Expression of recombinant 3-beta hydroxysteroid dehydrogenase protein in *E. coli*

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Abstract
3-beta hydroxysteroid dehydrogenase (3BHSD) is secreted by the cortex of adrenal gland functioning in stress conditions. The gene encoding the 3-beta hydroxysteroid dehydrogenase protein was PCR amplified from a λgt11 cDNA library using specific primers. The amplified PCR product was then cloned into pGEX-4T-1 expression vector under Ptac *promoter and the expression of the enzyme was examined in E. coli (BL21). Upon optimization of the expression condition, the enzyme was produced as a glutathione S-transferase (GST) fusion protein, which was purified by affinity chromatography using glutathione sepharose column. The GST part was then removed by selective proteolytic digestion with thrombin. The purified recombinant enzyme could be used in construction of diagnostic kits for screening the patients with premature ovarian failure (POF) for the presence of autoantibodies against 3BHSD as an important molecular target.*
Keywords: *3-beta hydroxysteroid dehydrogenase, Recombinant Protein, Autoantibody*

INTRODUCTION

Premature ovarian failure (POF) is a disease associated with steroid cell autoantibodies (SCA), comparising of several different patterns of autoantibodies against ovarian tissues (Valloton and Forbes, 1996; Sotsious, 1980, Pal and Santoro, 2002). It has been estimated that approximately one fifth of POF cases are the results of an autoimmune process, which is difficult to identify (Conway *et al.*, 1996). The main problem is the low analytical sensitivity of immunofluorescence technique, which is normally used (Falorni *et al.*, 2002). Identification of the molecular targets of SCA would enable the development of more sensitive approaches (e.g. immunoassays) for detection of the autoantibodies.

In our previous study we reported that autoantibodies against 3-beta hydroxysteroid dehydrogenase (3BHSD) produced in a large number of these patients. Therefore detection of the level of the enzyme could be of diagnostic value in POF patients. Several molecular targets of these autoantibodies were identified using a high titer serum with the SCA pattern typical of POF. The most reactive target was found to be the enzyme, 3BHSD (Arif *et al.*, 1996). The result suggested that the 3BHSD enzyme is a novel target for steroid cell autoantibody, which demonstrates the association of autoantibodies with endocrine autoimmune diseases. 3BHSD is involved in catalyzing the conversion of pregnenolone to progesterone in the biosynthesis of steroid sex hormones (Rheaume *et al.*, 1991). The enzyme is the fourth enzyme to be identified as an autoantigenic target in the biosynthesis of steroid hormones pathway. Deficiency in the enzyme activity has been reported in association with some form of menstrual dysfunction, typically oligo- or amenorrhea (Griffin *et al.*, 1994).

In the present study, the cloning and expression of 3BHSD was investigated. The gene was isolated from a λgt11 complementary DNA (cDNA) expres-

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sion library. The expression of the gene was then analyzed under p*tac* promoter in *E. coli*. This protein can be used to develop sensitive immunoassay techniques based on immunoblotting, enzyme-linked immunoabsorbent assay (ELISA) or radioimmuno assay (RIA) formats in diagnostic kits for screening the SCA patients.

MATERIALS AND METHODS

Polymerase chain reaction (PCR) amplification and sequencing of 3BHSD gene: Human adrenal λgt11 cDNA expression library (Dr. Krohn, university of Tampere, Finland) was screened with a single serum sample from a patient with polyendocrinopathy on the basis of high titer positivity for the SCA pattern, typically associated with POF. The positive clones were selected, partially amplified and sequenced. Several clones showed above 85 to 93% similarity to the sequences reported for 3BHSD gene using the blast system (Karlsen *et al.,* 1991; Rheaume *et al.,* 1991).

Clones with the highest similarity were chosen and subjected to PCR amplification using λσgt11 common primers (Invitrogen Corp., San Diago, CA). A standard PCR was performed using *Taq* DNA polymerase enzyme. The PCR cycles were as follows: 1 cycle at 94°C followed by 35 cycles consisting of 1 min at 94°C; 1 min at 57°C, 2 min at 72°C and one cycle of 72°C, for 7 min The amplified DNA fragments were subcloned into a TA cloning vector (Invitrogen Corp., San Diago, CA). The recombinant plasmids were transformed into *E. coli* (XL1-blue) and the positive clones were selected based on the blue/white assay on X-gal plates as described previously (Vallian *et al.,* 1998). The clones containing the recombinant plasmids (the plasmids with inserts) were cultured separately and the plasmids were isolated and purified using the Qiaex minipreparation kit following the instruction provided by the manufacturer (Qiagen Inc., Germany). The purified plasmids were then sequenced using the common primers flanking the inserted DNA fragments in the TA cloning vector and three sets of primers to different regions of 3BHSD cDNA. The sequence of the primers was:

F1= (5´-GTGTGATGGAGAAAGG-3´)
R1= (5´-ACTAAAGAAAATGAGAG-3´)
F2= (5´-TGAAGCCAAACAGAAAG -3´)
R2= (5´-TCCTACCTCTTTCAGC-3´)
F3 = (5´-CATCTCAAACCCAGCGG-3´)
R3 = (5´-ATGGACAAAGAAGGCAC-3´).

The primers used in this study were designed based on the published sequences and were made at King's College oligo service, King´s College London, UK. The sequencing was performed on an automated sequencing machine (ABI Inc., USA).

Cloning, expression and purification of 3BHSD: The full-length cDNA encoding the 3BHSD protein was expressed using glutathione S-transferase (GST) gene fusion system (Pharmacia, Milton Keynes, UK). The cDNA was sub-cloned into the pGEX4T-1 vector. Two mutagenic primers flanking the coding sequence of 3BHSD introducing *Bam*HI and *Not*I restriction sites at the 3´- and 5´-ends of the sequence, respectively, were used in PCR reactions. The sequence of the primers were as follows:
Forward primer:
5´- CGTGGATCCATGGTCTCGGGCCTCTACG-3´
Reverse primer:
5´- ATGCGGCCGCTATTGTGCGAC-3´

The vectors were transformed into *E. coli* (BL21) cells and expressed. Transformation and expression of GST fusion proteins were performed as described previously (Maniatis *et al.,* 1989). In this system the 3BHSD protein was expressed fused to the GST in the form of GST-3BHSD fusion protein. The expressed proteins were released from bacterial cells by three cycles of freezing in liquid nitrogen and thawing at 37°C. The released proteins were purified by affinity chromatography kit containing mini spin columns coated with glutathione-sepharose beads (Pharmacia Biotech Inc., UK). The purified enzyme was cleaved from the GST part using thrombin protease, which has a site at the fusion point following the instructions provided by the manufacturer.

SDS-PAGE and Immunoblotting: The recombinant 3BHSD protein was analyzed by SDS-PAGE as described previously (Wu *et al.,* 2001). The presence and level of expression of 3BHSD protein was analyzed by densitometer (Bio-Rad, USA). To analyze the specific expression of the protein, a sample was run on a SDS-PAGE and transferred onto nitrocellulose membrane. The sample was then resolved and subjected to immunoblotting. The antibody used against 3BHSD was a rabbit polyclonal antibody provided by Dr. J. S. Peterson, King´s College London.

RESULTS

PCR amplification and cloning of 3BHSD cDNA: In order to express the full-length 3BHSD cDNA, the preserved clones from the previous study were analyzed. The insert was PCR amplified using the common specific primers flanking the cDNA inserts in λgt-11 vectors. As shown in figure 1, eight positive clones were amplified. The PCR products from clone 1, 2 and 5-8 were about 800 bp and from clones 3 and 4 were about 2000 bp. In addition to the major bands, few low mobility bands were produced in the PCR products, which were considered as nonspecific amplification and only the major bands were purified on gel and used for further analysis (Fig. 1). The PCR products from clones 3 and 4, which might contain the full length 3BHSD cDNA were cloned into a TA cloning vector and transformed into XL1-blue bacterial host cells. The white cells containing the insert were isolated on X-gal plates. The isolated cells were cultured, and subjected to plasmid isolation. Eight colonies were isolated and the presence of the insert was confirmed by enzymatic digestion. As illustrated in figure 2, all the clones contained the insert with the expected size of about 2000 bp (lanes 1-6). The plasmid DNA from first three colonies (lanes 1-3) were purified and sequenced (Fig. 2).

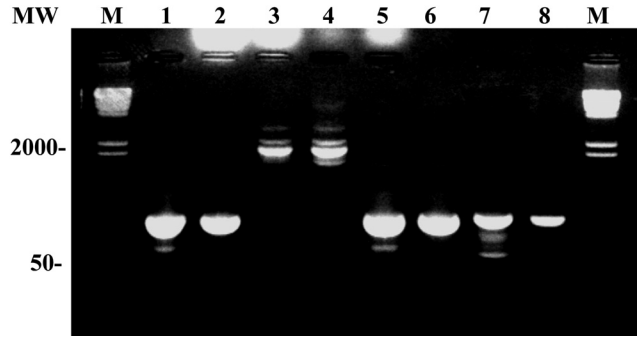

Figure 1: PCR amplification of 3-beta hydroxysteroid. Eight positive clones from screening of an adrenal cDNA expression library with patient sera with POF were PCR amplified. The PCR products were analysed on 1% agarose gel in Tris-Boric acid Buffer (TBE, Maniatis *et al.*, 1989). M represents the lambda *Hind*III DNA size marker, and the numbers indicate different clones. Lanes 1-2 and 5-8 are almost 800 bp and lanes 3-4, 2000 bp.

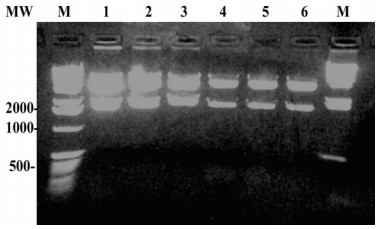

Figure 2: Restriction digestion analysis of clones. Six colonies were analyzed for the presence of the 3BHSD coding sequences. The plasmid DNA was extracted from each bacterial clone and digested with *Bam*HI and *Not*I enzymes. This double digestion releases the 3BHSD cDNA fragment of about 1980 bp. As shown, all the clones (lanes 1-6) contain the cDNA insert. M (left) represents the 1 Kb DNA size-marker, and M (right) the lambda *Hind*III DNA size marker. The gel condition was as in figure 1.

Isolation and subcloning of the coding sequences for 3BHSD: The cDNA containing the coding sequences for the 3BHSD protein was then amplified by PCR using engineered primers containing *Bam*HI and *Not*I sites to introduce these sites at the 3'- and 5'-end of the resulting PCR product, respectively. The introduction of the *Bam*HI site was according to the site present in the pGEX4T-1 expression vector. This could result in an in-frame cloning of the 3BHSD cDNA fragment to the sequences of the GST protein present in the vector.

This vector is part of the glutathione S-transferase gene fusion system, which contains a *Ptac* promoter for chemically inducible expression of recombinant proteins. The vector itself contains an internal *lac* I^q gene, which makes it suitable for using in any *E. coli* host. The recombinant vectors were transformed into *E. coli* cells (DH5α strain) and propagated.The colonies containing the insert were selected using the enzymatic digestion with the *Bam*HI/*Not*I digestion (Fig. 3, lanes 3, 4, 6, 7). This digestion produced the expected band with 1986 bp. The colonies with the insert were further digested with *Bgl*II/*Eco*RI (lane 2, an internal) and *Bgl*II/*Xho*I (lane 5, external) restriction sites to check the correct orientation of the insert. These digestions produced bands of 950, 700 and 1286 bp bands, respectively (Fig. 3).

Analysis of 3BHSD expression: The recombinant pGEX4T-1/3BHSD plasmid was purified from DH5α cells and transformed into BL21 cells. This strain is recommended for expression of GST fusion proteins, as it lacks certain proteases. The cells were cultured

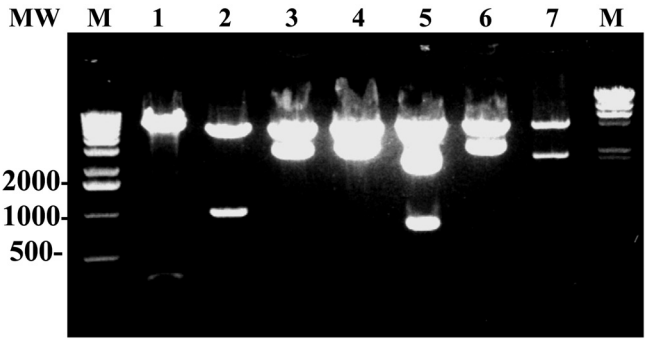

Figure 3: Restriction digestion analysis of the pGEX/3BHSD recombinant vector. The correct cloning of the 3BHSD cDNA into pGEX-4T-1 vector was confirmed by digestion of the plasmid with *Bam*HI/*Not*I enzymes (lane 3, 4, 6 and 7) and with *BglII*/*Eco*RI (lane 2), *BglII*/*Xho*I (lane 5). M (left) represents the 1 Kb DNA size marker, and M (right) the lambda *Hind*III DNA size marker. The gel condition was as in figure 1.

and the protein expression was induced by adding isopropyl-α-D-thiogalactoside (IPTG) to the culture medium.

Following expression, the total cell lysate was prepared and analyzed on SDS-PAGE. A major band with 68 KD was observed indicating the presence of the GST-3BHSD protein (Fig. 4A, lane 2). The relative expression of 3BHSD, was assessed when the affinity-purified protein from bacterial cells expressing the 3BHSD were separated on SDS-PAGE and stained with Coomassie blue. The bands were analyzed on a densitometer. The relative density of the bands show that approximately 30% of the total bacterial proteins contained the 3BHSD protein (data not shown), which was relatively high.

In order to examine the specificity of the expressed 3BHSD protein, an immunoblotting assay was performed on the protein, which was purified on glutathione 4B column and cut with thrombin to release the GST fusion part. The results indicated the presence of a major reactive band with molecular weight of 42 KD resembling 3BHSD protein. A few weak bands with low mobility bands, which could result from degradation of the protein, were also observed (Fig. 4B, lanes 1, 2).

DISCUSSION

In the present study cloning and expression of the gene encoding 3BHSD was carried out in *E. coli.* the 3BHSD protein was previously reported as an autoantigen in patients with POF (Arif *et al*, 1996). This finding suggested that 3BHSD could be used as a molecular target in developing sensitive immunoassay techniques based on immunoblotting, enzyme-linked immunoabsorbent assay (ELISA) or radioimmuno assay (RIA) formats in diagnostic kits for screening the patients with POF. In fact one of the main problems in identifying the patients with POF has been the low sensitivity of immunofluorescence techniques available (Conway *et al.*, 1996).

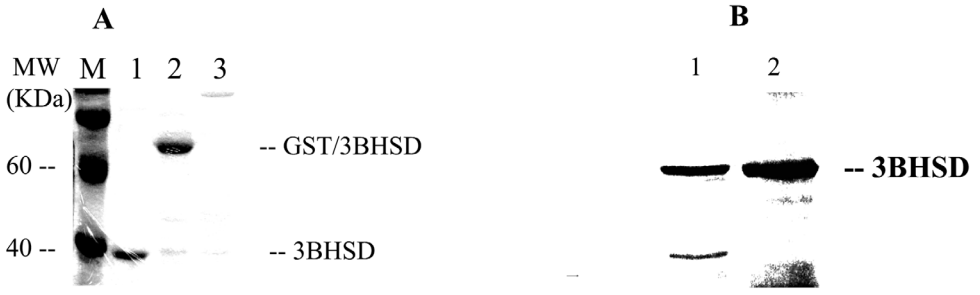

Figure 4: SDS-PAGE analysis of the expression of 3BHSD protein. The expressed proteins were affinity purified on glutathione 4B columns and subjected to SDS-PAGE analysis. A) Coomassie blue staining. The GST and 3BHSD proteins were analyzed on 10% polyacrylamide/bis-acrylamide gel. The gel was stained in 0.5% Coomassie briliant dye. Lane 1 contains the thrombin-digested GST/3BHSD protein with the molecular weight of 42 KDa. Lane 2 contains the undigested GST/3BHSD protein complex with 68 KDa. Lane 3 contains the eluate with no proteins. B) Immunoblotting of 3BHSD with a polyclonal antibody. The GST/3BHSD protein was dialysed using dialysis membrane with cut-off 30 KDa to remove small degraded proteins. Lane 1 contains the proteins before dialysis and lane 2 after dialysis.

The expression of 3BHSD was performed in a GST fusion system, as this system provides an integrated environment for the expression and purification of the fusion proteins. The specificity of 3BHSD purified with this system was verified using a polyclonal antibody (Fig. 4B). The protein was first produced as fusion with the GST protein, purified on affinity columns containing the glutathione 4B beads that would specifically bind the GST part. Then, the bound proteins were released from the affinity matrix in a very mild elution condition. The main disadvantage of this system could be the presence of the GST protein linked to the 3BHSD protein. However, the system has been designed in such a way that the GST part can be cleaved away from 3BHSD by the site-specific protease, thrombin. The cost of the thrombin enzyme and the lack of complete digestion of the fusion protein may seem to be problematic. However, since a small quantity of the protein is required in immuno-based assays, this system may prove to be sufficient.

Recombinant proteins produced in bacteria lack sufficient post-translational modifications (Makrides, 1996). The 3BHSD enzyme, which was produced in *E. coli* BL21, might lack some of the modifications that normally take place in human. These modifications may require for the biological function of the enzyme. However, since the produced enzyme retained its antigenicity and remained reactive to the antibodies to the native enzyme, it seems that post-translational modifications are not so critical for the antigenicity of the enzyme. The human kidney 11-beta-hydroxyseroid dehydrogenase (11-HSD2) has been successfully expressed in *E. coli* (Nunez *et al.,* 1999). Moreover, functional recombinant human interleukin-6 has been expressed in *E. coli* (Ejima *et al.,* 1999). Therefore, the lack of post-translational modifications may have no serious harm to the function of the protein as a molecular target in immuno-based assays. Alternatively, mammalian or insect cells could be used as host cells for expression of the functional enzyme, as theses systems provide sufficient modifications to the expressed proteins. However, they require their own expression vectors with suitable eucaryotic promoters.

Together, in this study recombinant human 3BHSD enzyme was expressed in *E. coli.* The protein was partially purified and analyzed by SDS-PAGE. The enzyme was found to be recognized by polyclonal antibodies raised against the enzyme. The enzyme could be further purified, characterized and used in diagnostic methods for specific detection of autoantibodies in patients with POF.

Acknowledgments

We are grateful to Dr. J. S. Peterson, King´s College London for providing the 3BHSD polyclonal antibody and Dr. Krohn, university of Tampere, Finland, for the adrenal cortex cDNA expression libraray.

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