Purification of Inhibin Variants from Bovine Fluid Extract Using the Whole Gel Eluter and the GS-670 Densitometer

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Introduction

Inhibins are produced predominately by the gonads as heterodimeric glycoproteins ($\alpha:\beta_A$, $\alpha:\beta_B$) that are processed into different molecular weight $\alpha\beta$ dimers[1] and free α[2] and β[3] subunits. The shortage of purified inhibin has impeded the ability to establish the roles that the various molecular weight forms of inhibin have in gonadal function. We recently reported a three step procedure incorporating immunoaffinity chromatography, SDS-PAGE, and electro-elution to isolate nine different molecular variants from bovine follicular fluid.[4] To improve our purification procedure, we chose to use Bio-Rad's Whole Gel Eluter to replace the SDS-PAGE and electro-elution steps of the aforementioned procedure. Advantages of the Whole Gel Eluter include reduction in amount of time preparations are kept at room temperature, omission of gel staining to locate protein bands, decrease in volume of buffer associated with the end product, and elimination of human error associated with excising bands out of multiple gels for subsequent electro-elution. We report here the use of the Whole Gel Eluter for purification of an array of molecular weight variants of inhibin (>160 to 29 kDa) from a bovine follicular fluid extract.

Methods

SAMPLE PREPARATION

Bovine follicular fluid was subjected to immunoaffinity chromatography.[4] The immunoaffinity extracted inhibin was concentrated (>1 mg/ml) and exchanged into 0.15 M Tris, 0.5 M NaCl, pH 6.5. Protein concentration was determined by using a spectrophotometer (O.D.=A_{280} nm) and samples were stored at -20 °C.

SDS-PAGE AND ELECTRO-ELUTION

Immunoaffinity extracted inhibin (5.0 mg total protein) was mixed (1:4, vol:vol) with sample buffer and subjected to 7.5–20% SDS-PAGE (14.1 cm wide, 1.5 mm thick) under non-reducing conditions. Total run time was 21.5 hours at constant current (15 mAmps). The gel was equilibrated in elution buffer 60 mM Tris, 40 mM 3-[cyclohexamino]-1-propanesulfonic acid (CAPS), 0.01% SDS, pH 9.4, for 20 minutes with gentle agitation. Following equilibration, the center portion of the gel was excised with a razor blade using the template. The Whole Gel Eluter (Bio-Rad) was assembled per manufacturer's instructions and protein was eluted from the gel for 30 minutes, constant current (250 mAmps). Following elution, the current was reversed for 15 seconds to aid in the removal of proteins sticking to the dialysis membrane. Fractions (n=30, ~3.0 ml each) were collected from channels using transfer pipets (DPTP, style D, Bio-Rad). Fractions (n=22) were dried, precipitated with methanol/acetone (50:50, vol:vol) to remove SDS, resuspended in 10% acetonitrile, 0.1% trifluoroacetic acid, and stored at -80 °C.

PROTEIN RECOVERY

Protein eluted from the gel was determined by two methods. The first method was % total protein recovered after elution based on O.D. at A_{280}. For the second method, the gel strips excised prior to elution and the center portion of the gel after elution were Coomassie blue stained for protein. Bands on gels were analyzed using the Molecular Analyst™ computer program and the Model GS-670 Imaging Densitometer (Bio-Rad). Total density of bands was expressed as arbitrary units.

α AND β IMMUNOBLOT ANALYSIS AND COLLOIDAL GOLD TOTAL PROTEIN STAIN

Samples (n=28, 0.5 µg/lane) were mixed (1:4, vol:vol) with sample buffer and subjected to 12% SDS-PAGE (n=6 gels) under non-reducing conditions. Proteins on gels were transferred to Immobilon P (30 minutes, 90 V). Membranes (n=3) were blocked with 0.01% BLOTTO for 1 hour, washed, and incubated with mink bINH α_C^{1-26} gly.tyr antiserum[5] (1:1,000) overnight at room temperature. Following incubation, membranes were washed with TTBS (0.05%, Tween-20, 50 mM Tris, 0.5 M NaCl, 0.02% NaN_3) and incubated with rabbit anti-mink bINH α_C^{1-26} gly.tyr antiserum (1:25,000) for 1 hour at room temperature. Membranes were washed, incubated with donkey anti-rabbit IgG HRP (1:1,000, Amersham) for 1 hour at room temperature, washed, and placed in a seal-a-meal pouch with western blotting detection reagents (1:1, Amersham) for 1 minute. Excess reagents were drained off and membranes were placed in a cassette and exposed to two pieces of Reflection film (8 x 10", Dupont, NEN) for 15 minutes at room

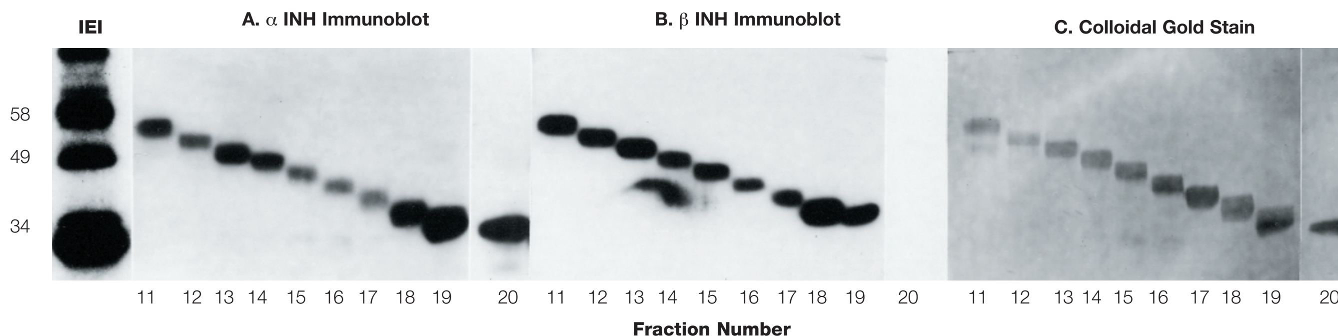

Fig. 1. *Immunoblot analysis and Colloidal Gold Stain of Whole Gel Eluter fractions A. For a immunoblots, immunoaffinity extracted inhibin (IEI, 10 µg), and 0.5 µg of fractions 11–20 were subjected to 12% SDS-PAGE and immunoblot analysis using mink bINH a_C1–26 gly.tyr antiserum. B. For b Immunoblots, 0.5 µg of fractions 11–20 were subjected to 12% SDS-PAGE and immunoblot analysis using human inhibin bA 82–114 monoclonal antibody. C. b immunoblot membrane was stained with Colloidal Gold Stain for protein.*

temperature. Membranes (n=3) were blocked with TTBS for 1 hour at room temperature and incubated with human inhibin $\beta_A^{82\text{-}114}$ monoclonal antibody[6] (1:500) overnight at room temperature. Following incubation, membranes were washed, incubated with sheep anti-mouse IgG HRP (Amersham, 1:1,000) for 1 hour at room temperature, washed, and placed in a seal-a-meal pouch with western blotting detection reagents (1:1, Amersham) for 1 minute. Excess reagents were drained off and membranes were placed in a cassette and exposed to two pieces of Reflection film for 22 hours at room temperature.[4] Membranes were stained for protein with Colloidal Gold Total Protein Stain (Bio-Rad) per manufacturer's instructions.

Results and Conclusion

Nine different molecular weight variants of inhibin were isolated from the preparation of immunoaffinity extracted inhibin: 58, 53, 49, 44, 41, 38, 36, 34, and 29 kDa (Figure 1). Immunoblot analysis and colloidal gold stain of fractions 11–20 demonstrated that each fraction was a single band. Fractions 11–19 cross-reacted with both α and β inhibin antibodies confirming that they are probably αβ dimers, whereas fraction 20 only cross-reacted with the α inhibin antibody indicating that it is a free α- subunit. Fractions 1–10 each contained 2–3 bands with a major band of activity ranging from 60 to >160 kDa (data not shown).

Total protein recovery after elution was 56% of the original 5 mg loaded on the gel. Following precipitation of each fraction, recovery was 20% of the original 5.0 mg and total protein ranged from 10–110 µg isolated for each inhibin variant.

We concluded that the Whole Gel Eluter is an efficient and useful apparatus for simultaneous purification of several molecular weight variants of inhibin. The eluter has several advantages over previous methods used in our laboratory. The overall time of the purification procedure was reduced by 22 hours. Stains were not necessary to identify proteins in gels, thus eliminating potential protein damage. The volume of each inhibin fraction was reduced from 20 ml to 3 ml. And processing of only one gel resulted in total recoveries of purified inhibin equivalent to 10 gels in our previous procedure.[4]

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