Soymetide, an immunostimulating peptide derived from soybean β-conglycinin, is an fMLP agonist

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Abstract A tridecapeptide (MITLAIPVNKPGR) that stimulates phagocytosis of human neutrophils was isolated from a trypsin digest of soybean proteins. This peptide is derived from the soybean β-conglycinin α′ subunit and was named soymetide-13. The N-terminal methionine residue of soymetide-13 is essential for its activity, and removal of C-terminal residues revealed that soymetide-4 (MITL) is the minimal structure required for phagocytosis stimulation. Although they are not formylated at their N-termini, soymetides have a weak affinity for the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) receptor and their phagocytosis-stimulating activity is inhibited by the fMLP antagonist Boc-MLP. Interestingly, soymetide-4 promotes tumor necrosis factor α production at a higher level than soymetide-13 following oral administration in mice.

Key words: *N*-Formyl-methionyl-leucyl-phenylalanine; Immunostimulating peptide; Phagocytosis; Soybean

1. Introduction

Many immunostimulating peptides have been isolated from enzymatic digests of various food proteins such as those found in milk [1,2]. These peptides, which have specific binding sites on human blood phagocytic cells, stimulate phagocytosis of human and murine macrophages in addition to protecting mice from *Klebsiella pneumoniae* infection.

We isolated the immunostimulating peptide His-Cys-Gln-Arg-Pro-Arg from a tryptic digest of soybean proteins [3]. This peptide, which has homology to tuftsin (Thr-Lys-Pro-Arg) [4], is derived from a soybean glycinin subunit and can activate phagocytosis of human neutrophils; furthermore, it stimulates tumor necrosis factor (TNF) production when it is orally administered to mice. In this study, we searched for other immunostimulating peptides that may be present in tryptic soybean protein digests. We describe the isolation of a peptide that acts as an agonist of the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) receptor even though it is not formylated at its N-terminus.

N-Formylmethionyl peptides are chemotactic for human neutrophils and macrophages [5]. fMLP is a synthetic *N*-formylmethionyl peptide that is strongly chemotactic for neutrophils [6], and a specific fMLP receptor has been identified on the surface of neutrophils and macrophages [7]. This receptor also mediates the generation of reactive oxygen species (ROS) from neutrophils and macrophages [8] as well as the phagocytosis-stimulating activity of neutrophils [9]. These functions appear to contribute to a rapid response to bacterial infection since bacterial proteins have an *N*-formylmethionine residue at their N-termini. This response leads to bacterial death by phagocytosis and ROS-induced bactericidal effects.

We investigated the basic properties of this immunostimulating peptide so that orally effective immunostimulating agents may eventually be developed.

2. Materials and methods

2.1. Reagents and chemicals

fMLP and Boc-MLP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soymetide-13 and its derivatives were synthesized by a solid phase peptide synthesizer (PS3, Protein Technologies, Inc., Tucson, AZ, USA) according to the 9-fluoroenyl methoxycarbonyl (Fmoc) method. Peptides were removed from the resin with trifluoroacetic acid and purified with high performance liquid chromatography (HPLC) and subsequent lyophilization.

2.2. Animals

Male ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan).

2.3. Enzymic digestion

5 g of the soybean protein isolate (SPI, Fuji Oil Co., Ltd., Osaka, Japan) were resuspended in 80 ml water and centrifuged at 3000 rpm for 20 min. The supernatant adjusted to pH 7.5 was boiled for 10 min. Bovine pancreas trypsin was then added, and the solution was incubated at 37°C for 5 h. The reaction was stopped by boiling for 10 min, and then the suspension was centrifuged at 10 000 rpm for 10 min. The supernatant was used after lyophilization as soybean trypsin digest.

2.4. Phagocytosis assay

Heparinized human venous blood was washed two times with phosphate-buffered saline (PBS; 136.9 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, and 8.04 mM Na_2HPO_4) containing glucose (GPBS; PBS containing 5.55 mM glucose, 0.33 mM sodium pyruvate, 0.49 mM $MgCl_2$ and 0.90 mM $CaCl_2$). 10 μl of sample solution were added to 100 μl of prepared human blood cells in GPBS and the resulting

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Abbreviations: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; TNF, tumor necrosis factor; ROS, reactive oxygen species

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mixture was incubated for 10 min at 37°C. Next, 10 μl of PBS-suspended fluorescent beads opsonized with human serum were added to the mixture, which was incubated for an additional 10 min at 37°C. The reaction was stopped by adding 3 ml of ice-cold PBS containing 4 mM ethylenediamine tetraacetic acid (EDTA) (EDTA-PBS). Erythrocytes were removed by lysing with NH_4Cl solution (77.2 mM NH_4Cl, 5 mM $KHCO_3$, and 0.041 mM EDTA-4Na). Neutrophils were analyzed by EPICS PROFILE (Beckman Coulter, Inc., Fullerton, CA, USA). The intensity of phagocytosis was shown as the phagocytic index, which is presented as the number of incorporated fluorescent beads per 100 neutrophils [10].

2.5. Purification of phagocytosis-stimulating peptides

100 mg of the soybean protein trypsin digest dissolved in 1 ml of Tris–HCl buffer (pH 7.2) were applied to a DEAE-cellulose column equilibrated with the same buffer, and the non-absorbed fraction was obtained. This fraction was passed over an ODS column (Cosmosil 5C18-AR, 20×250 mm, Nacalai Tesque, Inc., Kyoto, Japan) and a Phenetyl column (Develosil PhA-T-5, 4.6×250 mm, Nomura Chemical Co., Ltd., Aichi, Japan) on a linear acetonitrile concentration gradient (1%/min) in the presence of 0.1% trifluoroacetic acid. The fraction obtained in this step was then passed over an ODS column on a linear acetonitrile concentration gradient (1%/min) in 10 mM phosphate buffer (pH 7.4) to obtain the final sample. A protein sequencer (447A, Applied Biosystems, Inc., Foster City, CA, USA) was used for structural determination of the obtained peptides.

2.6. fMLP receptor binding assay

Saline containing 3% (w/v) dextran (Nacalai Tesque, Inc., Kyoto, Japan) was added to heparinized human peripheral blood. After 30 min, the supernatant fraction was collected, and contaminating erythrocytes were hypotonically lysed. The neutrophil pellet was obtained by centrifugation on Ficoll Paque (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) at 500×*g* for 20 min and was then suspended in PBS (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_2, and 9.35 mM Na_2HPO_4 (pH 7.4)). Samples plus 25 nM tritiated fMLP (NEN Life Science Products, Inc., Boston, MA, USA) were added to 1×10^6 neutrophils in PBS and incubated on ice for 60 min. The cells were then filtered onto GF/B glass microfiber filters (Whatman International, Ltd., Maidstone, UK) and washed two times with PBS. The filters were transferred to scintillation vials, and 3 ml Aquasol-2 (Packard Bioscience Co., Meriden, CT, USA) were added to each vial. After shaking overnight, radioactivity was measured with a liquid scintillation counter [11].

2.7. Superoxide anion generation assay

Superoxide anion ($O_2^{\bullet-}$) production was measured by superoxide dismutase inhibitable cytochrome *c* reduction [12]. Human neutrophils were isolated by the same method as described in Section 2.6. Cytochrome *c* solution (5 mM) and the isolated phagocytosis-stimulating peptides were added to neutrophils (4×10^6 cells/ml) suspended in HEPES-buffered saline (150 mM NaCl, 5 mM HEPES, 1 mM $CaCl_2$, and 2 mM glucose) and incubated at 37°C for 15 min with or without superoxide dismutase (200 U/ml). The amount of $O_2^{\bullet-}$ in the supernatant was calculated using the following formula: nmol of $O_2^{\bullet-} = 100/21\times$(difference in absorbance between 550 and 468 nm).

2.8. Determination of TNFα production

Peptides dissolved in 200 μl saline were intraperitoneally or orally administered to male ICR mice following 2 h of fasting (TNFα priming). 3 h later, 0.3 mg of the streptococcal preparation OK-432 (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) suspended in 200 μl saline were intravenously injected (TNFα triggering). Blood was collected 2 h later, and the TNFα concentration was measured with a TNFα enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Inc., Camarillo, CA, USA) [13].

3. Results

3.1. Purification and structure of the phagocytosis-stimulating peptide

Phagocytosis-stimulating activity was detected in the flow-through fraction obtained from a DE52 column used to purify a tryptic digest of soybean proteins. The flow-through fraction was separated on an ODS column (Fig. 1). The active fraction was further purified with reversed-phase HPLC on Phenetyl and ODS columns (Fig. 1). The active peptide is a tridecapeptide (Met-Ile-Thr-Leu-Ala-Ile-Pro-Val-Asn-Lys-Pro-Gly-Arg) that corresponds to residues 173–185 of the soybean β-conglycinin α′ subunit. The α and β subunits of soybean β-conglycinin also contain sequences similar to this tridecapeptide except that the N-terminal Met is replaced by Leu and Ile, respectively. Synthetic peptides corresponding to these sequences devoid of Met exhibited no activity (data not shown).

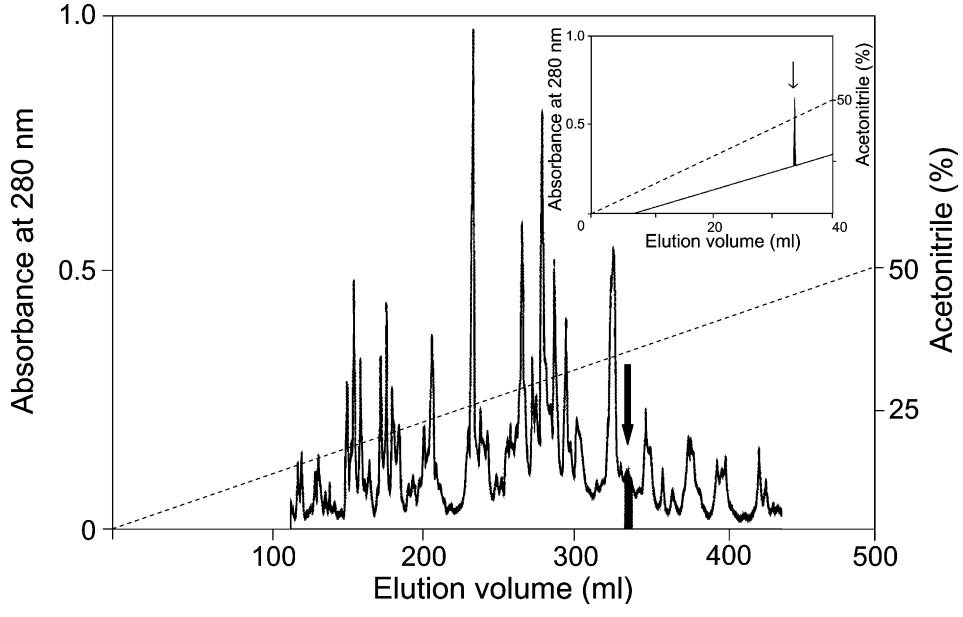

Fig. 1. Purification of a phagocytosis-stimulating peptide. The flow-through fraction from a DEAE column was subjected to HPLC on an ODS column and eluted on an acetonitrile gradient. Fractions that exhibited phagocytosis-stimulating activity were further purified on Phenetyl and ODS columns (inset). Fractions containing soymetide-13 are indicated by arrows.

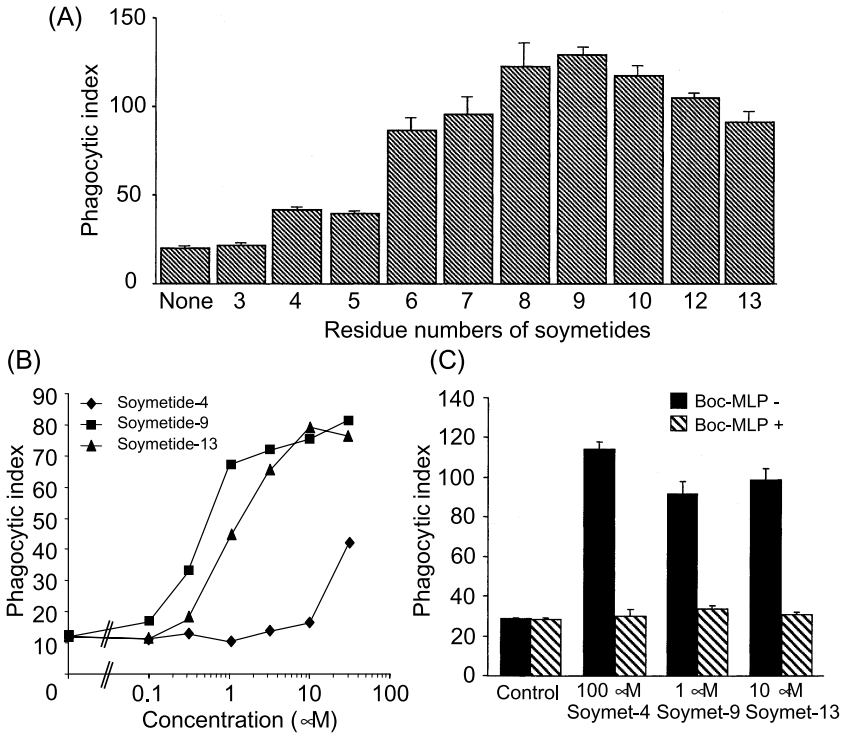

Fig. 2. A: Activation of phagocytosis by soymetides with C-terminal deletions. Human neutrophils were incubated with opsonized fluorescent beads and 30 μM soymetides ($n = 3$). Values are expressed as the mean ± S.E.M. B: Concentration-dependent activation of phagocytosis by soymetide-4, -9, and -13 ($n = 2$). C: Effect of the fMLP antagonist Boc-MLP (100 μM) on phagocytosis activated by soymetides ($n = 4$). Values are expressed as the mean ± S.E.M.

This active peptide was named soymetide-13, since the Met at its N-terminus is essential for its activity.

3.2. Structure–function relationship of the phagocytosis-promoting soymetides

To confirm the minimum structure of soymetide-13 required for its immunostimulatory activity, we synthesized peptides with amino acid residues deleted from either the N- or C-termini. Phagocytosis-stimulating activity disappeared by deletion of Met at the N-terminus. In contrast, activity gradually increased as residues were removed from the C-terminus. Although a statistical analysis was not performed, soymetide-9 (Met-Ile-Thr-Leu-Ala-Ile-Pro-Val-Asn) exhibited the highest immunostimulatory activity. Further deletion of residues from the C-terminus resulted in decreased activity. Met-Ile-Thr displayed no activity, suggesting that Met-Ile-Thr-Leu (soymetide-4) is the minimum sequence required for activity (Fig. 2A). The EC_{50} values (the concentration required for 50% maximum activation) for phagocytotic activation of soymetide-13, -9, and -4 were approximately 1, 0.5, and 30 μM, respectively (Fig. 2B).

3.3. Identification of soymetides as fMLP agonists

We attempted to identify the receptor that mediates the immunostimulatory activity of the various soymetides. fMLP is a typical immunostimulating peptide that contains a Met residue. A receptor binding assay with tritiated fMLP and human neutrophils demonstrated that the soymetides can bind to the fMLP receptor. The IC_{50} values of soymetide-4, -9 and -13 were 450, 25, and 50 μM, respectively (Table 1), indicating that the soymetides are weak ligands for the fMLP receptor.

Next, we tested the effects of Boc-MLP, an fMLP antagonist [14], on the phagocytosis-stimulating activity of the soymetides. As shown in Fig. 2C, Boc-MLP completely inhibited this activity. Together with the results described above, these findings reveal that immunomodulation of soymetides is mediated by the fMLP receptor.

3.4. Stimulation of $O_2^{\bullet-}$ release by soymetides

fMLP stimulates superoxide anion ($O_2^{\bullet-}$) release from neutrophils at nanomolar levels [8]. We found that the soymetides also weakly stimulated $O_2^{\bullet-}$ release from human neutrophils. At 10 μM, soymetides longer than soymetide-5 weakly stimulated $O_2^{\bullet-}$ generation, whereas soymetide-4 and -5 were inactive (data not shown). Stimulation of $O_2^{\bullet-}$ production by soymetide-13 was also blocked by Boc-MLP (data not shown).

3.5. Promotion of TNFα production

Following stimulation by fMLP, macrophages and neutrophils produce inflammatory cytokines such as TNFα and interleukin-1 (IL-1) [15]. TNFα production consists of two steps: induction (priming) and release (triggering). Following intraperitoneal administration in mice, soymetide-9 and fMLP

Table 1
Affinities of soymetide-13 and its derivatives for fMLP receptor

Peptides		IC_{50} (μM)
MITLAIPVNKPGR	(soymetide-13)	50
MITLAIPVN	(soymetide-9)	25
MITL	(soymetide-4)	450
fMLF	(fMLP)	0.03

Binding assay was performed in the presence of [^{3}H]fMLP (25 nM) using human neutrophils.

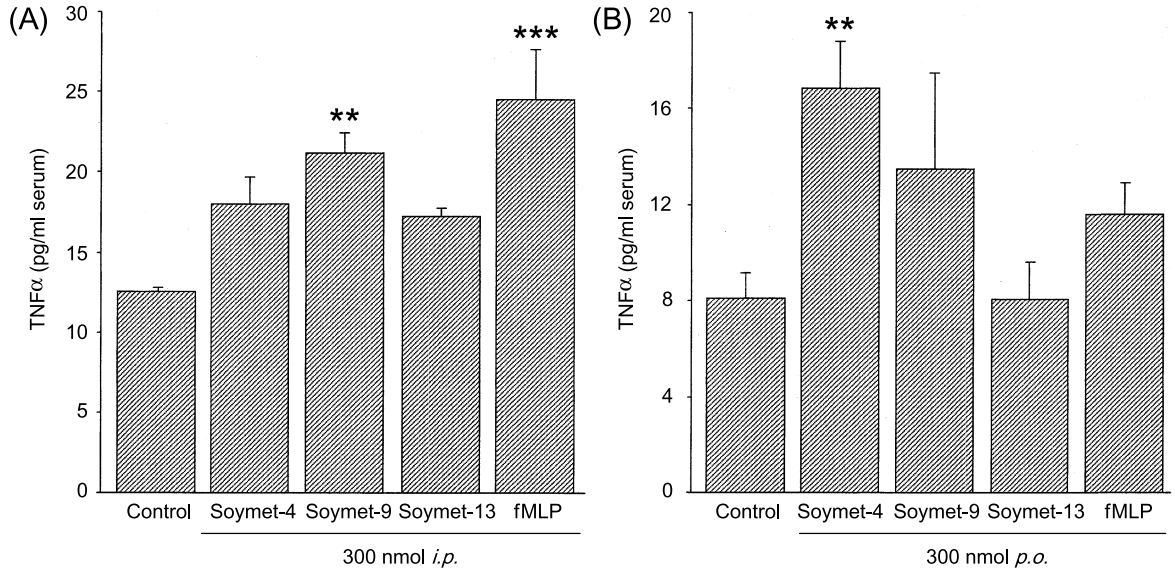

Fig. 3. Induction of TNFα by soymetides and fMLP. Six-week-old ICR mice were primed by peptides (300 nmol/mice) administered intraperitoneally (A) or orally (B) and given 0.3 mg/mice of OK-432 intravenously. Blood samples were collected 2 h later and serum TNFα levels were measured by a mouse TNFα ELISA kit. Values are expressed as the mean ± S.E.M. ($n = 4$). A statistical analysis of the data was carried out using the Fisher test (**$P < 0.01$, ***$P < 0.001$).

exhibited statistically significant priming activity (Fig. 3A), while soymetide-4 and -13 were inactive. In sharp contrast, orally administered soymetide-4, which had the weakest phagocytosis-stimulating activity in vitro (Fig. 2A), displayed statistically significant priming of TNFα (Fig. 3B), unlike soymetide-9, -13, and fMLP, which had no such effect.

4. Discussion

Peptides produced in the mitochondrion and chloroplasts of prokaryotes are usually formylated at their N-termini. *N*-formylmethionyl peptides bind to the fMLP receptor on the surface of macrophages and neutrophils in mammals and thereby activate their immune systems. Immunostimulation following binding of *N*-formylmethionyl peptides to the fMLP receptor alerts the body that a bacterial infection is occurring. Although soymetides derived from soybean protein are not formylated at their N-termini, they exhibit weak affinity for the fMLP receptor. Therefore, following dietary ingestion of soybeans, our immune systems receive a signal that is similar to that received upon bacterial infection. Soymetide is the first fMLP agonist peptide obtained from food protein.

fMLP induces three different responses in neutrophils depending on its concentration [9]. It activates chemotaxis at a peak concentration of 10^{-10} M, elicits phagocytosis at 10^{-9} M, and activates ROS production from 10^{-8} M to a peak at 10^{-6} M. At higher concentrations, fMLP stimulates both phagocytosis and ROS production, which results in inflammation of the injured tissue. Soymetide-4 did not induce ROS production in neutrophils in vitro, probably because its affinity for the fMLP receptor is much lower than that of fMLP. Soymetide-4 could therefore theoretically be safely used as an immunostimulatory agent without causing inflammation.

Among the soymetides, soymetide-9 exhibited the highest affinity for the fMLP receptor as well as the strongest phagocytosis-stimulating activity in vitro. However, soymetide-4 induces a stronger TNFα priming activity than soymetide-9, -13, or fMLP following oral administration to mice. These findings could be explained if the larger peptides are not absorbed as well by the digestive system as the smaller peptides. In support of this idea, the permeability of an *N*-formylmethionyl peptide through the intestinal mucosa was reported to be very restricted [16]. The smaller soymetide-4 is therefore more desirable than the other soymetides and fMLP for oral administration. We found that soymetide-13 was converted to soymetide-4 following incubation with pancreatic elastase in vitro, suggesting that soymetide-4 may be released in the intestine following soybean ingestion.

In conclusion, soymetides with an affinity for the fMLP receptor are weak but safe immunostimulatory peptides. Soymetide-4 is the smallest but most effective immunostimulatory soymetide following oral administration. The various physiological effects that occur following oral administration of soymetide-4 are currently under investigation.

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