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Characterization of immunoaffinity purified peptidoglycan-associated lipoprotein of *Actinobacillus actinomycetemcomitans*

Riikka Ihalin[a,b], Maribasappa Karched[a], Kjell Eneslätt[a], Sirkka Asikainen[a,*]

[a] *Division of Oral Microbiology, Umeå University, Umeå SE-90185, Sweden*
[b] *Institute of Dentistry, University of Turku, Turku FIN-20520, Finland*

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Abstract

Peptidoglycan-associated lipoprotein (PAL) is a highly conserved structural outer membrane protein among Gram-negative bacteria. In some species, it is proinflammatory and released extracellularly. We purified a newly identified PAL (AaPAL) of a periodontal pathogen *Actinobacillus actinomycetemcomitans* by using AaPAL antipeptide antibodies coupled to immunoaffinity chromatography column. No protein impurities originating in *A. actinomycetemcomitans* were found in the final product. Sera from patients infected by *A. actinomycetemcomitans* recognized the purified AaPAL. The present purification method seems to be suitable for isolation of AaPAL and probably PALs of other bacterial species, and applicable in studies investigating proinflammatory mechanisms of *A. actinomycetemcomitans*.
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Keywords: Peptidoglycan-associated lipoprotein; *Actinobacillus actinomycetemcomitans*; Immunoaffinity chromatography; Antipeptide antibodies

1. Introduction

Actinobacillus actinomycetemcomitans is a small (<1 μm) Gram-negative, non-motile facultatively anaerobic oral bacterium. It is a major pathogen in aggressive periodontitis that destroys tooth-supporting tissues, but can also cause severe non-oral infections, such as endocarditis and abscesses in brains and lungs [1–3].

A structural component of *A. actinomycetemcomitans*, well known of its pathogenic traits, is lipopolysaccharide (LPS) [4–6]. The functions of other structural cell-surface components, such as outer membrane proteins (AaOMPs), are only poorly known. Six AaOMPs have been reported [7] in addition to the recently found 17-kDa AaOMP of *A. actinomycetemcomitans* [8]. The 100-kDa outer membrane protein (OMP100) is thought to play a role in adherence and invasion to human keratinocytes, resistance to serum, and induction of IL-1β and TNFα production from mouse macrophages [9]. In addition to the OMP100, a heat modifiable 29-kDa OMP (OMP29) is a target of serum antibody response in patients with periodontitis [7,10,11]. We have found that sera from patients with periodontitis more frequently recognized a 17-kDa AaOMP than sera from periodontally healthy subjects [8]. However, in contrast to earlier findings [7,11], the 17-kDa OMP, identified as the peptidoglycan-associated lipoprotein of *A. actinomycetemcomitans* (AaPAL), was the immunodominant AaOMP in most patients [8].

In earlier studies, the peptidoglycan-associated lipoprotein (PAL) of *Haemophilus influenzae*, a phylogenetic relative of *A. actinomycetemcomitans*, has been purified by exploiting the insolubility of the peptidoglycan and the differences in the dissociation conditions of different peptidoglycan binding proteins [12–14]. In the present study, we report purification of AaPAL by using immunoaffinity chromatography to further purify the crude AaPAL preparation. N- and C-terminal peptide sequences were chosen from AaPAL, antibodies against these peptides were produced in rabbit, and the C-terminal antipeptide antibodies were used in immunoaffinity chromatography.

2. Experimental

2.1. Bacteria and growth conditions

For the purification of AaPAL, *A. actinomycetemcomitans* strain D7SS (serotype a), a gift from Dr. Casey Chen (University

* Corresponding author. Tel.: +46 90 785 6166; fax: +46 90 785 6053.
E-mail address: Sirkka.Asikainen@odont.umu.se (S. Asikainen).

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of Southern California, Los Angeles, USA), was revived from pure cultures preserved in skimmed milk at −80 °C by using supplemented blood agar plates (4.25% Columbia agar [Acumedia, Baltimore, MD, USA], 0.65% Bacto-Agar [Difco, Detroit, MI, USA], 0.05% de-fibrinated horse blood, 5 mg/l hemin [Sigma, St. Louis, MO, USA], 10 mg/l Vitamin K [Sigma]) incubated in 5% CO_2 in air at 37 °C for 3 days. After cultivation on plate, the bacteria were grown in supplemented Trypticase soy broth (TSB) (30 g/l TSB [Difco/Becton Dickinson, Sparks, MD, USA], 6 g/l yeast extract [Merck, Darmstadt, Germany], 8 g/l glucose) by stepwise increasing the volume until the total volume of the final culture was 2.26 l and $OD_{600} = 0.565 \pm 0.051$. Each time a 10-μl sample from inoculates as well as the final broth culture was cultured on blood agar plates to assure the purity. The typical smooth-colony conformation of the strain D7SS was confirmed under a stereo microscope. Gram staining was used to verify the typical coccoid *A. actinomycetemcomitans* cell morphology and Gram-negative staining characteristics.

2.2. Production of AaPAL antipeptide antibodies

The peptide synthesis, rabbit immunization, and antibody purification were done in co-operation with AgriSera (Vännäs, Sweden). Two 14-amino acid peptides, one from the N-terminal (4–17) and one from the C-terminal (123–136) end of the AaPAL sequence [8], were selected as immunization antigens based on the assumption that the ends of the native protein are most likely recognized by the antipeptide antibodies because they are less conformationally constrained than other regions of the protein [15]. In addition, these sequences were shown to have antigenic potential as analyzed with various computer softwares. One rabbit was immunized with both N- and C-terminal peptides of the AaPAL bound individually to a carrier protein (keyhole limplet hemocyanin). The rabbit was immunized four times with 1:1 mixture of peptide conjugates at 1-month intervals. Polyclonal antipeptide antibodies were separated from the produced antiserum using two affinity chromatography columns containing either the N-terminal or C-terminal peptides. The peptides were bound to UltraLink® Iodoacety Gel (Pierce Biotechnology, Rockford, USA). The serum (20.7 ml) from the final bleeding was first run to the N-terminal peptide column and the flowthrough then run to the C-terminal peptide column.

2.3. ELISA for detecting the AaPAL antipeptide antibodies

The antibody titers of serum samples taken at different time points during the immunization as well as of the immunoaffinity purified AaPAL samples were measured by a modification of ELISA [15]. The C-terminal and N-terminal AaPAL peptides served as antigens. Serial five-fold dilutions were made from the pre-immune (1:100–1:200,000) and immune (1:2500–1:7,812,500) sera. The immune serum samples analyzed were those taken 2–4 weeks after the third immunization (III + 2W, III + 4W) and the final bleeding occurred 2 weeks after the fourth immunization (IV + 2W). Aliquots of each serum dilution were added as duplicates on the ELISA plates and incubated at 37 °C overnight. The secondary antibody, AP-conjugated AffiniPure $F(ab')_2$ fragment of polyclonal goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, USA), was diluted to 1:5000 and 100 μl of the dilution was added to each well and incubated at 37 °C for 2 h. The bound secondary antibody was detected using freshly made 4-nitrophenyl phosphate (Roche, Mannheim, Germany) solution, and the absorbances were read at 405 nm with SpectraMAX 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA) after incubating in the dark at RT for 30 min.

2.4. Estimation of the 3-D structure of the periplasmic part of AaPAL

A model for the 3-D structure of the periplasmic part of AaPAL was created according to the amino acid sequence of AaPAL [8] and the resolved structure of the periplasmic part of the *E. coli* PAL (1OAP) using the Swiss-Pdb Viewer and Swiss-Model server (www.expasy.org/spdbv/ [16–18]).

2.5. Purification of AaPAL

2.5.1. Crude preparation

The crude preparation of AaPAL was done using a modified method from Karalus and Murphy [13]. Briefly, bacterial cells, grown as described above, were harvested by centrifugation (9000 × *g*, 4 °C, 30 min). The bacterial pellet was washed with 65 ml phosphate buffered saline (PBS; pH 7.4) and centrifuged as above. The washed bacteria were re-suspended in 65 ml Buffer B (0.1 M Tris, 0.5 M NaCl, 1% sodium dodecyl sulphate, 0.1% β-mercaptoethanol, pH 8.0) and sonicated six times for 10 min. The suspension was incubated at 37 °C for 30 min in a shaker (150 rpm), after which it was centrifuged (21,000 × *g*, 25 °C, 30 min) to pellet the insoluble peptidoglycan and unbroken cells. The supernatant was discharged and the pellet sonicated three times with fresh solution of Buffer B containing 10 μg/ml RNAse A (Sigma) as above, and then two times using Buffer B without enzyme. The above described isolation of peptidoglycan was repeated totally three times to get enough material. After storage on ice at 4 °C overnight or −20 °C for longer periods of time, the final peptidoglycan pellet was re-suspended in 60 ml Tris–buffered saline (TBS; 0.01 M Tris, 0.15 M NaCl, pH 7.4) using a 19G needle attached to a 5-ml pipette tip. The suspension was incubated in 65 °C water bath for 30 min to dissociate the AaPAL from the peptidoglycan. The suspension was ultra-centrifuged (100,000 × *g*, 30 °C, 60 min) to remove the peptidoglycan. The supernatant containing the AaPAL was collected and concentrated with Vivaspin 20 concentrator (Vivascience AG, Hannover, Germany), molecular weight cut off (MWCO) 10 kDa, (3000 × *g*, 4 °C) to approximately 7.5 ml. The solution was dialyzed against TBS at 4 °C overnight using Spectra/Por 6® (Spectrum Laboratories, Rancho Domingnez, CA, USA) dialyze tube having nominal molecular weight cut off (NMWCO) 10 kDa. The dialyzed product was concentrated with Vivaspin 20 concentrators.

2.5.2. Immunoaffinity purification

For further purification of the crude AaPAL preparation, an affinity chromatography column was made using purified AaPAL antipeptide antibodies against the C-terminal peptide, since their yield and affinity to AaPAL were higher than those of the antibodies against the N-terminal peptide. The antibodies (IgG) were bound covalently to protein A-Sepharose CL-4B matrix (5 mg/ml of matrix, total volume of 1 ml) according to the instructions by Baldwin [15]. The column was washed with 10 ml of washing buffer (10 mM Tris, 0.14 M NaCl, 0.025% NaN_3, 0.5% Triton X-100, 0.5% sodium deoxycholate, pH 8.0 at 4 °C), 5 ml of Tris/Triton/NaCl buffer pH 8.0 (50 mM Tris, 0.1% Triton X-100, 0.5 M NaCl, pH 8.0), 5 ml of Tris/Triton/NaCl buffer pH 9.0 (50 mM Tris, 0.1% Triton X-100, 0.5 M NaCl, pH 9.0), 5 ml of triethanolamine solution (50 mM triethanolamine, 0.1% Triton X-100, 0.15 M NaCl, pH ~11.5), and finally with 5 ml of washing buffer. The crude AaPAL preparation (5 ml, 4.0 mg protein) was applied to the washed column, the flowthrough collected for further runs, and the column was washed with 10 ml of washing buffer, 5 ml of Tris/Triton/NaCl buffer pH 8.0, and 5 ml of Tris/Triton/NaCl buffer pH 9.0. The AaPAL was eluted with 5 ml of triethanolamine solution into a tube containing 1 ml of 1 M Tris–HCl pH 6.7 to neutralize the fraction collected. The flowthrough from the first sample application was re-applied to the column, the column was washed and the AaPAL eluted as in the first run. This was repeated totally 10 times, since the binding capacity of the affinity column was approximately 300 μg AaPAL. The fractions were pooled, dialyzed against 5 l of TBS pH 7.4 using Spectra/Por 6® dialysis tube with a NMWCO 10 kDa, and the final product was concentrated with Vivaspin 20 concentrators.

2.5.3. Removal of antipeptide antibodies

Since the immunoaffinity purified AaPAL preparation contained AaPAL antipeptide antibodies detached from the column, the AaPAL preparation was further purified using Dynabeads M-280 sheep anti-Rabbit IgG magnetic beads (Dynal Biotech ASA, Oslo, Norway). The immunoaffinity purified AaPAL preparation (1.1 ml) was incubated with PBS (pH 7.4) washed beads ($24–28 \times 10^7$) with slow tilt rotation at 4 °C for 1 h, the AaPAL containing supernatant was removed, and bound antigens were eluted from the beads using 1 ml 0.1 M citrate (pH 2–3) at RT for 2 min. The beads were washed once with 1 ml PBS (pH 7.4), after which the incubation, elution, and washing steps were repeated twice until no signal higher than background was detected from 1:250 dilution in ELISA with AaPAL C-terminal peptide as antigen.

2.5.4. Exclusion filtration

Since the purified AaPAL preparation contained an extra protein having an approximate molecular size of 65 kDa after treatment with magnetic beads, the AaPAL preparation was fraction filtrated first with 30 kDa, and then with 50 kDa MWCO concentrators (Centricon −30 and −50; Millipore Corporation, Bedford, MA, USA).

2.6. Bioinformatics

CLUSTAL FORMAT for T-COFFEE software was used for sequence alignment analyses (http://igs-server.cnrs-mrs.fr/Tcoffee/), and the NCBI protein–protein Blast (http://www.ncbi.nlm.nih.gov/BLAST) for searching identical short peptide sequences to the selected AaPAL N- and C-terminal peptides.

2.7. Outer membrane protein extraction

The outer membrane proteins of *A. actinomycetemcomitans* strain D7SS were extracted according to the method by Wilson [10].

2.8. LPS purification

LPS was purified from *A. actinomycetemcomitans* strain D7SS using a modification of the method by al-Hendy et al. [19]. Briefly, a 3-day plate culture was suspended to PBS in final OD_{600} = 2, 1.5 ml was taken, and the cells were harvested by centrifugation (16,000 × *g*, RT, 30 s). The bacterial pellet was suspended to 1.5 ml TEA-buffer (2 mM EDTA, 40 mM Tris–acetate pH 8.5), and centrifuged again as above. After suspending the bacterial pellet to 200 μl TEA-buffer, 400 μl alkaline solution (3% SDS, 0.6% Trizma-base, 128 mM NaOH) was added, and the suspension incubated at 60 °C for 70 min. Pre-heated phenol–chloroform (1:1 v/v; 600 μl) was mixed with the suspension, which was then heated at 60 °C for 15 min. The mixture was centrifuged (16,000 × *g*, RT, 10 min), and the upper phase digested with proteinase K (final concentration 0.19 mg/ml) at 60 °C for 60 min. After digestion, 1/10 vol of sodium acetate solution (3 M, pH 5.2) was added, vortexed, and precipitated with 2 × vol of ice-cold 99.5% ethanol at −80 °C for 20 min. The precipitate was separated by centrifugation (16,000 × *g*, 4 °C, 10 min) and suspended in 200 μl of Tris–buffered sodium acetate (100 mM sodium acetate, 50 mM Tris pH 8.0) by vortexing. LPS was precipitated with ice-cold ethanol and separated by centrifugation as described above, after which the LPS pellet was dried using SpeedVac concentrator (Savant, Farmingdale, NY, USA) for 5 min. The purified LPS was suspended in 50 μl H_2O and stored at −80 °C until used.

2.9. Human sera

Sera from three subjects were used to test whether human antibodies recognized the affinity-purified AaPAL preparation. Two of the subjects exhibited *A. actinomycetemcomitans*-associated infection, periodontitis or suspected infective endocarditis, and the third subject was periodontally and systemically healthy and culture-negative for *A. actinomycetemcomitans*. The patient with periodontitis was subgingivally *A. actinomycetemcomitans* culture-positive and had several pathologically deepened periodontal pockets with clinical and radiographic loss of periodontal attachment. The other patient had blood samples repeatedly culture-positive for *A. actinomycetemcomitans* due to the suspected infective endocarditis.

2.10. Characterization of the products

2.10.1. Chemical assays

The method by Lowry [20] was used to determine the total protein concentration of the preparation at various purification steps. The LPS concentration was estimated using Limulus assay (Cape Cod., E. Falmouth, MA, USA) for endotoxins of Gram-negative bacteria, in addition to the visual determination in silver-stained SDS–PAGE gels.

2.10.2. SDS–PAGE and silver staining

SDS–PAGE and silver staining that shows both proteins and LPS were used to estimate the purity of the samples (2–7 μg of protein in 10 μl). Protocols were modified from those in Current Protocols in Molecular Biology. Precision Plus Protein™ Standard (Bio-Rad) (1:20 diluted in H_2O; 10 μl dilution + 2 μl glycerol to inhibit fluctuation of standard to neighboring wells) was used as molecular weight standard in each gel (Criterion Tris–HCl 8–16% linear gradient gel [Bio-Rad]). The gel was stained using non-ammonical silver staining.

2.10.3. SDS–PAGE and immunoblotting with AaPAL antipeptide antiserum

SDS–PAGE and immunoblotting with AaPAL antipeptide antiserum were used to confirm the presence of AaPAL in the protein preparation. The SDS–PAGE was run as described above except that the amount of protein in each sample was approximately 10% of the silver-stained sample. A protocol modified from Current Protocols in Molecular Biology was used for immunoblotting. The proteins were transferred to PVDF membrane using discontinuous transfer buffer in semi-dry transfer system (Bio-Rad). The membrane was treated with 15 ml of 1:100,000 dilution of AaPAL antipeptide serum (III + 2W) at RT for 60 min to detect the AaPAL, and the bound primary antibodies were detected with HRP-conjugated donkey anti-rabbit IgG (1:10 000) (Jackson ImmunoResearch) at RT for 60 min together with SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and X-ray film.

2.10.4. SDS–PAGE and immunoblotting with purified antipeptide antibodies against N- and C-terminal AaPAL sequences separately

The immunoblotting was done as described above, except that the total protein amount in the sample was 12.5 ng. Dilutions containing 7, 0.7, and 0.07 μg/ml of primary antibodies, i.e. purified antipeptide antibodies against the N- and C-terminal AaPAL sequences separately, were used to detect cross-reactions with other proteins. In addition to the crude AaPAL preparation, also the whole-cell lysate containing 1 μg total protein was used in parallel with each sample in the gel to see the overall cross-reactions with *A. actinomycetemcomitans* proteins. Furthermore, the AaOMP preparation was immunoblotted using the anti-C-terminal peptide antibodies to see if the OMP preparation could be used as starting material in immunoaffinity purification.

2.10.5. MS analysis of the major constituents in the immunoaffinity purified AaPAL preparation

The immunoaffinity purified AaPAL was heated in a Laemmli sample buffer at 99 °C for 5 min, and the proteins (40 μg per lane) were separated on 8–16% gradient gels as described above. The gel was fixed (30% ethanol, 10% acetic acid, at RT for 30 min, on a shaker), rinsed once with water, stained (0.1% Coomassie blue, 49.5% ethanol, 10% acetic acid, at RT for 2 h, on a shaker), and destained (10% acetic acid, at RT overnight, on a shaker). The 17-kDa and ∼65-kDa bands were excised from the gel and subjected to trypsin digestion. Peptides for protein identification by a peptide mass fingerprint were created essentially as described by Shevchenko et al. [21] using sequencing grade modified trypsin from Promega. MALDI-TOF mass spectra were acquired by Thomas Kieselbach at the Umeå Life Science Platform using a Voyager DE-STR mass spectrometer from Applied Biosystems. Database searches were performed using an in-house Mascot server that was licensed to Umeå University by Matrixscience (www.matrixscience.com).

2.10.6. Antigenic reactivity of immunoaffinity purified AaPAL with human sera

To confirm the presence of antigenic epitopes of AaPAL in the purified protein, the final AaPAL preparation was slot blotted, a method that should not denature the 3-D structure of the protein extensively, using sera from two patients with *A. actinomycetemcomitans* culture-positive periodontitis or suspected endocarditis. Serum from a healthy subject was used as a negative control. AaPAL sample containing 280 ng of protein (one sample per each tested serum) and the same volume (30 ng protein) of <10 kDa fraction from the concentration step of immunoaffinity purified AaPAL preparation were applied into pre-wetted (water) and TBS (20 mM Tris, 500 mM NaCl, pH 7.4) equilibrated nitrocellulose membrane using MilliBlot™ Systems slot blot equipment (Millipore Corporation). After blotting, the membrane was blocked (TBS, 0.01% Tween 20 [VWR International, Fontenay Bois, France], 10% non-fat milk [Merck]) at 4 °C overnight. The membrane was washed three times for 5 min each (TBS + 0.01% Tween 20), cut into strips for detection using each serum individually, and incubated with human sera (1:1000) diluted in antibody buffer (TBS + 0.01% Tween 20, 3% bovine serum albumine (BSA) fraction V [Roche]) at 4 °C overnight. The strips were washed three times as described above, and incubated with HRP conjugated anti-human IgG (1:10,000 in antibody buffer; DakoCytomation, Solna, Sweden) at RT for 1 h. The reactions were detected using SuperSignal® West Pico Chemiluminescent Substrate and X-ray film.

2.11. Statistical analysis

The difference in the ELISA results between the immune and pre-immune sera were analyzed by Bonferroni corrected paired two-tailed *t*-test. *P*-values <0.05 were considered to be statistically significant.

2.12. Ethics

Permission for the production of AaPAL antipeptide antibodies in rabbit was obtained from the Ethics Committee for animal experiments in Umeå (Umeå djurförsöksetiska nämnd, Umeå, Sweden) in 2004. For the use of human sera for research purposes, informed written consents were obtained from the subjects and permission from the Ethics Committee (Institute of Dentistry, University of Helsinki, Helsinki, Finland) in 1991.

3. Results and discussion

3.1. AaPAL antipeptide antibodies, production and selectivity

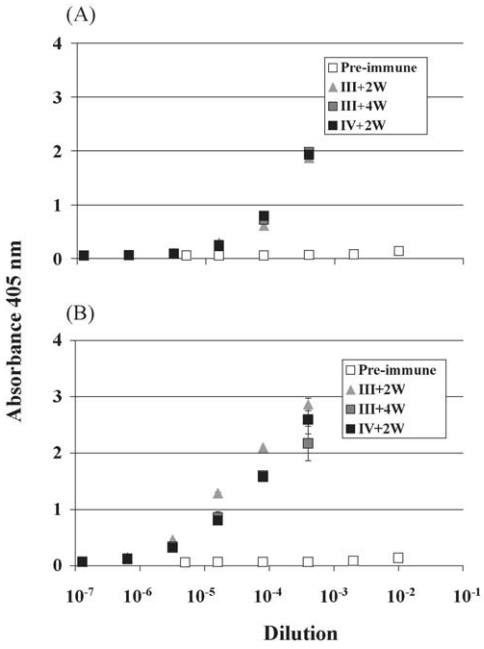
Only the first amino acid (serine) of the 14 amino acids long N-terminal peptide sequence of AaPAL was conserved in the respective sequences of its phylogenetic relatives, *H. influenzae*, *Pasteurella multocida*, *A. pleuropneumoniae*, and *H. ducreyi*. In fact, the interspecies comparison further showed that the selected sequence was the most variable part of the whole PAL protein in these species. However, the C-terminal peptide sequence was highly conserved; 71% of the amino acids were identical in all analyzed PAL sequences. Surprisingly, the N- and C-terminal sequence regions of AaPAL differed less from those of *E. coli* PAL than from those of the above-mentioned relative species, the percentage of identical amino acids being 57 and 79% for N- and C-terminal peptides, respectively. In addition, the alignment indicated that the C-terminal peptide sequence is a highly conserved region also among PALs of a variety of other bacterial species, such as *Photorhabdus luminescens*, *Salmonella typhimurium*, *Erwinia carotovora*, *Idiomarina loihiensis*, *Vibrio cholerae*, *Shigella flexneri*, *Yersinia pseudotuberculosis*, and *Y. pestis*, the presence of identical amino acids varying from 92 to 78%.

In addition to the high N- and C-terminal sequence identity, the AaPAL and *E. coli* PAL have high overall amino acid sequence identity and similarity values of 53 and 79%, respectively [8]. Thus, the modeling of the 3-D structure of the periplasmic part of the AaPAL could be done according to the resolved structure of *E. coli* PAL. The C-terminal peptide sequence of AaPAL, used in the rabbit immunization, was not conformationally constrained in the obtained model (Fig. 1), which suggests that the C-terminal AaPAL antipeptide antibodies most probably recognize the peptide sequence in the protein.

The titers of both N-terminal and C-terminal antipeptide antibodies reached the maximum already 2 weeks after the third immunization (Fig. 2). Thus, the fourth immunization did not increase the antibody titers. The titers of the N-terminal and C-terminal antipeptide antibodies in each immune serum (III + 2W, III + 4W, IV + 2W) differed statistically significantly from the titers of the pre-immune serum, as measured from the respective serum dilutions (1:2500, 1:12,500, 1:62,500) (Fig. 2C). The ELISA-measured antibody titers suggested that the antibody production was greater against the C-terminal than the N-terminal peptide (Fig. 2), which was confirmed by the higher yield of the C-terminal (8.3 mg) than the N-terminal antipeptide antibodies (2.6 mg) from the immune serum IV + 2W (20.7 ml).

Fig. 1. A model of the 3-D structure of the periplasmic part of AaPAL. The ribbon diagram of the periplasmic part of the AaPAL was created according to the resolved structure of the comparable part of the *E. coli* PAL (1OAP) using the Swiss-Pdb Viewer and SwissModel server (www.expasy.org/spdbv/ [16–18]). The C-terminal peptide sequence used in the rabbit immunization is shown in dark.

In order to examine the applicability of the N-terminal and C-terminal antipeptide antibodies in the immunoaffinity purification of AaPAL, the whole-cell lysate of *A. actinomycetemcomitans* and the crude AaPAL preparation were immunoblotted with different concentrations of the respective antibodies. The C-terminal antibodies showed significantly higher affinity than the N-terminal antibodies against AaPAL in immunoblot (Fig. 3). To subsequently choose appropriate starting material for the immunoaffinity chromatography, *A. actinomycetemcomitans* whole-cell lysate, OMP preparation, and the crude AaPAL preparation were immunoblotted with the C-terminal antibodies. In the immunoblot, the AaPAL antipeptide antibodies against the C-terminal sequence cross-reacted with several proteins in the whole-cell lysate (Fig. 3, lane A) as well as in the OMP preparation of *A. actinomycetem-*



(C)

N-terminal			
dilution	*III+2W*	*III+4W*	*IV+2W*
1:2 500	8.4×10^{-7}	9.6×10^{-7}	3.0×10^{-6}
1:12 500	2.8×10^{-2}	1.1×10^{-3}	1.6×10^{-5}
1:62 500	4.3×10^{-5}	4.0×10^{-4}	1.5×10^{-4}
C-terminal			
dilution	*III+2W*	*III+4W*	*IV+2W*
1:2 500	5.4×10^{-6}	8.6×10^{-4}	2.8×10^{-5}
1:12 500	1.9×10^{-4}	2.3×10^{-3}	4.8×10^{-4}
1:62 500	5.8×10^{-6}	5.8×10^{-3}	3.3×10^{-3}

Fig. 2. Antibody production in rabbit against N- (A) and C-terminal (B) peptides of AaPAL. Antibody amounts were measured using ELISA with N- or C-terminal AaPAL peptides as antigens. The measured time points were before immunization (Pre-immune), 2 weeks (III + 2W) and 4 weeks (III + 4W) after the third immunization, and final bleeding 2 weeks after the fourth immunization (IV + 2W). Each point represents mean ± S.D. from three repetitions. Each repetition contained duplicates. Statistically significant differences between immune sera and pre-immune serum are given as *p*-values of Bonferroni corrected paired two-tailed *t*-test (C).

comitans (Fig. 3, lane C), whereas only with two proteins, AaPAL and an unknown 25–37-kDa protein, in the crude AaPAL preparation (Fig. 3, lane B). However, in the crude AaPAL preparation (Fig. 3, lane B), the extra band of 25–37-kDa was visible only when the highest primary antibody concentration (7 μg/ml) and a long exposure time (135 min) were used.

The findings suggest that antibodies against the particular C-terminal sequence of AaPAL could be applicable not only to the purification of AaPAL but also to the purification of PALs of other bacterial species. In addition, the usage of the purified C-terminal antipeptide antibodies in immunoaffinity chromatography was favored by their larger yield and higher affinity as shown in the immunoblot results (Fig. 3).

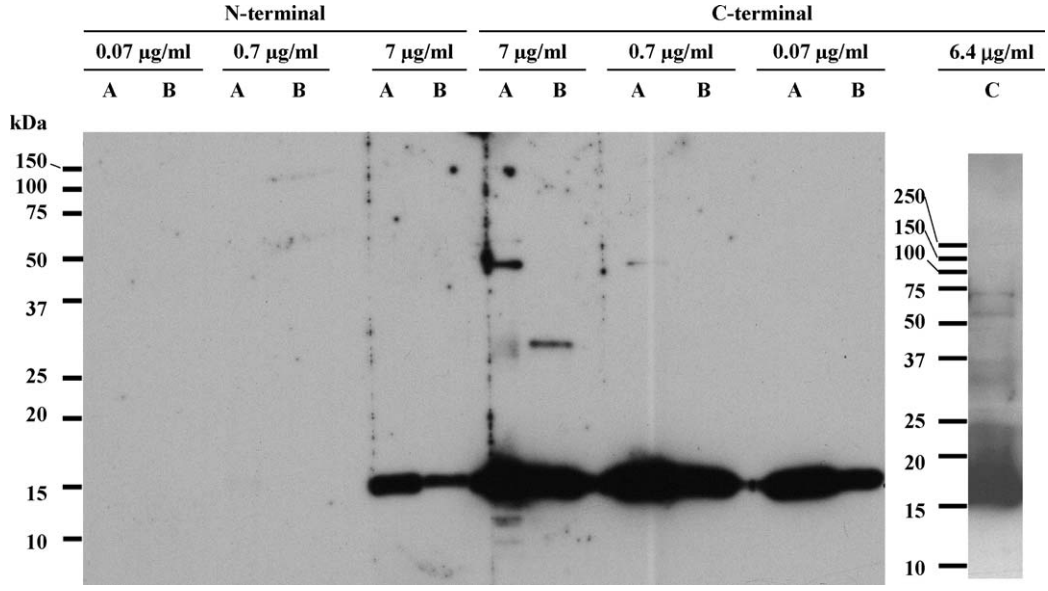


Fig. 3. Reactions of purified N- and C-terminal AaPAL antipeptide antibodies with the whole-cell lysate (A) and crude AaPAL preparation (B) of *A. actinomycetemcomitans* strain D7SS. The protein amount in each lane was 12.5 ng and the concentrations of N- and C-terminal AaPAL antipeptide antibodies varied from 0.07 to 7 μg/ml. The OMP preparation [10] of strain D7SS (C) was immunoblotted using C-terminal AaPAL antipeptide antibodies (6.4 μg/ml) to see if it could be used as a starting material in immunoaffinity purification. The X-ray film was slightly overexposed (2 h 15 min) in immunoblot of the whole-cell lysate (A) and crude AaPAL preparation (B).

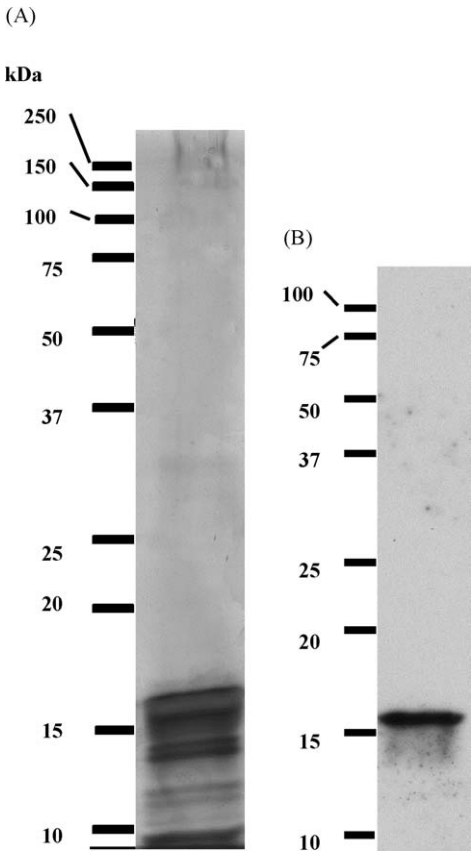


Fig. 4. Characterization of the crude AaPAL preparation. Sample of crude AaPAL was run in SDS–PAGE, which was silver-stained (A), or immunoblotted using AaPAL antipeptide antiserum (III + 2W; 1:100,000 dilution) (B). The amount of protein was 8 and 0.4 μg in silver staining and immunoblotting, respectively.

3.2. Crude preparation of AaPAL

The crude purification of AaPAL resulted in a total yield of approximately 4.0 mg of protein. In addition to the 17-kDa band, the size of the AaPAL, six faint bands were seen in the silver-stained SDS–PAGE gel of the crude AaPAL preparation (Fig. 4A). Four of the bands were of sizes smaller than 15 kDa, one was slightly bigger than 17 kDa, and one was between 25 and 37 kDa. The LPS concentration in the crude AaPAL was 147 ng/ml, corresponding to 0.037 ng/μg of the total protein.

An immunoblot analysis of the crude AaPAL preparation using antipeptide antiserum (III + 2W) revealed a strong reaction at 15–17 kDa (Fig. 4B), as anticipated according to the above results (Fig. 3) from the immunoblot analyses.

The crude purification of AaPAL before the immunoaffinity purification was essential because of the following reasons. Firstly, whole-cell lysate of *A. actinomycetemcomitans* could have blocked the affinity column and most probably would have required a pre-column to reduce the blockage risk. In addition, the whole-cell lysate of *A. actinomycetemcomitans* contained various other proteins reacting with the antibodies against the C-terminal peptide sequence selected for the immunoaffinity chromatography (Fig. 3, lane A). Secondly, using the OMP preparation as a starting material in the immunoaffinity chromatography could have caused problems due to the cross-reactions of the other AaOMPs with the antibodies against the C-terminal peptide of AaPAL (Fig. 3, lane C).

The used crude purification method is based on the insolubility of the peptidoglycan and the differences in the dissociation conditions of different peptidoglycan binding proteins. It has earlier been used as a part of a purification protocol of an unidentified 16.6-kDa AaOMP [11], which most probably is OMP 18/16 [7]. Since it is likely that one of the 15–18-kDa bands in our crude AaPAL preparation was OMP 18/16 [7], a final purification method based on the size of the target protein would have been questionable.

3.3. Immunoaffinity purification of AaPAL

The immunoaffinity purified AaPAL preparation had one prominent 17-kDa band in the 15–18 kDa region in silver-stained SDS–PAGE (Fig. 5A), whereas the crude AaPAL preparation contained at least two proteins of 15–18 kDa size (Fig. 4A). It is likely that one of these 15–18 kDa proteins is OMP 18/16 [7]. Since the AaPAL and OMP 18/16 do not share any significant sequence similarity, the immunoaffinity chromatography was an ideal option for the final purification of AaPAL.

In addition to the 17-kDa band, a band of >150-kDa molecular mass appeared in the same sample of the affinity purified AaPAL (Fig. 5A) although it was not visible in the crude AaPAL preparation (Fig. 4A). The >150 kDa band was removed with anti-Rabbit IgG magnetic beads (Dynabeads M-280), which suggests that the band contained AaPAL antipeptide antibodies. This was further confirmed by ELISA using the C-terminal AaPAL peptide as antigen. In ELISA, the absorbance values

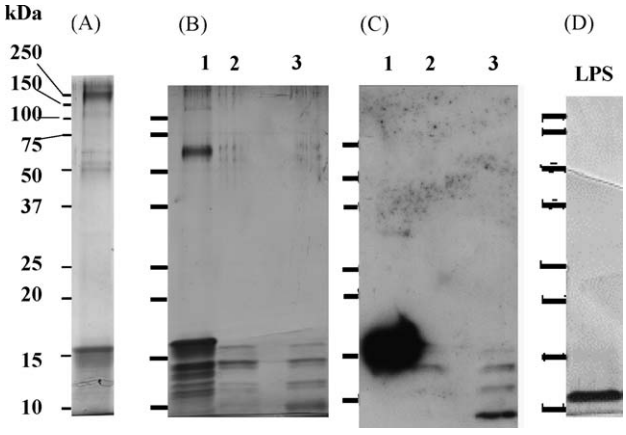


Fig. 5. Characterization of the immunoaffinity purified AaPAL (A). After removing antipeptide antibodies with sheep anti-rabbit IgG-coated magnetic beads (Dynal Biothech ASA, Oslo, Norway), the preparation was fractionated using filtration through different cut off (MWCO: 10, 30, and 50 kDa) centrifugal filtration units. Sample of each fraction was run in SDS–PAGE, which was silver-stained (B), or immunoblotted using AaPAL antipeptide antiserum (III + 2W; 1:100,000 dilution) (C). The amount of protein was 1.4, 1.1, and 1.7 μg in silver staining, for fractions 1 (>50 kDa), 2 (<50 and >30 kDa), and 3 (<30 and >10 kDa), respectively, and 1:10 of these for immunoblotting. Fraction 1 (>50 kDa) corresponds the final purification product of AaPAL. Purified LPS [19] from the same *A. actinomycetemcomitans* strain was run in a similar SDS–PAGE gel and was silver-stained (D) for LPS size determination.

(A_{405}) decreased significantly (from 0.57 to 0.06; measured from 1:250 dilution) after the IgG removal. The final absorbance value did not exceed the background level in ELISA. When preparing the immunoaffinity column, the antipeptide IgG antibodies were bound first non-covalently via the Fc region to the affinity column matrix (protein A-Sepharose CL-4B), after which the IgGs were covalently crosslinked to the protein A using dimethylpimelimidate. The method decreases sterical hindrance in the binding of the purified antigen, in this case AaPAL, to the AaPAL antipeptide antibody [15]. Although the AaPAL antipeptide antibodies were covalently bound to the immunoaffinity column, and the column was thoroughly washed before use, the results indicated that antibodies still could detach from the column during the AaPAL elution.

After the IgG removal a new ∼65-kDa protein had appeared to the purified AaPAL. To remove the ∼65-kDa protein from the purification product fraction filtration was applied using 30- and 50-kDa cut off concentrators. Although some of the proteins smaller than 17 kDa were removed, the ∼65-kDa band and 17-kDa AaPAL remained in the same >50-kDa fraction (Fig. 5B, lane 1) containing a total amount of 255 μg protein. The contaminating ∼65-kDa protein was identified using the MALDI-TOF MS, which results are reported and discussed below.

In the final AaPAL purification product, i.e. the main purification fraction >50 kDa, the silver-stained SDS–PAGE (Fig. 5B) as well as the Limulus assay showed LPS (0.005 ng/μg protein). The migration size of LPS from the strain D7SS was confirmed by running purified LPS from this strain on SDS–PAGE gel with subsequent silver staining (Fig. 5D). The presence of small amounts of LPS in the AaPAL preparation was expected, since tight PAL-LPS complexes have been reported [22]. For instance, several commercial LPS preparations contain PAL as impurity, and in some cases, the PAL cannot be removed from the complex with protease K treatment [22]. However, the LPS/protein ratio was relatively low (0.005 ng/μg) in our immunoaffinity purified AaPAL preparation.

3.4. Identification of the major proteins in the immunoaffinity purified AaPAL preparation

The identities of the major protein bands in SDS–PAGE, i.e. the 17-kDa and the ∼65-kDa bands (Fig. 5C), were determined with MALDI-TOF MS. When comparing the obtained mass spectra (Fig. 6) with the spectra from databases, the results showed that the 17-kDa band was AaPAL as expected, and the ∼65-kDa band was BSA (data not shown). The likely source of BSA was the solution containing 0.1% BSA where the magnetic beads were stored, although the beads were washed according to the manufacturer's instructions before use. The BSA contamination most probably can be avoided by extensive washing of the magnetic beads before use.

The identity of proteins smaller than 15 kDa (Fig. 5B) could not be determined with the present MALDI-TOF. It is probable that they represented degradation products of AaPAL as also suggested by the immunoblot (Fig. 5C), since the <15-kDa proteins were recognized by the AaPAL antipeptide antibodies in the smaller molecular size fractions <50 kDa >30 kDa and <30 kDa >10 kDa (Fig. 5C, lanes 2 and 3, respectively). In these two fractions, the <15 kDa proteins represented the majority of the total protein (Fig. 5B lanes 2 and 3).

3.5. Antigenic reactivity of purified AaPAL with human sera

The sera of *A. actinomycetemcomitans* culture-positive periodontitis and endocarditis patients reacted with the >50-kDa fraction of the immunoaffinity purified AaPAL preparation, whereas the serum of a healthy control subject did not (Fig. 7). Although only a limited number of sera were tested and the

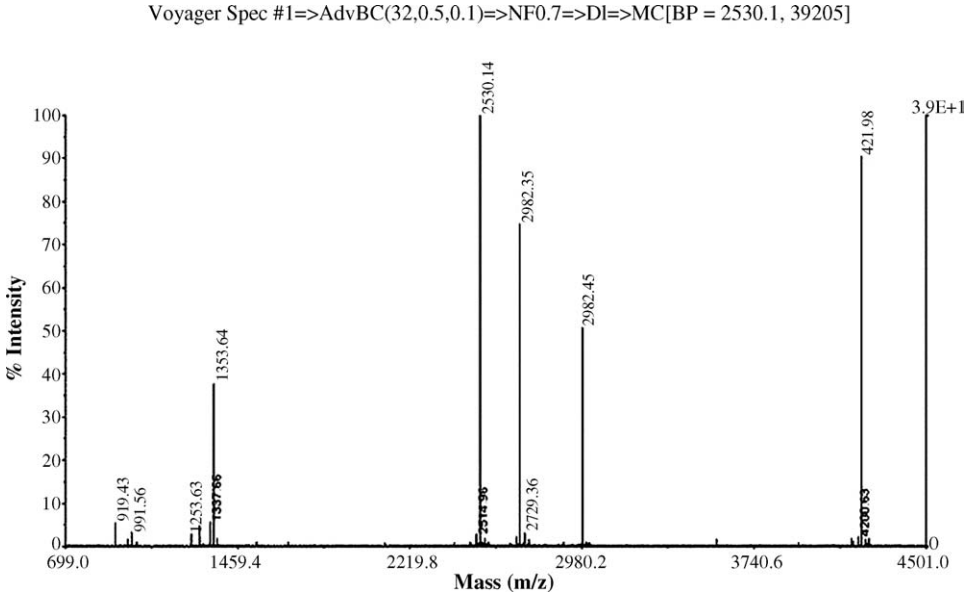


Fig. 6. MALDI-TOF peptide mass fingerprint of the trypsin digested major 17-kDa band in the immunoaffinity purified AaPAL preparation. The 17-kDa band was obtained from the Coomassie blue-stained 8–16% gradient SDS–PAGE gel. Peptides for protein identification by a peptide mass fingerprint were created essentially as described by Shevchenko et al. [21]. MALDI-TOF mass spectra were acquired using a Voyager DE-STR mass spectrometer (Applied Biosystems).

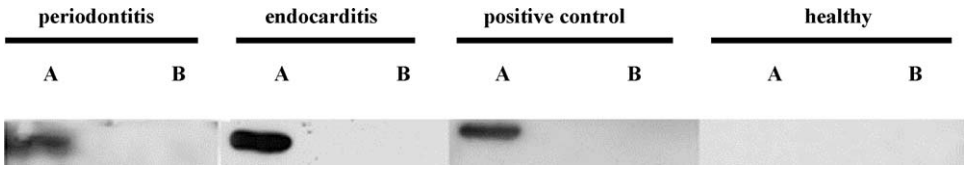


Fig. 7. Antigenic reactivity of purified AaPAL with human sera and AaPAL antipeptide antiserum (III + 2W). Immunoaffinity purified AaPAL (>50 kDa fraction; 280 ng protein) (A), as well as similar volumes (30 ng protein) of <10 kDa fraction (B) from the concentration step of immunoaffinity purified AaPAL preparation, was applied to the pre-wetted nitrocellulose membrane using Slot blot equipment. The membrane was treated with 1:1000 dilutions of sera of periodontits or endocarditis patients culture-positive for *A. actinomycetemcomitans*. Serum from a healthy subject (1:1000) was used as a negative control and AaPAL antipeptide rabbit serum (III + 2W; 1: 100,000) as a positive control. The bound primary antibodies were detected with appropriate HRP-conjugated secondary antibodies. The exposure times of the X-ray films were 1 h for human sera and 1 min for the positive control.

specificity of the immune reaction was not studied, it seems obvious that human antibodies recognized the purified AaPAL preparation. Additionally, the recognized antigen was in the >50-kDa fraction, since the <10-kDa flowthrough fraction from the same AaPAL preparation did not react with any of the sera (Fig. 7, lane B).

Harsh elution conditions in immunoaffinity chromatography may denature membrane proteins [15]. However, as confirmed with the slot blot assay the antibodies produced against AaPAL in *A. actinomycetemcomitans* culture-positive patients recognized the epitopes in the immunoaffinity purified AaPAL. Thus, at least some of the immunogenic epitopes were present in the purified AaPAL as determined by a method that should not denature the 3-D structure extensively. Although AaPAL was recognized by human serum IgG antibodies, the native conformation of the purified AaPAL is difficult to confirm due to the lacking natural enzymatic activity of the soluble protein. Since the natural environment of the protein is the outer membrane of a Gram-negative bacterium, the conformation of the soluble form most likely differs from that of the membrane situated AaPAL. However, it is conceivable that the conformation of the immunoaffinity purified soluble AaPAL resembles more the soluble PAL released by bacterial cells in vivo [23,24] than the membrane situated AaPAL. The 3-D structure of the released AaPAL, as well as other PALs, still remains unknown.

4. Conclusions

This is the first study reporting purification of AaPAL, a conserved outer membrane protein in Gram-negative bacteria. Our protocol consisted of two steps, the crude preparation, using a previously reported method for purification of an OMP P6, a homolog of AaPAL, of *H. influenzae* [12,13] and a further purification of the crude preparation by immunoaffinity chromatography using AaPAL antipeptide antibodies. The first purification step was essential, since the AaPAL antipeptide antibodies cross-reacted with various proteins in the whole-cell lysate of *A. actinomycetemcomitans*. The C-terminal sequence of AaPAL, used for production of antibodies to be used in immunoaffinity chromatography, was highly conserved among PALs of various species. Therefore, our method, combining the crude preparation and immunoaffinity, seems applicable for the purification of AaPAL but also PALs of other Gram-negative species. Since no significant amounts of other bacterial proteins than AaPAL were found in the immunoaffinity purified AaPAL as confirmed by silver-stained SDS–PAGE and MALDI-TOF mass spectrometry, the preparation could be used in studies investigating biological activities of AaPAL.

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