Characterisation of opacity factor from group-A streptococci

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Summary. Extracellular opacity factor (OF) from group-A *Streptococcus* M-type 22 was purified by ammonium sulphate precipitation followed by ion-exchange on DE-52 cellulose and gel filtration on sephacryl S-400. OF was eluted near the void volume and shown to be heterogenous by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Antiserum to ammonium sulphate-purified OF from a cell-free culture supernate was prepared in rabbits. All preparations of OF from supernate and cell-extract were inhibited by the antiserum. No M protein was detected in the OF samples from various purification steps. The purified OF showed activity at a broad pH range with optimal activity at pH 6; it was inactivated considerably at high temperatures. Enzyme activity was inhibited by pepstatin A, but was unaffected by serine proteinase inhibitor, aprotinin, ethylene diamine trichloroacetic acid, N-ethylmaleimide, iodoacetamide and mercaptoethanol. This suggests that OF is an aspartic proteinase.

Introduction

Several infections are associated with streptococci of Lancefield's group A (*Streptococcus pyogenes*) two of the most common being pharyngitis and impetigo. Post-streptococcal diseases such as acute glomerulonephritis and rheumatic fever, although less common, are more serious, and are important health problems in New Zealand.^{1,2} The ability of group-A streptococci to cause infection in man is primarily attributed to the M protein of the cell wall that enables streptococci to resist phagocytosis. Immunity is mediated by antibodies directed against the M proteins. Identification of group-A streptococci based on specific M protein type is essential in studies of the epidemiology, pathogenesis and therapy of streptococcal infection. Some M types are poorly immunogenic in animals and antisera against them are difficult to prepare.^{3,4} Most of these serotypes produce opacity factor (OF), an enzyme which produces opacity in serum. The opacity-inhibition test is used as a supplementary typing system for the identification of OF-producing M types⁵ but OF antisera, like M antisera, are difficult to prepare.^{4,6} Therefore, we examined the physicochemical nature of OF as a first step in the development of a better method for antiserum production.

Materials and methods

OF-producing strain

S. pyogenes; R68/3116, T12, M22⁴ was used in these experiments. For OF production, the organism was grown at 37°C in Todd-Hewitt broth supplemented with neopeptone 1% according to the method of Hallas and Widdowson.⁷

OF purification from culture supernate

The cells from 1L of culture were sedimented by centrifugation (4000 g, 30 min). The supernate was filtered through a 0.45-µm membrane filter to remove residual cells. Protein in the cell-free supernate (CFS-OF) was precipitated by adding ammonium sulphate to 60% saturation at 4°C. This precipitated protein containing the extracellular OF (ASP-OF) was dialysed against 0.01 M Tris-HCl, pH 7.5, and then applied to a DE-52 cellulose column (1.6 × 40 cm) equilibrated with 0.1 M Tris-HCl, pH 7.5, containing 0.1 M NaCl. OF was eluted with a linear salt gradient of 0.1–0.6 M NaCl in 0.1 M Tris-HCl, pH 7.5. OF fractions were pooled, dialysed against 0.01 M Tris-HCl, pH 7.5, and freeze-dried. The lyophilised enzyme was dissolved in 3 ml of distilled water and applied to a sephacryl S-400 gel filtration column (2.7 × 100 cm) equilibrated with 0.1 M Tris-HCl, pH 7.5, containing 0.1 M NaCl; 6-ml volumes were collected.

OF purification from cell-extract

The cells from 1L of culture were washed three times

with saline, deposited by centrifugation, resuspended in 7 ml of saline and sonicated for 8 min in ice, with a Heat Systems Ultrasonics Model W-375 instrument. Sonicates were then centrifuged at 17 000 *g* for 1 h. The supernate was purified as described for OF from culture supernate. OF from sedimented cells was also extracted by sodium dodecyl sulphate and sodium deoxycholate methods.⁴

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of purified OF in a polyacrylamide vertical 10% gel slab was by the method of Laemmli.⁸

M protein detection

Samples from various stages of purification were tested against the reference M22 antiserum by the double gel diffusion technique. A reference acid extract of M22 was included as a control.

Protein determination

Protein concentration was determined by the method of Lowry.⁹

Assay of OF activity

Opalescence in human or horse serum was assayed by the serum-agar slide⁵ and optical density (OD)¹⁰ methods. In the OD method, 100 μ l of horse or human serum was incubated with 10 μ l of OF sample at 37°C. This method was used to quantify OF activity. The OF activity unit (OFU) was defined as the opacity absorbance of the reaction mixture at 450 nm after incubation for 4 h. Specific activity was derived from the quotient of the total OF activity and the protein concentration of the OF sample.

Time-dependent OF activity

The activity of the OF preparations, CFS-OF and ASP-OF, was compared. OD readings were taken at 30-min intervals for 6 h, an extra reading was taken after 15 min for ASP-OF.

Serial dilution of OF samples

The preparations ASP-OF and CFS-OF were each serially diluted and the activity of a 10- μ l sample of each dilution was determined after 2 h and 22 h.

pH optimum

The influence of pH on OF activity of the purified preparation (PP-OF) (42 μ g/ml) was examined by adjust-

ing the pH of horse serum with concentrated HCl to give a range of pH values from 4 to 11.

Heat stability

Samples of PP-OF (42 μ g/ml) in 0.1 M Tris-HCl, pH 7.5, containing 0.1 M NaCl were incubated at 55°C and 95°C in Kimax Hungate tubes in a heating block. Samples incubated at 55°C were removed at 10-min intervals and those incubated at 95°C at 5-min intervals; these were immediately chilled in an ice-water bath and the residual OF activity was compared with that of an unheated sample. OD readings were obtained after 2 h.

The effect of inhibitors, denaturing and reducing agents on OF activity

PP-OF (42 μ g/ml) was incubated for 2 h at room temperature in given concentrations (table II) of various inhibitors, denaturing agents and reducing agents. The residual OF activity of each sample was determined after incubation with horse serum for 4 h.

The effect of various metals on OF activity

The PP-OF sample (42 μ g/ml) was dialysed against distilled water at 4°C and the effect of metals on OF activity was determined as described for inhibitors.

Preparation of antiserum

The following preparations were used as antigens at concentrations given: (i) ASP-OF (5 mg/ml); (ii) PP-OF (0.82 mg/ml); (iii) sephacryl S-400 purified OF from cell extract (1.2 mg/ml). For the initial injection, a 1-ml sample of antigen preparation mixed with Freund's complete adjuvant was injected into a 5-month-old Rex rabbit. ASP-OF was injected intraperitoneally and PP-OF and sephacryl-purified OF subcutaneously. After 5 days a course of 1-ml of antigen preparation was given on 3 consecutive days for 2 weeks, intravenously for ASP-OF and intraperitoneally for the other two preparations. The serum was tested 5 days after the last injection and if antibody was detected a further 1-ml booster injection was given 5 days before final bleeding. In the absence of antibody, three further injections were given before bleeding. Antibody was detected by both slide⁵ and optical density¹⁰ methods for the opacity-inhibition test.

Results

OF purification from cell-free supernate

A large portion of non-enzyme protein and coloured material was removed during the ammonium-sulphate precipitation and ion-exchange chromatography stages, and *c.* 58% of the original OF activity was lost. When OF was further purified

by sephacryl S-400 gel-filtration chromatography, the enzyme was eluted near the void volume suggesting OF aggregation. The OF obtained from the last stage of purification had a specific activity of 2986 OFU/mg of protein. The results of enzyme purification are shown in table I.

The purified OF was shown to be heterogeneous by SDS-PAGE; it comprised four major protein bands of 30–65 K da. No M protein was detected in the OF samples at the various stages of purification.

Time-dependent OF activity

With the purified and concentrated OF, maximum serum opacity was obtained within 1 h, whereas with the less concentrated OF, opacity increased over 6 h (fig. 1).

Serial dilution

ASP-OF at a dilution of 1 in 256 after incubation for 22 h produced a serum opacity (OD reading) similar to that given by a dilution of 1 in 2 incubated for only 2 h. Similarly, OF from culture supernate produced opacity at a 1 in 2 dilution in 2 h, similar to that of a 1 in 64 dilution in 22 h (fig. 2).

pH optimum

The PP-OF enzyme had a broad pH profile of activity, the optimum was *c.* pH 6 (fig. 3).

Heat stability

The PP-OF activity after heating to 55°C and 95°C is shown in fig. 4. The enzyme was unstable at high temperatures; *c.* 90% of OF activity was lost after 5 min at 95°C (fig. 4).

Fig. 1. Time-dependent OF activity. The enzyme activity of OF preparations ASP-OF (○); and CFS-OF (●) compared at 30-min intervals up to 6 h by OD measurement. (For ASP-OF an extra reading was taken after 15 min).

Fig. 2. Serial dilution of OF samples. The enzyme activity of each dilution of OF preparation was measured by OD after incubation for 2 h and 22 h ASP-OF—2 h (●), 22 h (○); CFS-OF—2 h (■), 22 h (□).

Table I. Purification of OF from M-type-22 *Streptococcus*, strain R68/3116

Sample	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (OFU)	Specific activity (OFU/mg of protein)
Cell-free supernate (CFS-OF)	1000	10.4	10 400	29 200	2.8
Ammonium sulphate precipitate (ASP-OF)	55	9.6	528	16 896	32
Cellulose DE 52 purified	68	0.72	48.96	12 185	248
Sephacryl S-400 purified (PP-OF)	60	0.042	2.52	7 524	2986

Fig. 3. The effect of pH on OF activity in horse serum.

Fig. 4. Determination of heat stability. Purified OF (42 µg/ml) in 0.1 M Tris-HCl, pH 7.5, containing 0.1 M NaCl was incubated at 55°C (●) and 95°C (○). Residual OF activity was measured by OD.

The effect of inhibitors, denaturing and reducing agents on OF activity

PP-OF was inhibited by 5 mM pepstatin A (aspartic proteinase inhibitor) but was unaffected by PMSF (serine proteinase inhibitor), aprotinin (trypsin, chymotrypsin and plasmin inhibitors), ethylene diamine trichloroacetic acid (EDTA), *o*-phenanthroline (metal chelator), mercaptoethanol and dithiothreitol (reducing agents active against disulphide bonds), iodoacetamide and N-ethylmaleimide (alkylating agents active against free sulphhydryl groups). The enzyme was completely inhibited by trypsin and proteinase K (table II).

Table II. The effect of inhibitors, denaturing and reducing agents on PP-OF activity

Treatment	Percentage residual activity
Untreated OF (control)	100
10 mM PMSF	104
Aprotinin (40 trypsin inhibitor units)	114
5 mM pepstatin A	11
1 mM pepstatin A	93
4 M urea	25
1% SDS	53
25 mM sodium-deoxycholate	46
10 mM EDTA	119
10 mM <i>o</i> -phenanthroline	97
1% mercaptoethanol	100
10 mM dithiothreitol	110
10 mM iodoacetamide	107
10 mM N-ethylmaleimide	97
Trypsin 0.25 mg/ml	0
Proteinase K 0.25 mg/ml	0

The effect of metals on OF activity

The PP-OF enzyme was sensitive to potassium cyanide and OF activity was reduced in the presence of cupric chloride, mercuric chloride and sodium citrate (table III).

Preparation of antiserum

Preparation of antisera against the sephacryl S-400 purified OF from both culture supernate and cell-extract was not successful, whereas immunising the rabbit with ASP-OF from the supernate of broth culture produced antiserum with a titre of 128. This antiserum specifically inhibited the activity of all OF preparations.

Discussion

Group-A streptococci can be divided into more than 80 distinct types, determined by the type-

Table III. The effect of various metals on PP-OF activity

Treatment	Percentage residual activity
Untreated OF (control)	100
10 mM calcium chloride	101
10 mM zinc sulphate	99
10 mM magnesium sulphate	98
10 mM cupric chloride	70
10 mM mercuric chloride	87
10 mM sodium citrate	66
10 mM potassium phosphate	106
10 mM potassium cyanide	42

specific M-protein antigens and OF.¹¹ Although recognition of the M protein of a strain has always been regarded as the definitive test,^{12,13} this requires M antisera of good quality. OF type-specificity parallels the M type,³⁻⁵ so that when good M antisera are not available the opacity-inhibition test has been the method of choice for the identification of OF-producing M types. However, OF antisera, like M antisera, have proved difficult to prepare. Fraser⁶ reported in her studies that only 61 (44%) of 138 rabbits vaccinated produced OF antibodies and at low titres (<16). She attributed these results to inconsistencies in the rabbits' response and described a much higher success rate with guinea-pigs. Attempts in our laboratory, before this study, to produce OF antisera in guinea-pigs by Fraser's method were not particularly successful; only 8 (24%) of 34 guinea-pigs responded;¹⁴ a heat-killed whole-cell vaccine was used. The current study shows that 75% of OF activity is inactivated within 20 min at 55°C (fig. 4), suggesting that previous failures to stimulate an immune response in guinea-pigs and rabbits may have been due to the inoculation of insufficient quantities of enzyme to serve as antigen.

Failure to evoke an antibody response may also relate to changes produced in the antigen during purification. Our attempts to prepare antiserum against sephacryl S-400-purified OF were unsuccessful. Martinez *et al.*¹⁵ similarly failed to obtain OF antibody against OF purified by isoelectric focusing from an M-type-2 streptococcus.

We were able to evoke a specific antibody response in rabbits with highly active preparation of OF. Response to less active OF preparations were poorer.

An important aspect of this study has been the demonstration that OF activity, in common with all enzyme activity, is time-dependent. The same opacity reaction occurs after overnight incubation in serum of very weak OF preparations that occurs

within 2 h with high concentrations of OF. In determining a strain's capacity to produce OF, overnight incubation of the supernate-serum mixture is useful but when testing the OF activity of vaccines as a measure of potency, overnight incubation may give misleading results.

This study has confirmed the findings of other workers that OF activity is inactivated by proteinases^{7,16} and by extremes of pH.¹⁶ The modification of the substrate at extremes of pH could have some effect on OF activity, however, OF activity was also reduced by SDS, sodium deoxycholate, and urea.

It has been shown that during purification of OF by gel filtration, OF forms aggregates which dissociate in the presence of SDS to reveal several protein bands in gel.^{7,15} Similar phenomena were observed in the present study during OF purification.

Previously we showed that OF cleaves the apoprotein A1 of serum high-density lipoprotein and the OF was designated as an apoproteinase.¹⁷ These studies showed that OF activity was inhibited by pepstatin A, an aspartic proteinase inhibitor, but not by serine, metal and sulphhydryl proteinase inhibitors, suggesting that OF is an aspartic proteinase. Because mercaptoethanol and dithiothreitol, both reducing agents active against disulphide bonds, had no effect on enzyme activity, we may infer that disulphide bonds do not contribute to OF stability. Urea and SDS detergents, known to dissociate hydrogen and hydrophobic bonds,¹⁸ disrupted the activity of OF. This finding supports the involvement of hydrophobic interaction and hydrogen bonds in OF stability.

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