

MICROBIAL PATHOGENESIS

# Isolation and characterisation of sialidase from a strain of *Streptococcus oralis*

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*Streptococcus oralis*, the most virulent of the viridans streptococci, produces a sialidase and this exo-glycosidase has been implicated in the disease process of a number of pathogens. The sialidase of *S. oralis* strain AR3 was purified in order to understand the characteristics of this putative virulence determinant. The enzyme isolated as a high mol. wt aggregate (c. 325 kDa) was purified 4520-fold from late exponential phase cultures by a combination of ultrafiltration, ammonium sulphate precipitation, ion-exchange and gel filtration chromatography. The sialidase component had a mol. wt of 144 kDa as determined by SDS-PAGE analysis. The purified sialidase released N-acetylneuraminic acid from a range of sialoglycoconjugates including human  $\alpha_1$ -acid glycoprotein, bovine submaxillary mucin, colominic acid and sialyl- $\alpha 2,3$ - and sialyl- $\alpha 2,6$ -lactose. Also, N-glycolylneuraminic acid was cleaved from bovine submaxillary mucin. The sialidase had a  $K_m$  of 11.8  $\mu\text{M}$  for  $\alpha_1$ -acid glycoprotein, was active over a broad pH range with a pH optimum of 6.0 and cleaved  $\alpha 2,3$ -,  $\alpha 2,6$ - and  $\alpha 2,8$ -sialyl glycosidic linkages with a marked preference for  $\alpha 2,3$ -linkages. The enzyme was competitively inhibited by the sialic acid derivative, 2,3-dehydro-N-acetylneuraminic acid, with a  $K_{IC}$  of 1.2  $\mu\text{M}$ . The characteristics of the purified sialidase would support a nutritional role for this enzyme that may be significant in the proliferation of this organism in the oral cavity and at extra-oral sites in association with life-threatening infections.

## Introduction

The viridans streptococci have been traditionally regarded as minor opportunist pathogens, being primarily associated with dental caries [1]. However, over the past decade these bacteria have emerged as significant pathogens of immunocompromised patients and those with haematological malignancies [2–5]. Primary bacteraemia in susceptible patient groups is associated with secondary complications that include infective endocarditis, adult respiratory distress syndrome and streptococcal shock resulting in mortality rates of up to 30% [6, 7]. Treatment of infections caused by viridans streptococci is becoming more problematic: therapy with penicillins was once a standard regimen but has become less effective because of the emergence of high levels of antimicrobial resistance within this group of organisms [8].

The viridans streptococci are a highly heterogeneous group of organisms and it is only with the advent of modern typing schemes that it has been possible to associate specific diseases with the isolation of a particular species of viridans streptococcus [9–11]. The results of these and similar schemes have shown clearly that within the viridans streptococci, *Streptococcus oralis* is a significant agent of infective endocarditis and a major pathogen in patients with attenuated host defence mechanisms [6, 10, 12–14]. The mechanisms by which *S. oralis* causes this wide range of infections are as yet unclear, but the sialidase produced by this bacterium has been proposed as contributing towards the pathogenicity of several other micro-organisms, including *S. pneumoniae*, *Salmonella typhimurium* and *Vibrio cholerae* [15–17]. Bacterial sialidases cleave terminal, non-reducing sialic acid residues from sialoglycoconjugates. The precise role of these enzymes is unknown, but it has been suggested that they act as generalised virulence determinants [18] in that the release of sialic acid may expose cryptic carbohydrate binding sites for the invading organism [19, 20] and break down mucosal defence barriers of the host [21]. Furthermore, production of sialidase may be a critical factor in the provision of free sialic acid, a

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fermentable carbohydrate source for bacterial proliferation [18, 22, 23]. As a secondary consequence, sialic acid removal by these enzymes leads to direct damage to the host because of the central role of this sugar in cellular and molecular interactions [24].

In an attempt to identify virulence determinants of viridans streptococci causing serious infections, the possible role of the *S. oralis* sialidase in disease processes was examined. As a first step towards understanding the role of sialidase as a putative virulence determinant, the sialidase was isolated from *S. oralis* and its properties were characterised.

## Materials and methods

### Bacterial strain and maintenance

*S. oralis* strain AR3, an isolate from infective endocarditis obtained from the London Hospital Medical College, was stored at  $-70^{\circ}\text{C}$  in vials containing cryopreservative (LabM, Salford, Lancs) and routinely maintained as described previously [22].

### Medium and growth conditions for extracellular sialidase production

Bacterial colonies were removed from agar plates into 200 ml of pre-reduced Tryptone Soya Broth (TSB; Oxoid) and incubated anaerobically until cultures had attained late exponential phase. This culture was used to inoculate 10 L of TSB and this culture was incubated aerobically at  $37^{\circ}\text{C}$  without agitation.

### Sialidase assay

Sialidase activity was monitored throughout the purification process with a synthetic fluorogenic substrate, 2'-4-methylumbelliferyl- $\alpha$ -D-N-acetylneuraminic acid (4-MU-NeuNAc; Sigma), as described previously [22]. The increase in relative fluorescence, caused by the release of 4-MU by the bacterial sialidase, was measured over time in a fluorimeter (Perkin Elmer LS-3B, Beaconsfield, Bucks) with excitation and emission wavelengths set at 380 nm and 460 nm, respectively. The release of 4-MU was quantified by comparison of relative fluorescence values with those obtained for standard concentrations of authentic methylumbelliferone.

### Protein determination

The total protein concentration at each stage of the purification was determined by a bicinchoninic acid assay (Sigma) according to the manufacturer's instructions. A more rapid protein assay, the Coomassie Blue dye-binding assay (Pierce and Warriner, Chester, Cheshire) was used to screen for the presence of protein in column fractions. Coomassie Blue protein reagent (100  $\mu\text{l}$ ) was added to 100  $\mu\text{l}$  of sample from

column fractions in a microtitration tray and the  $A_{620}$  was determined.

### Purification of the extracellular sialidase of *S. oralis*

**Stage I:** culture supernate. Cells were removed from the culture of *S. oralis* by means of an ultrafiltration unit fitted with a 0.16- $\mu\text{m}$  cut-off membrane (Minisette, Flowgen Instruments, Lichfield, Staffordshire). EDTA, leupeptin, phenylmethylsulphonyl fluoride and pepstatin A (all purchased from Sigma) were added to the cell-free culture supernate at final concentrations of 100  $\mu\text{M}$ , 1  $\mu\text{M}$ , 200  $\mu\text{M}$  and 1  $\mu\text{M}$ , respectively. All subsequent stages of the purification were performed at  $4^{\circ}\text{C}$  in 50 mM Tris-HCl buffer (pH 7.0) containing the listed protease inhibitors (buffer A). In the absence of protease inhibitors, extensive proteolytic degradation of the sialidase was observed.

**Stage II:** concentrated culture supernate. The culture supernate from stage I was concentrated 50-fold to a volume of c. 200 ml with an ultrafiltration unit (Minisette) fitted with a 10-kDa cut-off membrane.

**Stage III:** ammonium sulphate precipitate. The culture supernate from stage II was brought to 50% ammonium sulphate saturation with stirring and allowed to stand for 3 h before the precipitate was collected by centrifugation at 17 600 g for 30 min. The protein pellet, which contained 97% of the total sialidase activity, was dissolved in a small volume of buffer A. The preparation was dialysed against 6 L of this same buffer over 12 h.

**Stage IV:** DEAE Trisacryl eluate. The retentate from stage III was applied to a column of DEAE Trisacryl M ( $4.8 \times 15$  cm; Sepracor SA, Villeneuve la Garenne Cedex, France) pre-equilibrated in buffer A. The column was washed with two bed volumes (600 ml) of buffer A and adsorbed proteins were eluted with 400-ml volumes of this same buffer, each containing increasing concentrations of NaCl (0.05 M, 0.10 M, 0.15 M and 0.20 M), at a flow rate of 200 ml/h. The eluted fractions (8 ml) were assayed for sialidase activity and protein. The fractions eluting with 0.15 M NaCl, containing >93% of the total sialidase activity, were pooled and concentrated to c. 4 ml in a stirred-cell ultrafiltration unit with a 10-kDa cut-off membrane (Flowgen).

**Stage V:** Ultrogel ACA-34 eluate. The concentrated preparation from stage IV was applied to a column of Ultrogel ACA 34 ( $2.5 \times 100$  cm; Sepracor SA) pre-equilibrated in buffer A and proteins were eluted by this same buffer at a flow rate of 24 ml/h. All fractions (4.5 ml) were assayed for sialidase activity and protein. Sialidase-active fractions, at a protein concentration of 50  $\mu\text{g}/\text{ml}$ , were pooled and stored at  $-70^{\circ}\text{C}$ , with no

significant loss of enzyme activity detected after storage for 6 months.

### Further purification of the *S. oralis* sialidase

Further purification of the *S. oralis* sialidase preparation from stage V attempted by affinity chromatography with N-(p-amino-phenyl) oxamic acid-agarose [25] and gel filtration on Sephacryl S-300 (Pharmacia, St Albans, Herts) in the presence of SDS resulted in no further purification of the enzyme when analysed by SDS-PAGE.

### Native PAGE and SDS-PAGE

Native PAGE and SDS-PAGE [26, 27] analysis of the stage V preparation and material from subsequent purification steps was performed in order to determine the purity of the enzyme and to estimate the mol. wt of the sialidase. Native and SDS-PAGE gels were run on two separate occasions in order to ascertain the purity and mol. wt of the purified sialidase. A self-casting, mini gel system (105 × 100 mm; model MV1 DC, Anachem, Luton) was used for PAGE analysis.

SDS-PAGE was performed with a resolving 7% gel. The purified sialidase (10 µg in the presence of SDS) was resolved at 100 V for 4 h. Sialidase was located in the gel with 4-MU-NeuNAc [26]. Briefly, the gel was incubated in 0.2 M sodium phosphate buffer (pH 7.0) containing Triton X-100 10% v/v at 37°C for 2 h before spreading 500 µl of 100 µM 4-MU-NeuNAc over the surface of the gel. Sialidase activity was detected by the appearance of a fluorescent band, visualised with a UV light box ( $\lambda = 302$  nm; Biovision Transilluminator; Biogene, Kimbolton, Cambridge-shire). The image was captured with a high performance CCD camera linked to Bioscan Snapshot software (Datacell, Reading, Berkshire). After localisation of the sialidase activity, the gel was fixed and stained with Coomassie Brilliant Blue.

Native PAGE was performed with a resolving 6% gel. The purified sialidase (10 µg in non-denaturing buffer) was resolved by electrophoresis and localised in the gel as described for SDS-PAGE, except that Triton X-100 was omitted from the gel washing solution. The gel was stained for protein with a silver staining kit (Sigma) according to the manufacturer's instructions.

### Mol. wt determination by gel filtration

The Ultrogel ACA-34 column used in the purification of the *S. oralis* sialidase was calibrated with mol. wt standards (Sigma) and the elution conditions described earlier. The void volume was determined from the elution position of blue dextran. Fractions (3 ml) were collected and assayed for protein, and the elution volumes of the protein standards and the purified sialidase were recorded. This procedure was performed

in triplicate to estimate the native mol. wt of the purified sialidase accurately.

### Substrate specificity of the *S. oralis* sialidase

Sialyl- $\alpha$ 2,3-lactose, sialyl- $\alpha$ 2,6-lactose, colominic acid (a polymer containing  $\alpha$ 2,8-linked N-acetylneuraminic acid – NeuNAc), bovine submaxillary mucin (BSM) (all purchased from Sigma),  $\alpha$ <sub>1</sub>-acid glycoprotein (AGP; Bio Products, Elstree) and 4-MU-NeuNAc were used to determine the substrate specificity of the purified enzyme. The sialic acids – NeuNAc and N-glycolylneuraminic acid (NeuNGc) – of the native glycoconjugates were quantified by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with a DX-500 system (Dionex UK, Camberley, Surrey) after mild acid hydrolysis [28].

The enzymic release of sialic acids from glycoconjugates was determined from reaction mixtures (1.0 ml) which contained 100 µl of the stage V enzyme preparation and 10 µM substrate with respect to bound NeuNAc or NeuNGc in 50 mM Tris-HCl buffer (pH 7.0). The assays were incubated at 37°C and the initial rate of NeuNAc or NeuNGc release was determined by HPAEC-PAD. The initial rate of release of 4-MU from 4-MU-NeuNAc was also quantified as described in the sialidase assay methodology; the concentration of 4-MU-NeuNAc present and the proportion of enzyme preparation added to the total volume of the assay was comparable with other substrate specificity assays.

### Enzyme kinetics of the *S. oralis* sialidase

The apparent  $K_m$  and  $V_{max}$  for the purified enzyme were determined with AGP as substrate. The initial reaction velocity of NeuNAc release from AGP over the substrate concentration range 10–300 µM NeuNAc was measured by HPAEC-PAD; the composition of the reaction mixture for the assay was as described above except that the total volume was scaled to 100 µl.

The fluorogenic assay for sialidase activity was employed to determine the pH optimum for the enzyme. Assays comprised: 35 µl of 0.2 M buffer (sodium citrate, pH 3.0–6.0; sodium phosphate, pH 6.0–8.0 and Tris-HCl, pH 8.0–9.0), 25 µl of 1 mM 4-MU-NeuNAc, an appropriate amount of enzyme preparation and distilled water to give a final volume of 100 µl. Assays were incubated at 37°C and the reaction was terminated by the addition of 100 µl of 0.5 M sodium carbonate buffer (pH 10.2) while the rate of increase in product formation was still linear with respect to time. Release of 4-MU was quantified and the pH optimum for sialidase activity was determined.

### Inhibition of the *S. oralis* sialidase

Inhibition of sialidase activity was examined with the fluorogenic assay for the enzyme described.

Compounds used in inhibition assays were the metal salts  $\text{CuSO}_4$ ,  $\text{ZnSO}_4$  and  $\text{HgCl}_2$ ; the thiols cysteine-HCl, dithiothreitol and iodoacetate; the metabolic inhibitors NaF and chlorhexidine; the NeuNAc derivative 2,3-dehydro-NeuNAc; oxamic acid and the glyco-protein-derived monosaccharides N-acetylglucosamine, mannose, galactose, N-acetylgalactosamine, fucose and NeuNAc (all purchased from Sigma).

Inhibition assays comprised 15  $\mu\text{l}$  of enzyme preparation and 15  $\mu\text{l}$  of 2 or 20 mM of the potential inhibitor in 50 mM Tris-HCl (pH 7.0). The enzyme solution was incubated at room temperature for 5 min before the addition of 100  $\mu\text{l}$  of 100  $\mu\text{M}$  4-MU-NeuNAc and 100  $\mu\text{l}$  of 2 or 20 mM of a listed compound or free sugar solution. Assays were incubated at 37°C and the rate of 4-MU release was quantified. Sialidase activity over a range of 2,3-dehydro-NeuNAc concentrations (0–5  $\mu\text{M}$ ) and at several substrate concentrations (20, 30 and 50  $\mu\text{M}$  4-MU-NeuNAc) was quantified to determine the type of inhibition and the  $K_{IC}$  for 2,3-dehydro-NeuNAc.

## Results

### Purification of the *S. oralis* sialidase

Extracellular sialidase was purified from a late exponential phase culture of *S. oralis* grown in TSB. Incubation of cultures beyond this stage led to degradation of the sialidase, due to the production of protease activities (data not shown). The enzyme was purified 4520-fold by a combination of ammonium sulphate precipitation, ion-exchange and gel filtration chromatography. Table 1 summarises the activity, yield and fold-purification of the sialidase for each step.

Fractionation of the enzyme by anion-exchange chromatography (stage IV) is shown in Fig. 1. A small proportion of the total sialidase activity (<6% of the total sialidase activity from stage III) eluted at 100 mM NaCl, but these fractions were not investigated further. Most of the enzyme activity eluted with application of 150 mM NaCl (fractions 175–188). Pooled active fractions were subjected to gel filtration chroma-

Table 1. Purification of the *S. oralis* sialidase

Stage	Sialidase preparation	Total volume (ml)	Protein concentration (mg/ml)	Total activity ( $\mu\text{mol}$ 4-MU released/min)	Yield (%)	Specific activity (nmol 4-MU released/min/mg of protein)	Fold purification
I	Culture supernate	10 000	3.59	2.87	100.0	0.08	1.0
II	Concentrated supernate	420	3.51	2.68	93.1	1.82	22.8
III	$(\text{NH}_4)_2\text{SO}_4$ precipitate	70	2.17	2.26	78.7	14.9	186.3
IV	DEAE Trisacryl	103	0.12	2.12	73.8	171.1	2138.8
V	Ultrogel ACA-34	47	0.05	0.85	29.6	361.7	4521.3

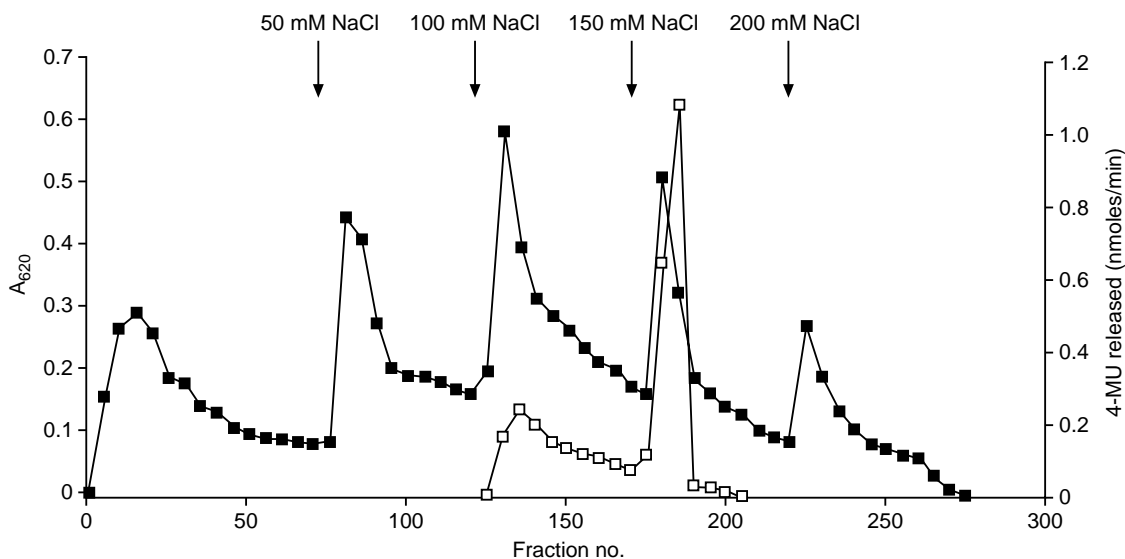


Fig. 1. Chromatography of the *S. oralis* sialidase on DEAE Trisacryl M. Proteins were eluted from a column of DEAE Trisacryl M at a flow rate of 200 ml/h by application of increasing concentrations of NaCl in buffer A (denoted by arrows). Fractions (8 ml) were collected and assayed for protein ( $A_{620}$ ; ■) and sialidase activity (4-MU released; □).

phy and the sialidase eluted as a distinct protein peak (fractions 127–136; Fig. 2).

### Purity and mol. wt of the *S. oralis* sialidase

The stage V sialidase preparation had an apparent mol. wt of c. 329 kDa as determined by gel filtration chromatography on Ultrogel ACA 34 calibrated with a range of mol. wt markers (Fig. 2). Native PAGE analysis of the enzyme demonstrated the presence of a single band with sialidase activity, as determined with the fluorogenic substrate (Fig. 3). After silver staining of the gel, one major band was apparent with a mol. wt of c. 322 kDa.

The enzyme exhibited a marked stability when heated at 100°C for 5 min in the presence of sample buffer containing SDS. A single band of enzyme activity was observed on gels stained with 4-MU-NeuNAc after reversal of SDS inhibition by treatment with Triton X-100 (Fig. 4). However, protein staining of this gel demonstrated the presence of eight major protein bands, the sialidase-positive band had a mol. wt of c. 144 kDa.

### Substrate specificity of the *S. oralis* sialidase and enzyme kinetics

The activity of the sialidase against AGP was studied and data indicated that substrate cleavage followed typical Michaelis-Menten kinetics. Further analysis of these data indicated that the apparent  $K_m$  value was

11.8  $\mu\text{M}$  and the estimated  $V_{\text{max}}$  was 0.12 nmol NeuNAc released/min.

The substrate specificity of the *S. oralis* sialidase for a range of sialylated substrates is summarised in Table 2. The rates of cleavage of NeuNAc from sialyl- $\alpha$ 2,6-lactose and colominic acid ( $\alpha$ 2,8-sialyl-linked) relative to sialyl- $\alpha$ 2,3-lactose (taken as 1.00) were calculated to be 0.54 and 0.44, respectively. This demonstrates that the enzyme cleaves NeuNAc which is  $\alpha$ 2,3-linked to the adjacent monosaccharide in preference to  $\alpha$ 2,6- or  $\alpha$ 2,8-linked. The relative rate of release of NeuNAc from the synthetic substrate 4-MU-NeuNAc was 0.86, which is comparable to the relative rate determined for the release of NeuNAc from the natural substrate AGP, calculated as 1.11. Therefore, 4-MU-NeuNAc is an appropriate substrate to assay the activity of this bacterial sialidase, as the enzyme displays a similar specificity for this synthetic substrate as it does for the natural, physiologically relevant AGP. It is interesting to note that the sialidase cleaves NeuNGc as well as NeuNAc from sialoglycoconjugates. However, the enzyme preferentially cleaved neuraminic acid containing an N-acetyl group over an N-glycolyl group, as the rate of NeuNGc release from BSM was seven-fold slower than the rate of release of NeuNAc from BSM.

The purified sialidase was active against 4-MU-NeuNAc over a wide pH range (4.0–9.0), but displayed >80% of the maximal activity between pH 5.5 and 7.0. The pH optimum was 6.0.

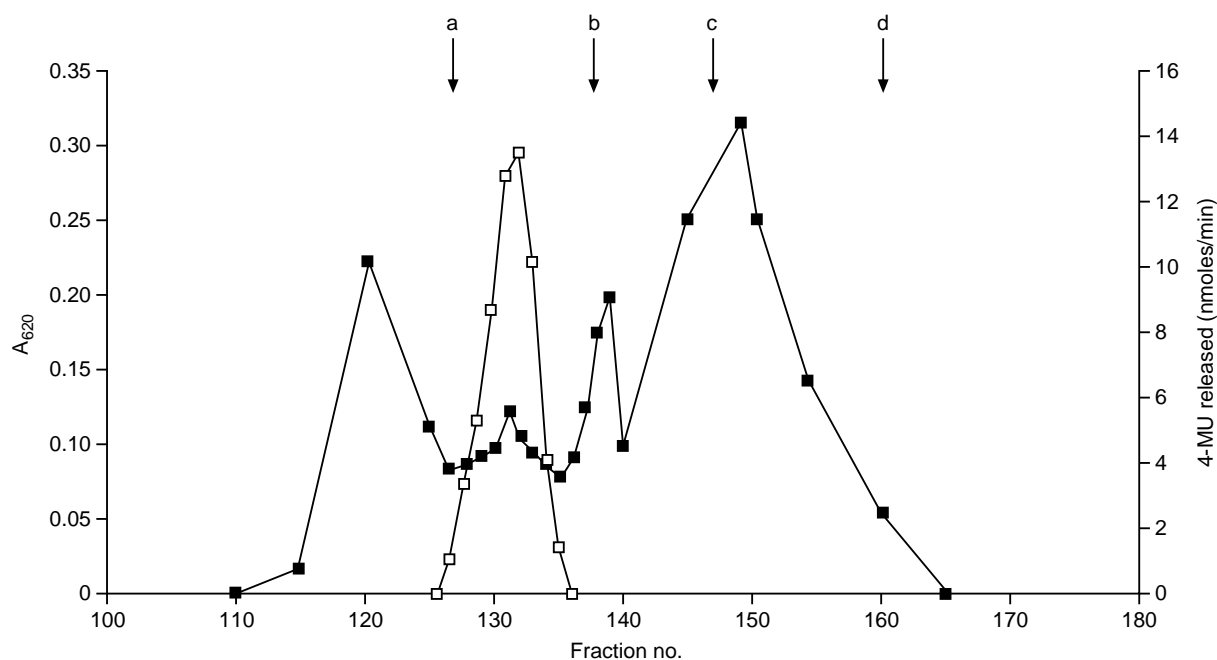


Fig. 2. Chromatography of the *S. oralis* sialidase on an Ultrogel ACA 34. Proteins were eluted from a column of Ultrogel ACA 34 at a flow rate of 24 ml/h in buffer A. Fractions (3 ml) were collected and assayed for protein ( $A_{620}$ ; ■) and sialidase activity (4-MU released; □). The column was calibrated with mol. wt markers: (a) apoferritin, 443 kDa; (b)  $\beta$ -amylase, 200 kDa; (c) serum albumin, 66 kDa; (d) carbonic anhydrase, 29 kDa; their elution positions are indicated by ↓.

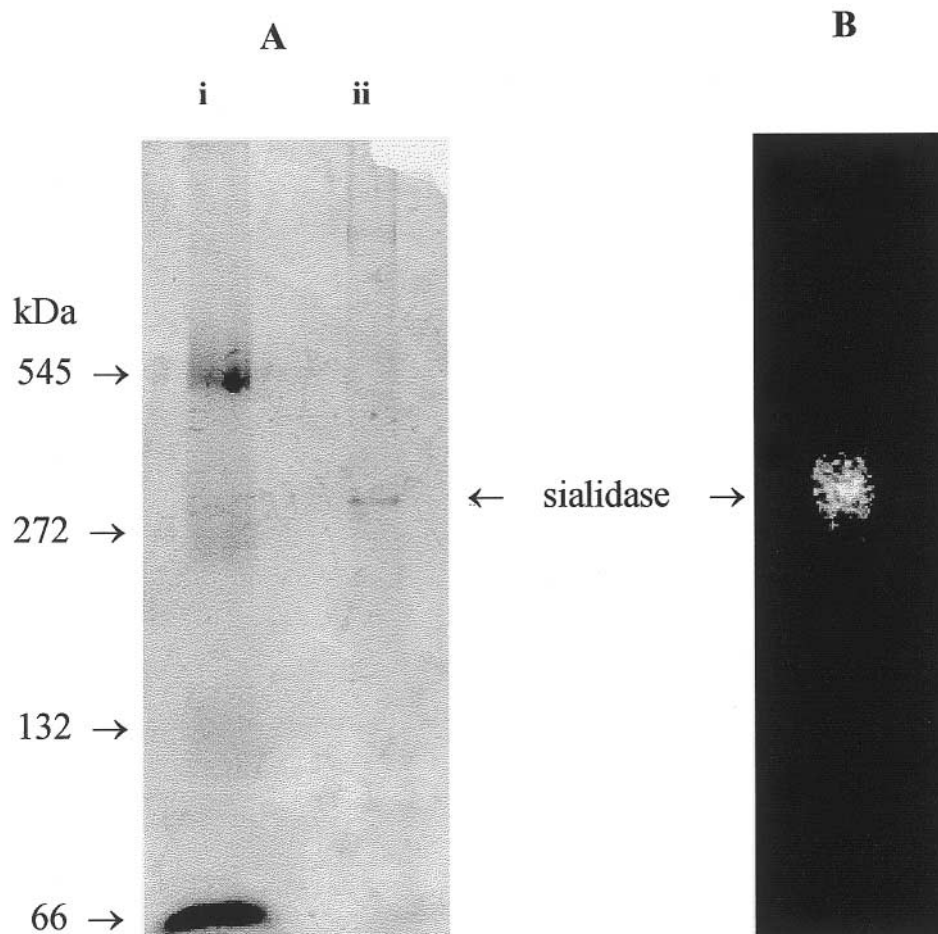


Fig. 3. Native PAGE analysis of the stage V sialidase. A, Lane (i) mol. wt markers (urease dimer, 545 kDa and monomer, 272 kDa and bovine serum albumin dimer, 132 kDa and monomer, 66 kDa); (ii) sialidase active band. The gel was stained for protein with silver stain. B, Sialidase activity, localised in the gel by the appearance of a fluorescent band when overlaid with 100  $\mu$ m 4-MU-NeuNAc.

### Inhibition of the *S. oralis* sialidase

N-Acetylglucosamine, mannose, galactose, N-acetylgalactosamine, fucose, NeuNAc, CuSO<sub>4</sub>, ZnSO<sub>4</sub>, HgCl<sub>2</sub>, cysteine-HCl, dithiothreitol, iodoacetate, NaF, chlorhexidine and oxamic acid at final concentrations of 1 or 10 mM had no effect on sialidase activity. However, the NeuNAc derivative 2,3-dehydro-NeuNAc completely inhibited sialidase activity at a concentration of 1 mM. The inhibitory effect of 2,3-dehydro-NeuNAc on sialidase was studied further by measuring enzyme activity against 4-MU-NeuNAc at various concentrations in the presence of this inhibitor over the concentration range 0–5  $\mu$ M (Fig. 5). It is evident from the Dixon plot that 2,3-dehydro-NeuNAc is a competitive inhibitor of sialidase, with a  $K_{IC}$  of 1.2  $\mu$ M.

### Discussion

The virulence determinants associated with diseases caused by viridans streptococci are not well understood, and to date most research has focused on the organisms' ability to adhere to host tissues, aggregate

platelets and resist host defences [6, 29–31]. Studies of several pathogenic micro-organisms have implicated sialidase as a major virulence determinant [32]. Sialidase is also produced by some viridans streptococci, including *S. oralis* [33], which is probably the most pathogenic species of this group, and the production of this enzyme may play a pivotal role in the preponderance of this organism in extra-oral infections. Therefore, the enzyme was isolated and purified to gain further insight into characteristics relevant to the disease process.

Earlier studies have indicated the inherent difficulties encountered in purifying sialidasases of viridans streptococci to homogeneity. This was most notable for an organism described as '*S. sanguis*', which could now be identified as *S. oralis* or *S. mitis*, where a high mol. wt aggregate containing sialidase was reported and purification to homogeneity was not achieved by conventional column chromatography [34, 35]. Likewise, in the present study a high mol. wt aggregate (c. 325 kDa), which possessed sialidase activity, was isolated as determined by native PAGE and gel filtration chromatography. It was not possible to purify

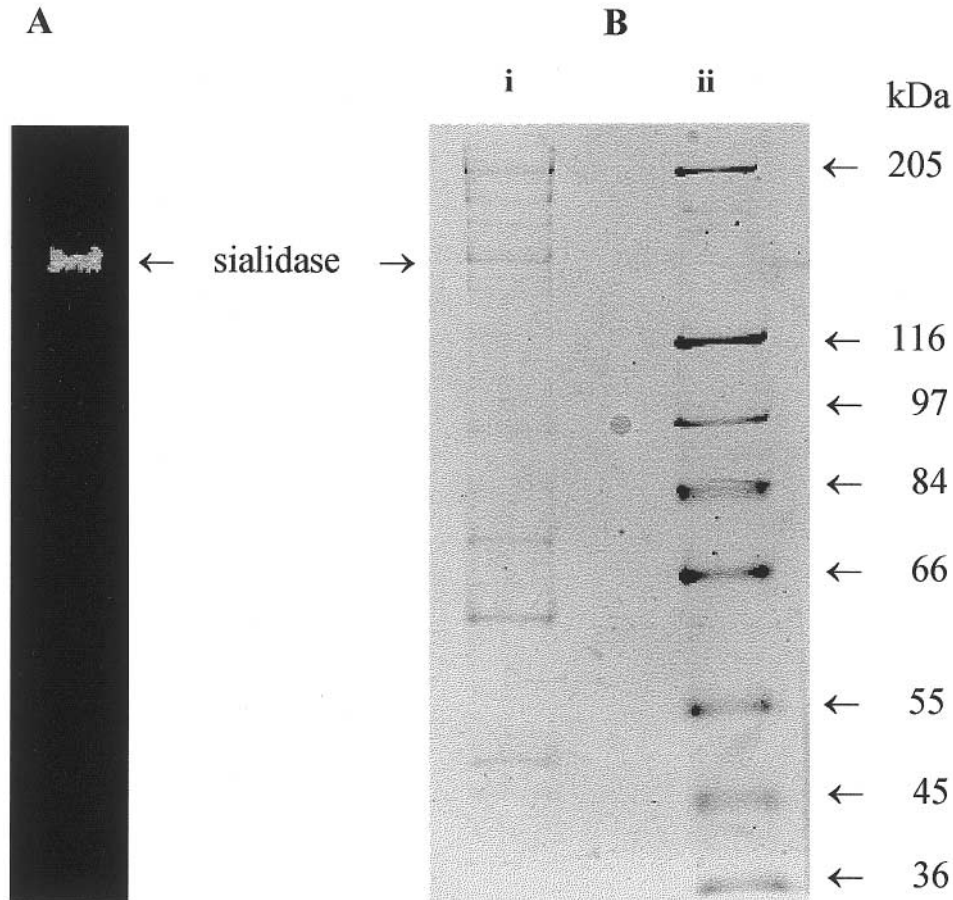


Fig. 4. SDS-PAGE analysis of the stage V sialidase. A, Sialidase activity; localised in the gel by the appearance of a fluorescent band when overlaid with  $100 \mu\text{m}$  4-MU-NeuNAc. B, Lane (i) sialidase active band and other purified proteins present in the stage V sialidase preparation; (ii) mol. wt markers (glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; ovalbumin, 45 kDa; glutamic dehydrogenase, 55 kDa; albumin, 66 kDa; fructose-6-phosphate kinase, 84 kDa; phosphorylase b, 97 kDa; galactosidase, 116 kDa; myosin, 205 kDa). The gel was stained with Coomassie Brilliant Blue.

Table 2. Substrate specificity of the *S. oralis* sialidase

Substrate	Sialyl-linkage	Relative rate of cleavage*
Oligosaccharides and polysaccharides		
Sialyl- $\alpha$ 2,3-lactose	$\alpha$ 2,3	1.00
Sialyl- $\alpha$ 2,6-lactose	$\alpha$ 2,6	0.54
Colominic acid	$\alpha$ 2,8	0.44
Glycoproteins		
AGP	$\alpha$ 2,3 and $\alpha$ 2,6	1.11
BSM (NeuNAc)	$\alpha$ 2,6	0.20
(NeuNGc)	$\alpha$ 2,6	0.03
Synthetic		
4-MU-NeuNAc	...	0.86

\*Cleavage rates of NeuNAc presented relative to that obtained with sialyl- $\alpha$ 2,3-lactose as substrate.

the sialidase any further from this aggregate. SDS-PAGE analysis revealed the presence of several distinct proteins, the sialidase-positive band had a mol. wt of c. 144 kDa. The results from the non-denaturing and denaturing determinations of mol. wt and the demonstration of additional protein components by SDS-PAGE suggest that sialidase is a component of a protein complex. The nature of this association is not

clear, but a study on the extracellular sialidase of *V. cholerae* has demonstrated that this enzyme forms a multi-enzyme complex, which contains other glycosidic and proteolytic activities [36]. Sialidase complexed with other enzymes, including  $\beta$ -galactosidase, is also found in human lysosomes [37]. A similar association may be present with respect to the *S. oralis* sialidase, whereby glycosidic and proteolytic activities form a multi-enzyme complex. This could have significant physiological benefit for the bacteria in that the hydrolytic enzymes could interact in a concerted manner in the breakdown of host glycoproteins, facilitating adhesion of the organism to epithelial surfaces and circulatory glycoproteins, as well as supplying a nutrient source for the bacteria.

The sialidase resolved by SDS-PAGE had an apparent mol. wt of 144 kDa. A comparison of the properties of a variety of bacterial sialidasases revealed that these enzymes are highly diverse with respect to mol. wt, isoelectric point, substrate specificity and specific activity [32]. However, despite these differences, conserved sequences have been demonstrated at the molecular level in bacterial sialidasases [38]. Interestingly, the

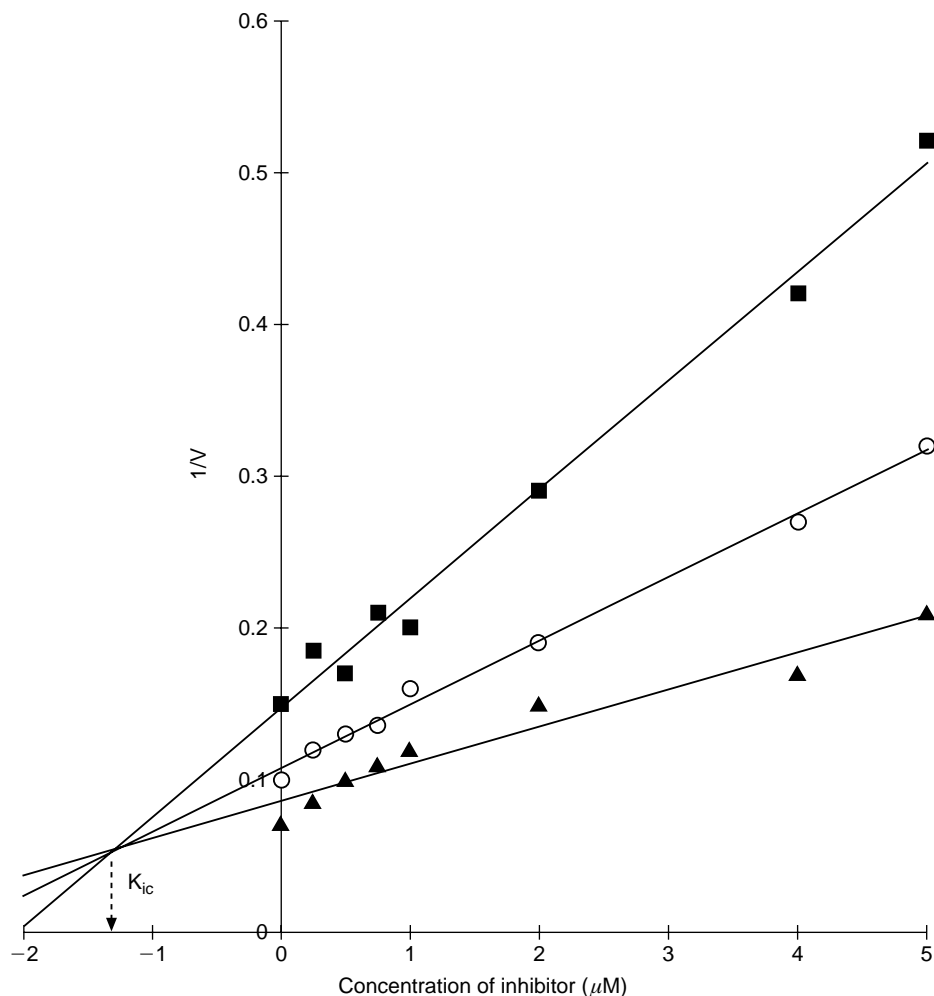


Fig. 5. Inhibition of the stage V *S. oralis* sialidase by 2,3-dehydro-NeuNAc. The Dixon plot is shown where concentration of inhibitor [I] is expressed in  $\mu\text{M}$  and the reaction velocity ( $V$ ) is expressed in nmol 4-MU released/min. Data are shown for 2,3-dehydro-NeuNAc over a range of substrate (4-MU-NeuNAc) concentrations: 10  $\mu\text{M}$  (●), 20  $\mu\text{M}$  (□) and 50  $\mu\text{M}$  (▲).

occurrence of sialidase in micro-organisms is frequently not in accordance with the phylogenetic relationship of bacterial species or strains. Highly related species or even strains of one species differ with respect to sialidase production [39, 40]. Furthermore, these enzymes – which share sequence homologies – are common in animals and in diverse micro-organisms that mostly exist as animal commensals or pathogens. Therefore, the hypothesis has been proposed that sialidases have originated in higher animals and that bacteria have acquired this genetic information during association with their animal hosts by horizontal gene transfer mechanisms [41]. The diverse biochemical properties of sialidases may be a result of the sialidase gene evolving to occupy a particular biological niche. In the case of the *S. oralis* sialidase this could be to remove sialic acids from O- and N-linked glycoproteins as substrates for growth.

A large pH range could be tolerated by the *S. oralis* sialidase (i.e., it was active between pH 4.0 and 9.0) and it exhibited a pH optimum of 6.0, which is typical of that of other bacterial sialidases [32, 42]. The

enzyme demonstrated a broad substrate specificity, cleaving  $\alpha 2,3$ -,  $\alpha 2,6$ - and  $\alpha 2,8$ -linked NeuNAc from a range of glycoprotein and oligosaccharide substrates. Furthermore, the purified enzyme cleaved NeuNGc from the naturally occurring substrate, BSM. However, NeuNGc (which is not found in healthy human tissues) was less susceptible to sialidase action than the N-acetyl derivative, the most abundant sialic acid in man [24]. Similar characteristics have also been observed for a number of other bacterial sialidases [43]. Comparison of the kinetic properties of the purified sialidase of *S. oralis* with other bacterial enzymes is difficult, as a range of substrates has been used and different assay conditions have been employed. Nevertheless, from the present data the sialidase has a low apparent  $K_m$  of 10.2  $\mu\text{M}$  of total bound NeuNAc against the natural substrate human AGP, indicating the high affinity of the enzyme towards N-linked, complex-type, sialylated glycoproteins.

It has been demonstrated that unconjugated NeuNAc will support growth of *S. oralis* when supplied as the sole source of fermentable carbohydrate, with the

concomitant induction of the intracellular enzymes required for the catabolism of this monosaccharide [22]. Furthermore, both the presence of an inducible transport system for NeuNAc and the pivotal role of sialidase in the degradation and utilisation of sialoglycoconjugates has recently been demonstrated for *S. oralis* [44, 45]. When the highly sialylated glycoprotein AGP was used as a model, it was shown that the constituent NeuNAc was released by sialidase and catabolised as a source of fermentable carbohydrate. Additionally, as a result of this exo-glycosidic activity the glycan chains were opened to degradation by other glycosidases produced by this bacterium, therefore extending the fermentable carbohydrate source available. The dependence on the production of sialidase for the growth of *S. oralis* on AGP was further supported by its inhibition in the presence of the sialidase inhibitor, 2,3-dehydro-NeuNAc. The important role of sialidase in the growth of bacteria on host glycoproteins *in vivo* has been demonstrated previously [23], where sialidase-deficient mutants of *Bacteroides fragilis* exhibited a reduced ability to grow in a rat pouch model. In the current study, a sialidase was isolated from *S. oralis* and was shown to possess properties which may facilitate bacterial persistence *in vivo*, including a broad pH optimum and substrate specificity. Taken together, these studies provide compelling evidence for the presence of a highly integrated system for the release and metabolism of NeuNAc, which is likely to be important in the nutrition of this organism.

Studies are now being pursued to further characterise the high mol. wt aggregate, which contains sialidase activity, and to identify any associated components. Understanding the detailed molecular mechanisms by which bacteria proliferate may be important in the development of future intervention strategies, at a time when the need for alternative antimicrobial therapies is becoming increasingly important with the emergence of penicillin resistance within this group.

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## References

- Bochud P-Y, Calandra T, Francioli P. Bacteremia due to viridans streptococci in neutropenic patients: a review. *Am J Med* 1994; 97: 256-264.
- Awada A, van der Auwera P, Meunier F, Daneau D, Klastersky J. Streptococcal and enterococcal bacteremia in patients with cancer. *Clin Infect Dis* 1992; 15: 33-48.
- Bochud P-Y, Eggiman P, Calandra T, Van Melle G, Saghafi L, Francioli P. Bacteremia due to viridans streptococcus in neutropenic patients with cancer: clinical spectrum and risk factors. *Clin Infect Dis* 1994; 18: 25-31.
- Classen DC, Burke JP, Ford CD et al. *Streptococcus mitis* sepsis in bone marrow transplant patients receiving oral antimicrobial prophylaxis. *Am J Med* 1990; 89: 441-446.
- Cohen J, Donnelly JP, Worsley AM, Catovsky D, Goldman JM, Galton DA. Septicaemia caused by viridans streptococci in neutropenic patients with leukaemia. *Lancet* 1983; 2: 1452-1454.
- Douglas CWI. Pathogenic mechanisms in infective endocarditis. *Rev Med Microbiol* 1993; 4: 130-137.
- McWhinney PHM, Gillespie SH, Kibbler CC, Hoffbrand AV, Prentice HG. *Streptococcus mitis* and ARDS in neutropenic patients. *Lancet* 1991; 337: 429.
- McWhinney PHM, Patel S, Whiley RA, Hardie JM, Gillespie SH, Kibbler CC. Activities of potential therapeutic and prophylactic antibiotics against blood culture isolates of viridans group streptococci from neutropenic patients receiving ciprofloxacin. *Antimicrob Agents Chemother* 1993; 37: 2493-2495.
- Beighton D, Hardie JM, Whiley RA. A scheme for the identification of viridans streptococci. *J Med Microbiol* 1991; 35: 367-372.
- Hardie JM, Whiley RA. Recent developments in streptococcal taxonomy: their relation to infections. *Rev Med Microbiol* 1994; 5: 151-162.
- Kilian M, Mikkelsen L, Henrichsen J. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). *Int J Syst Bacteriol* 1989; 39: 471-484.
- Beighton D, Carr AD, Oppenheim BA. Identification of viridans streptococci associated with bacteraemia in neutropenic cancer patients. *J Med Microbiol* 1994; 40: 202-204.
- Jacobs JA, Schouten HC, Stobbering EE, Soeters PB. Viridans streptococci isolated from the bloodstream. Relevance of species identification. *Diagn Microbiol Infect Dis* 1995; 22: 267-273.
- West PWJ, Al-Sawan R, Foster HA, Electricwala Q, Alex A, Panigrahi D. Speciation of presumptive viridans streptococci from early onset neonatal sepsis. *J Med Microbiol* 1998; 47: 923-928.
- Berry AM, Paton JC, Glare EM, Hansman D, Catchside DE. Cloning and expression of the pneumococcal neuraminidase gene in *Escherichia coli*. *Gene* 1988; 71: 299-305.
- Galen JE, Ketley JM, Fasano A, Richardson SH, Wasserman SS, Kaper JB. Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. *Infect Immun* 1992; 60: 406-415.
- Hoyer LL, Roggentin P, Schauer R, Vimr ER. Purification and properties of cloned *Salmonella typhimurium* LT2 sialidase with virus-typical kinetic preference for sialyl $\alpha$ 2-3 linkages. *J Biochem* 1991; 110: 462-467.
- Corfield T. Bacterial sialidases - roles in pathogenicity and nutrition. *Glycobiology* 1992; 2: 509-521.
- Cacalano G, Kays M, Saiman L, Prince A. Production of the *Pseudomonas aeruginosa* neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. *J Clin Invest* 1992; 89: 1866-1874.
- Gibbons RJ, Hay DI, Childs WC, Davis G. Role of cryptic receptors (cryptitopes) in bacterial adhesion to oral surfaces. *Arch Oral Biol* 1990; 35 Suppl: 107S-114S.
- Gottschalk A. Correlation between composition, structure, shape and function of a salivary mucoprotein. *Nature* 1960; 186: 949-951.
- Byers H, Homer KA, Beighton D. Utilization of sialic acid by viridans streptococci. *J Dent Res* 1996; 75: 1564-1571.
- Godoy VG, Dallas MM, Russo TA, Malamy MH. A role for *Bacteroides fragilis* neuraminidase in bacterial growth in two model systems. *Infect Immun* 1993; 61: 4415-4426.
- Kelm S, Schauer R. Sialic acids in molecular and cellular interactions. In: A survey of cell biology (*Int Rev Cytology* 175). San Diego, Academic Press. 1997: 137-240.
- Cuatrecasas P, Illiano, G. Purification of neuraminidases from *Vibrio cholerae*, *Clostridium perfringens* and influenza virus by affinity chromatography. *Biochem Biophys Res Commun* 1971; 44: 178-184.
- Berg W, Gutschker-Gdaniec G, Schauer R. Fluorescent staining of sialidases in polyacrylamide gel electrophoresis and ultrathin-layer isoelectric focusing. *Anal Biochem* 1985; 145: 339-342.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-685.
- Lifely MR, Hale C, Boyce S, Keen MJ, Phillips J. Glycosylation and biological activity of CAMPATH-1H expressed in different cell lines and grown under different culture conditions. *Glycobiology* 1995; 5: 813-822.

29. Baddour LM. Virulence factors among gram-positive bacteria in experimental endocarditis. *Infect Immun* 1994; 62: 2143–2148.
30. Gossling J. Occurrence and pathogenicity of the *Streptococcus milleri* group. *Rev Infect Dis* 1988; 10: 257–285.
31. Herzberg MC. Platelet–streptococcal interactions in endocarditis. *Crit Rev Oral Biol Med* 1996; 7: 222–236.
32. Saito M, Yu RK. Biochemistry and function of sialidases. In: Rosenberg A (ed) *Biology of the sialic acids*. New York, Plenum Press. 1995: 261–313.
33. Beighton D, Whiley RA. Sialidase activity of the “*Streptococcus milleri* group” and other viridans group streptococci. *J Clin Microbiol* 1990; 28: 1431–1433.
34. Straus D, Portnoy-Duran C. Neuraminidase production by a *Streptococcus sanguis* strain associated with subacute bacterial endocarditis. *Infect Immun* 1983; 41: 507–515.
35. Varki A, Diaz S. A neuraminidase from *Streptococcus sanguis* that can release O-acetylated sialic acids. *J Biol Chem* 1983; 258: 12465–12471.
36. Stewart-Tull DES, Ollar RA, Scobie TS. Studies on the *Vibrio cholerae* mucinase complex. I. Enzymic activities associated with the complex. *J Med Microbiol* 1986; 22: 325–333.
37. Potier M, Michaud L, Tranchemontagne J, Thauvette L. Structure of the lysosomal neuraminidase- $\beta$ -galactosidase-carboxypeptidase multienzymic complex. *Biochem J* 1990; 267: 197–202.
38. Roggentin P, Schauer R, Hoyer LL, Vimr ER. The sialidase superfamily and its spread by horizontal gene transfer. *Mol Microbiol* 1993; 9: 915–921.
39. Hoyer LL, Hamilton AC, Steenbergen SM, Vimr ER. Cloning, sequencing and distribution of the *Salmonella typhimurium* LT2 sialidase gene, nanH, provides evidence for interspecies gene transfer. *Mol Microbiol* 1992; 6: 873–884.
40. Roggentin P, Kleineidam RG, Schauer R. Diversity in the properties of two sialidase isoenzymes produced by *Clostridium perfringens* spp. *Biol Chem Hoppe Seyler* 1995; 376: 569–575.
41. Schauer R, Vliegthart JFG. Introduction. In: Schauer R (ed) *Sialic acids – chemistry, metabolism and function*. New York, Springer. 1982: 1–3.
42. Homer KA, Whiley RA, Beighton D. Production of specific glycosidase activities by *Streptococcus intermedius* strain UNS35 grown in the presence of mucin. *J Med Microbiol* 1994; 41: 184–190.
43. Corfield AP, Veh RW, Wember M, Michalski J-C, Schauer R. The release of N-acetyl- and N-glycoloyl-neuraminic acid from soluble complex carbohydrates and erythrocytes by bacterial, viral and mammalian sialidases. *Biochem J* 1981; 197: 293–299.
44. Byers HL, Homer KA, Tarelli E, Beighton D. N-acetylneuraminic acid transport by *Streptococcus oralis* strain AR3. *J Med Microbiol* 1999; 48: 375–381.
45. Byers HL, Tarelli E, Homer KA, Beighton D. Sequential deglycosylation and utilization of the N-linked, complex-type glycans of human  $\alpha_1$ -acid glycoprotein mediates growth of *Streptococcus oralis*. *Glycobiology* 1999; 9: 469–479.