CHAPTER III:

Development Of A Polyclonal Anti-Sperm Protein 17 Antibody

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1. Abstract

Polyclonal antibodies were developed for the detection of Sp17 in human tissues. A recombinant Sp17 protein was expressed and purified for use as an antigen. Polyclonal anti-sera was harvested after seven immunizations with the Sp17 recombinant protein. ELISA analysis detected positive anti-recombinant Sp17 titers as low as 1:500 dilution of the rabbit anti-sera. Furthermore, dot blot analysis revealed that the polyclonal anti-Sp17 sera exhibited high affinity for concentrated Sp17 recombinant protein.

2. Introduction

Previous studies have presumed the Sp17 mRNA to be testes specific by northern blot analysis (1, 2). Similar studies have described the Sp17 protein in the sperm and testes using western blot analysis (1). In addition, an intronless Sp17 gene variant has been detected and characterized in human genomic DNA (Chapter 2). However, because mRNA levels are not an indicator of gene expression and translation, it is crucial to examine Sp17 protein expression in normal and diseased tissues as compared to normal testes tissues.

Thus, to investigate Sp17 protein expression in various tissues, an immunohistochemical approach was employed. In this study, the Sp17 coding region (482bp) was amplified from normal human testis by RT-PCR and in human genomic DNA by PCR. A Sp17 expression vector was then created to express Sp17 coding region to generate Sp17 recombinant protein. Subsequently, two rabbits were immunized with the Sp17 recombinant protein to generate anti-Sp17 polyclonal sera. The polyclonal anti-sera was characterized using ELISA and dot blot analysis.

3. Materials and Methods

3.1 Construction of a Sp17 expression vector

3.1.1 PCR amplification

Sp17 coding region specific sense and anti-sense primers were created with BamH1 and KpnI restriction sequences, respectively (Biosynthesis, Inc., Lewisville, TX) (Table 3.1). Sp17 nucleotide sequence was amplified from normal human testis cDNA and human genomic DNA (Chapter 1, section 3.8) by RT-PCR and PCR, respectively. These procedures were performed as described in Chapter 1, section 3.3 with the following primer and template modifications: 200nM Shu sense primer, 200nM Ahu anti-sense primer, 4uL human testis cDNA template or 1uL human genomic DNA template. The PCR amplified DNA products were resolved by agarose gel electrophoresis, visualized with UV light and photographed. The appropriately sized PCR bands were excised from the agarose gel and purified using GeneClean (Bio 101, Vista, CA).

3.1.2 Subcloning into pQE vectors

The purified DNA fragments were subcloned into a pQE-30 vector (Quiagen, Inc., Valencia, CA) according to the manufacturer's guidelines. Briefly, the vector and the Sp17 specific PCR amplified DNA fragment were double-digested with the restriction enzymes BamH1 (Life Technologies) and KpnI (Life Technologies). The pQE-30 vector was dephosphorylated and the DNA fragment was inserted into the vector by sticky-end ligation. The pQE-30 vector, containing the Sp17 DNA insert, was transformed into *E. coli* XL-1 Blue bacteria (Stratagene, LaJolla, CA). Ten plasmids of the testis and genomic DNA template were purified using the Wizard plasmid purification system (Promega, Madison WI).

The insertion of the PCR amplified DNA fragment was visualized with respect to the T5 promoter of the plasmids by restriction digestion with BamH1 and KpnI. The digested samples were resolved adjacent to an empty pQE-30 vector and 250bp DNA molecular weight marker by agarose gel electrophoresis. The results were visualized with UV light and photographed. Clones exhibiting appropriately sized DNA fragments were sequenced.

3.1.3 DNA sequencing

The orientation of the DNA fragment was confirmed by DNA sequencing. One positive clone of each of the testis and genomic DNA templates were sequenced from the T3 and T7 promoters using ABI BigDye Terminator chemistry (Applied Biosystems; Foster City, CA) by the DBS Automated DNA Sequencing Facility (University of California, Davis, CA). DNA sequence analysis, assembly and alignment was performed using Gene Jockey 1.3 software (BioSoft Ltd., Cambridge, UK). Clones exhibiting forward reading orientation were transformed and expressed.

3.2 Expression of recombinant protein in *Escherichia coli*

3.2.1 Sp17 recombinant protein expression in *E. coli*

The pQE-30 plasmids, containing the Sp17 DNA insert, were transformed into the M15 [pREP4] *Escherichia coli* (*E. coli*) host strain. The *E. coli* bacterial cultures were grown in Luria-Bertani (LB) medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl) containing antibiotics (ampicillin (100ug/mL; Sigma) and kanamycin (25ug/mL, Sigma)) at 37°C overnight with constant agitation (200rpm).

To promote uniform growth and density, the overnight culture (500uL) was diluted in LB medium (10mL) containing antibiotics and grown at 37°C until the spectrophotometric optical density measured 0.5 - 0.7 at an absorbance of 600nm on a BioSpec-1601 DNA/Protein/Enzyme Analyzer (Shimadzu, Kyoto, Japan).

The expression of the recombinant Sp17 protein was induced by addition of Isopropyl b-D-thiogalactopyranoside (IPTG; Sigma) to a final

concentration of 1mM. The induced cultures were grown at 37°C for 3.5 hours. The bacterial pellets were collected by centrifugation (6,000 rpm) at 4°C for 10 minutes and were stored at -20°C. The bacterial lysate was resolved on a 12% polyacrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.2 Sodium dodecyl sulfate polyacrylamide gel-electrophoresis

The SDS-PAGE polyacrylamide gel was prepared as described by Laemmli, 1970 (3). The bacterial pellets from bacterial culture aliquots (1mL) were resuspended in 4X SDS-PAGE sample buffer (10% D-Saccharose, 0.1% Bromophenol blue, 200mM Tris-HCl (pH 8.0), 5mM EDTA (pH 8.0), 2% Sodium dodecyl sulfate, 10mM DTT). The samples were boiled at 95°C for 5 minutes and cooled on ice for 5 minutes. The samples were centrifuged (13,000 rpm) at room temperature for 10 minutes and the supernants (15uL) were resolved adjacent to a 230kDa kaleidoscope pre-stained protein marker (BioRad Laboratories, Hercules, CA) on a 12% polyacrylamide gel by SDS-PAGE. The gels were run at 20mAmps each for 2.5 hours and were stained with Coomassie blue (50% methanol, 10% acetic acid, 40% water, commassie blue 250 (Sigma)). The gels were visualized after destaining with Comassie destain (50% methanol, 10% acetic acid, 40% water) and were digitally scanned into a computer.

3.2.3 Sp17 protein production time course study

To determine the optimal time for the production of the Sp17 recombinant protein, a positive colony of the *E. coli* host strain was grown as described in section 3.2.1. Expression of Sp17 recombinant protein was induced by the addition of IPTG (Sigma) to a final concentration of 1mM.

Aliquots (1mL) were removed at time 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 hours after induction with IPTG. The aliquot taken at time 0 served as the non-induced control. The bacterial pellets cells were collected by centrifugation (13,000 rpm) at room temperature for 2 minutes and resuspended in 1X SDS Page buffer (50uL). The samples were stored at -20°C. The expressed Sp17 recombinant protein was resolved adjacent to a 230kDa kaleidoscope pre-stained protein marker (BioRad) on a 12% polyacrylamide gel by SDS-PAGE.

In addition, to monitor the bacterial growth curve, the optical density of each aliquot (1mL) was spectrophotometrically measured at an absorbance of 600nm on a BioSpec-1601 DNA/Protein/Enzyme Analyzer (Shimadzu, Kyoto, Japan).

3.2.4 Sp17 recombinant protein solubility

To determine the solubility of the Sp17 recombinant protein a positive colony of the *E. coli* host strain was grown in LB medium (5mL) as described

in section 3.2.1. At time zero, a 1mL aliquot of was removed to serve as the non-induced control. The expression of recombinant Sp17 protein was induced by the addition of IPTG (Sigma) to a final concentration of 1mM. The culture was incubated at 37°C for 3.5 hours. At which time another 1mL aliquot was removed to serve as the induced control. The non-induced and induced samples were centrifuged (14,400 rpm) at room temperature for 2 minutes and the pellet was resuspended in 1X SDS-PAGE buffer (50uL).

The 3.5 hour culture was centrifuged (4000 rpm) at 4°C for 15 minutes and the pellet was resuspended in lysis buffer (400 uL; 300mM NaCl, 50mM NaH_2PO_4, 10mM imidazole; pH 8.0). Lysozyme reagent (1mg/mL) was added to the suspension and the sample was sonicated on ice for sixty seconds in ten-second intervals at 200-300W. The sample was centrifuged (13,000 rpm) at 4°C for 30 minutes. The supernant was collected as the soluble fraction. The pellet was resuspend in lysis buffer (400uL) and collected as the insoluble fraction. Aliquots (15uL) of the soluble and insoluble fractions were resuspended in 4X SDS-PAGE buffer (5uL). The non-induced, induced, soluble and insoluble fractions (20uL) were resolved adjacent to a 230kDa kaleidoscope pre-stained protein marker (BioRad) on a 12% polyacrylamide gel by SDS-PAGE.

3.2.5 Purification of the Sp17 recombinant protein

To determine the optimum conditions for purification, a preliminary purification was performed on the expressed Sp17 recombinant protein collected from the bacteria in section 3.2.3.

Briefly, to produce Sp17 recombinant protein, the *E. coli* host strain was grown in LB medium containing antibiotics. The Sp17 recombinant protein, containing a histidine tag at the NH_2-terminus, was purified by nickel chelate affinity chromatography (Ni-NTA resin; Quiagen, Inc.) under native and denaturing conditions according to manufacturer guidelines (Quiagen).

The optimum elution conditions for the tagged Sp17 from the column was determined by the presence of imidazole (20mM), which competes with the tagged recombinant Sp17 protein for histidine binding sites within the Ni-NTA resin. The column fractions were analyzed adjacent to a 230kDa kaleidoscope pre-stained protein marker (BioRad) on a 12% polyacrylamide gel by SDS-PAGE. Optimal purification conditions were experimentally determined to be those described in the manufacturer guidelines.

Additionally the purified Sp17 recombinant protein was dialyzed against PBS in dialysis tubing (12-15 MW). The dialyzed Sp17 recombinant protein was analyzed as compared to non-dialyzed protein adjacent to a 230kDa kaleidoscope pre-stained protein marker (BioRad) on a 12% polyacrylamide gel. The dialyzed protein was then sterilized through a 0.2 micron filter. The

protein concentration was spectrophotometrically calculated using a BioSpec-1601 DNA/Protein/Enzyme Analyzer (Shimadzu, Kyoto, Japan).

Similarly, large scale cultures (2 liters) were grown as described in section 3.2.3 for 3.5 hours; the determined time for optimal expression of the Sp17 recombinant protein. The large scale cultures were subsequently batch purified under the optimal native and denatured conditions, dialyzed and sterilized. The protein concentration was spectrophotometrically calculated.

3.3 Production of anti-Sp17 polyclonal antibodies

3.3.1. Anti-Sp17 polyclonal antibody

An anti-Sp17 polyclonal antibody was generated by subcutaneously immunizing 2, 4-week-old female rabbits (Covance, Denver, PA) with Sp17 recombinant protein. Approval for the use of animals was obtained from the University of California, Davis (Animal Use Protocol #8803). The rabbits were housed and cared for by the UCD Animal Care Facility (UCD, Davis, CA).

The pre-immune rabbits were anesthetized with a subcutaneous injection (0.4mL) of a 5:1.5 solution of Oxymorphone Hydrocloride (1.5mg/ml; Endo Laboratories, Chadds Ford, PA) and Acepromazine (10mg/ml; Vedco, St. Joseph, MO). The rabbits were initially bled (10mL) via marginal ear vein. The whole blood sample was allowed to coagulate at room temperature for 1 hour and then at 4°C, overnight. The sera samples were collected and stored at -20°C until screening for anti-Sp17 activity by ELISA analysis.

While anesthetized, the rabbits were administered a subcutaneous injection (500uL) of Sp17 recombinant protein (500ug) emulsified in complete Freund’s adjuvant (Sigma). Subsequent Sp17 antigen injections were prepared in incomplete Freund’s adjuvant (Sigma). The test bleeds and antigen injections were repeated every two weeks until the desired anti-Sp17 titer was detected by ELISA analysis.

After 16 weeks (seven Sp17 antigen boosts), both rabbits exhibiting anti-Sp17 antibody activity, as measured by ELISA, were euthanized with a subcutaneous injection of a 2.5:0.5 ratio of Ketamine (50mg/kg; Ketaflo Abbott Laboratories, North Chicago, IL) and Xylazine (5mg/kg; Bayer Corporation, Shawnee Mission, KN) and an inter-cardiac injection of Euth-6 (2ml; Western Medical Supply, Arcadia, CA). The rabbits were exanguanated via cardiac puncture. The sera was collected and screened for anti-Sp17 activity by ELISA analysis.

3.3.2. ELISA analysis of anti-Sp17 sera

Briefly, a 96 well, flat-bottom plate (Corning, Corning, NY) was coated (50ul/well) with native Sp17 recombinant protein (10ug/mL) dissolved in

50mM carbonate buffer (35mM Sodium carbonate, 15mM Sodium bicarbonate, anhydrous, pH 9.6) and incubated at 4°C overnight. The plate was washed with wash solution (phosphate buffered saline (PBS): 150mM NaCl, 8mM sodium phosphate, dibasic-anhydrous, 2mM sodium phosphate, monobasic, pH 7.4 containing 0.05% Tween20) and blocked with blocking reagent (200uL/well; 3% BSA in PBS) at room temperature for two hours.

The rabbit pre-immune and anti-Sp17 sera samples were serially diluted with blocking reagent to concentrations of 1:10, 1:100, 1:500, 1:1000, 1:5000, 1:10000. The sera samples were loaded (25uL) onto the plate and incubated at room temperature for one hour. The plate was washed three times with wash solution. The secondary antibody, horseradish peroxidase (HRPO) conjugated goat anti-rabbit IgG (Caltag; Burlingame, CA) was diluted 1:2000 with blocking reagent and added to each well (50uL/well). The plate was incubated at room temperature for one hour. The plate was washed three times with wash solution.

The samples were developed with developing solution (50uL/well; 10mL=5mg 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt (Sigma), 20uL 30% hydrogen peroxide, 2mL 5X citrate buffer (5% Citric acid, 0.74% sodium hydrosulfate (pH 4.2)) and 8mL deionized water). The plate was allowed to stand for 10 minutes after which the reaction was stopped with 1% SDS in PBS (50uL/well).

The optical density of the samples were spectrophotometrically measured at an absorbance of 405nm with a Wallac Victor^2 1420 Multilabel Counter (Wallac, Inc., Norton, Ohio) linked to a PC-programmed with Wallac Victor^2 data analysis software version 2.00, release 8. The immune sera samples were graphically analyzed and compared to the background and pre-immune values.

3.3.3. Dot blot analysis

Stock concentrations of recombinant Sp17, purified under native and denatured conditions, were serially diluted in PBS to final concentrations of 1, 0.5, 0.25, 0.125, 0.625, 0.3125, 0.155, 0.075 and 0.0037 ug per 0.5uL. To control for non-specific binding, a 1mg/mL solution of BSA in PBS was also prepared. A 0.5uL aliquot of each protein dilution was applied to each strip of nitrocellulose paper (Micron Separations, Inc., Westborough, MA). The strips were allowed to air dry for 30 minutes and were blocked at room temperature for one hour with blocking reagent (3% non-fat skim milk in PBS).

Rabbit pre-immume sera and anti-Sp17 sera were serially diluted in blocking reagent to concentrations of 1:10, 1:100, 1:250, 1:500, 1:1000, 1:2500 and 1:5000. Each dotted nitrocellulose strip was incubated with a different concentration of either pre-immune sera or anti-Sp17 sera. The strips were incubated with agitation at room temperature for one hour. Each strip

was washed with agitation four times for 5 minutes with wash solution (PBS with 0.05% Tween 20).

The secondary antibody, HRPO goat anti-rabbit IgG (Caltag), was diluted 1:2000 with blocking reagent, applied to each strip and incubated with agitation at room temperature for one hour. The blots were washed with agitation four times for 5 minutes with wash solution. The blots were developed using SuperSignal chemiluminescent reagents (Pierce) according to manufacturer guidelines and exposed to film.

4. Results

4.1 Analysis of Sp17 expression vector

The Sp17 coding region nucleotide sequence was examined in normal human testis by RT-PCR and in human genomic DNA by PCR (primers Shu and Ahu). Analysis of the PCR products revealed DNA fragments at 482bp corresponding to the coding region of Sp17 (Figure 3.1).

Insertion of the PCR amplified DNA fragment into the pQE-30 expression vector was detected by restriction digest analysis. Restriction digestion of the human testis and human genomic DNA pQE-30 expression vectors revealed bands at 3417bp and at 482bp, compared to the single band at 3417bp of an empty pQE vector (Figure 3.2). The double band pattern confirmed the insertion of a DNA fragment whose size corresponded to the inserted Sp17 PCR amplified DNA fragment (482bp). Clones exhibiting a double DNA band pattern were sequenced.

Sequence analysis of five human testes Sp17 clones confirmed the insertion, sequence and forward orientation of the Sp17 DNA fragments. The DNA insert 100% identical to the published Sp17 nucleotide sequence (Chapter 1, Figure 1.2, (4)). Similarly, sequence analysis of five genomic DNA Sp17 clones revealed a forward oriented DNA insert homologous to the Sp17-2 gene sequence (Chapter 2, Figure 2.1).

4.2 Characterization of the Sp17 recombinant protein in *E. coli*

To detect the expression of the Sp17 recombinant protein, non-induced and induced cultures of the Sp17 expression vector were evaluated by SDS-PAGE. The induced fraction from the testis Sp17 expression vector revealed a strong band at approximately 24.5 - 26kDa as compared to the non-induced control (Figure 3.3A). Conversely, protein expression from the induced genomic Sp17 expression vector was not detected as compared to the non-induced control (Figure 3.3B).

To determine the time needed for the optimal expression of the Sp17 recombinant protein, the host bacteria were monitored for growth and Sp17 protein expression every 30 minutes for five hours (Figure 3.4 and Figure 3.5AB). The time course analysis of Sp17 expression detected the Sp17 recombinant protein as early as 30 minutes after induction as compared to the non-induced control. Although the Sp17

recombinant protein was expressed throughout the five-hour time course, the optimal time for Sp17 protein expression was determined to be 3.5 hours.

The solubility of the Sp17 recombinant protein was determined by evaluating the cytosolic (soluble) and membrane (insoluble) bacterial fractions as compared to the non-induced and induced controls on SDS-PAGE. The Sp17 recombinant protein was strongly detected in the induced and soluble cytosolic fraction as compared to the non-induced and insoluble membrane fraction suggesting that the Sp17 recombinant protein is a highly soluble (Figure 3.6).

The Sp17 recombinant protein was purified under non-induced or induced and native or denaturing conditions. The purification fractions (lysate, flow-through, washes and elutant) were evaluated by SDS-PAGE (Figure 3.7AB). Comparison of the elutants from the non-induced, native and denatured conditions confirmed the expression of the Sp17 recombinant protein. The lysate, flow though and wash fractions confirmed the application, presence and purification of the Sp17 recombinant protein. Elutants from the induced, native and denatured conditions revealed a purified protein band at approximately 24.5kDa.

In addition, the purified Sp17 recombinant protein was dialyzed against PBS. SDS-PAGE analysis of the dialyzed fractions confirmed the presence of the Sp17 recombinant protein before and after the procedure (Figure 3.8).

4.3 Characterization of the rabbit polyclonal anti- recombinant Sp17 sera

ELISA analysis was used to detect anti-recombinant Sp17 reactivity in the rabbit anti-sera. Spectrophotometric analysis revealed anti-Sp17 reactivity as compared to the pre-immune sera (Figure 3.9). Additionally, the anti-Sp17 sera reacted equally toward the Sp17 recombinant protein purified under native and denatured conditions.

Dot blot analysis was performed to determine the activity and sensitivity of the anti-recombinant Sp17 sera for the Sp17 recombinant protein purified under native or denatured conditions, as compared to pre-immune and BSA controls. Anti-Sp17 reactivity was observed against both native and denatured purified Sp17 protein in anti-sera titers as low as 1:1000 dilution (Figure 3.10AB).

5. Discussion

The goal of this study was to create a human Sp17 recombinant protein in order to generate rabbit anti-Sp17 polyclonal antibodies. The anti-Sp17 antibodies would be subsequently used for the immunohistochemical detection of sperm protein 17 in normal tissues.

RT-PCR, PCR and DNA sequence analysis were used to amplify the Sp17 nucleotide sequence from human testis tissue and human genomic DNA. The human testis DNA fragment (482bp) was 100% identical to the published Sp17 sequence (5). As a result, this fragment was presumed to be Sp17-1. Conversely, the genomic DNA fragment exhibited

90% homology to the corresponding 482bp published human Sp17 coding region sequence. This fragment was presumed to be the Sp17-2 pseudo gene.

To generate a Sp17 recombinant protein both DNA fragments were subcloned into pQE-30 and expressed in *E. coli*. The expression of the DNA fragment amplified from testis cDNA resulted in a recombinant protein at approximately 26 kDa. Conversely, expression of a recombinant protein from the genomic DNA fragment was not detected. These results suggest that the Sp17-2 gene does not encode a full length Sp17 protein.

SDS-PAGE is a commonly accepted method to effectively resolve protein purity and size (3). The native Sp17 protein molecule is known to be a 17kDa protein (5). Similarly, the Sp17 recombinant protein, with the addition of a histidine tail, was calculated to be 18.8kDa. However, on SDS-PAGE, the native recombinant Sp17 protein was resolved at approximately 24.5 – 26kDa. This variation in size was explained by Richardson, et. al. (1994) and Yamaski, et. al. (1995) to be the result of the high acidity of the Sp17 protein (1, 6). Thus, due to the dynamics of SDS-PAGE the recombinant Sp17 protein is observed to be variably larger than its' actual size.

Polyclonal antibodies are commonly used in immunohistochemical studies to detect protein expression (1, 6). For this study, rabbits were immunized with recombinant Sp17 protein to generate polyclonal anti-recombinant Sp17 antibodies. The reactivity and sensitivity of these antibodies were determined by ELISA and dot blot analysis. The polyclonal anti-recombinant Sp17 sera exhibited reactivity toward Sp17 recombinant protein purified under both native and denatured conditions. However, anti-recombinant Sp17 reactivity was slightly greater against the denatured recombinant Sp17 protein, suggesting that the anti-recombinant Sp17 polyclonal antibodies recognize epitopes on a denatured Sp17 protein that may not be sterically available to bind to the native protein. Consequently, the anti-recombinant Sp17 antibodies may detect the Sp17 protein and its degradation products. Furthermore, dot blot analysis revealed the optimal anti-recombinant Sp17 sera concentration to detect a known Sp17 protein concentration.

6. Conclusion

The rabbit polyclonal anti-recombinant Sp17 serum exhibits anti-Sp17 reactivity against both the native and denatured recombinant Sp17 protein. Furthermore, the anti-recombinant Sp17 sera may be used to detect the native Sp17 protein and its degradation products in future immunohistochemical studies targeting Sp17 protein expression.

7. Tables and Figures

Table 3.1. Sp17 specific sense and anti-sense primers used to amplify the Sp17 coding region from normal human testis by RT-PCR and in human genomic DNA by PCR.

Sense (Shu)	5'	CGCGGATCCATGTCGATTCCATTCTCC	3'
Anti-Sense (Ahu)	5'	CGGGGTACCAACCAGTGTCCTCACTTG	3'

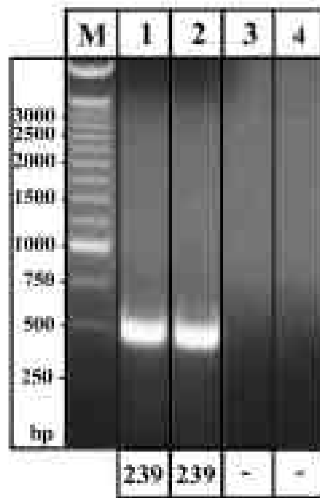

Figure 3.1. DNA amplification of the Sp17 coding region from normal human testis by RT-PCR and in human genomic DNA by PCR The PCR cocktails were prepared with the following templates: 1: cDNA; 2: gDNA; 3: RNA; 4: none (Chapter 1, Table 1.1). The PCR products were measured in bp adjacent to a DNA marker (M). All controls (3 and 4) were negative for genomic DNA.

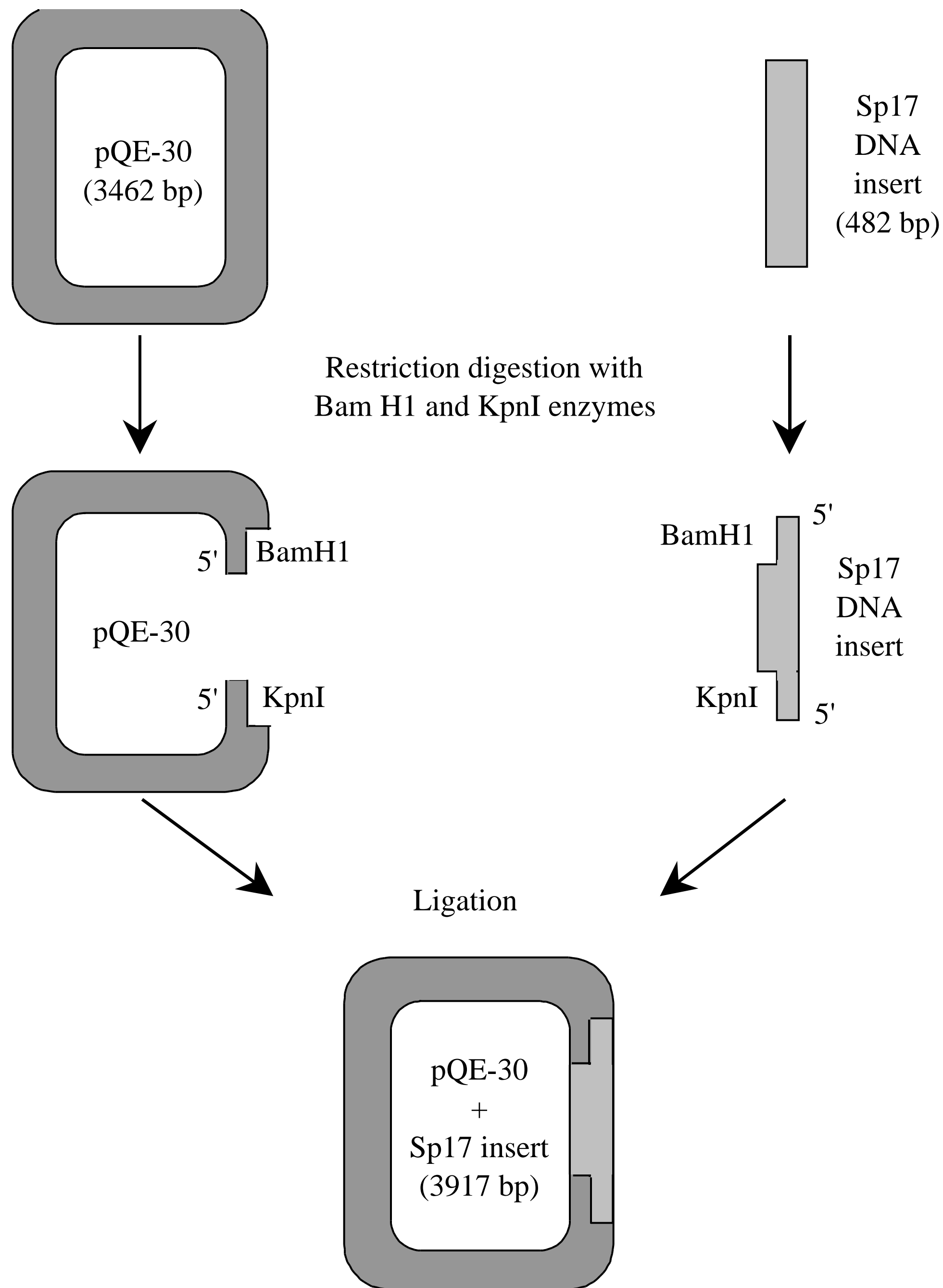

Figure 3.2. A schematic of the Sp17-pQE cloning strategy. An empty pQE-30 vector (3462bp) and the RT-PCR amplified Sp17 DNA insert (482bp) will be double digested with BamH1 and KpnI restriction enzymes. The pQE vector and DNA insert will be ligated together forming a pQE-30/Sp17 expression plasmid (3917bp).

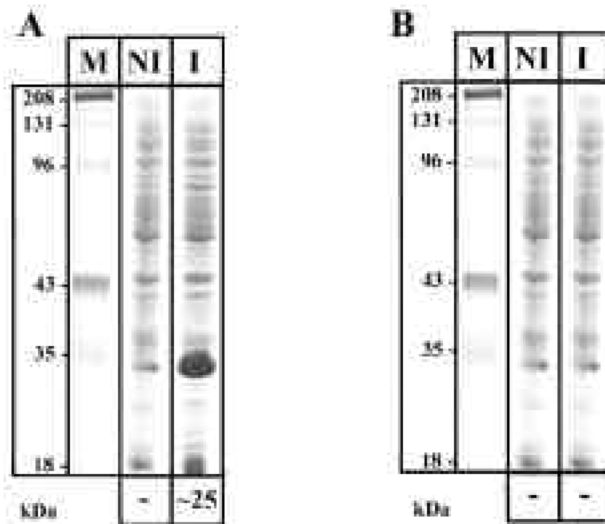

Figure 3.3. Recombinant Sp17 protein expression from pQE-30/Sp17 expression cultures. SDS-PAGE was used to evaluate the expression of Sp17 recombinant protein in non-induced (NI) and induced (I) pQE-30/Sp17 expression cultures generated from a human testis template (A) and genomic DNA template (B). The protein products were measured in kDa adjacent to a protein marker (M). **A**. The induced fraction from the testis Sp17 expression vector revealed a strong band at approximately 24.5 - 26kDa as compared to the non-induced control. **B**. Protein expression from the induced genomic Sp17 expression vector was not detected as compared to the non-induced control.

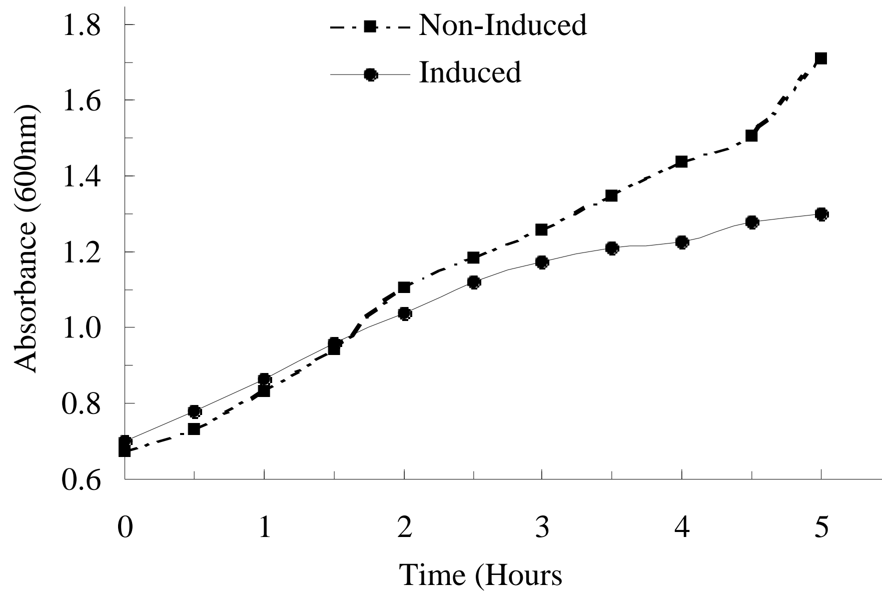

Figure 3.4. Growth rate of pQE-30/Sp17 expression cultures. To monitor the growth rate of pQE-30/Sp17 cultures, bacterial aliquots of non-induced and induced cultures were spectrophotometrically (600nm) analyzed every 30 minutes for five hours.

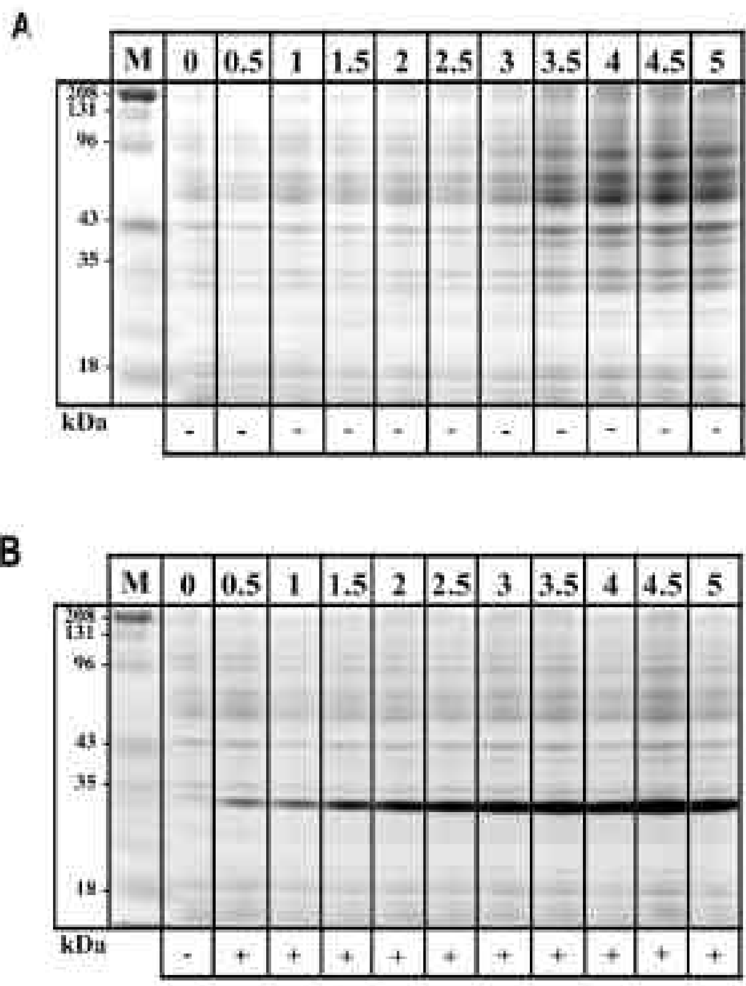

Figure 3.5. Time course evaluation of Sp17 recombinant protein expression from pQE-30/Sp17 expression cultures. To determine the optimal expression of the Sp17 recombinant protein, pQE-30/Sp17 cultures were analyzed every 30 minutes for five hours on SDS-PAGE. The protein products were measured in kDa adjacent to a protein marker (M). **A**. Non-induced cultures served as a negative control. **B**. Recombinant protein expression was induced with IPTG (1mM).

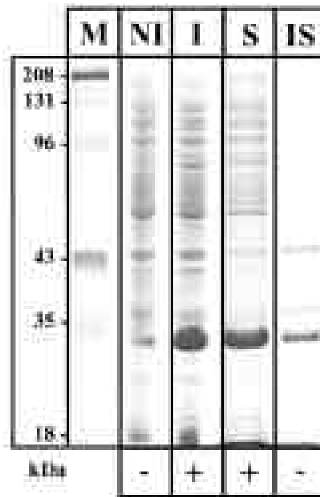

Figure 3.6. Solubility of Sp17 recombinant protein from pQE-30/Sp17 expression cultures. The solubility of the Sp17 recombinant protein was determined by evaluating the cytosolic (soluble; S) and membrane (insoluble; IS) bacterial fractions as compared to the non-induced (NI) and induced (I) controls on SDS-PAGE. The protein products were measured in kDa adjacent to a protein marker (M). The Sp17 recombinant protein was strongly detected in the induced and soluble cytosolic fraction suggesting that the recombinant Sp17 protein was effectively solubilized.

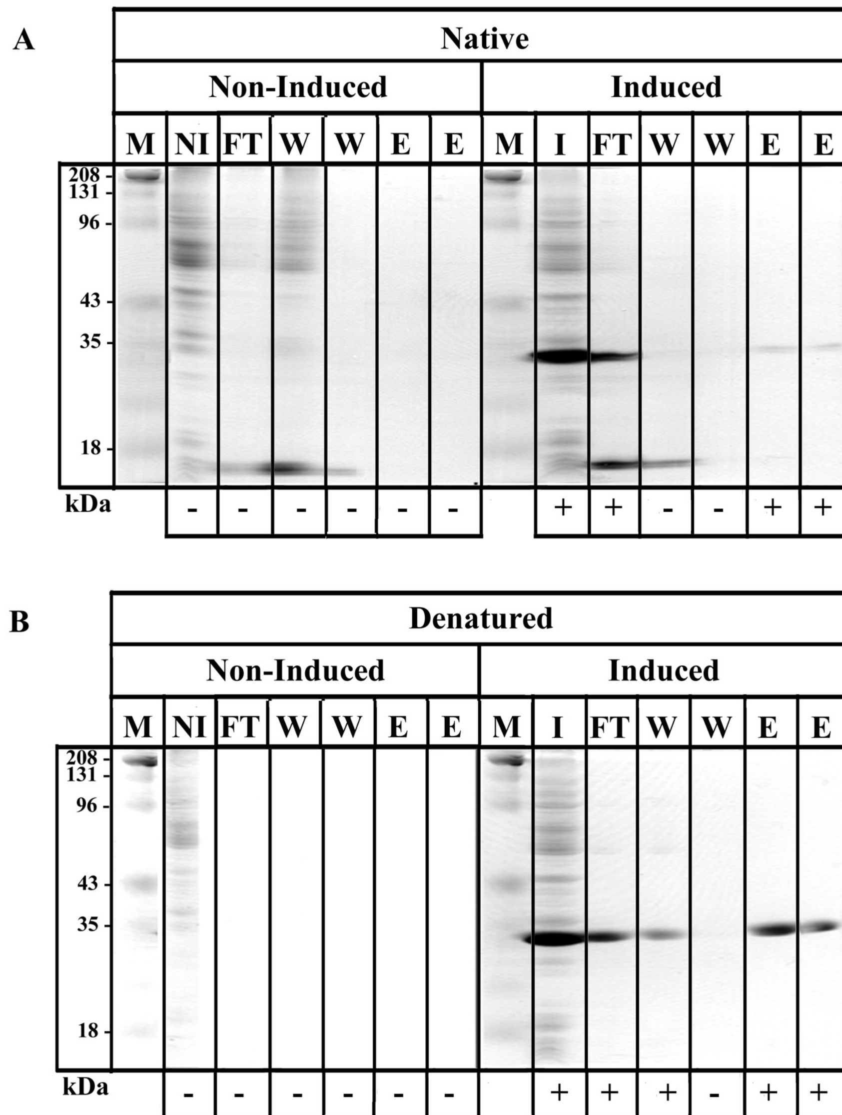

Figure 3.7. Purification of Sp17 recombinant protein from pQE-30/Sp17 expression cultures. The Sp17 recombinant protein was purified from non-induced and induced cultures by nickel chelate affinity chromatography (Ni-NTA resin; Quiagen, Inc.) under native (**A**) and denaturing (**B**) conditions. The non-induced (NI), induced (I), flowthrough (FT), wash (W) and elution (E) fractions were resolved by SDS-PAGE. The protein products were estimated in kDa from an adjacent protein/molecular size standards (M).

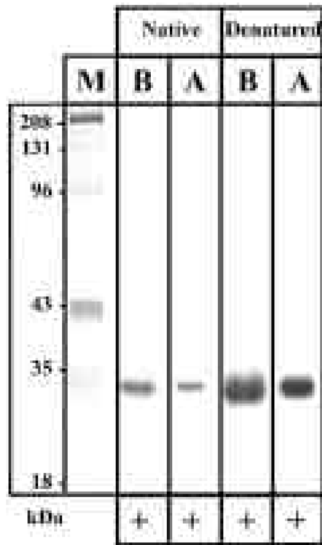

Figure 3.8. Dialysis of purified recombinant Sp17 protein. The Sp17 recombinant protein was purified under native and denaturing conditions. The purified Sp17 recombinant protein fractions were dialyzed against PBS. The fractions were resolved by SDS-PAGE before (B) and after (A) dialysis. The resulting Sp17 protein was measured in kDa adjacent to a protein marker (M).

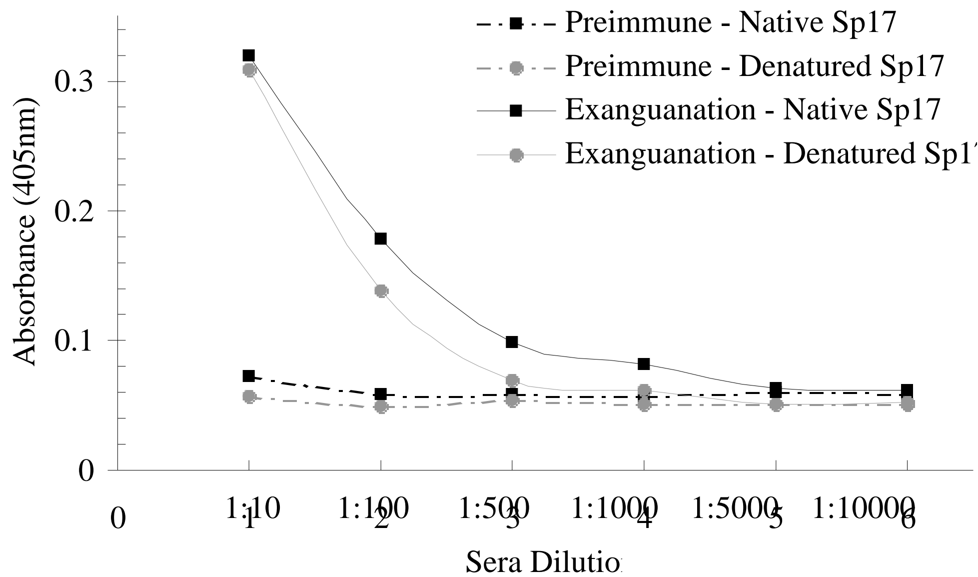

Figure 3.9. ELISA analysis of rabbit anti-recombinant Sp17 polyclonal sera. Two rabbits were immunized with recombinant Sp17 protein. After seven boosts, the rabbits were exanguanated and the anti-sera were analyzed for anti-Sp17 reactivity using Sp17 recombinant protein purified under native and denaturing conditions. Pre-immune rabbit sera served as the negative control. The results for the two rabbits were averaged.

A **Native Purified Recombinant Sp17 Protein**

[Protein] ug	1:100	1:250	1:500	1:1000	1:2500
BSA (1mg/mL)	-	-	-	-	-
0.015	+	-	-	-	-
0.031	+	-	-	-	-
0.0625	+	+	-	-	-
0.125	+	+	-	-	-
0.25	+	+	+	-	-
0.5	+	+	+	+	-
1.0	+	+	+	+	-

Anti-Sp17 Sera Dilution

B **Denatured Purified Recombinant Sp17 Protein**

[Protein] ug	1:100	1:250	1:500	1:1000	1:2500
BSA (1mg/mL)	-	-	-	-	-
0.015	-	-	-	-	-
0.031	+	-	-	-	-
0.0625	+	+	-	-	-
0.125	+	+	+	-	-
0.25	+	+	+	-	-
0.5	+	+	+	+	-
1.0	+	+	+	+	-

Anti-Sp17 Sera Dilution

Figure 3.10. Dot blot analysis of rabbit anti-recombinant Sp17 polyclonal sera. The Sp17 recombinant protein purified under native (**A**) and denatured (**B**) conditions was applied to nitrocellulose paper and incubated with serially diluted rabbit anti-Sp17 sera. BSA served as a negative control. Positive results are indicated by a + and negative results are indicated by a -. All rabbit pre-immune sera controls were negative for anti-Sp17 reactivity.

8. References

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