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A new type of mitogenic factor produced by *Streptococcus pyogenes*

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A new type of mitogenic factor (protein) was purified from the culture supernatant of a strain of *Streptococcus pyogenes* by SP-Sephadex C-25 column chromatography, preparative isoelectric focusing and reversed-phase high-performance liquid chromatography. The purified factor, showing marked mitogenic activity in rabbit peripheral blood lymphocytes, gave a single-band staining for protein on SDS-PAGE. The molecular weight of the purified mitogenic factor was determined to be 25,370, which was different from those calculated from reported amino acid sequences deduced from 4 different nucleotide sequences of 3 kinds of streptococcal pyrogenic exotoxins (two SPEAs, SPEB and SPEC). The amino acid sequence of the N-terminal region of the purified mitogenic factor was determined to be Gln-Thr-Gln-Val-Ser-Asn-Asp-Val-Val-Leu-Asn-Asp-Gly-Ala-Ser-Lys-Tyr-Leu-Asn-Glu-Ala-, which was also different from the reported N-terminal sequences deduced from the 4 different nucleotide sequences. These data indicate that this mitogenic factor is distinct from the already described streptococcal pyrogenic exotoxins.

Streptococcus pyogenes; Mitogenic activity; Streptococcal pyrogenic toxin; SPEA; SPEB; SPEC

1. INTRODUCTION

Group A streptococci produce several extracellular proteins such as streptolysin O, streptolysin S and erythrogenic toxins [1]. Erythrogenic toxin, or streptococcal pyrogenic exotoxin (SPE), considered to be a pathogenic agent of scarlet fever, is characterized by biological activities such as pyrogenicity, mitogenicity, enhancement of susceptibility to endotoxin shock, suppression of IgM and enhancement of IgG production [1]. Three serologically distinct streptococcal pyrogenic exotoxins, SPEA, SPEB and SPEC have so far been reported [2–4], and nucleotide sequences of the genes of these toxins have been determined [5–8]. While attempting to purify the streptococcal pyrogenic exotoxins, we found a new type of mitogenic factor differing from these three kinds of streptococcal pyrogenic exotoxins in its N-terminal amino acid sequence. Here we report the purification of this new type of mitogenic factor produced by *Streptococcus pyogenes* and its N-terminal amino acid sequence.

2. MATERIALS AND METHODS

2.1. *Bacterial strain and culture medium*

Streptococcus pyogenes strain NY-5 provided by Dr. T. Nakahara (Saitama College of Health, Urawa, Japan) was used. Bacterial cells were cultured at 37°C for 24 h with intermittent stirring in the medium as described by Igarashi et al. [9], which contained 4% NZ-amine (Wako Pure Chemical, Osaka), 0.15% yeast extract (Difco Laboratories, USA), and 0.2% glucose.

2.2. *Assay of mitogenic activity*

Mitogenic activity was assayed as described by Abe et al. [10]. Briefly, cells from heparinized peripheral venous blood from a rabbit were separated from plasma, mixed with an equal volume of 1% polyvinyl alcohol in phosphate-buffered saline, pH 7.0 (PBS), and incubated at 37°C for 30 min in a water bath. The leukocyte-rich fraction was collected and washed 3 times with PBS. The cells were suspended at a cell density of 1×10^6/ml in Dulbecco's modified Eagle Medium containing 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid, 100 μg/ml of kanamycin and 20% inactivated autologous or homologous serum. Then 0.2 ml aliquots of the cell suspension were introduced into wells of a 96-well microtiter plate and cultured for 4 days in a CO_2 incubator. Test materials were added at the start of culture. Con A at a concentration of 5 μg/ml was used as a positive control. Seventeen hours before the end of culture, [*methyl*-^{3}H]-thymidine (^{3}H-TdR, Amersham-Japan, Tokyo) was added at a concentration of 9.25 Bq/ml. Cells were harvested on glass fiber filters (Printed filter mat A, Pharmacia, Sweden) with a cell harvester (Type 1295-001, LKB, Sweden) and radioactivity was determined in a liquid scintillation spectrometer (Type 1205 Betaplate, LKB, Sweden).

2.3. *SP-Sephadex C-25 column chromatography*

Culture supernatants obtained by centrifugation at 10,000 × *g* for 20 min were filtered through a membrane filter (Nihon Millipore Ltd., Tokyo) and diluted 5-fold with distilled water. They were then mixed with SP-Sephadex C-25 gel equilibrated with 0.01 M citrate phosphate buffer (pH 4.6) and kept overnight at 4°C. After adsorption of the factor, the SP-Sephadex C-25 gel was collected by filtration, and introduced into a column of 5 × 30 cm. The adsorbed material was then eluted successively with 2,000 ml of 0.01 M citrate phosphate buffer (pH 4.6), 1,000 ml of 0.02 M citrate phosphate buffer (pH 6.7), and 1,000 ml of 0.01 M citrate phosphate buffer (pH 4.6) containing 1 M NaCl. Fractions with mitogenic activity were collected and concentrated to a small volume by ultrafiltration on an Amicon YM-10 membrane (Amicon Corporation, Ireland).

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2.4. *Preparative isoelectric focusing*

The eluate from the SP-Sephadex C-25 column was dialyzed against distilled water and applied to an Ampholine column type 8101 (LKB, Sweden). Isoelectric focusing was performed at 200 V for 48 h. After electrophoresis, the material was fractionated and fractions with mitogenic activity were collected.

2.5. *Reversed-phase high performance liquid chromatography*

The preparation from the preparative isoelectric focusing column was subjected to reversed-phase high-performance liquid chromatography (HPLC) on a Cosmosil $5C_{18}$-P300 column (4.6 × 150 mm, Nacalai Tesque, Kyoto) in a Waters HPLC system, model LC-204 (Waters, Nihon Millipore Ltd., Tokyo). Material was eluted with a linear gradient of acetonitorile (20–80%, 1%/min) in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min, and the absorbances of the eluate at 214 nm and 280 nm were monitored.

2.6. *Chromatofocusing column chromatography*

The eluate from the SP-Sephadex C-25 column was dialyzed against 25 mM imidazole-HCl buffer (pH 7.4) and applied to a column (1 × 40 cm) of 30 ml of polybuffer exchanger PBE94 (Pharmacia, Sweden) that had been equilibrated with 25 mM imidazole-HCl buffer (pH 7.4) and eluted with polybuffer 74 (pH 4.0) to obtain a gradient of pH 7.0 to 4.0. Fractions with mitogenic activity were collected.

2.7. *Polyacrylamide gel electrophoresis*

Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was carried out as described by Laemmli [11] in 12% acrylamide gel.

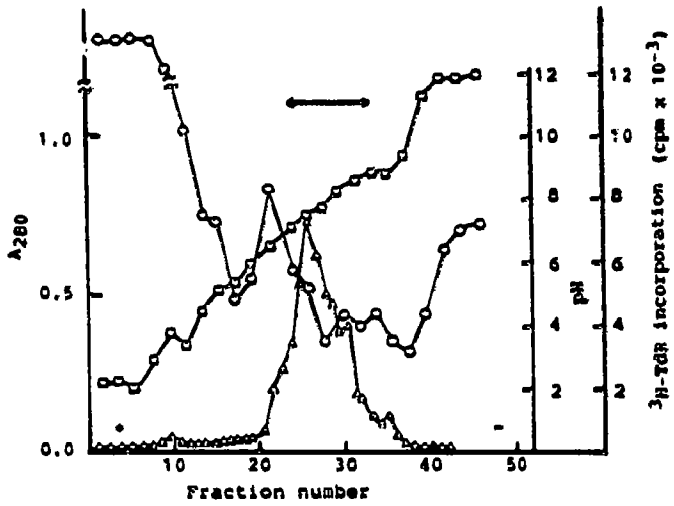

Fig. 1. Preparative isoelectric focusing of the mitogenic factor. Preparative isoelectric focusing was performed as described in the text. After electrophoresis, fractions of 2.8 ml were collected. Samples were diluted 100-fold with PBS and tested for mitogenic activity. Assays were done in triplicate. A negative control (PBS) and positive control (Con A) gave about 90–140 and 11,000–13,000 cpm, respectively. The bar with arrowheads shows the fractions collected for further purification. (○) Absorbance at 280 nm (A_{280}); (□) pH; (△) ^{3}H-TdR incorporation.

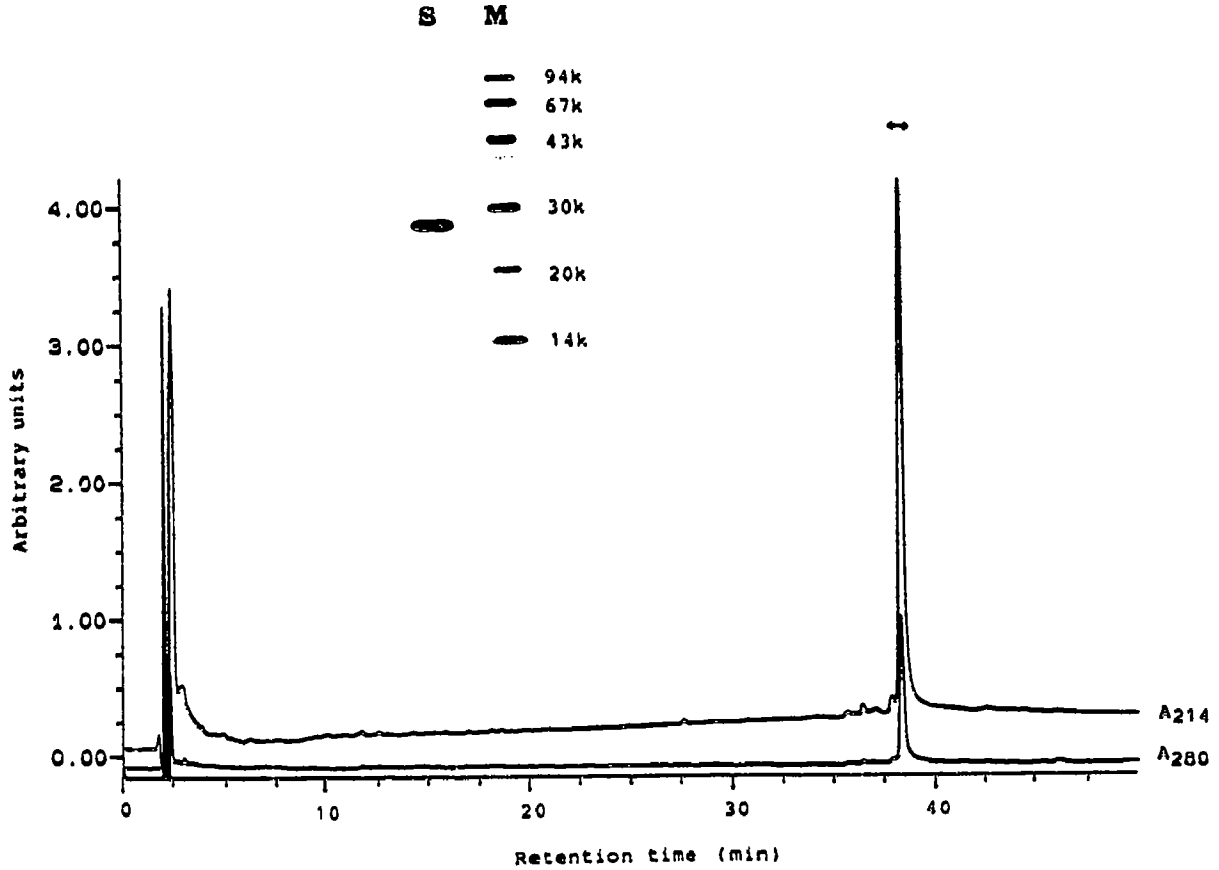

Fig. 2. Reversed-phase HPLC of the mitogenic factor. The absorbances at 214 nm (A_{214}) and 280 nm (A_{280}) of the eluate were monitored simultaneously. The fractions shown by a bar with arrowheads were collected and subjected to SDS-PAGE as described in the text. Insert: the result of the SDS-polyacrylamide gel electrophoresis. Lane S, purified factor; lane M, molecular weight markers. Numbers on the right margin represent the molecular weight of the markers.

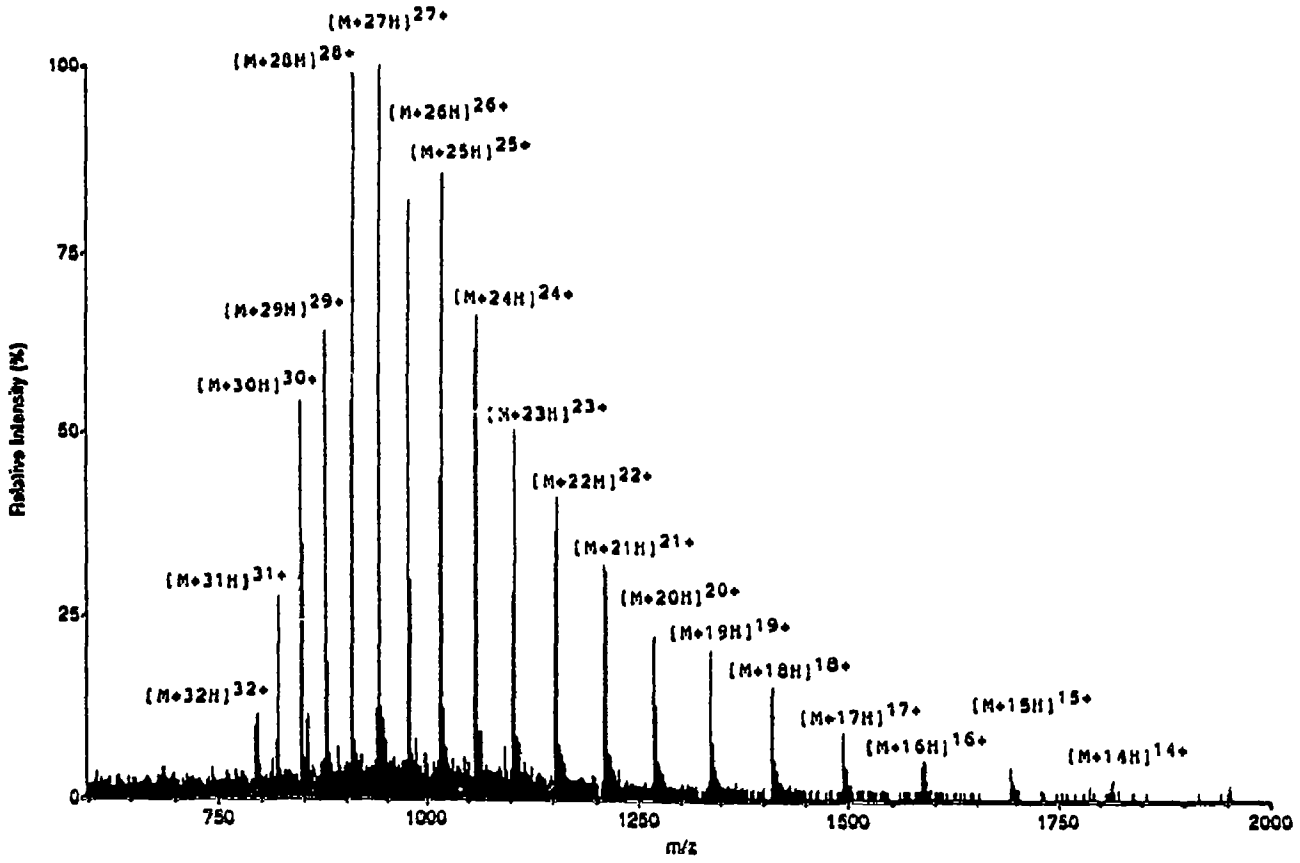

Fig. 3. Ion-spray mass spectrum of the purified mitogenic factor. The mitogenic factor isolated by reversed-phase HPLC was directly subjected to ion-spray mass spectrometry as described in the text. From each *m/z* value of the multiply charged ion signals ($[M+nH]^{n+}$), the molecular weight of the mitogenic factor was calculated to be 25,370 ± 3.

2.8. *Amino acid composition and sequence analysis*

The amino acid composition of the purified toxin was determined in an L-8500 amino acid analyzer (Hitachi Ltd, Tokyo) after hydrolysis of samples in 5.7 N hydrochloric acid at 110°C for 48 h in evacuated sealed tubes. For analysis of the amino acid sequence, the purified factor was dissolved in 3 vols. of a solution of 37% acetonitrile, 63% acetic acid, and one volume of 25% aqueous trifluoroacetic acid was added. The mixture was then examined in an automated gas-phase amino acid sequencer (Model PSQ-1, Shimadzu Corporation, Kyoto).

2.9. *Determination of protein*

Protein content was determined with a protein assay kit (Bio-Rad Laboratories, USA). The protein content of the purified factor was calculated from the values determined by amino acid composition analysis.

2.10. *Measurement of ion-spray mass spectra*

Ion-spray mass spectra were measured on a triple quadruple mass spectrometer (SCIEX APIII, Canada) equipped with a standard atmospheric pressure ionization source. The purified factor isolated by HPLC was dissolved in 0.1% aqueous trifluoroacetic acid containing 50% acetonitrile and infused into the ion source at a flow rate of 20 μl/min. Details of the procedure were described previously [12].

3. RESULTS AND DISCUSSION

The culture supernatant prepared as described in section 2 was chromatographed on an SP-Sephadex C25 column. Materials with mitogenic activity were eluted with 0.02 M citrate phosphate buffer (pH 6.7) and with 0.01 M citrate phosphate buffer (pH 4.6) containing 1 M NaCl. The former eluate was found to contain SPEA by further chromatography on a chromatofocusing column and amino acid sequence analysis (data not shown). The latter eluate was collected and applied to a preparative isoelectric focusing column. As shown in Fig. 1, material with mitogenic activity was eluted between pH 7.6 and 8.4. The fractions with activity were collected and subjected to reversed-phase HPLC. As shown in Fig. 2, this material gave a sharp peak of protein with several minor protein peaks. The sharp peak indicated by a bar with arrowheads showed marked mitogenic activity, whereas the other peaks did not. On SDS-PAGE (insert in Fig. 2), the purified factor gave a single band staining for protein with a molecular weight of 27,000–28,000. Thus, this fraction was collected as the purified factor. The optimal dose (ED_{50}) of the purified mitogenic factor for rabbit peripheral blood lymphocytes was about 300 ng/ml. In contrast, the optimal dose of SPEA for a mitogenic effect on C57BL/6 spleen cells is reported to be 3 to 10 μg/ml [13]. Although we did not examine the mitogenic activity of

Table I

Comparison of amino acid compositions of the purified mitogenic factor and streptococcal pyrogenic exotoxins produced by *Streptococcus pyogenes*

	Molecular ratio (%)				
	MF	SPEA-W	SPEA-J	SPEB	SPEC
Asp/Asn	16.1	14.0	12.3	13.0	17.3
Thr	8.0	6.3	7.3	3.6	5.7
Ser	4.8	6.8	7.7	8.7	7.7
Glu/Gln	9.8	12.7	10.9	9.1	8.2
Gly	10.4	4.1	4.1	11.9	5.3
Ala	8.4	1.8	1.4	7.1	2.4
Val	6.9	6.3	6.4	7.5	3.4
Met	0.0	1.3	1.4	1.6	1.4
Ile	3.7	5.9	6.8	4.3	10.1
Leu	8.4	9.0	8.6	5.5	6.2
Tyr	5.8	7.7	7.3	5.9	8.2
Phe	2.1	4.1	5.0	4.0	5.3
Lys	4.8	10.0	10.0	5.5	10.1
His	1.6	2.7	2.3	2.8	2.9
Arg	5.4	1.3	2.7	2.8	3.4
Pro	3.7	4.1	3.6	4.7	1.9
Trp	ND	0.5	0.5	1.6	0.0
Cys	ND	1.4	1.8	0.4	0.5

The amino acid composition of the mitogenic factor purified in this study (MF) was analysed as described in the text. The amino acid compositions of SPEA, SPEB, and SPEC were calculated from reported amino acid sequences deduced from the nucleotide sequences of the respective toxins [5–8]. SPEA-W and SPEA-J were both reported as streptococcal exotoxin type A by Weeks and Ferretti [5] and Johnson et al. [6], respectively. ND, not determined.

the purified mitogenic factor on mouse spleen cells, we suppose that this mitogenic factor has potential biological activity on mammalian lymphocytes.

Ion-spray mass spectrometry of the purified factor showed that its molecular weight was 25,370 ± 3 (Fig. 3). This value is similar to, but not the same as, those of SPEA reported by Weeks and Ferretti [5] (M_r = 25,787), SPEA reported by Johnson et al. [6] (M_r = 25,805), SPEB (M_r = 27,588) [7] and SPEC (M_r = 24,354) [8].

The amino acid composition of the purified factor shown in Table I is quite different from those of SPEAs, SPEB and SPEC. The sequence of the first 21 N-terminal amino acids of the purified factor was determined to be Gln-Thr-Gln-Val-Ser-Asn-Asp-Val-Val-Leu-Asn-Asp-Gly-Ala-Ser-Lys-Tyr-Leu-Asn-Glu-Ala-. This sequence is also different from those of SPEAs, SPEB and SPEC (Table II): neither the N-terminal sequence nor the sequences of other regions of these already reported toxins were identical with the N-terminal sequence of the new mitogenic factor. A computer search of data bases also showed that this amino acid sequence of this mitogenic factor differs from those of known proteins. From these results we conclude that the purified factor is a novel mitogenic factor produced by *Streptococcus pyogenes*.

Recently pyrogenic exotoxins from *Streptococcus pyogenes* and certain enterotoxins from *Staphylococcus aureus* were reported to comprise a family of superantigens [13–15], defined as bifunctional molecules binding to MHC class II structures and activating T cells expressing appropriate V beta segments of the T cell receptor [16–19]. Further studies to characterize the novel mitogenic factor as a superantigen are now in progress.

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Table II

Comparison of N-terminal amino acid sequences of the novel mitogenic factor and streptococcal pyrogenic exotoxins produced by *Streptococcus pyogenes*

MF:	Gln-Thr-Gln-Val-Ser-Asn-Asp-Val-Val-Leu-Asn-Asp-Gly-Ala-Ser-Lys-Tyr-Leu-Asn-Glu-Ala
SPEA-W:	Gln-Gln-Asp-Pro-Asp-Pro-Ser-Gln-Leu-His-Arg-Ser-Ser-Leu-Val-Lys-Asn-Leu-Gln-Asn-Ile
SPEA-J:	Ser-Thr-Arg-Pro-Lys-Pro-Ser-Gln-Leu-Gln-Arg-Ser-Asn-Leu-Val-Lys-Thr-Phe-Lys-Ile-Tyr
SPEB:	Gln-Pro-Val-Val-Lys-Ser-Leu-Leu-Asp-Ser-Lys-Gly-Ile-His-Tyr-Asn-Gln-Gly-Asn-Pro-Tyr
SPEC:	Asp-Ser-Lys-Lys-Asp-Ile-Ser-Asn-Val-Lys-Ser-Asp-Leu-Leu-Tyr-Ala-Tyr-Thr-Ile-Thr-Pro

MF=the mitogenic factor purified in this study. The reported N-terminal amino acid sequences of SPEA, SPEB and SPEC which were deduced from the nucleotide sequences of the respective toxins [5–8] are shown. SPEA-W and SPEA-J were both reported as streptococcal exotoxin type A by Weeks and Ferretti [5], and Johnson et al. [6], respectively.

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