Purification of *Treponema pallidum*, Nichols strain, by two-step column chromatography

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Abstract

A rapid and simple purification method for *Treponema pallidum*, Nichols strain, the etiological agent of venereal syphilis, was developed. A 40-ml suspension of organisms ($1 \cdot 10^9$/ml) was extracted from rabbit testicular tissue and solubilized with a non-ionic detergent, 1-O-*n*-octyl-β-D-glucopyranoside. Solubilized antigens were purified by cation-exchange and hydroxyapatite column chromatography. The overall recovery of immunoreactive material was 48.3% and the specific activity increased. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting analysis confirmed the purity and species specificity of the purified antigen.

1. Introduction

Treponema pallidum, Nichols strain, a bacterium of the family Spirochaetaceae, is the causative agent of venereal syphilis [1]. It is important to investigate the biology and to characterize the pathogen-specific antigens of such agents. Because they are responsible for eliciting a protective immune response in the infected host, the study of these antigens would be the useful for solving the mechanisms of syphilitic infection. However, the analysis of *T. pallidum* antigens has been limited by the difficulties of growing the organism in vitro. *T. pallidum* propagated in the testicles of rabbits was extracted from minced host tissue and was supplied for research [2–4]. These suspensions of *T. pallidum* contain various amounts of host

material, including serum proteins, spermatozoa, host-derived lipids and cellular tissue debris.

Hanff et al. [5] and Sato and Kubo [6] described a density gradient centrifugation method to remove serum protein and non-cellular components from *T. pallidum* suspensions. Radolf et al. [3] and Stamm et al. [4] used a detergent to solubilize and extract *T. pallidum* antigens. However, these methods do not provide sufficient amounts of purified antigens for their utilization in the serodiagnosis of syphilis.

Recently, the expression of treponemal polypeptides in *Escherichia coli* has been performed by several laboratories [7–15]. This technique has the advantage of providing unlimited amounts of individual antigens for pathogenesis investigation. However, the uncertainty of the precise localization of the pathogenic antigens has hindered selection from genomic libraries of clones expressing recombinant protein.

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In this work, we developed a rapid and large-scale purification procedure for *T. pallidum* antigens, which includes extraction with a non-ionic detergent, 1-O-*n*-octyl-β-D-glucopyranoside, and two-step chromatographic separation using cation-exchange and hydroxyapatite column chromatography.

2. Experimental

2.1. Reagents and materials

The protein concentration was measured with BCA protein assay reagent (Pierce, Rockford, IL, USA) [16]. The TPHA kit (*Treponema pallidum* haemagglutination assay kit for detecting treponemal antibodies, Seroclit-TP; Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was used.

S-Sepharose Fast Flow (Pharmacia–LKB Biotechnology, Uppsala, Sweden) was used as a cation-exchange carrier. Bio-Gel HTP (DNA grade; Bio-Rad Labs., Richmond, CA, USA) was used as the hydroxyapatite gel for column chromatography. The chromatography conditions were controlled by the Pharmacia FPLC system.

2.2. T. pallidum

The organisms were grown and isolated by the following procedure. A 1-ml volume of pathogenic standard Nichols strain of *T. pallidum* ($6.0 \cdot 10^7$/ml) was propagated in rabbit testicular tissue. The testes were then removed, sliced and incubated for 30 min at 37°C in 2.2% (w/v) sodium citrate solution (100 ml). Thereafter, 100 ml of organism suspension were extracted. The extract was centrifuged for 5 min at 200 g to remove the precipitate of rabbit tissues. The supernatant was centrifuged for 30 min at 3000 g to precipitate *T. pallidum* microorganisms. The cells thus obtained were extensively washed with phosphate-buffered saline (PBS) (0.023 M potassium dihydrogenphosphate–0.013

M disodium hydrogenphosphate dodecahydrate–0.126 M sodium chloride, pH 6.50) and suspended in 40 ml of PBS containing 35% (w/v) sucrose for the pretreatment step and the number of *T. pallidum* was adjusted to $1 \cdot 10^9$/ml by counting with a dark-field microscope.

2.3. Purification of T. pallidum antigens

Pretreatment

A stepwise gradient of sucrose consisting of 7 ml of 45%, 42.5%, 40%, 37.5% and 35% (w/v) sucrose in PBS was prepared in four polycarbonate tubes (3 × 10 cm), and 10 ml of the *T. pallidum* suspension containing 35% sucrose were layered on the upper layer. The tubes were centrifuged at 10 000 g at 4°C for 12 h.

Solubilization and extraction of antigens

A 40-ml volume of the treponemal visible band was divided into four polycarbonate tubes, washed three times with 40 ml of PBS and finally suspended in 25 ml of PBS. The suspension was sonicated at 120 W in a sonicator (Astrason Model W-385; Heat Systems Ultrasonic, New York, USA) for 5-min intervals at 50% output in an ice-bath. The disrupted organisms were centrifuged for 30 min at 12 600 g to remove the precipitate. The precipitate was washed twice with 10 mM potassium phosphate buffer (KPB) (pH 7.0) by centrifugation for 30 min at 12 600 g. Thereafter, KPB containing 1% (w/v) of 1-O-*n*-octyl-β-D-glucopyranoside (OG) (reagent grade, Nacalai Tesque, Kyoto, Japan) was added in an amount of 25 ml to each precipitate. The suspensions were then sonicated for 5 min in the sonicator under the same conditions as described above. After storage at 4°C for 16 h, the mixture was centrifuged for 1 h at 10 000 g at 4°C. The supernatant was filtered with a membrane filter (pore size 0.22 μm, Millex-GS; Japan Millipore, Tokyo, Japan) and the extracted antigen was obtained. The extracted antigen was dialysed against 1 l of 10 mM KPB containing 1% (w/v) OG (pH 6.0). The dialysis buffer was changed three times.

Chromatography

A 40-ml volume S-Sepharose Fast Flow gel suspension was washed batchwise three times with 200 ml of eluent A [10 mM KPB containing 1% (w/v) OG, pH 6.0]. The gel was then resuspended in eluent A and the slurry was packed into an SR 25/45 column (Pharmacia–LKB Biotechnology). A 105.5-ml volume of extracted antigen was applied to the column and the passed-through fractions, exhibiting over 0.015 absorbance at 280 nm, were collected (volume 126 ml) as the partially purified antigen.

A 15.0-g amount of hydroxyapatite gel powder was washed with eluent A and the gel was resuspended in 200 ml of the same buffer. The gel slurry was packed into an SR 25/45 column. After washing with 400 ml of eluent A, the partially purified antigen (126 ml) was loaded on to the gel. Thereafter, the column was washed until the absorbance of the eluate solution became 0.010 or less at 280 nm and the pH value was confirmed to be within 6.0 ± 0.1. Linear gradient elution was conducted by gradually increasing the ratio of eluent B [350 mM KPB containing 1% (w/v) OG, pH 6.0] to eluent A from 0 to 40%, and finally increasing B to 100%. Fractions of 3 ml were collected.

The antigen activity of each fraction was measured by the immunoreaction assay. The fractions exhibiting an antigen activity of 512 titre/ml or more were collected. The collected fractions were then concentrated under reduced pressure by using a seamless cellulose tube (Wako, Osaka, Japan) until the protein concentration was approximately 60 μg/ml [17]. The fractions obtained were considered to be the purified antigen.

2.4. Immunoreaction assay of T. pallidum antigens

Antigen solutions of *T. pallidum* were absorbed by syphilitic rabbit sera and the remaining treponemal antibodies were determined with the TPHA kit according to the instructions of the manufacturer. A final dilution ratio of the rabbit serum which caused the haemagglutination was defined as the antigen activity. This antigen

activity was represented by titre (titre/ml). Specific activity (titre/μg protein) was calculated from the antigen activity and the protein concentration of the sample.

2.5. Reiter treponemes

T. phagedenis, biotype Reiter, was supplied by the National Institute of Health (Tokyo, Japan). A 1.5-ml volume of a suspension of the organism ($1 \cdot 10^9$/ml) was prepared by growth in 15 ml of spirolate medium [15] with 10% (v/v) foetal calf serum and was incubated for 1 week at 25°C. After growth, Reiter treponemes were harvested by centrifugation at 10 000 g for 60 min and washed four times with PBS. The final pellet was suspended in PBS and the number of the organisms was adjusted to $1 \cdot 10^9$/ml.

2.6. Production antisera to T. pallidum and to Reiter treponemes antigens

Purified *T. pallidum* antigens (60 μg/ml) and Reiter treponemes antigens (100 μg/ml) were used as immunogens. A 1-ml volume of Reiter treponemes was mixed with PBS containing 1% (w/v) Tween-80 (EIA grade, Bio-Rad), and the protein concentration was adjusted to 100 μg/ml with the same buffer. A 0.5-ml volume of each immunogen was mixed with 0.5 ml of complete Freund's adjuvant (H-37 Ra, Bacto; Difco Labs., Detroit, MI, USA). A 1.0-ml volume of the mixture was injected intradermally along the backs of three rabbits. After 4 weeks, the rabbits were again boosted with 1 ml of each immunogen, except that the antigen solution was mixed with 3 ml of incomplete Freund's adjuvant. Ten days after the booster injection, the rabbits were exsanguinated.

Antiserum against Reiter treponemes was serially diluted in glass tubes. A 50-μl volume of diluted serum was mixed with 50 μl of an extracted solution of immunogen on the glass slide. Observing visually, the maximum dilution exhibiting agglutination was defined as the antibody titre. The titre of antisera against purified *T. pallidum* was determined with Seroclit-TP.

2.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotting

SDS-PAGE was performed with intact cells, extracted, partially purified and purified antigens of T. pallidum and extracted Reiter treponemes, using the method of Laemmli after slight modifications [18]. Polyacrylamide gradient gels of 10–20% (w/v) were used. The polypeptides were revealed in the gel by silver staining (Sil-Best Stain for Protein/PAGE; Nacalai Tesque).

Immunoblotting analysis was performed by the method of Towbin et al. [19]. After blocking the blotted nitrocellulose membrane with 10 mM Tris–HCl saline (TBS, pH 8.0) containing 3% (w/v) BSA, the membrane was exposed to the rabbit antiserum diluted 1:50 in TBS containing 1% (w/v) BSA (1% BSA–TBS). The membrane was incubated for 1 h with anti-rabbit IgG + A + M antibodies raised in goat and conjugated to horseradish peroxidase (Binding Site, Birmingham, UK) diluted 1:2000 in 1% BSA–TBS. After washing with TBS, the membrane was stained with 4-chloro-1-naphthol.

3. Results

3.1. Pretreatment and extraction of treponemal antigens

The T. pallidum organisms were found at approximately the 40.0–42.5% sucrose concentration layer. A less prominent band containing tissue debris, fibrin and a minimal number of T. pallidum was visible above the T. pallidum band. Rabbit erythrocytes, spermatoza and other testicular cells were precipitated at the bottom of the tube. Host-derived lipids were located at the top of the gradient layer. The discontinuous gradients were separated into three fractions, containing lipid components, T. pallidum organisms and the precipitation of tissue debris. The three fractions were extracted with 1% (w/v) OG and the T. pallidum fraction showed immunoreactivity.

The solubilization time was tested at 16, 24 and 48 h and re-extraction from solubilized residue was also tested. However, no more immunoreactive antigen fractions were obtained. The immunoreactivity of intact T. pallidum and just sonicated T. pallidum without OG was assayed, but no antigenicity was found.

3.2. Purification of T. pallidum antigens by two-step column chromatography

The elution profile and the immunoreactivity of the fractions are shown in Fig. 1. Passed-through fractions 11–45 were collected and assayed for immunoreactivity. The specific activity was increased from 11 to 16 after ion-exchange chromatography (Table 1). The adsorbed fractions that were eluted after washing the column with 10 mM KPB (pH 6.0) containing 0.50 M NaCl did not have any immunoreactivity (Fig. 1).

The partially purified antigens were then chromatographed on hydroxyapatite. Three major immunoreactive fractions were eluted at KPB concentrations of 37.2–146.0 mM (fractions 5–42, Fig. 2). The other peak, which was eluted with 350 mM KPB, did not have any reactivity.

The overall recovery after the last concentration step was 48.3%. The stepwise recoveries
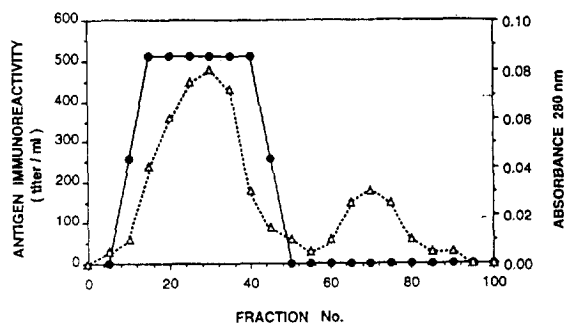

Fig. 1. Chromatography of extracted T. pallidum antigen on an S-Sepharose column. Chromatographic conditions: flow-rate, 3.0 ml/min; detection, absorbance at 280 nm; sensitivity, 0.1 AUFS; chart speed, 0.25 cm/min. All parameters were programmed with the FPLC system (Pharmacia–LKB). Fractions (3.0 ml) were assayed for (●) immunoreactivity and (△) absorbance at 280 nm.

Table 1
Purification steps and recovery of T. pallidum antigens

	Immunoreactivity (titre/ml)	Protein concentration (μg/ml)	Volume (ml)	Total antigen activity (titre)	Specific activity (titre/μg)	Purification (fold)	Antigen activity, recovery (%)
Extracted	1024	93.1	105.5	108 032	11	1.0	100.0
Partially purified (S-Sepharose)	512	31.4	126.0	64 512	16	1.5	59.7
Purified (hydroxyapatite)	512	13.6	126.5	64 768	38	3.4	60.0
Concentrated	2048	64.0	25.5	52 224	32	2.9	48.3

and antigen-specific immunoreactivity are summarized in Table 1.

3.3. SDS-PAGE and blotting analysis

As shown in Fig. 3, intact T. pallidum exhibited (lane c) numerous protein bands of M_r between 14 000 and 90 000. Extracted (lane d) and partially purified T. pallidum antigen (lane e) contain twenty and eleven polypeptides, respectively, with M_r between 14 000 and 65 000. The purified T. pallidum antigens (lane f) showed two major bands (M_r 47 000 and 42 500) and several minor bands with M_r between 65 000 and 17 000.

Lane b shows the electrophoresis profile of Reiter treponemes extracts, with apparent molecular masses of eleven protein bands between
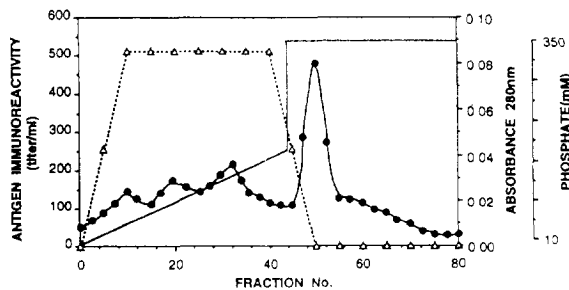

Fig. 3. SDS-PAGE analysis of T. pallidum and T. phagedenis, biotype Reiter. T. pallidum and T. phagedenis, biotype Reiter, were prepared by the procedure described under Experimental. Lanes: a = molecular mass marker proteins; b = extracted Reiter treponemes; c–f = T. Pallidum; c = intact cells solubilized with SDS; d = antigens extracted with OG; e = antigens partially purified by S-Sepharose; f = antigens purified by hydroxyapatite column chromatography.

Fig. 2. Chromatography of a partially purified T. pallidum S-Sepharose eluate on a hydroxyapatite column. Chromatographic conditions as in Fig. 1. Fractions (3.0 ml) were collected every 3 ml per tube and assayed for (●) immunoreactivity and (△) absorbance at 280 nm. (—) Phosphate buffer gradient.
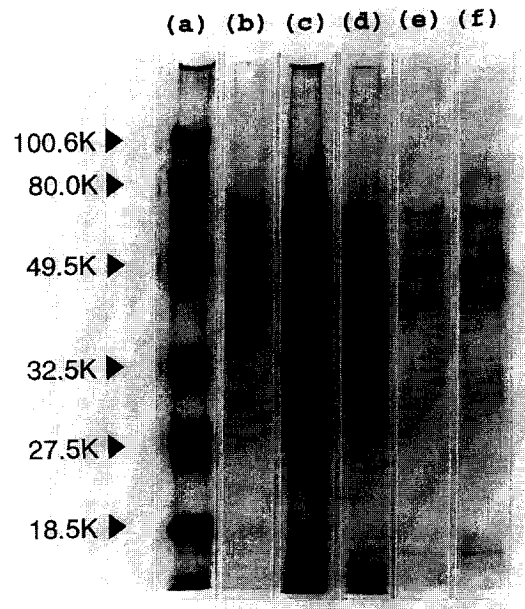
65 000 and 17 000. The M_r 65 000, 55 000 and 17 000 polypeptides were common with those of purified *T. pallidum* in line f.

Fig. 4 shows the immunoblotting analysis. Extracted *T. pallidum* showed four common antigenic M_r 65 000, 30 000, 29 000 and 26 000 polypeptides with Reiter treponemes (lanes b and c). From lane a, it was apparent that the purified *T. pallidum* antigens did not show any cross-reactivity with anti-Reiter antisera. The four common antigenic polypeptides were completely eliminated during two-step chromatography. The M_r 47 000, 42 500 and 37 000 polypeptides of purified *T. pallidum* were most immunoreactive (lane d). In lane e, anti-*T. pallidum* antiserum was reacted with four polypeptides, the M_r 65 000, 47 000, 42 500 and 37 000 polypeptides of extracted *T. pallidum*.
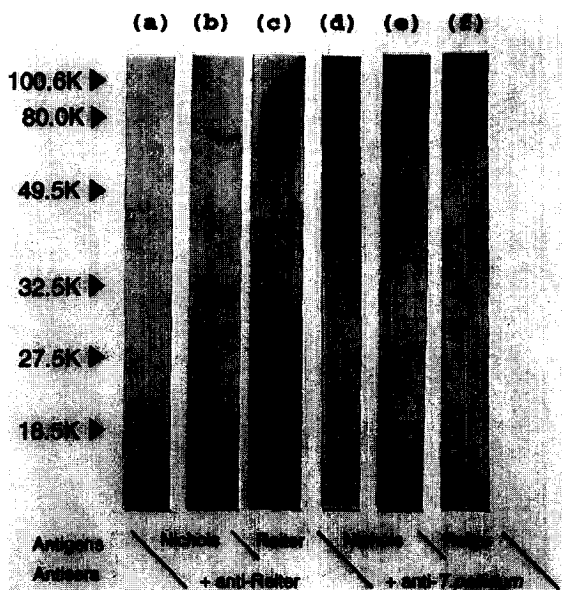

Fig. 4. Immunoblotting analysis of *T. pallidum* and Reiter treponemes. Extracted and purified antigens were subjected to SDS-PAGE followed by immunoblotting analysis according to the procedure described under Experimental. Lanes: a and d = purified *T. pallidum* antigens; b and e = *T. pallidum* extracted with OG; c and f = extracted Reiter treponeme antigens. The blotted membranes of lanes a–c were reacted with rabbit antiserum against Reiter treponemes; lane d–f were reacted with antiserum against purified *T. pallidum* antigens.

4. Discussion

In recent years, the application of modern biological techniques has greatly improved the identification and functional characterization of pathogen-specific *T. pallidum* antigens. In this paper we have presented a method for the purification of *T. pallidum* antigens involving the use of the non-ionic detergent OG and successive two-step column chromatography.

The separation of *T. pallidum* organisms from host tissue components has been accomplished by sucrose gradient centrifugation. Hanff et al. [5] reported a purification method involving percoll density gradient centrifugation. However, it was difficult to eliminate percoll from organisms completely for the next procedure. In our method, sucrose should be eliminated easily by washing with KPB.

To determine the optimum and mild solubilization conditions, we tried a variety of ampholytic and non-ionic detergents, e.g., CHAPS {3 - [(3 - cholamidopropyl)dimethylammonio] - 1 - propanesulphonate}, Triton X-100 [2] and OG. The resultant immunoreactive antigen fraction was obtained with CHAPS, Triton X-100 and OG as low as 1.0%. We selected OG because it did not show any absorption at 280 nm, unlike Triton X-100, so we could monitor the protein elution by measuring the absorbance at 280 nm throughout the procedures.

After solubilization and extraction of antigen, the buffer was changed to 10 mM KPB (pH 6.0) for next cation-exchange column chromatographic step. Preliminary studies have shown that the antigen activity was stable between pH 5.5 and 9.0, so they might be acidic proteins. Hence around pH 6.0, antigenic proteins are negatively charged, and non-antigenic, soluble proteins which are positively charged would be absorbed on the cation-exchange gel, so effective separation would be expected.

After cation-exchange chromatography, we applied partially purified antigen to hydroxyapatite column chromatography for the following reasons. First, in the previous step we used ion-exchange chromatography, so we needed a different separation mode such as hydrophobic

binding interaction to eliminate non-antigenic materials. Second, for purified antigen to be applied as a reagent in serological test kits on a large scale, chromatography would be suitable for these industrial purposes.

As shown in Fig. 2, immunoreactive antigen fractions were eluted from 37.2 to 146 mM KPB and gave three major peaks. The first-eluted fractions between 10 and 20 mM and a sharp peak at 350 mM KPB (Fig. 2) did not show immunoreactivity. The number of polypeptide bands revealed was not greatly decreased (Fig. 3), but the specific activity was increased after hydroxyapatite chromatography.

SDS-PAGE and blotting analysis demonstrated that our purified antigen would be identified as previously reported by other investigators. The most immunoreactive and abundant M_r 47 000 protein should be the well characterized M_r 47 000 T. pallidum antigen [9,20–22]. Penn et al. [2] and Radolf et al. [7] also identified the M_r 47 000 protein as the sole polypeptide component. They reported that the M_r 47 000 protein may be located in both the outer and cytoplasmic membranes of T. pallidum, which is consistent with results obtained for the recombinant M_r 47 000 antigen [8]. The immunogenic M_r 42 500 and 37 000 proteins could be similar proteins to those described by several other workers [23–26]. As described under Results, the extracted and partially purified antigens contain numerous proteins. Probably they were actively incorporated into the cell wall of T. pallidum during growth in the experimental host.

By immunoblotting analysis, purification of specific antigens, M_r 47 000, 42 500 and 37 000 proteins, was confirmed (lane d in Fig. 4). Four proteins, with M_r 65 000, 30 000, 29 000 and 26 000, of extracted T. pallidum protein reacted with anti-Reiter antisera (lane b). They should be common immunoreactive polypeptides of T. pallidum and Reiter treponemes.

As shown in lane e (Fig. 4), the M_r 65 000 polypeptide of extracted T. pallidum was reactive with anti-T. pallidum antisera, but it was not revealed in lane d. On further exposure of purified antigens to much higher titre antisera, or prolonged treatment with SDS before electro-

phoresis, no other bands were observed except the M_r 47 000, 42 500 and 37 000 major antigens. We guessed that the M_r 65 000 polypeptide might be one of the specific proteins associated with host-derived proteins, and was dissociated or reduced to a single antigenic polypeptide during the two-step chromatographic procedure.

Current seological tests for syphilis have specific problems with regard to the presence of cross-reactive antibodies in the serum of normal individuals. The use of T. pallidum or pathogen-specific molecules as the basis for a serological test could significantly increase the reliability and sensitivity of syphilitic serology. In a subsequent study, we intend to separate purified antigens to single polypeptide bands and to confirm the precise cellular localization and function of the antigens. Further, the availability of purified antigens should allow direct assessment of their vaccinogenic potential and provide a useful tool for pathogenic research in microbiology.

Acknowledgements

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