Common Antigens of Streptococcal and Non-streptococcal Oral Bacteria: Isolation and Biochemical Characterization of the Extracellular Protein Antigen

By MARIE SCHÖLLER*, J. PAUL KLEIN, PASCAL SOMMER AND ROBERT FRANK

Groupe de Recherches, INSERM U 157 Faculté de Chirurgie Dentaire, 67000 Strasbourg, France

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An extracellular soluble common protein (ECP) has been purified from extracellular soluble fractions of exponential phase cultures of *Streptococcus sanguis* OMZ9, of a representative strain of each of Bratthall's seven serological groups of *Streptococcus mutans*, and of one strain each of *Lactobacillus salivarius* and *Actinomyces viscosus*. The ECP antigens from the different strains were prepared from SDS-dissociated immunoprecipitates by affinity chromatography on an anti-rabbit immunoglobulin column. The identity of such purified ECP antigens was demonstrated by their behaviour in immunodiffusion analysis, in SDS-PAGE, in which an identical molecular weight (60000) was found, and by virtue of their similar amino acid and sugar compositions. This common antigen (ECP) consisted of 90% protein and 10% sugar.

INTRODUCTION

Streptococcus mutans, an important bacterium in the aetiology of dental caries, has been immunologically grouped into seven serotypes, *a* to *g* (Bratthall, 1970; Perch *et al.*, 1974). The serotype antigens have been characterized as wall-associated polysaccharides, and have been extensively studied by several investigators (Burgess & Edwards, 1973; Iacono *et al.*, 1975; Wetherell & Bleiweis, 1978; Brown & Bleiweis, 1979; Hamada & Slade, 1980). However, very little is known about the surface proteins of *S. mutans* strains. Mukasa & Slade (1973) found that certain proteins were simultaneously extracted with the group antigen of *S. mutans* FA-1 by treatment with cold trichloroacetic acid or lysozyme. Several *S. mutans* strains were found to possess protein antigens which appeared to bind more or less strongly to cell walls (Russell, 1979; Nesbitt *et al.*, 1980). Thus, glucosyltransferase as well as dextran-binding proteins were found to be associated with the cell surface of *S. mutans* (Kelstrup & Funder-Nielsen, 1974; Kuramitsu & Ingersoll, 1978). Examination of culture supernatants and cell wall extracts of *S. mutans* serotype *c* showed the presence of at least four proteins (I to IV), in addition to the serotype antigen (Russell & Lehner, 1978). Some of these proteins were shown to be common to other serological groups of *S. mutans* (Russell *et al.*, 1980*a*).

In a previous investigation of the antigenic structure of *S. sanguis* OMZ9 we reported the existence of at least three extracellular soluble antigens (ESA) present in culture fluids and common to different oral bacteria including strains representative of each serological group of *S. mutans*, and a strain each of *Actinomyces viscosus* and *Lactobacillus salivarius* (Schöller *et al.*, 1981). Two of these antigens were also present on the cell surface of all bacteria tested. The third antigen was only found in the cell-free culture supernatant and was identified as a soluble protein common to all bacterial species studied. This extracellular common protein (ECP) has

Abbreviations: ECP, extracellular common protein; ESA, extracellular soluble antigen; PBS phosphate-buffered saline; WEA, wall-associated antigen.

now been purified from *S. sanguis*, *S. mutans*, *A. viscosus* and *L. salivarius* culture supernatants. Isolation and purification of ECP antigen was performed by dissociation of the antibody-antigen complexes followed by an affinity chromatography step. This paper describes these procedures, together with a biochemical comparative analysis of the protein purified from each strain.

METHODS

Organisms. The following strains, *S. sanguis* OMZ9, *S. mutans* strains E49 (serotype *a*), OMZ51 (*b*), OMZ70 (*c*) OMZ176 (*d*), B-2 (*e*), OMZ175 (*f*) and KIR (*g*), *L. salivarius* ATCC 11741 and *A. viscosus* OMZ104, were described previously (Schöller *et al.*, 1981). Strains were maintained in Brain Heart Infusion Broth (BHI, Difco).

Antigen preparation. Antigens were prepared from exponential phase cultures grown in synthetic medium D1-20 (Carlsson 1972). Extracellular soluble antigens were precipitated from culture supernatants with ammonium sulphate at 90% saturation and then desalted on a Bio-Gel P6 column (Bio-Rad). Wall-associated antigens (WEA) were extracted from the bacterial pellet with 0.5 M-phosphate buffer pH 6.0 (Schöller *et al.*, 1981).

Heat-killed *S. sanguis* cells were prepared from cells grown in BHI supplemented with glucose, according to the technique described by McCabe & Smith (1973).

Antisera. Antisera against ESA and WEA from *S. sanguis* were raised in rabbits by administering four subcutaneous injections of an equal mixture of antigens and Freund's incomplete adjuvant at 10 d intervals (Schöller *et al.*, 1981); animals were bled 10 d after the last injection. After ammonium sulphate precipitation at 50% saturation, the IgG fraction of anti-ESA gamma globulin was obtained by chromatography on protein A-Sepharose CL4B (5 × 1 cm) (Pharmacia) equilibrated with 0.1 M-phosphate buffer pH 7.2. The IgG fraction was eluted in the second peak with 0.2 M-glycine/HCl buffer pH 2.2 (Ey *et al.*, 1978).

Sheep IgG (Institut Pasteur Production, Paris) directed against rabbit immunoglobulins were also prepared by chromatography on protein A-Sepharose. Sheep IgG (30 mg) was coupled to CNBr-activated Sepharose 4B (Pharmacia) as described by the manufacturers. The immunosorbant was packed into a column (5 × 1 cm) and equilibrated with phosphate-buffered saline (PBS).

Preparation and dissociation of immune complexes. Anti-*S. sanguis* ESA IgG was first absorbed with *S. sanguis* heat-killed cells and then with *S. sanguis* WEA to eliminate the cross-reactive antigens, as previously described (Schöller *et al.*, 1981). In immunodiffusion, the double cross-absorbed IgG fraction thus obtained gave only one precipitin band common to all bacterial species and was further used for antigen purification.

The extracellular common protein was purified from immune complexes by mixing ESA from each bacterial strain with anti-*S. sanguis* ESA cross-absorbed IgG fraction. The equivalence point for antigen-antibody precipitation was determined by reacting together several dilutions of antigen and antibody in ID tests. Concentrations of antigen and antibody corresponding to the equivalence point were then used to prepare immunoprecipitates. After incubation for 1 h at 25 °C to avoid absorption of non-specific IgG, the immunoprecipitate was sedimented (20 min, 10000 *g*, 4 °C) and washed twice with PBS. The washed complexes were suspended in 100 µl electrophoresis buffer containing various dissociating agents, mixed for 10 min and submitted to PAGE. Various concentrations of Triton X-100, SDS and urea were tested for their ability to dissociate the immune complexes.

Antigen purification. The ECP antigen was purified from immune complexes obtained by mixing 10 ml ESA from each bacterial strain with 10 ml of the anti-ESA absorbed IgG fraction. After sedimentation and washing, the immune complexes were dissociated in a minimal volume (0.5 ml) of PBS containing 0.5% SDS, boiled for 3 min and immediately diluted 50-fold with cold PBS. The diluted samples were applied to the sheep anti-rabbit immunoglobulin immunosorbant and eluted with PBS at a flow rate of 20 ml h⁻¹. Fractions of 5 ml were collected until *A*₂₈₀ was lower than 0.02. The column could be used again after elution with 0.2 M-glycine/HCl buffer pH 2.2 and equilibration with PBS.

Analytical methods. The total carbohydrate content was evaluated by the anthrone method of Morris (1948) with D-glucose as a standard. Protein concentration was measured by the Lowry procedure with bovine serum albumin as a standard.

Amino analysis was carried out on ECP purified by affinity chromatography. ECP from each strain was hydrolysed in 6 M-HCl at 110 °C for 24 h under vacuum and then analysed with a Beckman Instruments model 119CL analyser. Cysteine was measured as cysteic acid after performic oxidation.

Samples were subjected to methanolysis with methanol/0.5 M-HCl under reflux during 24 h; liberated sugars were submitted to trimethylsilylation and subjected to GLC on a Hewlett-Packard 7620A chromatograph with a flame ionization detector on a silicone OV17 column. Derivatives were separated on an Innox column (4 m) containing the following stationary phase: Chromosorb 80-100 mesh, WAD-DMCS 3%, and silicone OV17, employing a linear temperature gradient of 115-200 °C at 4 °C min⁻¹; the carrier nitrogen flow was 20 ml min⁻¹.

The effectiveness of antigen-antibody dissociation was checked by PAGE according to the method of Davis (1964), in the presence of 0.1% Triton X-100, 0.1% SDS or 6 M-urea. Gels were scanned in a Beckman Acta CII

Spectrophotometer. SDS-PAGE was done by the method of Weber & Osborn (1969), with 7.5% (w/v) polyacrylamide gels. Prior to electrophoresis, the different samples were treated for 5 min at 100 °C in the presence of 1% SDS. Molecular weights were estimated by co-electrophoresis of ECP samples with standard proteins (electrophoresis calibration kit, Pharmacia). Immunodiffusion was performed in 1% (w/v) agarose (Ouchterlony, 1958).

RESULTS

Immune complex dissociation

It has been observed previously that the ESA fraction obtained from representative strains of the different serotypes of *S. mutans*, as well as from *L. salivarius* and *A. viscosus*, contained a single common protein antigen (ECP), which reacted with *S. sanguis* ESA antiserum that was absorbed with both whole cells and WEA. The ECP antigen appeared to be selectively excreted during bacterial growth (Schöller *et al.*, 1981). Attempts were made to use the immunoprecipitates thus obtained to purify ECP. Dissociation of antigen-antibody complexes was achieved with all reagents tested [Triton X-100, 1% (v/v); SDS, 0.5 to 1% (w/v); and urea, 6 M].

A mixture of proteins (Fig. 1) was obtained with all three dissociating procedures. However, maximal dissociation was obtained by 0.5% SDS as judged by PAGE analysis of the immunoprecipitates (Fig. 1, C) and was used throughout this study. In these profiles, the ECP was not resolved from IgG heavy chain, but was well separated from IgG light chain.

Purification of the ECP antigen

Immunoprecipitates were prepared from each strain by mixing 10 ml ESA (2 mg ml⁻¹) with 10 ml of the rabbit IgG fraction at the required concentration. Antigen-antibody complexes, after dissociation were immediately diluted 50-fold and applied to a column containing immobilized sheep anti-rabbit IgG: rabbit IgG was retained, whereas the ECP antigen passed through with the starting buffer (PBS). ECP antigens were dialysed against 10-fold diluted PBS and concentrated by lyophilization to a final protein concentration of 1 mg ml⁻¹.

The purity of the ECP antigens prepared from each strain by affinity chromatography was checked by gel electrophoresis and by immunodiffusion. The electrophoresis pattern of ECP antigens from different strains showed a single band of Coomassie brilliant blue positive

Fig. 1. Electrophoretic pattern of *S. sanguis* OMZ9 immunoprecipitates analysed according to Davis (1964). Precipitates were obtained by mixing *S. sanguis* OMZ9 ESA with absorbed anti-ESA IgG (see Methods). The complexes were dissolved in 6 M-urea (A), 1% (v/v) Triton X-100 (B), or 0.5% (w/v) SDS (C). Scans of Coomassie blue stained gels are shown. ECP, extracellular common protein; H, IgG heavy chains; L, IgG light chains.

Fig. 2. SDS-PAGE of samples of purified ECP from the following strains: A, *S. sanguis* OMZ9; B, *S. mutans* serotype *a*; C, *S. mutans* serotype *b*; D, *S. mutans* serotype *c*. Arrows indicate the positions of standard reference proteins (sizes shown in kDal).

Fig. 3. Immunodiffusion of different ECP fractions against whole anti-*S. sanguis* ESA serum. Wells 1, 2, 3, 4, 5 and 6 contained, respectively, purified ECP from *S. sanguis* OMZ9 and from *S. mutans* serotypes *a*, *b*, *c*, *d*, and *e*.

material (Fig. 2) for all antigens tested. The molecular weight of the ECP antigens was estimated by SDS-PAGE as approximately 60 000 for all strains.

In immunodiffusion tests, ECP antigens from all the strains reacted with anti-*S. sanguis* ESA serum to give a single common precipitin line (Fig. 3). Absorption of anti-ESA IgG with *S. sanguis* ECP abolished the precipitin reaction with all ECP antigens. However, the ECP antigens did not react with either *S. sanguis* WEA antiserum absorbed with ESA or sheep anti-rabbit IgG serum. All these results are in favour of total identity of the ECP antigens derived from all oral strains and confirm their monomeric nature.

Biochemical characterization of the ECP antigens

Amino acid analysis of the ECP antigens isolated from the different bacterial strains revealed a mixture of the common amino acids except methionine, which was not detected, and tryptophan, which was destroyed during hydrolysis (Table 1). The amino acid composition of the ECP

Table 1. Amino acid and amino sugar composition of the ECP antigens derived from the different bacterial strains

Amino acid	Number of residues per mol*									
	<i>S. sanguis</i>	<i>S. mutans</i> serotype:							<i>L. salivarius</i>	<i>A. viscosus</i>
		<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>		
Lysine	33	29	29	32	32	31	30	31	32	31
Histidine	11	8	8	12	8	7	9	13	9	9
Arginine	14	11	12	13	12	10	14	17	14	12
Aspartic acid	41	42	42	38	44	40	43	40	44	47
Threonine	45	44	42	47	45	39	43	42	46	42
Serine	44	44	43	42	46	44	46	44	47	42
Glutamic acid	44	55	56	53	47	53	52	44	44	49
Proline	39	43	43	40	40	41	40	41	42	48
Glycine	—	—	—	—	—	—	—	—	—	—
Alanine	34	43	40	42	39	39	36	33	37	36
Half cystine	17	14	14	11	14	12	12	10	11	13
Valine	41	47	42	43	42	43	45	44	43	47
Methionine	—	—	—	—	—	—	—	—	—	—
Isoleucine	14	13	14	13	15	21	14	15	14	17
Leucine	26	26	28	24	26	32	27	27	27	31
Tyrosine	11	5	7	6	7	6	9	10	8	7
Phenylalanine	24	23	23	19	24	22	21	21	22	20
Glucosamine	12	9	10	10	8	9	10	11	11	10
Galactosamine	7	10	9	8	12	13	9	13	8	10

* Calculated numbers of residues based on assumed molecular weight of 60000.

Table 2. Neutral and amino sugar composition of the ECP antigens derived from the different bacterial strains

Sugar(s)	Sugar composition (percentage of total)									
	<i>S. sanguis</i>	<i>S. mutans</i> serotype:							<i>L. salivarius</i>	<i>A. viscosus</i>
		<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>		
Neutral sugars	5	5	5	5	5	5	5	5	5	5
Amino sugars	4	4	4	4	4	4.5	4	5.5	4	4
	Sugar composition (molar ratio)									
Glucose	10	10	10	10	9	9	10	9	10	10
Galactose	2	3	2	3	2	3	3	2	3	3
Mannose	4	3	5	4	5	5	4	3	4	3
Fucose	4	4	3	3	4	3	3	5	4	4
Glucosamine	10	8	9	9	6	7	8	9	9	8
Galactosamine	6	8	7	7	10	11	8	11	7	8

antigens was characterized by high concentrations of aspartic and glutamic acids, as well as threonine, serine, proline and alanine. Cysteine, measured as cysteic acid, was present at a concentration of only 3%. All samples were characterized by a high level of glycine, probably due to residual residues derived from the glycine/HCl buffer used for column regeneration. These values were not further considered for the total amino acid content and are omitted from Table 1.

The neutral carbohydrate content of the ECP antigens in each case was about 5% as estimated by the anthrone method (Table 2). From GLC analysis of ECP antigens, glucose, galactose, mannose and fucose were identified in similar molar ratio. ECP antigens also contained about

4% amino sugars which consisted of glucosamine and galactosamine in molar ratio varying between 0.8 to 1.7.

The UV light absorption spectra of ECP antigens exhibited a maximum absorption at 280 nm, consistent with a 91% protein content. In all cases, no significant difference appeared between ECP purified from the different strains.

DISCUSSION

Recently special attention has been given to the extracellular and wall-associated protein antigens of *S. mutans* (Russell & Lehner, 1978; Russell, 1979; Nesbitt *et al.*, 1980; Russell *et al.*, 1980*a, b*), which could play an important role in caries protection studies (Russell *et al.*, 1980*b*; Lehner *et al.*, 1980). In a previous report, we described the presence of a protein antigen (ECP) selectively released in the culture media in the exponential phase of growth by *S. sanguis* OMZ9. This antigen appeared to be common to some oral streptococci including representative strains of the seven serotypes of *S. mutans*, as well as a strain of *L. salivarius* and a strain of *A. viscosus* (Schöller *et al.*, 1981). Monospecific antisera to *S. sanguis* ECP antigen was obtained by extensive cross-absorption of the anti-ESA serum with both heat-killed *S. sanguis* cells and WEA fraction. The data from immunodiffusion analysis of ESA obtained from the different oral strains with antisera cross-absorbed with *S. sanguis* showed the presence of only one precipitin band.

Several bacterial protein antigens were studied by PAGE analysis of solubilized immunoprecipitates (Dankert & Hofstra, 1978; Archer, 1979). However, the resulting protein profiles always showed contamination by some serum albumin.

Recently, Stone *et al.* (1979) reported the possibility of purifying IgM by dissociating brucella immune complexes. Dissociation of antigen-antibody complexes may yield proteins other than specific antibody, and such contaminant proteins, principally albumin-like proteins, can be eliminated by an additional gel filtration step. To overcome contamination by non-specific serum proteins, we used only the IgG fraction obtained after protein A-Sepharose chromatography. The existence of a single precipitin band in ID analysis of ESA with absorbed antisera, and the possibility of easily dissociating the antigen-antibody complexes, led us to purify the ECP antigens from crude ESA fractions obtained from the different oral strains.

Maximal dissociation of the immune complexes was obtained by SDS as judged by PAGE analysis. A high concentration of the ionic detergent SDS is known to interfere with antigen-antibody reaction in immunochemical techniques such as immunodiffusion or immunoelectrophoresis (Bjerrum & Lundahl, 1976). However, SDS at concentrations lower than 0.2% did not affect the activities of some bacterial enzymes (Owen & Doherty, 1979) and has been used previously for immunoprecipitation experiments to determine antibodies against free lipoprotein of the *E. coli* outer membranes (Inouye *et al.*, 1976); the method appeared to be very specific.

In our study, there were two consequences of the step that involved the 50-fold dilution of the dissociated immunoprecipitates. Firstly, the initial SDS concentration was reduced to 0.01%, which did not affect the interactions between the rabbit IgG and the anti-rabbit globulin immunosorbant. Secondly, the protein concentration in the solution was too low for immediate formation of antibody-antigen complexes.

Starting with the crude mixture of extracellular antigens, the ECP antigen was purified to homogeneity, as demonstrated by SDS-PAGE, by chromatography on an anti-rabbit IgG immunosorbant which removed rabbit IgG. ECP antigen recovered from the column was also immunologically homogeneous since immunodiffusion showed that ECP antigen reacted with both total or absorbed anti-ESA IgG to give a single precipitin line.

ECP antigens purified by affinity chromatography from *S. sanguis*, the seven serological groups of *S. mutans*, and individual strains of *L. salivarius* and *A. viscosus*, were identical as judged by PAGE analysis and by immunodiffusion. The apparent molecular weight of the ECP antigen determined by SDS-PAGE, was approximately 60000, regardless of the strain from which it was isolated. Positive cross-reaction of the different ECP antigens against anti-*S. sanguis* ESA or absorbed anti-ESA serum established the identity of ECP antigens from the

organisms tested. These results were supported by analysis of the amino acid and sugar composition of the different ECPs.

Another extracellular protein, antigen II, was described by Russell *et al.* (1980*a*) with a molecular weight of 48 000 and an acidic amino acid composition similar to that of ECP antigen, but antigen II differs from ECP in several respects. It is reported to be protease resistant, whereas ECP antigen is protease sensitive (Schöller *et al.*, 1981). Antigen II is also associated with cell wall of only some *S. mutans*, while the ECP antigen seems to be secreted by all bacteria tested; however, it may be present in undetectable amounts on the bacterial cell surface (Schöller *et al.*, 1981).

The presence of such a widely distributed antigen among oral bacteria may be of great importance for future caries protection studies. Recently, however, some *S. mutans* antigens were reported to cross-react with human heart tissue (Russell, 1979; Hughes *et al.*, 1980), and a further comparison of the ECP antigen and human heart tissue antigens will be necessary before it could be used in human vaccination.

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