terminal domain of MIS

The carboxy-terminal domain of MIS was cleaved from holo-MIS using plasmin [17,18]. Holo-MIS, purified after anion-exchange chromatography and dialyzed against PBS, was treated with plasmin in a ratio 1:25 (w/w) (plasmin/MIS) for 90 min at room temperature. The reaction was quenched with 10 m*M* PMSF. In order to dissociate the carboxy-terminal from the amino-terminal domain, the pH of the plasmin-treated sample was reduced to pH 3.5 with acetic acid. The mixture was then loaded onto wheat germ lectin column equilibrated in 25 m*M* glycine, 0.5*M* acetic acid, pH 3.5 to bind the carbohydrate containing amino-terminus. The flow-through fraction containing the carboxy-terminal domain and the amino terminus eluted with 0.5 *M* *N*-acetyl-glucosamine were analyzed by PAGE, Bradford [25], and MIS ELISA [26], then tested for activity in MIS bioassays [24].

2.7. Bioassays

Two different assays were used to test the bioactivity of conditioned media and purified MIS. One is based on the signature activity of MIS, regression of the Müllerian duct in organ cultures of 14.5-day-old female fetal rat urogenital ridges [24], and the other is the MIS-mediated inhibition of cytochrome P450c17 hydroxylase/lyase (Cyp17) mRNA expression in MA-10 Leydig tumor cells [27] as previously described [8].

3. Results and discussion

3.1. MIS expression in serum-free media

CHO cells transfected with the human MIS gene provide a consistent, albeit costly, source for the isolation of MIS [20], making it important to optimize bioactivity and yield to achieve a product that can meet US Food and Drug Administration (FDA) standards for use in patients and secondarily, provide a source for crystallography. MIS production by CHO cells was adapted to serum-free conditions using CD CHO, a chemically defined media optimized for growth of CHO cells and expression of recombinant proteins in culture. When produced in serum containing media, MIS measured by ELISA is less than 0.2% of the total protein present in the media [20]. CD CHO medium does not contain protein or peptides from animal, plant, or synthetic origin and it is made without hypoxanthine and thymidine for use in dihydrofolate reductase amplification systems. After rotation through media containing 5% FBS and then 2.5% FBS, the cells were placed in serum-free CD CHO media. To this media we added the protease inhibitor benzamidine (4 m*M*) to minimize destruction of MIS by proteolysis and the conditioned media collected 2 days later. Longer incubations did not improve the expression of MIS but increased the presence of contaminants or degraded forms. MIS expression ranged from 1 to 2 μg/ml as measured by ELISA, which is less than that measured for CHO cells in serum containing media. Silver stained sodium dodecyl sulfate (SDS)–PAGE examination showed a major band of ~70 kDa under reducing conditions (Fig. 2, lane 1). Other minor contaminants of degraded products can also be found; therefore, we subjected the media to chromatography for further purification.

Purification protocols from serum-free media, as summarized in Fig. 1, include immunoaffinity purification, or anion-exchange chromatography after ammonium sulfate precipitation or lectin-affinity chromatography. Holo-MIS could then be cleaved by plasmin followed by wheat germ lectin affinity chromatography to separate amino-terminal from the active carboxy-terminal MIS.

3.2. Purification of holo-MIS

3.2.1. Immunoaffinity purification from serum-free media

Previously, MIS was purified from serum-containing conditioned media by immunoaffinity chromatography as described [20]. Immunoaffinity purification of MIS from serum-free media resulted in a product with fewer cleavage fragments than that from serum containing media when the proteins were

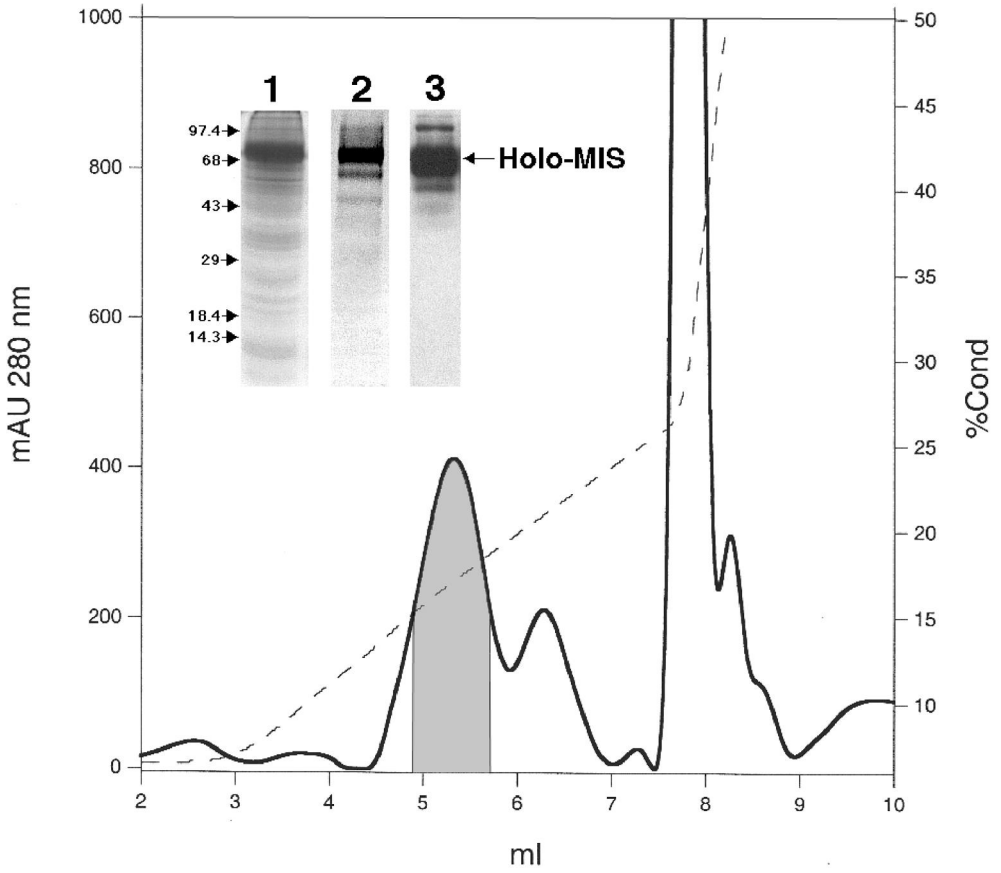

Fig. 2. Anion-exchange FPLC chromatography (MonoQ HR5/5) after 40% ammonium sulfate precipitation of serum-free conditioned medium of CHO cells expressing MIS. The column was equilibrated in 25 m*M* glycine, 4 m*M* benzamidine, pH 10.5. The resolubilized precipitate was loaded onto the column and MIS eluted (shaded peak) by applying a linear gradient 0–300 m*M* NaCl. The solid line represents the absorbance at 280 nm. The dashed line represents the conductivity. Inset: silver-stained SDS–PAGE gel (reducing conditions) of approximately 1 μg of total protein of serum-free medium of MIS expressing CHO cells (lane 1), purified peak (lane 2), and the corresponding Western blot against holo-MIS (lane 3).

compared by electrophoretic analysis. Their recoveries (15%) and bioactivities in the Müllerian duct regression assay, however, were comparable (data not shown). Consequently, there is no distinct advantage to using immunoaffinity purification from serum-free over serum-containing media. Moreover, the use of this technique for large-scale purification of MIS for clinical use in particular, has limitations, such as the requirement of large amounts of specific antibody and the relatively short useful life of antibody columns, as well as the potential to introduce bovine serum proteins and mouse antibodies into protein preparations to be used in clinical application in humans. To circumvent these restraints we developed new procedures for MIS purification without mouse antibody from serum-free conditioned media using commercially available chromatographic columns.

3.3. Ammonium sulfate precipitation/anion-exchange

Previous studies from our group reported that MIS precipitates in 30–40% ammonium sulfate [14,20]. Use of fractions from the 40% ammonium sulfate as a first step of concentration and purification from

serum-free media improved recovery over lower concentrations of ammonium sulfate (data not shown). After precipitation, resuspension in the presence of protease inhibitors and dialysis into 25 m*M* glycine, pH 10.5, the protein was loaded onto a MonoQ column and eluted with a linear 0–300 m*M* NaCl gradient, MIS activity was recovered in a 120–180 m*M* NaCl peak (Fig. 2). Analysis of this eluted peak by SDS–PAGE under reduced conditions as well as by Western blot, revealed that the majority of the protein that migrated at an apparent molecular mass of 70 kDa under reducing conditions was uncleaved-MIS (Fig. 2, insert), with a lower proportion of fragments, presumably products of degradation of MIS, since they are recognized by Western blot analysis. More shallow gradients dilute the protein into broader peaks, thereby reducing yield.

3.4. Wheat-germ lectin affinity/anion-exchange

Analysis of the primary structure of MIS reveals several glycosylation sites in the amino-terminal domain that permit binding to selected lectins [14]. Taking advantage of these characteristics, MIS in serum-free media was loaded onto a wheat germ lectin Sepharose column. After extensive washing, the protein was stripped with 0.5 *M* *N*-acetylglucosamine, dialyzed and loaded onto a MonoQ column under similar conditions, and eluted in a major peak at 120–180 m*M* NaCl (Fig. 3). Silver staining of SDS–PAGE and Western blot analysis revealed the presence in this peak of holo- (70 kDa band) and amino-terminal (57 kDa band) MIS (Fig. 3, insert), which is probably produced by cleavage in CHO cells by endogenous biosynthetic proteases. Bioactivity of this product was quantitatively comparable to that found after ammonium sulfate and higher than immunoaffinity purified MIS when compared both in Müllerian duct regression (Fig. 4A), and steady state mRNA levels of Cyp17 assays (Fig. 4B).

3.5. Recovery and bioactivity of purified MIS

In the present work, the recovery of MIS from serum-free media was 15% of total MIS after immunoaffinty chromatography and 36% after ammonium sulfate precipitation followed by ion-exchange chromatography using a MonoQ column. Of note is that the largest enhancement in yield was observed after wheat-germ lectin affinity plus MonoQ chromatography where approximately a 75% of total MIS was recovered.

Bioactive holo-MIS purified by the above mentioned protocols were incubated for 3 days in an organ culture as described in Experimental. MIS purified either by ammonium sulfate precipitation or wheat germ lectin followed by anion-exchange chromatography exhibited similar dose–response profiles (Fig. 4). The first histological signs of Müllerian duct regression (mesenchymal condensation, basement membrane alterations or a smaller duct) can be observed at concentrations of 4 n*M* (Fig. 4A) of holo-MIS purified either by ammonium sulfate precipitation/MonoQ or wheat-germ lectin affinity/MonoQ, and total regression was accomplished at 15 n*M* MIS. MIS purified by the immunoaffinity method from either serum containing or serum-free media required 35 m*M* MIS for complete regression (Fig. 4A). We also found quantitatively similar results for MIS inhibition of Cyp17 expression, where, again, immunoaffinity purified MIS was less active (Fig. 4B). Quantitation by phosphorimager showed 80–85% inhibition for the non-immunoaffinity purified MIS, while less than 50% was found after immunoaffinity purification. This reduced activity of immunoaffinity purified MIS can be explained by the presence of aggregated forms of MIS that are recognized by the antibody (data not shown) or misfolded forms of MIS following acidification needed for elution of the protein and re-neutralization, leading to a heterogeneous product.

3.6. Purification of the carboxy-terminal domain of MIS

MIS contains two cleavage sites that are sensitive to plasmin; the primary monobasic site which is located at amino acid position 427–428 releases the active carboxy-terminal domain of MIS resembles the furin primary cleavage site of TGFβ [28]. A secondary dibasic cleavage site, identified by amino-terminal sequencing of MIS fragments is located at residues 254–255 in the amino-terminal domain and does not follow the consensus Arg–X–(Arg/Lys)–Arg for furin cleavage [29]. Separation of purified

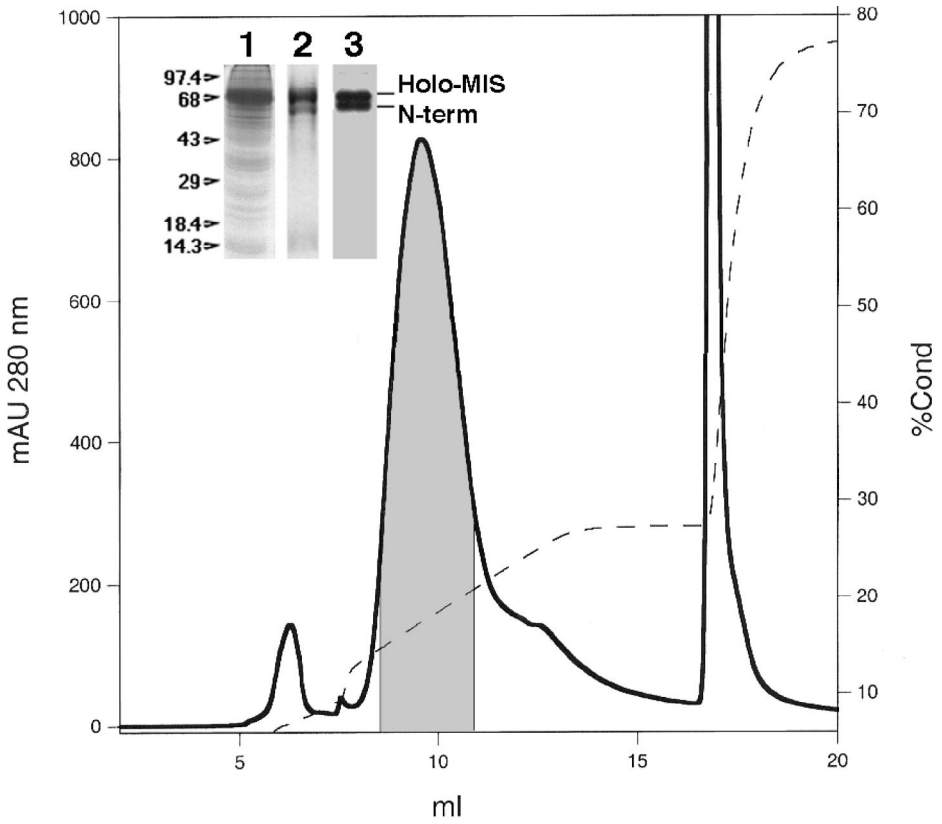

Fig. 3. Anion-exchange FPLC (MonoQ HR5/5) of the eluate from the wheat-germ lectin Sepharose column loaded with serum-free conditioned medium of MIS expressing CHO cells. The column was equilibrated in 25 m*M* glycine, 4 m*M* benzamidine, pH 10.5. The wheat-germ lectin eluate was loaded onto the column and MIS eluted (shaded peak) by applying a linear gradient 0–300 m*M* NaCl. The solid line represents the absorbance at 280 nm. The dashed line represents the conductivity. Inset: silver-stained SDS–PAGE gel (reducing) of approximately 1 μg of total protein of serum-free medium of MIS expressing CHO cells (lane 1), purified peak (lane 2), and the corresponding Western blot against holo-MIS (lane 3).

carboxy-terminal from amino-terminal MIS after digestion with exogenous plasmin previously used molecular size-exclusion chromatography under acidic conditions [18]. This technique requires extreme care to control MIS digestion, since long incubations of MIS in plasmin produced the carboxy-terminal MIS domain plus a 22 kDa fragment [20] due to cleavage both at the primary and secondary sites; it is extremely difficult to separate them from one another by size exclusion. Since all fragments generated after plasmin digestion are glycosylated, except the carboxy-terminal domain, wheat-germ lectin affinity was used as an alternative to size chromatography separation to purify the carboxy-terminal domain of MIS. After plasmin cleavage the resulting fragments were loaded onto a wheat germ lectin column at pH 3.5 in order to dissociate the amino- and carboxy-terminal domains. As shown in Fig. 5, holo- and amino-terminal domains of MIS bound to the column, while the carboxy-terminus was recovered in the flow-through. This 25 kDa protein appeared as a single protein band on SDS–PAGE under non-reducing conditions (Fig. 5, lane 4) and a single 12.5 kDa band by staining after reduction (Fig. 5, lane 3) and Western analysis with an antibody to C-terminal MIS (Fig. 5, lane 6). Size-exclusion chromatography examination of this sample produced a single peak corresponding to a size of 25 kDa, with a $K_{av}=0.26$ and Stoke's radius of 19.24 Å that is consistent with the dimeric form of the

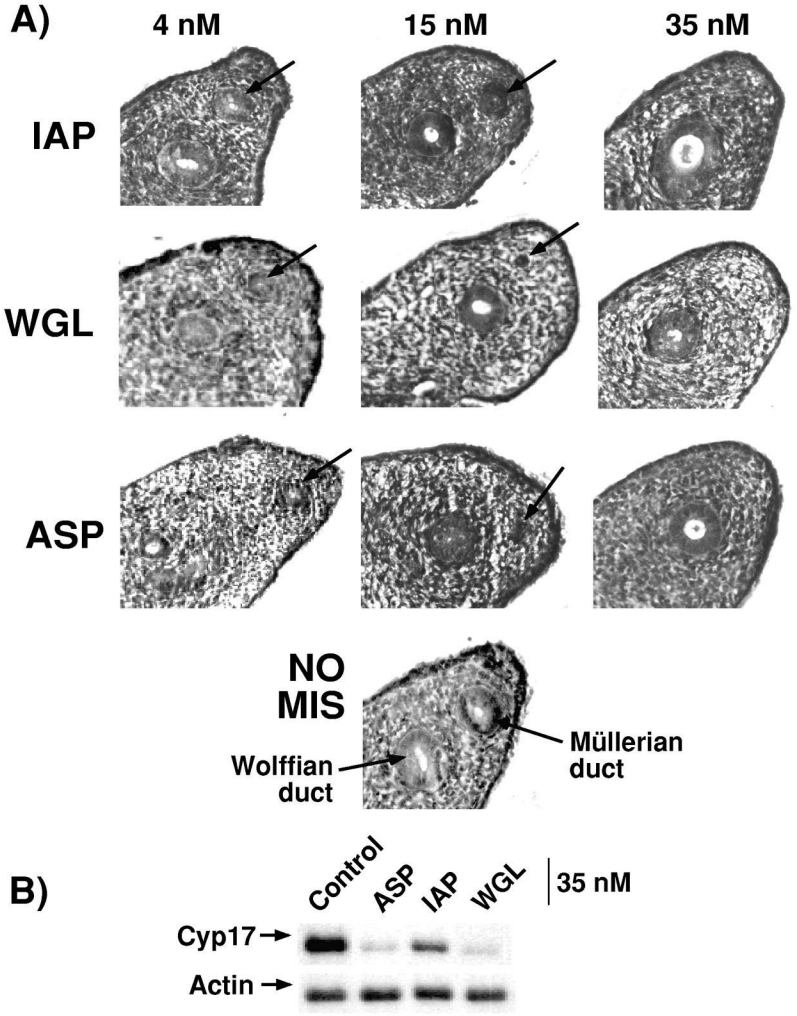

Fig. 4. Bioactivity of purified MIS. (A) Müllerian duct regression bioassay. Rat urogenital ridges (14 1/2 day gestation) are incubated with different concentrations of MIS purified after immunoaffinity (IAP), ammonium sulfate precipitation followed by MonoQ (ASP), or wheat-germ lectin affinity followed by MonoQ (WGL). (B) Northern blot showing inhibition of Cyp17 expression after incubation with immunoaffinity purified MIS, ammonium sulfate precipitation/MonoQ purified MIS, and wheat-germ lectin/MonoQ purified MIS. The MIS concentration was 35 n*M*. MA-10 cells incubated with buffer was used as control.

carboxy-terminal domain of MIS. The resultant protein was active in the Müllerian duct regression assay (data not shown).

Previous methods of MIS purification used tissue extracts or serum-containing media that required multiple chromatographic steps or immunoaffinity chromatography. Attempts to express either holo-MIS or carboxy-terminal MIS alone in non-mammalian systems were unsuccessful, or produced MIS which was unstable or aggregated. In the present communication, we have shown that recombinant human MIS, expressed and secreted by eukaryotic cells into serum-free media, provides a reliable source that can be expanded for large-scale purification using commercially available columns. Given its greater bioactivity, holo-MIS purified either by ammonium sulfate precipitation/anion-exchange chromatography or wheat-germ lectin affinity/anion-exchange chromatography provides a product suitable for both in vivo and in vitro studies and for use in

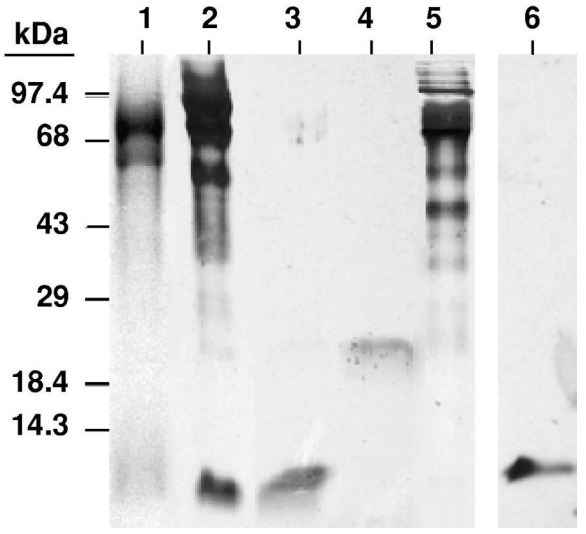

Fig. 5. Purification of carboxy-terminal domain of MIS (SDS–PAGE). Lane 1: holo-MIS purified after WGL and MonoQ. Lane 2: holo-MIS incubated 60 min at room temperature with plasmin. The plasmin-digested MIS was loaded again onto a new wheat-germ lectin column. Carboxy-terminal domain of MIS is not glycosylated and, therefore, recovered in the flow-through displaying a ~12 kDa under reducing conditions (lane 3) or ~25 kDa in non-reducing conditions (lane 4). Lane 5: Product stripped with 0.5 *M N*-acetylglucosamine from the wheat-germ lectin column. Lane 6: Western blot against C-terminal domain of MIS corresponding to similar conditions to those of the lane 3.

humans. Although the carboxy-terminus is the bioactive domain of MIS, the presence of the amino-terminus in bioactive holo MIS may be useful in vivo for clinical trials since it may be required as a scaffold for the proper dimerization of the carboxy-terminal domain, its presentation to the receptor and to increase its serum half life. Also important is the fact that the homogeneity of the carboxy-terminal domain purified by the new methods described above approaches the homogeneity required for crystallographic purposes.

Acknowledgements

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