

# Identification of the functional region on the superantigen *Yersinia pseudotuberculosis*-derived mitogen responsible for induction of lymphocyte proliferation by using synthetic peptides

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**Abstract** *Yersinia pseudotuberculosis*-derived mitogen (YPM) is the unique Gram-negative bacillary superantigen known. In order to identify the regions on the YPM molecule involved in its superantigenic activity, seven overlapping peptides of the entire YPM molecule were synthesized and tested to evaluate their effects on the YPM-induced proliferation of human peripheral blood lymphocytes. A peptide corresponding to the N-terminal amino acid sequence (1–23) was found to inhibit YPM-induced lymphocyte proliferation in a concentration-dependent manner. The N-terminal peptide was found to show no inhibition of the proliferation induced by the other superantigen (staphylococcal enterotoxin B) or the other T-cell mitogen pertussis toxin, indicating that the inhibition is specific to YPM-induced proliferation. Thus, we have identified the N-terminal region (1–23) of the YPM as one of the functional regions responsible for its superantigenic activity.

**Key words:** Superantigen; YPM; Structure-function relationship; Lymphocyte proliferation; *Yersinia pseudotuberculosis*

## 1. Introduction

*Yersinia pseudotuberculosis*-derived mitogen (YPM) is known as a superantigen which is a highly potent immunostimulatory molecule [1–6]. Unlike conventional peptide antigen, a superantigen binds directly as an intact protein to major histocompatibility complex (MHC) class II molecules on antigen-presenting cells at a site distinct from the antigen binding groove. The superantigen/MHC complex then reacts with the T-cell receptors (TCR) through the particular  $\beta$  chain of a variable region (V $\beta$ ), rather than with the normal antigen-binding groove on TCR. Therefore, each superantigen reacts with all T-cells that express a particular V $\beta$  gene. The repertoire of V $\beta$  activated has a pattern that is characteristic for each superantigen. The result of these interactions is proliferation of a large number of T-cells with consequent release of cytokines. This ability of superantigens directly accounts for their pathogenic effects and clinical symptoms of the infection [7,8].

Several bacterial superantigens, such as toxic shock syndrome toxin-1, staphylococcal enterotoxins and streptococcal

pyogenic exotoxins, are known. Although almost all of members are produced by the Gram-positive cocci *Staphylococcus aureus* and *Streptococcus pyogenes* [9], YPM is the only known Gram-negative bacillary superantigen whose primary structure substantially differs from other bacterial superantigens [4–6].

In the present study, we have used a competitive inhibition approach using synthetic YPM peptide fragments for identification of the functional sequences on the YPM molecule involved in the proliferation of human peripheral blood lymphocytes. These findings provide insight into how YPM functions as a superantigen.

## 2. Materials and methods

### 2.1. Superantigens and pertussis toxin

YPM was purified from the cell lysate of a *Y. pseudotuberculosis* strain by the previously reported procedure [4]. Staphylococcal enterotoxin B (SEB) and pertussis toxin (PT) were obtained from Sigma Chemical (St. Louis, MO, USA).

### 2.2. Peptide synthesis

Overlapping peptides corresponding to the entire sequence of YPM were synthesized by using a PerSeptive Biosystems 9050 peptide synthesizer. The peptide chains were elongated stepwise on polyethylene glycol polystyrene resin with 4-fold equivalent of an equimolar mixture of 9-fluorenylmethoxycarbonyl (Fmoc)-amino acid and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in *N,N*-dimethylformamide (DMF) containing 8.5% diisopropylethylamine. The Fmoc group was removed by treatment with 2% 1,8-diazabicyclo[5.4.0]undec-7-ene in DMF. In general, the protected peptide resin was treated with a mixture of trifluoroacetic acid (TFA)/thioanisole/*m*-cresol/1,2-ethanedithiol (90/5/3/2, v/v) for 1 h at room temperature. After removal of TFA, the crude peptides were extracted with 1 M acetic acid, and then washed with diethyl ether. The synthetic peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC).

### 2.3. Peptide competition studies

Human peripheral blood mononuclear cells (PBMC) were obtained from heparinized peripheral blood of healthy donors by density gradient centrifugation using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMC suspended in RPMI 1640 medium with 100  $\mu$ g/ml of streptomycin, 100 U/ml of penicillin and 10% (v/v) of fetal bovine serum were dispensed into a 96-well microtiter plate at a density of  $2 \times 10^5$  cells per well (200  $\mu$ l). To assess the ability of peptides to inhibit superantigen-induced proliferation, peptides were added to the wells. Then, the superantigen (YPM or SEB) or PT was added at a final concentration of 100 pg/ml or 2.5  $\mu$ g/ml, respectively. The PBMC were incubated for 72 h and pulsed with 37 kBq of [*meth*-<sup>3</sup>H]thymidine per well for the final 7 h of incubation. The cells were harvested onto glass fiber filters and the [*meth*-<sup>3</sup>H]thymidine incorporation of cells was determined.

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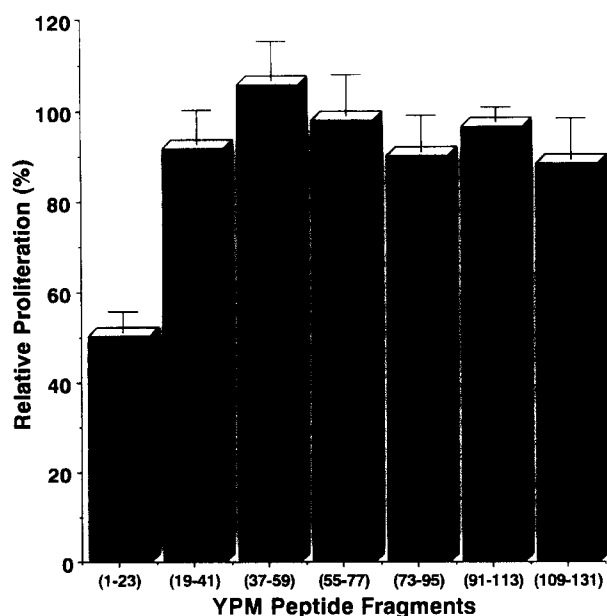


Fig. 1. Inhibition of YPM-induced proliferation of PBMC by synthetic peptide fragments of YPM. YPM peptide fragments were used at a final concentration of 50  $\mu$ M. The superantigen YPM was used at a final concentration of 100 pg/ml. Data are presented as the relative value (%) of net cell proliferation in the presence of YPM and an indicated peptide as compared with that in the presence of YPM alone. Each bar represents the mean  $\pm$  S.E. of six individual experiments.

### 3 Results and discussion

In an attempt to relate structure to function, seven overlapping peptides of the entire YPM molecule were chemically synthesized. The amino acid sequences of the seven peptide fragments are presented in Table 1. Synthetic peptides purified by RP-HPLC were characterized by amino acid analysis, Edman degradation and fast atom bombardment mass spectrometry, giving the expected data (not shown). Of all the peptides at a concentration of 50  $\mu$ M tested for stimulation of PBMC proliferation, no peptide showed a significant increase over the normal value.

The synthetic peptides at a concentration of 50  $\mu$ M were examined for direct functional competition with YPM for inhibition of human PBMC proliferation stimulated by YPM. It was observed that only the peptide YPM(1–23) was able to significantly inhibit the YPM-induced proliferation (Fig. 1). Dose-response study of this peptide showed a significant inhibition of YPM-induced proliferation at a concentration as low as 10  $\mu$ M (Fig. 2). Fifty percent inhibition of the proliferation was observed for the peptide YPM(1–23) at a concentration of 50  $\mu$ M. These results suggest that the N-terminal

region at residues 1–23 on the YPM molecule is a region responsible for the induction of PBMC proliferation by YPM.

The peptide YPM(1–23) was tested with the other superantigen SEB or with the other T-cell mitogen PT to determine if the displayed inhibitory effect is specific to YPM. The peptide at a concentration as high as 200  $\mu$ M was unable to inhibit the SEB-induced or PT-induced PBMC proliferation (data not shown), indicating that the inhibitory effect of the peptide YPM(1–23) is specific to the YPM-induced proliferation.

In the present study, we have identified through competition studies using synthetic peptide fragments that a region of YPM encompassing amino acid residues 1–23 is involved in the induction of human PBMC proliferation. Neither the N-terminal sequence nor the other regions of the other superantigen molecules were identical to the N-terminal sequence (1–23) of YPM [4]. Several investigators showed the sequences that are important for superantigenic activity of Gram-positive coccal superantigens using synthetic peptide fragments [10–14], the present study performed the characterization of the bacterial superantigen produced by a Gram-negative bacillus. It is also the first report of the structure-function relationship of YPM.

The use of synthetic peptide fragments is valuable in studies of protein-protein interaction, but a potential problem exists. Only a small percentage of peptide may mimic the binding site at any one time; thus, a large molar excess of peptide may be needed to demonstrate the inhibition. In addition to this, if the three-dimensional structure of the binding site is specific by virtue of its conformation, then a synthetic peptide may not mimic the binding site. Therefore, the results in the present study demonstrate that the N-terminal region (1–23) of YPM is one of the regions responsible for the superantigenic activity, but not only one.

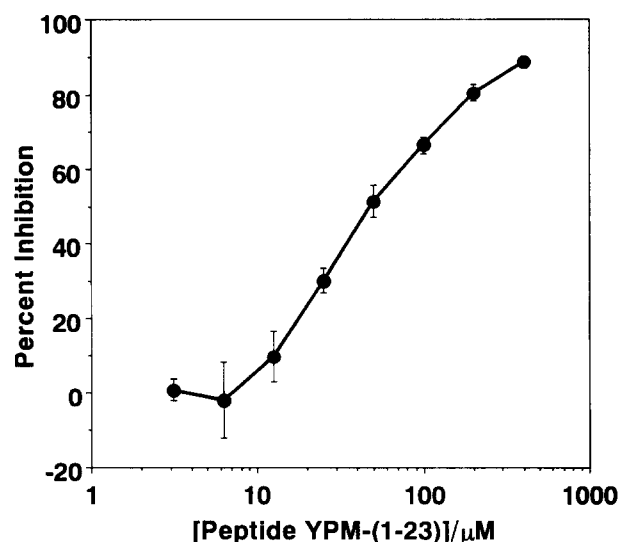


Fig. 2. Concentration-dependent inhibition of the peptide YPM(1–23) to YPM-induced PBMC proliferation. The superantigen YPM was used at a final concentration of 100 pg/ml. Data are presented as the relative value (%) calculated by the formula as  $100(A-B)/C$ ;  $A$  and  $B$ : gross of radiolabel incorporated by cell proliferation induced by YPM in the presence ( $B$ ) and the absence ( $A$ ) of the peptide YPM(1–23);  $C$ : net of radiolabel incorporated by cell proliferation induced by YPM alone. Each bar represents the mean  $\pm$  S.E. of six individual experiments.

Table 1  
Amino acid sequences of the synthetic YPM peptide fragments

YPM(1–23)	TDYDNTLNSIPSLRIPNIATYTG
YPM(19–41)	ATYTGTIQGGKEVA*IIIGNKEGKT
YPM(37–59)	KEGKTRGGELYAVLHSTNVNADM
YPM(55–77)	VNADMTLILLRNVGNGWGGEIKR
YPM(73–95)	GEIKRNDIDKPLKYEDYYTSGLS
YPM(91–113)	TSGLSWIWKIKNNSSETSNYSLD
YPM(109–131)	NYSLDATVHDDKEDSDVLTKA*PV

\* Alanine residue was substituted for two cysteine residues (Cys<sup>32</sup> and Cys<sup>129</sup>).

In order to stimulate T-cells, a superantigen has to interact with both the MHC class II molecule and the V $\beta$  domain of TCR. Therefore, two kinds of interface are present on a superantigen molecule, one is for the class II MHC molecule and the other is for the TCR V $\beta$  domain [7,8]. At present, we cannot conclude whether the N-terminal region of the YPM molecule constitutes the interface for the class II MHC molecule, the V $\beta$  domain of TCR, or both.

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