

Replacement of the Reactive Site Loop of Human Antithrombin III with that of Protein C Inhibitor.

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ABSTRACT

Antithrombin and protein C inhibitor are essential factors in the coagulation cascade and function in the maintenance of homeostasis. Maintaining homeostasis is often the most important factor in the prognosis of patients undergoing surgery or suffering from severe trauma. The reactive-site loop of Human Antithrombin III was manipulated through site-directed mutagenesis to contain the amino acids of the reactive site loop of protein C inhibitor. The recombinant proteins were expressed in *Trichoplusia ni* cells using the Baculovirus Vector Expression System. Recombinant proteins were purified, assayed for activity, and found to be inactive. Upon further review of the DNA sequence it was discovered that an essential amino acid had been changed which prevented the protein from assuming the proper tertiary confirmation rendering the protein inactive.

INTRODUCTION

Antithrombin III is a plasma serine protease inhibitor which functions to inactivate proteins by attacking the protein at the PI position resulting in the formation of an ester linkage with the PI residue with a serine residue in its active site (1,2). Human Antithrombin III (AT III) has been previously expressed using the Baculovirus Vector Expression System using *Spodoptera fugiperda* cells. This protein was immunologically reactive with antibodies against AT III from human plasma and reacted with heparin at the same molar concentration as nascent antithrombin (1).

ATIII inhibits factors IXa, Xa, XIa, XIIa, and Thrombin in the coagulation cascade (1). Inactivation of thrombin by antithrombin prevents the cleavage of fibrinogen into fibrin which is necessary for blood clot formation. Thrombomodulin is also an important factor in the coagulation cascade. Thrombomodulin (TM) is a glycoprotein expressed on the surface of endothelial cells that binds thrombin. Thrombin bound to TM serves as a means of negative feedback, shunting the production of more thrombin (3). Thrombin bound to TM also activates protein C which can inactivate factors Va and VIIIa preventing the cleavage of prothrombin into thrombin (4). Protein C inhibitor can bind thrombin bound to TM preventing the activation of protein C, and therefore allowing factors Va and VIIIa to cleave prothrombin into thrombin.

Site-directed mutagenesis. was employed to change the amino acid sequence of the reactive-site loop of AT III to those of the reactive-site loop of protein C inhibitor (PCI). Another point mutation was made at the P3 position which changed the Threonine residue in the reactive-site of PCI to an Arginine residue. This change was made to influence the binding strength of the recombinant molecule to its substrate (Figure 1). The resulting recombinant molecules could have a dual function of inhibiting thrombin, acting as an anticoagulant to

prevent venous thrombus formation, but not thrombin bound to TM, acting as a pro-coagulant allowing clot formation at the endothelial surface.

	P4	P3	P2	P1	P1'	P2'	P3'	P4'
PCI:	Phe	Thr	Phe	Arg	Ser	Ala	Arg	Leu
AT:	Ile	Ala	Gly	Arg	Ser	Leu	Asn	Pro
rAT 6A:	Ile	Thr	Phe	Arg	Ser	Ala	Arg	Pro
rAT 6B:	Ile	Arg	Phe	Arg	Ser	Ala	Arg	Pro

Figure 1: Mutations made to the Reactive-Site Loop of Human AT III.

METHODS

AT III cDNA obtained from the ATCC was clone into plasmid pKT218 with a GC clamp in the N-terminal end of the gene. The gene was PCR amplified and cloned into pGEM by former students.

Site-Directed Mutagenesis. Plasmid containing the AT III cDNA obtained from culture using Qia Spin Miniprep Kit, Qiagen. Quik Change Site-Directed Mutagenesis Kit, Stratagene was used to direct the mutagenesis. Developed mutagenesis primers AT 6: AGT-CACCCTGTTGGGTCTTGCCGA, and AT 6 RC: ACGGAATSTAATCACAACAGC. (All primers used in this investigation were manufactured by Integrated DNA Technologies). Mutagenesis reaction run in 16 cycles of : 95°C 30s, 55°C 60s, 68°C 2/min per kb plasmid (9min); using *Pfu* DNA polymerase. Products digested with *Dpn* I to remove unmutagenized strands. Mutations were confirmed using Thermosequenase Kit, Amersham Life Sciences and the following primers : pUC/M13 reverse sequencing primer: GGAAAACAGCTATGAC-CATC; Sp6: GATTTAGGTGAC ACTATAG; AT 9: TAAGACCGAAGGCCG; and AT 10: CAGTGAGCTCATTGATGG. Primers AT9 and AT10 were developed to sequence through the *Nco* I site, Sp6 and pUC/M13 were used to sequence the gene from both directions to ensure no other mutations had been introduced during the mutagenesis. Sequencing reaction was run for 30 cycles of: 95°C 30s, 55°C 30 s, 72°C 60s. Products were electrophoresed on a glycerol tolerant gel, exposed to film, and sequenced.

Homologous Recombination. The mutagenized region of AT III was located in between the *Nco*I and *Bam*HI restriction endonuclease sites. This region was subcloned out of pGEM and into the plasmid pVL-1392 which contained a wild type copy of the AT III cDNA. This new construct was used in the homologous recombination with Baculoviral DNA. The Baculovirus Vector Expression System, PharMingen was used to facilitated the homologous recombination of the mutagenized AT III-pVL1392 with Baculoviral DNA. Recombinant virus was amplified in *Spodoptera fugiperda* cells. *Tricopsulina ni* cells (0% serum) were infected with the amplified recombinant virus to produce the recombinant protein.

Analysis of Recombinant Protein. Recombinant proteins purified from media using HiTrap Heparin Affinity Chromatography. The concentration of produced proteins determined using ELISA with Rabbit - anti AT III and Alkaline phosphatase conjugated Goat - anti- Rabbit antibodies with para-Nitro phenyl phosphate as substrate. Recombinant proteins visualized by Immunoblot utilizing same antibodies as in ELISA with Nitro Blue Tetrazoleum/ Bromo Chloro Indoyl Phosphate as substrate. The activity of the recombinant proteins analyzed using a time course assay with 2 nM thrombin, 100nM protein, 1 μ g/ml Heparin, and 10 mM Thrombin chromogenic substrate.

RESULTS

Preliminary sequencing results indicate that the desired mutations had been made to the reactive site loop. The homologous recombination of the Baculoviral DNA and AT III-pVL1392 was successful as evidenced by the formation of a CaHPO₄ precipitate upon mixing of the two species of DNA in appropriate Buffer. The disappearance of this precipitate approximately 4 hours after introduction to the *Tricopsulina ni* cells indicate its endocytosis by the cells. *Tricopsulina ni* cells were successfully infected with the recombinant Baculovirus as evidenced by rounding of the cell, swollen nuclei, and detachment from culture flask. Uninfected cells grow attached to the culture flask, have small nuclei, and are relatively long and thin in appearance.

Results from the ELISA show that the recombinant protein 6B was produced in microgram quantities. However, the recombinant protein 6A was undetected by the ELISA. Visualization of the recombinant proteins show that 6B was produced at the correct molecular weight of approximately 58 kDa. Recombinant 6A was detected very faintly as a series of smaller molecular weight bands indicating the protein was susceptible to protease activity *in vivo* (Figure 2). No activity was detected in either of the recombinant proteins.

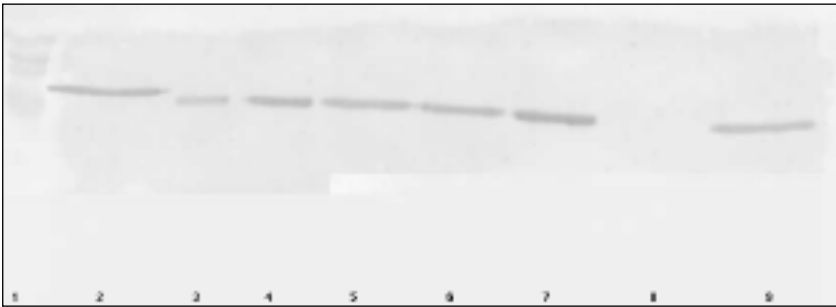


Figure 2: Immunoblot analysis of recombinant proteins. Lane 1: Bio-Rad prestained Standards: Myosin, 206kDa; (-)Galactosidease, 121 kDa; Bovine Serum Albumin, 74 kDa; Ovalalbumin, 47 kDa; Lane 2: Human Antithrombin III; Lane 3: wild type recombinant AT; Lane 8: recombinant protein 6A; Lane 9: recombinant protein 6B.

DISCUSSION

Further analysis of the DNA sequence using BLAST analysis with the original AT cDNA sequence (5) and the experimentally derived sequence revealed that a key amino acid in the N-terminal portion of the gene had been changed. A point mutation was made at base 2746

which change amino acid #95 from a Cysteine to an Arginine residue. It is believed that these mutations were introduced during the PCR amplification from the original plasmid. It has been previously reported that the Cysteine residue at amino acid #95 forms a disulfide bond with amino acid #21 and it has been suggested that this structure is essential in binding the serine protease to its substrate (6). This analysis also showed that an Arginine residue at amino acid position 46 was missing. The absence of this residue could also presumably affect the protein's ability to assume the proper tertiary confirmation due to disruption of charge interactions.

The degradation of the recombinant protein 6A due to the one amino acid substitution of a Threonine residue versus the Arginine residue in the 6B recombinant is very interesting. Perhaps the presence of the Arginine residue in the 6B recombinant allows the protein to assume a tertiary confirmation such that in combination with the protein confirmation resulting from the other two amino acid mutations described above makes this protein resistant to the protease which degraded 6A.

Future plans for this investigation are to subclone the mutagenized segment of the AT III cDNA (the segment between the *Nco* I and *Bam* HI restriction endonuclease sites) back into the original plasmid, confirm the sequence, and continue on with protein expression.

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