Mitogenic factor secreted by *Streptococcus pyogenes* is a heat-stable nuclease requiring His¹²² for activity

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The gene encoding a mitogenic factor, termed MF, was cloned from *Streptococcus pyogenes* and the recombinant MF was overexpressed in *Escherichia coli*. Both the natural and recombinant MF had heat-resistant nuclease activity. The nuclease activity of MF was characterized using the recombinant protein. MF showed endonuclease activity, digesting ssDNA, dsDNA and tRNA. The optimal pH for the DNase activity of MF was 9.5. The DNase activity was enhanced approximately tenfold by the simultaneous presence of two divalent cations, Mg²⁺ and Ca²⁺, compared to either alone and was inhibited by EDTA or NaCl. The heat stability of MF was biphasic; the DNase activity was heat-stable from 0 to 50 °C and over 80 °C but very unstable at around 60 °C. DNA digested by MF possessed 5'-phosphorylated and 3'-hydroxylated termini, identical to those obtained by digestion of DNA by pancreatic deoxyribonuclease I. A mutant clone revealed that His¹²² was a residue essential to the nuclease activity.

Keywords: mitogenic factor, heat-stable nuclease, DNA hydrolysis, *Streptococcus pyogenes*

INTRODUCTION

The group A streptococcus *Streptococcus pyogenes* is a multifactorial pathogen which elaborates both cell-associated virulence factors, such as M-protein and protein F, and extracellular virulence factors, including the cytolytic toxins streptolysin O and streptolysin S, and hydrolytic enzymes such as proteinase, NADase, deoxyribonucleases and hyaluronidase. It also produces a number of superantigens, proteins which exert their effects by forming a bridge between the major histocompatibility complex (MHC) class II of macrophages or other antigen-presenting cells and receptors on T-cells that interact with the class II MHC (Bisno, 1991; Schlievert, 1993).

Three serologically distinct streptococcal pyrogenic exotoxins (SPEs), SPEA, SPEB and SPEC, have thus far been reported. Their genes have been cloned and their nucleotide sequences have been defined (Goshorn & Schlievert, 1988; Hauser & Schlievert, 1990; Weeks & Ferretti, 1986). Recently, SSA was reported as a new

streptococcal superantigen and was also cloned (Reda *et al.*, 1994). We also reported a novel mitogenic factor (MF) purified from the culture supernatant fraction of *S. pyogenes* (Yutsudo *et al.*, 1992), and the nucleotide sequence of the gene encoding MF was determined (Iwasaki *et al.*, 1993). The superantigenic property of MF has also been reported (Norrby-Teglund *et al.*, 1994; Toyosaki *et al.*, 1996). In these papers, MF was characterized as a superantigen that stimulates T-cells by cross-linking variable parts of T-cell receptors with major histocompatibility class II molecules on accessory cells.

We examined the distribution of the MF gene in various strains by PCR and found that it exists only among group A streptococcal strains (Yutsudo *et al.*, 1994). When crude DNA extracts were used as templates to detect the MF gene by PCR, the amplified PCR fragments were rapidly degraded and did not appear on agarose gels. Other PCR products, such as SPEA, SPEB, SPEC and streptolysin O genes, were also degraded. The degradation may have been due to heat-stable nuclease activity of the MF protein which was present in the PCR mixture. Removal of culture supernatant fluid by centrifugation or treatment with proteolytic enzymes, such as proteinase K or trypsin, was effective in pre-

Abbreviations: GST, glutathione S-transferase; MF, mitogenic factor; SPE, streptococcal pyrogenic exotoxin.

venting degradation of the PCR products, as described before (Yutsudo *et al.*, 1994).

At least four immunologically and electrophoretically distinct nucleases, designated DNase A, B, C and D, are known to be secreted by many strains of group A streptococci (see Ferreira *et al.*, 1992). Several reports have described the genes encoding nucleases belonging to other groups of streptococci (Ferrieri *et al.*, 1980; Georgatsos *et al.*, 1962; Lindler & Macrina, 1987; Puyet *et al.*, 1989; Smyth & Fehrenbach, 1974; Wolinowska *et al.*, 1991). However, little information is available on the genes encoding nucleases derived from group A streptococci. Here the nuclease activity of recombinant MF protein is characterized.

METHODS

Bacterial strain. The *Streptococcus pyogenes* strain NY-5 was used as described previously (Yutsudo *et al.*, 1992).

Purification of recombinant MF. Previously, we reported that the mature MF protein was recombinantly expressed as a fusion protein with glutathione S-transferase (GST) in *Escherichia coli* (Iwasaki *et al.*, 1993). Briefly, the DNA fragment comprising the coding region for the mature MF protein was amplified by PCR, ligated with a pGEX-2T expression vector (Pharmacia) and transformed into *E. coli*. The cultured bacteria were disrupted by sonication. The bacterial lysates were applied to a prepacked glutathione-Sepharose 4B column (Pharmacia), then the bound material was eluted with 5 mM glutathione in 50 mM Tris/HCl buffer (pH 8.0). The obtained GST-MF fusion protein was cleaved at the junction region by digesting with human thrombin (Sigma), and the recombinant MF separated by ion-exchange chromatography. However, as sufficient recombinant MF protein could not be obtained even with this system a new plasmid (designated as pSPMF) was constructed to over-express the MF protein. The DNA fragment comprising the coding region for mature MF protein was amplified by PCR with primers 5'-GCGAATTCTGATTAGCTAGAAAA-GAGGACAAGCA-3' (34-mer with an *EcoRI* restriction endonuclease recognition sequence) and 5'-CTGGATCCAC-TTTTGGACAAATCATA-3' (26-mer with a *BamHI* restriction endonuclease recognition sequence). The PCR product was digested with *EcoRI* and *BamHI* endonucleases, and ligated with the expression vector pSPORT1 (Life Technologies) which had been treated with the same enzymes. *E. coli* cells were transformed with the ligated mixture and several transformants were isolated. The selection of the MF transformants was done by PCR between the T7 primer whose sequence is dependent on the pSPORT1 vector and the primer comprising the coding region for the mature MF. The transformants were cultured in Terrific broth (Iwasaki *et al.*, 1993) at 37 °C overnight with shaking. Expression of the MF protein was induced with 5 mM IPTG during the last 3 h of incubation of the culture. The cells were harvested by centrifugation and disrupted by sonication. The bacterial lysate was then centrifuged at 100 000 g for 3 h and the supernatant fraction was collected. The supernatant fraction was diluted fivefold with 25 mM Tris/HCl buffer (pH 7.8), and ammonium sulfate (56.1 g per 100 ml) was added. The resulting precipitate was dissolved in 10 mM citrate/phosphate buffer (pH 4.6) and dialysed against same buffer. The crude material was subjected to SP-Sephadex C-25 column chromatography (Pharmacia), followed by chromato-

focusing (PBE94; Pharmacia) and then DEAE-Sepharose CL-6B column chromatography (Pharmacia). A PBE94 polybuffer exchanger gel for chromatofocusing was equilibrated with 25 mM ethanolamine/acetate buffer (pH 9.6) and fractionation was performed by eluting with polybuffer 96 (Pharmacia). Fractions containing the nuclease activity were collected and concentrated by ultrafiltration using an Amicon YM-10 membrane. The concentrated material was dialysed against 25 mM triethylamine/HCl buffer (pH 9.8) and was applied to a DEAE-Sepharose CL-6B column equilibrated with the same buffer. The material was eluted with a linear gradient of NaCl (0–0.4 M) in triethylamine/HCl buffer.

N-terminal amino acid sequence analysis revealed that this recombinant MF protein had an additional arginine residue compared to the N-terminus of mature MF protein. To delete the N-terminal Arg from the recombinant MF protein, a plasmid designated as pSPMFΔR, with the three nucleotides corresponding to the Arg codon deleted, was constructed using a site-directed mutagenesis kit (Clontech Laboratories). From *E. coli* cells transformed by this plasmid pSPMFΔR, the recombinant MF protein was purified by the method described above.

The homogeneity of the purified recombinant MF protein was examined by SDS-PAGE. The physico-chemical properties of this recombinant MF protein were identical to those of the natural MF protein when compared by SDS-PAGE, mass spectrometry of lysyl endopeptidase-treated peptide fragments, and reactivity with rabbit antibodies raised against the natural MF protein. This recombinant MF protein (rMF) was used in the characterization studies.

Determination of nuclease activity. Nuclease activity was determined by agarose gel electrophoresis and spectrophotometry. For agarose gel electrophoresis, rMF protein and substrate DNA, such as phage M13mp18 DNA (Takara Shuzo, Japan), pBluescript II plasmid DNA (Stratagene), λ bacteriophage DNA (Takara Shuzo), calf thymus DNA (Sigma) or yeast tRNA (Life Technologies) were incubated in 0.1 M Tris/HCl buffer (pH 8.0), 1 mM MgCl₂ and 1 mM CaCl₂ at 37 °C for 15 min. The reaction was terminated by addition of excess EDTA. The samples were subjected to electrophoresis in 0.8% agarose gels and the gels were stained with ethidium bromide and visualized with a UV trans-illuminator.

For spectrophotometry, the activity of nuclease was determined by measuring the maximum increase in A_{260} min⁻¹ during incubation for 25 min using calf thymus DNA (40 µg) as the substrate. The nuclease activity was assayed at 25 °C in 1 ml reaction volumes using different buffers with varying concentrations of cations as described below. Specific activity was expressed as ΔA_{260} min⁻¹ (mg protein)⁻¹. Results were obtained from at least three independent experiments.

Neutralization test for nuclease activity. The antiserum against natural MF (anti-nMF) was prepared by immunization of rabbits with nMF protein as described by Iwasaki *et al.* (1993). The antiserum against recombinant streptococcal pyrogenic exotoxin C (anti-rSPEC) was used as a control serum. SPEC was prepared by using PCR to amplify the DNA fragment comprising the coding region for the mature SPEC (Goshorn & Schlievert, 1988). The amplified DNA fragment was ligated with the pGEX-2T expression vector (Pharmacia) and transformed into *E. coli*. The lysate of the transformant was applied to a glutathione-Sepharose 4B column (Pharmacia). The GST-SPEC fusion protein was cleaved at the junction region by digestion with human thrombin (Sigma)

and SPEC was separated from GST, thrombin and other proteins by HPLC using a DEAE-glass column (Cosmo Bio). Anti-rSPEC was prepared by immunization of rabbits with the purified rSPEC. To avoid the endogenous nucleases present in sera, the IgG fraction was purified by a HiTrap Protein G column (Pharmacia) before use in the following neutralization test. rMF (0.1 µg) or bovine pancreatic deoxyribonuclease (DNase I, 0.2 µg protein, Takara), used as a reference nuclease, was preincubated with IgG fraction at 37 °C for 10 min and the remaining nuclease activity was measured as described above. The substrate was 350 µg λ phage DNA (Takara).

Analysis of 5' and 3' ends of oligonucleotides generated by digesting plasmid DNA with rMF. The nature of the 5' and 3' ends of products of DNA digested by rMF were examined as described by Habraken *et al.* (1993). Briefly, to characterize the 3' end, rMF-digested pBluescript II DNA (2 µg) was incubated with 15 U calf thymus terminal deoxynucleotidyl transferase (TdT; Life Technologies) and 370 kBq [α -³²P]dCTP (111 TBq mmol⁻¹; Amersham) at 37 °C for 30 min. Such treatment resulted in 3'-end labelling of the DNA fragment whose 3' end had been dephosphorylated. To analyse the status of the 5' end, rMF-digested pBluescript II DNA was incubated with or without bacterial alkaline phosphatase (BAP, 0.4 U; Takara) at 65 °C for 30 min, and then treated with 10 U T4 polynucleotide kinase (T4PNK; Takara) and 370 kBq [γ -³²P]ATP (111 TBq mmol⁻¹; Amersham). DNA was purified from the 3'- and 5'-labelling mixtures by phenol extraction, followed by ethanol precipitation and electrophoresis on 0.8% agarose gel. The fractionated DNA was transferred to a nylon membrane (Amersham) and visualized by exposure to X-ray film (Eastman Kodak).

RESULTS

MF nuclease activity

The nuclease activity of rMF on several different substrates was examined. As shown in Fig. 1, rMF digested both single-stranded circular DNA (bacteriophage M13 mp18 DNA) and double-stranded circular DNA (plasmid pBluescript DNA), indicating that it has an endonuclease activity. rMF also digested yeast tRNA (Fig. 1c) and showed nuclease activity in the presence of divalent cations, a reaction which was inhibited by EDTA. This result indicates that the divalent cations play a role in the nuclease activity of MF. Degradation of closed circular pBluescript II DNA was not inhibited as readily as degradation of open circular plasmid DNA. At 2 mM EDTA the closed circular DNA was totally degraded; at 4 mM EDTA, about 50% of the DNA seemed to be degraded.

Activation conditions for MF nuclease

The effect of pH and cations on the nuclease activity of rMF was examined by the spectrophotometric method. rMF activity was observed in the presence of Mg²⁺ as well as Ca²⁺. The DNase activity of rMF was enhanced in the presence of both divalent cations (Mg²⁺ and Ca²⁺); activity was significantly greater than that observed in the presence of a single cation. The nuclease activity of DNase I, used as a reference nuclease, was also enhanced in the presence of the two cations, but the

Fig. 1. Nuclease activity of rMF in the presence of divalent cations. rMF (10 µg ml⁻¹) was incubated with bacteriophage M13mp18 single-stranded circular DNA (a), plasmid pBluescript II double-stranded circular DNA (b) or yeast tRNA (c) as substrates at 37 °C for 15 min. The reaction was performed with (+) or without (–) 1 mM MgCl₂ and CaCl₂, or 2–16 mM EDTA or both, as indicated above each panel. Thereafter, the reaction mixtures were subjected to 0.8% agarose gel electrophoresis. Undigested substrates are indicated by an arrowhead (a and c) or arrows (b: oc, open circular DNA; cc, closed circular DNA). M, bacteriophage λ DNA digested with restriction endonuclease *Eco*T14I as molecular size markers.

level of the enhancement was less than that observed in the case of rMF. DNase I activity was observed in the presence of Mg²⁺ but was totally inhibited by the addition of EGTA, which selectively chelates Ca²⁺. This suggested that Ca²⁺, a known cofactor of DNase I, might have contaminated the buffer and enhanced the activity of DNase I in cooperation with Mg²⁺. On the other hand, EGTA showed no effect on the DNase activity of MF. Thus, Ca²⁺ may not act as a cofactor of the DNase activity of rMF.

In the presence of both Mg²⁺ and Ca²⁺, the optimal pH for MF nuclease was observed at a narrow range around pH 9.5, while that of DNase I appeared over a broad range between pH 6.5 and pH 8.0.

The optimal concentrations of Mg²⁺ and Ca²⁺ for the rMF DNase activity were determined. The highest activity of MF was manifested in the buffer (pH 9.5) containing Mg²⁺ and Ca²⁺ (0.5 mM each). Enhancement

Table 1. Characteristics of nuclease activity of rMF compared with DNase I

Characteristic	rMF	DNase I
Optimal pH	9.5	6.5–8.0
Heat stability	Stable*	Labile
Effect of NaCl/KCl	Inhibitory	Inhibitory
Optimal ion concn	Mg ²⁺ + Ca ²⁺ (0.5 mM each)	ND

ND, Not determined.

* Inactivated at 60 °C but activity restored above 80 °C.

of rMF activity by various divalent cations was further examined. In addition to Mg²⁺ and Ca²⁺, we found that Mn²⁺, Sr²⁺, Cd²⁺, Cu²⁺, Co²⁺, Ni²⁺, Zn²⁺ and Fe²⁺ were effective, whereas Ba²⁺ was not (data not shown).

Heat stability of MF nuclease

The nuclease activity of rMF seems to be heat stable (Yutsudo *et al.*, 1994). To confirm this, the heat stability of rMF was examined. After preincubation at 100 °C for various periods of time, the remaining nuclease activities of rMF and DNase I were determined. DNase I was readily inactivated by the preincubation at 100 °C for 5 min, but rMF retained more than 30% of its activity even after such heat treatment for 10 min. The activity decreased gradually with longer incubation, but did not completely disappear even after incubation at 100 °C for 60 min.

The heat stability profile of the nucleases was also examined. After preincubation at various temperatures for 10 min, the activity of DNase I decreased with a rise in temperature and was completely inactivated at 80 °C. On the other hand, the activity of rMF was constant up to 50 °C but dropped to a minimum (6%) at 60 °C and then recovered up to about 50% at 80 °C. The heat-inactivated rMF at 60 °C was reincubated at either 60 °C or 80 °C for 10 min, and then the remaining nuclease activity was determined. Reheating at 60 °C led to a further decrease of the activity (4.5%), whereas a slight increase occurred by further incubation at 80 °C (11%). These results suggest that the nuclease activity of rMF is almost completely inactivated at 60 °C but that the activity can be restored at 80 °C.

Inhibition effect of ionic strength

NaCl is known to be an inhibitor of several nucleases (Citak & Gray, 1980; Kunitz, 1950). Its effects on the DNase activity of rMF were tested. Inhibition greater than 90% was observed with more than 60 mM NaCl. KCl also inhibited the nuclease activity of rMF in an identical manner. These properties of the nuclease activity of rMF are summarized in Table 1.

Neutralization of MF nuclease activity by antibodies to MF

The nuclease activity of rMF was neutralized by antibodies raised against the natural MF but not by control serum (anti-rSPEC). DNase I was not affected by the antibodies to MF, confirming the specificity of the anti-MF antibodies.

Mode of DNA hydrolysis

To determine the mode of hydrolysis in the oligonucleotide products resulting from the digestion of calf thymus DNA by rMF, the cleavage site was analysed as described by Habraken *et al.* (1993). ³²P was incorporated into the 5' terminus of the digested products by treatment with T4 polynucleotide kinase (T4PNK) in the presence of [γ -³²P]ATP only after they had been treated with bacterial alkaline phosphatase (BAP) (Fig. 2, left panel). This indicated that rMF cleaves DNA between the 3' position of deoxyribose and phosphate, thus generating oligonucleotides that possess the 5'-phosphorylated terminus. The fact that the rMF-digested DNA product was efficiently labelled by the terminal deoxynucleotidyl transferase (TdT) in the presence of [α -³²P]dCTP showed that the digested product possesses a 3'-hydroxylated terminus. These results paralleled those of DNase I (Fig. 2, right panel).

His¹²² requirement for nuclease activity of MF

Two mutant GST–MF fusion proteins, DJ-6 and DB-1, obtained during PCR amplification of the MF gene described previously (Iwasaki *et al.*, 1993), enabled us to identify an amino acid residue required for the activity of this enzyme. The recombinant protein DB-1 with an amino acid mutation H122R (His¹²² changed to Arg¹²²; the number represents the position of the residue relative to the N-terminal of mature MF) showed almost no DNase activity, whereas the recombinant protein DJ-6 with two amino acid mutations S195P and I218V (Ser¹⁹⁵ and Ile²¹⁸ changed to Pro¹⁹⁵ and Val²¹⁸, respectively) showed about 25% of the activity of the wild-type. When the codon corresponding to the Arg¹²² in DB-1 was substituted for the His codon by site-directed mutagenesis, the reconstituted rMF showed restoration of full nuclease activity. These results suggest that His¹²² is critical for MF nuclease activity.

DISCUSSION

Streptococci of serological groups A and C are known to produce substantial amounts of extracellular deoxyribonucleases (Ferreira *et al.*, 1992). The extracellular DNase activity produced by *S. pyogenes* has been shown to consist of at least four distinct enzymes (Marker & Gray, 1972; Yasmineh & Gray, 1968). Two of these enzymes, originally described as DNase B and DNase D, have been demonstrated to possess RNase activity (Gray & Yasmineh, 1968). All four enzymes are endonucleases producing fragments ending in 5'-phosphate (Winter & Bernheimer, 1964). We recently purified the MF protein

Fig. 2. Generation of oligonucleotides having 5'-phosphorylated and 3'-hydroxylated termini by digesting plasmid DNA with rMF nuclease. After digestion of pBluescript DNA with either rMF or DNase I, the DNA digestion products were reacted with calf thymus terminal deoxynucleotidyl transferase (TdT) in the presence of [α - 32 P]dCTP as described in Methods. The products were incubated with or without bacterial alkaline phosphatase (BAP) and then reacted with T4 polynucleotide kinase (T4PNK) in the presence of [γ - 32 P]ATP. The reaction mixtures were subjected to electrophoresis in 0.8% agarose gel and DNA in the gels was stained with ethidium bromide (a) and visualized by autoradiography (b). Undigested substrate DNA is indicated by arrows (upper band, open circular DNA; lower band, closed circular DNA). Digestion products are indicated by arrowheads. M, bacteriophage λ DNA digested with restriction endonuclease EcoT14I as molecular size markers.

MF	MNLLGSRVFSKCKRLVKFSMVALVSATMAVTTVTLENTALARQTVS-NDVVLNDGASK	59
SDC	MS--KKLRNFLVRIIVAAFAFVMAIPPYHHNTVLAKTVSVNQTYGEYKDYTYVIGESN	58
MF	YLNEALAWTFNDSPNYKTLGTSQITPALFPKAGDILYSKLDLGRTTARGTLTYANVE	119
SDC	IDQSAPFKIYKTERVYKGGTSEKRVTV---SDVVYNPLDGYKRSTGAYGVVTKDMID	114
MF	GSYGVRSQSGKNQNPAGW-----TGPNPHVK-----YKIEW-----LNGLS	155
SDC	MSKGYREKWEINPEPSGWFYFNADNEEISEKEYDSRRTSKYKVTNNVPVLTTLKGKK	174
MF	YVGDFWNRSHLIADSLGGDALRVNAVGTGRTQNVGGRDQKGGMRYTEQRAQEWLEANRDG	215
SDC	YNSHLFVASHLFADSLGGKSIRKNAITGTQMNVGTR--KGGMQYIEKKVLSHITKNPDV	232
MF	YLYEAAPIYNADLIPRAVVSMQSSDNTINEKVLVYNTANGYTINYHNGT-----	267
SDC	YVFYSALPEYQGAELLARSVLVSALSSDGVINETVRVNTADGFNINYEKGGLLTSPVS	292
MF	-----PTQK	271
SDC	EIDNIEDSTTDEIENSVDSEEIVYNDTTTEEEEN	327

Fig. 3. Amino acid sequence comparison of MF with DNase of group C streptococcus *S. equisimilis*. MF, MF sequence including signal peptide. SDC, streptococcal DNase derived from *S. equisimilis* (Wolinowska et al., 1991). Amino acids that are identical or show consensus between MF and group C streptococcal DNase are indicated by a star or a dot, respectively. Gaps inserted to maximize alignment are indicated by dashes. His¹²² or Ser¹⁹⁵ and Ile²¹⁸ (His¹⁶⁵, Ser²³⁸ and Ile²⁶¹ in premature MF, respectively) are indicated by an arrowhead or an arrow, respectively. CLUSTAL V was used as the amino acid sequence comparison program. The database accession numbers of MF and SDC are D13428 and X17241, respectively.

as a novel superantigen from the culture supernatant fluids of *S. pyogenes* strain NY-5 (Yutsudo et al., 1992). In the present study, MF protein exhibited endonuclease activity cleaving DNA to generate oligonucleotides that possess 5'-phosphorylated and 3'-hydroxylated termini. Moreover, MF degraded the yeast tRNA, indicating that MF has RNase activity (Fig. 1c). Although there is no information about the sequences of the four nucleases of *S. pyogenes* described above, MF seemed to correspond to one of these nucleases. It might be a candidate for either DNase B or DNase D because they showed RNase activity as described above. The optimal pH of DNase B is reported to be 8 to 9 (Winter & Bernheimer, 1964), which is consistent with that of MF, whereas that of DNase D is 7.0. Therefore MF seems to correspond to DNase B rather than DNase D.

A haemoprobe-B test kit for determining the level of anti-DNase B antibodies in human sera (Kyorin Pharma-

ceutical) was available to try to determine anti-DNase B activity with our rabbit anti-nMF and anti-rMF antibodies. Both anti-MF antibodies demonstrated neutralizing titres of 1:240, whereas preimmune serum and anti-rSPEC serum used as negative controls showed neutralizing titres of <1:40. However, the critical positive dilution for human sera is 1:320 in this immunoassay. Consequently, by comparison our rabbit anti-MF antibodies might be deemed to fail to or only partially neutralize DNase B activity. More study is needed to identify the MF with DNase B.

The amino acid sequences of MF and streptococcal DNase (SDC) produced by the group C streptococcus *S. equisimilis* (Wolinowska et al., 1991) are aligned in Fig. 3. The maximum amino acid sequence identity of these proteins was calculated as 34%. His¹²² (His¹⁶⁵ when calculated from the beginning of the premature MF protein) was determined to be a residue critical to the

nuclease activity as described above, and Ser¹⁹⁵ or Ile²¹⁸ or both may also be important. These three residues are located in highly conserved regions between these nucleases. In agreement with this, some reports have suggested that a histidine residue plays an important functional role in the active site of nucleases (Paudel & Liao, 1986; Weston & Suck, 1993; Worrall & Connolly, 1990; Yakovlev *et al.*, 1994).

The biphasic pattern of the heat stability of the MF nuclease seems to be unique. A similar phenomenon called the Arrhenius effect has been observed with α -haemolysin produced by *Staphylococcus aureus* (Arbuthnott, 1970), thermostable direct haemolysin produced by *Vibrio parahaemolyticus* (Miwatani *et al.*, 1972; Takeda *et al.*, 1974, 1975), and exotoxin A produced by *Pseudomonas aeruginosa* (Vasil *et al.*, 1976). The phenomenon was presumed to be due to protein aggregation occurring at 60 °C or activation of a contaminating proteinase at 60 °C which was inactivated at temperatures above 80 °C. It is very unlikely that this phenomenon is due to a proteinase contaminant in the MF materials used here, because the activity of rMF inactivated at 60 °C is restored by reheating at 80 °C. This rather suggested the possibility that temperature-dependent conformational change might occur, affecting the nuclease activity of rMF. Additional experiments are needed to clarify this issue.

S. pyogenes produces more than 20 extracellular proteins, some of which are cytolytic toxins, superantigens or enzymes (such as proteinases and nucleases). Of the streptococcal pyrogenic exotoxins, SPEB is known to be commonly distributed among group A streptococcal isolates and is a cysteine protease (Berge & Björck, 1995; Yutsudo *et al.*, 1994). MF may play a role in pathogenesis similar to the other pyrogenic exotoxins but as shown here it is also a nuclease. These extracellular proteins may have more than one role in the pathogenesis of group A streptococcal infection.

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