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Analysis of antigenic domain of GST fused major surface protein (p30) fragments of *Toxoplasma gondii*

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Abstract: Antigenic domain of major surface protein (p30) of *Toxoplasma gondii* was analyzed after polymerase chain reaction (PCR) of its gene fragments. Hydrophilic or hydrophobic moiety of amino acid sequences were expressed as glutathione S-transferase (GST) fusion proteins. Fragments of p30 gene were as follows: T37, total p30 open reading frame (ORF); S28, total ORF excluding N-terminal signal sequence and C-terminal hydrophobic sequence; A19, N-terminal 2/3 parts of S28; P19, C-terminal 2/3 of S28; X9, N-terminal 1/3 part of S28; Y10, middle 1/3 of S28; and Z9, C-terminal 1/3 of S28, respectively. Primer of each fragment was synthesized to include clamp sequence of *EcoR* I restriction site. PCR amplified DNA was inserted into GST (26 kDa) expression vector, pGEX-4T-1 to transform into *Escherichia coli* (JM105 strain). GST fusion proteins were expressed with IPTG induction as 63, 54, 45, 45, 35, 36, and 35 kDa proteins measured by SDS-PAGE. Each fusion protein was confirmed with GST detection kit. Western blot analysis with the serum of a toxoplasmosis patient revealed antigenicity in proteins expressed by T37, S28, and A19 but not those by P18, X9, Y10, and Z9. Antigenicity of p30 seems to be located either in N-terminal 1/3 part in the presence of middle 1/3 part or in the oligopeptides between margins of the first and second 1/3 parts.
Key words: *Toxoplasma gondii*, p30, PCR, expression, western blot, antigenic domain

INTRODUCTION

Toxoplasma gondii, an obligate intracellular protozoan parasite, has long been recognized as a pathogen of congenital defects or abortion and an opportunistic pathogen of acquired toxoplasmosis in immune compromised patients of any cause (Luft, 1989; Buxton, 1990).

p30 is a major immunogenic protein of surface membrane of *T. gondii* (Kasper *et al.*, 1983; Darcy *et al.*, 1992; Decoster *et al.*, 1992; Kasper *et al.*, 1992; Ossorio *et al.*, 1992). Recently p30 is found to play an important role in invasion to host cells (Mineo *et al.*, 1993). Immunization using purified p30 as a vaccine protects mice from a lethal parasite challenge (Bulow & Boothroyd, 1991). This immunity appears to be primarily mediated by $CD8^+$ cells specific for p30 (Kasper *et al.*, 1992). Therefore, intramolecular analysis of p30 seems to be necessary to explain its role as a strong antigen.

Since Burg *et al.* (1988) have reported the DNA sequence of p30, many vectors and host cells are used to express p30 to obtain large amount of the antigen (Makioka & Kobayashi,

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1991; Xiong *et al.*, 1993; Kim *et al.*, 1994). In this study, fragments of p30 of *T. gondii* were examined to determine its antigenic domain. Gene fragments equivalent to hydrophilic or hydrophobic moieties of peptide sequences were expressed as GST fusion proteins.

MATERIALS AND METHODS

Genomic DNA preparation: *T. gondii* (RH strain), maintained by peritoneal passage in mice, was purified by centrifugation (1,800 g, 30 min) on 40% Percoll (Pharmacia Biotech, Sweden) in phosphate buffered saline (PBS)(Choi *et al.*, 1988). Tachyzoites (5×10^8) were washed three times with PBS and were pretreated with 1 ml K buffer (0.1 M Tris-Cl, pH 7.5, 12.5 mM EDTA, 0.15 M NaCl, 1% SDS, 20 mg/ml proteinase K) at 58°C for 1 hr and 37°C for 16 hrs. Then DNA was extracted with phenol/chloroform/isoamyl alcohol and washed with 70% alcohol (Ausubel *et al.*, 1992).

Amplification of p30 gene fragments by PCR: Oligonucleotide primers were synthesized according to the coding sequence of Burg *et al.* (1988) as in Fig. 1. The sequences of synthesized primers were shown in Table 1. Each primer was designed to have clamp sequence of *EcoR* I restriction site including coding sequence. Reaction mixture was composed of 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxyribonucleotide triphosphate (dNTPs), 0.2 mM each primer, and 2.5 U *Taq* DNA polymerase (Boehringer Mannheim Biochemica, Germany). PCR was done in a thermal cycler (M/J , USA) for 32 cycles (1 min at 94°C, 1-2 min at 58°C, and 1-2 min at 72°C) and finally for 5 min at 72°C for full extension. Amplified DNA was electrophoresed in 1.2% agarose.

Construction of recombinant plasmid: Amplified DNA was digested with *EcoR* I (Gibco BRL, USA) to insert them into plasmid of pGEX-4T-1 (GST expression vector, Pharmacia Biotech, Sweden) which was digested with EcoR I and dephosphorylated by CIAP (calf intestinal alkaline phosphatase, Gibco BRL, USA). After ligation with T4 DNA ligase (Gibco BRL, USA), DNA was used to transform host *Echerichia coli* (JM105).

Expression of fusion protein for western blot: JM105 cells of log phase were treated with 0.5 mM isopropyl β-D-thiogalactoside

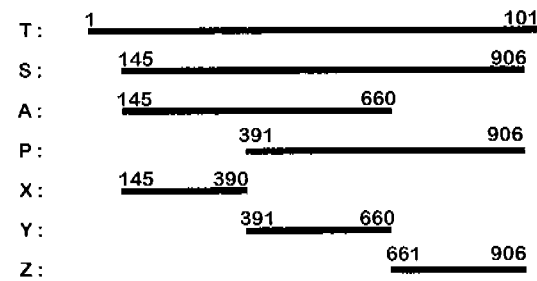


Fig. 1. Design of fragmentation of p30 into hydrophilic and hydrophobic moieties.

Table 1. Primers designed for the amplification of p30 gene fragments

Name		Sequence of primers
T	sense	5'-GTTG*AATTC*ATGTCGGTTTCGCTGCAC-3'
	antisense	5'-GTGG*AATTC*ACGCGACACAAGCTGC-3'
S	sense	5'-GTTG*AATTC*GATCCCCCTCTTGTTGCC-3'
	antisense	5'-GTGG*AATTC*GGCAAACTCCAGTTTCAC-3'
A	sense	5'-GTTG*AATTC*GATCCCCCTCTTGTTGCC-3'
	antisense	5'-GTGG*AATTC*GACTCCATCTTTCCCGCA-3'
P	sense	5'-GTTG*AATTC*GGGGATTCTGCTAGTCTC-3'
	antisense	5'-GTGG*AATTC*GGCAAACTCCAGTTTCAC-3'
X	sense	5'-GTTG*AATTC*GATCCCCCTCTTGTTGCC-3'
	antisense	5'-GTGG*AATTC*CGTCCACCAGCTATCTTC-3'
Y	sense	5'-GTTG*AATTC*GGGGATTCTGCTAGTCTC-3'
	antisense	5'-GTGG*AATTC*GACTCCATCTTTCCCGCA-3'
Z	sense	5'-GTTG*AATTC*AAAGTTCCTCAAGACAAC-3'
	antisense	5'-GTGG*AATTC*GGCAAACTCCAGTTTCAC-3'

(IPTG) for 3 hrs at 30°C to induce expression of the fusion proteins. After SDS-PAGE on 10% gel (Laemmli, 1970) fusion protein was identified with GST detection kit (Pharmacia Biotech, Sweden). Western blot was performed on the proteins which were transferred electrophoretically onto nitrocellulose paper. Serum of a toxoplasmosis patient, confirmed by indirect latex agglutination test and ELISA, was used as the first antibody (1:100) and peroxidase-conjugated anti-human IgG antibody (Cappel Co., USA) as the second antibody (1:1,000). Color development was done with H_2O_2 and 4-chloro-1-naphthol (4C1N, Sigma Chem. Co., USA) as substrates.

Fig. 2. PCR-amplified p30 coding gene fragments of *T. gondii* separated in 1.2% agarose gel.
M: pBR328 DNA/*Bgl* I + *Hinf* I fragments; **1:** PCR product of 1,011 bp for T37; **2:** 762 bp for S28; **3** and **4:** 516 bp for A19 and P19; **5:** 246 bp for X9; **6:** 270 bp for Y10; **7:** 246 bp for Z9; and **m:** pBR322 DNA/*Hae* III fragments.

Fig. 3. Selected p30 DNA fragments for insertion into pGEX-4T-1 after *EcoR* I digestion.
M: lambda DNA/*Hind* III fragments; **G:** pGEX-4T-1 only; **1:** pGEX-4T-1 with 1,011 bp for T37; **2:** with 762 bp for S28; **3** and **4:** with 516 bp for A19 and P19; **5:** with 246 bp for X9; **6:** with 270 bp for Y10; **7:** with 246 bp for Z9; **m1:** pBR328 DNA/*Bgl* I + *Hinf* I fragments; and **m2:** pBR322 DNA/*Hae* III fragments.

RESULTS

Amplification of p30 gene fragments by PCR: As designed, p30 gene fragments were amplified as long as the sequences including *EcoR* I clamp sequences. According to the primers used, DNA fragments were synthesized as 1,011 bp for total ORF of p30 (T37), 762 bp for S28 (144 bp N-terminal signal sequence and 105 bp C-terminal sequence were excluded), 516 bp for A19, 516 bp for P19, 246 bp for X9, 270 bp for Y10, and 246 bp for Z9, respectively (Fig. 2).

Construction of recombinant plasmid in pGEX-4T-1: After digested with *EcoR* I, DNA fragments were inserted into pGEX-4T-1. The plasmid was transformed into host *E. coli* of JM105 strain. The plasmids, obtained from each colony, were digested with *EcoR* I. The colony with the desired insert length of DNA, was chosen for the expression and confirmed

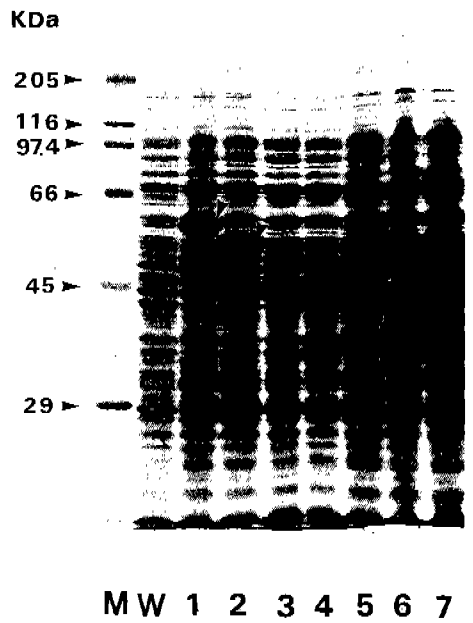
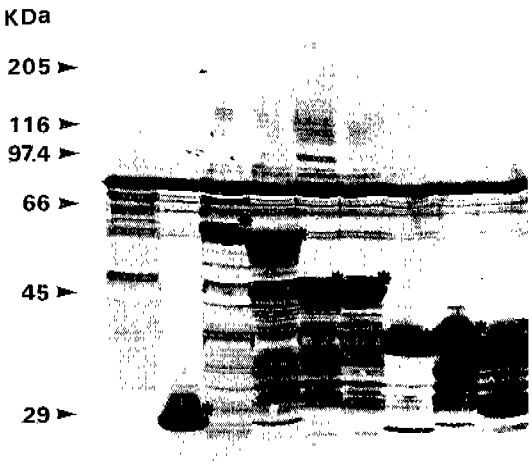


Fig. 4. SDS-PAGE findings of expressed GST-p30 fusion proteins.
W: wild JM105 cells; **1:** GST-T37 of MW of 63 kDa; **2:** GST-S28, 54 kDa; **3** and **4:** GST-A19 and GST-P19, 45 kDa; **5:** GST-X9, 35 kDa; **6:** GST-Y10, 36 kDa; and **7:** GST-Z9, 35 kDa, as indicated with arrow heads. **M:** Molecular mass markers.



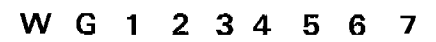


Fig. 5. Confirmation of GST fusion protein of p30 fragments by western blot.
W: wild JM105 cells; and **G:** GST only. Others are described in Fig. 4 as marked with asterisk (*).

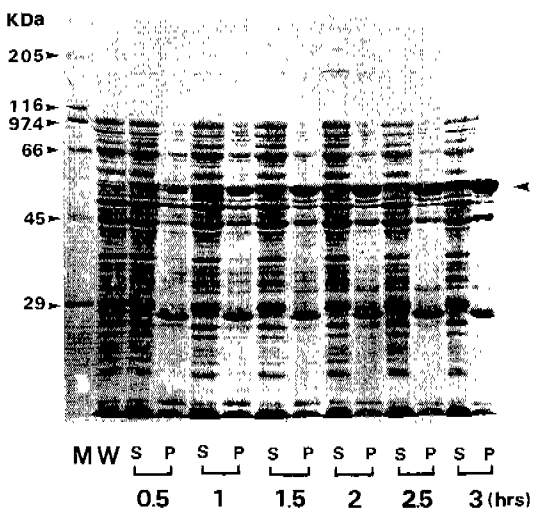


Fig. 6. Demonstration of rapid formation of inclusion body of GST-S28 fusion protein.
S: supernatant after sonification and centrifugation in PBS and **P:** precipitant. Numerals of x-axis are induction time of hr at 30°C. GST-S28 fusion protein is indicated with right arrow head.

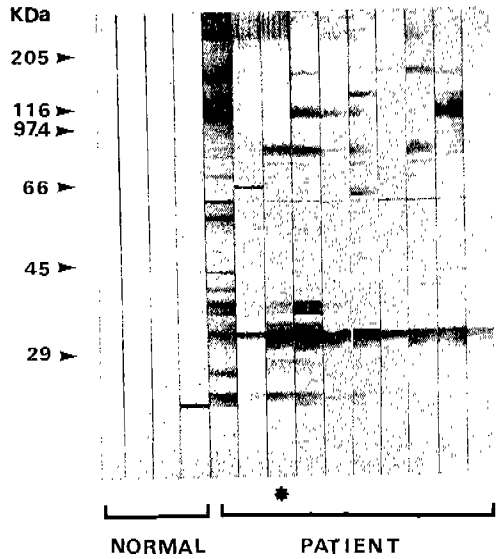


Fig. 7. Western blot analysis of RH tachyzoite extracts with patients sera of toxoplasmosis.
NORMAL: reaction with normal human sera and **PATIENT:** reaction with positive human toxoplasmosis sera. Arrow head indicates reaction of sera to p30 of tachyzoites. *: selected serum for western blot in Fig. 8.

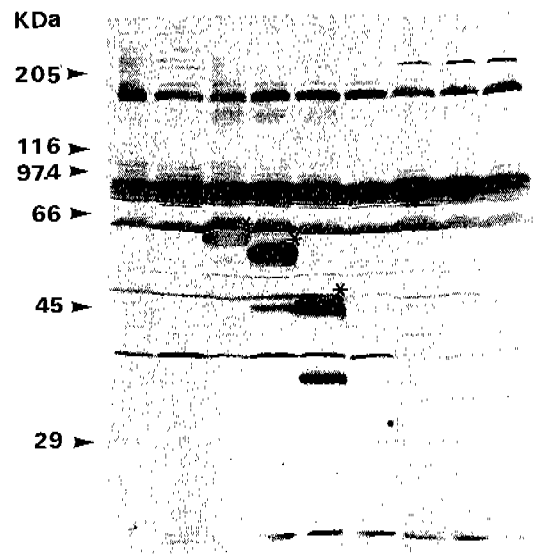


Fig. 8. Western blot analysis of GST fusion proteins of p30 fragments using the patient serum.
Refer to the legend of Fig. 5. GST fusion proteins of p30 fragments are marked with asterisk (*).

by electrophoresis (Fig. 3). The orientation of the insert was disregarded in this step.

Expression of GST fusion protein from JM105: pGEX-4T-1 and recombinant plasmids transformed into JM105 were induced to express GST or GST fused fragments of p30 by treating IPTG. As shown in Fig. 4, GST was expressed as 26 kDa protein. GST fusion proteins were expressed as 63 kDa protein for T37, 54 kDa for S28, 45 kDa for A19, 45 kDa for P19, 35 kDa for X9, 36 kDa for Y10, and 35 kDa for Z9 in SDS-PAGE. Each fusion protein was confirmed with GST detection kit (Fig. 5). T37, S28, A19, and P19 formed inclusion body as rapid as within a hour after IPTG induction. Fig. 6 exhibited the formation of inclusion body of S28 protein as an example. Proteins of X9, Y10, and Z9 did not form inclusion bodies (data not shown).

Analysis of antigenic domain in p30: By western blot, patients sera of toxoplasmosis were confirmed to show high reactivity with p30 band of RH strain of *T. gondii* (Fig. 7). Of them, the serum shown by asterisk (*) in Fig. 7 was selected in analyzing the antigenic domain of the recombinant proteins. The serum showed indirect latex agglutination titer of 1:4,096 and ELISA absorbance of 1.09. p30 fragments of T37, S28, and A19 reacted with the patient serum while those of P19, X9, Y10, and Z9 together with GST did not (Fig. 8).

DISCUSSION

Synthesized primers used in this study amplified the exact sequence of p30 fragments. GST fusion proteins were expressed well in JM105 cells transformed with either pGEX-4T-1 alone or with those containing p30 gene fragments. Fusion proteins were confirmed with GST detection kit. By western blot analysis, antigenicity of p30 was revealed in the polypeptide of N-terminal 1/3 part in the presence of middle 1/3 or in the oligopeptides between margins of the first and second 1/3 parts.

It is desirable that recombinant protein has the same epitope as its original form. But the recombinant protein of S28 is designed not exactly same as expressed by total ORF. In this connection, 47 amino acids in highly hydrophobic N-terminal is a signal sequence which disappeared when located at surface membrane (Xiong *et al.*, 1993). p30 is anchored to the membrane via glycosyl-phosphatidylinositols (GPIs)(Tomavo *et al.*, 1992). Predicted amino acid sequence at hydrophobic C-terminal domain is thought to be removed during anchor addition to membrane of *T. gondii* (Nagel & Boothroyd, 1989; Ferguson *et al.*, 1994). Therefore, S28 without the hydrophobic N- and C-terminal fragments is suitable for analyzing the antigenic domain of p30.

The fusion proteins of GST, produced in this study, were incorporated into inclusion body in *E. coli* which was insoluble in PBS (Thatcher & Hitchcock, 1994). In addition, the sequence of p30, identified by Burg *et al.* (1988), exhibited a large number of cysteine residues (12 in the mature form), suggesting a tertiary structure, tightly held together by disulfide bonds. In the process of analysis, however, detergent (SDS) and reducing agent (2-mercaptoethanol) in the sample buffer of SDS-PAGE was sufficient to solubilize and denature the inclusion body and the tertiary structure of the fusion proteins.

p30 is a highly conformational antigen, whose recognition by antibody is dependent upon correct disulfide bonding (Kim *et al.*, 1994). In this connection, Godard *et al.* (1994) reported that rat and mouse showed antibody responses when immunized with oligopeptide of 48-67 amino acids in p30 itself or its multiple antigenic peptide form prior to be boosted by infection with tissue cyst forming strain of *T. gondii*. We think that the antigenic domain of p30 is located in the first 1/3 of S28. But the expression of antigenicity requires the presence of the second 1/3, because the first 1/3 alone did not reveal antigenicity. This kind of interpretation on the antigenicity is possible when the blotted, fused fragments retain their tertiary structure. On the other hand, the B cell epitopes in p30 are reported to be located between 125-276 amino acid sequence, and for the structural conservation of this protein the amino acid sequence between 125-165 residues is essential (Velge-Roussel *et al.*, 1994). These results suggest strongly that the antigenic

domain in p30 is located between margins of the first and second 1/3 parts. Of the above two possible explanations on antigenic domain of p30, exact mechanism is yet to be determined.

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=초록=

융합단백질로 발현된 톡소포자충의 주요막단백질(p30) 절편의 항원성

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톡소포자충(*Toxoplasma gondii*) 주요막단백질의 하나인 30 kDa 단백질(p30)의 항원부위를 결정하고자 p30의 아미노산 분석에 따른 친수성 부위 및 협수성 부위에 맞게 유전자를 증폭하고 발현시켜 항원성을 검토하였다. p30의 절편으로는 p30 전체, p30의 N-말단 signal sequence와 C-말단의 협수성 부위를 제거한 S28, S28의 N-말단 2/3부위인 A19, S28의 C-말단 2/3부위인 P19, S28의 N-말단 1/3부위인 X9, 중앙 1/3부위인 Y10 및 C-말단 1/3부위인 Z9로 구성하였다. 각절편에 대한 primer에는 *EcoR* I의 clamp sequence를 포함시켜 중합효소반응으로 증폭시켰으며, GST를 발현하는 pGEX-4T-1 vector에 삽입시킨 후 *Escherichia coli*(JM105 strain)에 형질변형시키고 IPTG로 각 절편이 GST와 융합단백질로 발현되도록 하였다. SDS-PAGE상에서 p30은 63 kDa, S28는 54 kDa, A19과 P19은 각각 45 kDa, X9은 35 kDa, Y10은 36 kDa 및 Z9은 35 kDa 단백질로 발현되었다. 각각의 단백질은 western blot상에서 GST detection kit와 잘 반응하여 융합단백질임을 확인하였다. 톡소포자충증 환자 혈청과 western blot에서 p30, S28 및 A19은 반응하여 항원성이 인정되었으나, P19, X9, Y10 및 Z9는 반응하지 않았다. 따라서, p30의 중간 1/3 부위의 존재하에 N-말단 1/3부위가 항원성을 나타내는 구조적 항원이거나, 첫 1/3부위와 중간 1/3부위의 경계에 위치한 polypeptide가 항원성을 발현하는 것으로 추정되었다.

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