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Active Fragments and Analogs of the Plant Growth Factor, Phytosulfokine: Structure–Activity Relationships

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Sulfated pentapeptide phytosulfokine-α (PSK-α) is the first chemically characterized peptidal plant growth factor that induces proliferation of the mesophyll cells of asparagus. To determine the active core involved in the sequence PSK-α, we synthesized PSK-α and twelve PSK-α analogs by the solid phase peptide synthesis and the direct sulfation to the peptide-resin using dimethylformamide-sulfurtrioxide (DMF-SO_3) complex. The truncated analogs of PSK-α without the first and second *C*-terminal amino acids retained 8% and 20% of the activity of the parent pentapeptide, respectively. Deletion of the sulfate groups of Tyr^1 and Tyr^3 resulted in compounds with 0.6% and 4% of the activity of PSK-α, indicating that the sulfate group of Tyr^1 is more important than that of Tyr^3 for the expression of its activity. In contrast, the *N*-terminal truncated analog and unsulfated analog exhibited little or no activity. Thus the *N*-terminal tripeptide fragment H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-OH has been identified as the active core of PSK-α.

Proliferation of dispersed plant cells in culture is strictly dependent on cell density, and cells in a low density culture can only grow in the presence of a conditioned medium (CM). Many efforts have been made to identify the active principle involved in this phenomenon (1-5). Recently, we isolated two growth factors contained in CM and determined their structures as disulfated pentapeptide, H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH, and disulfated tetrapeptide, H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-OH (1). The pentapeptide named phytosulfokine-α (PSK-α) induced proliferation of the mesophyll cells of *Asparagus officinalis* L. at a concentration above 1.0 nM and the ED_{50} value was 4 nM. The *C*-terminal truncated peptide named PSK-β showed 10-fold lower activity than that of PSK-α. The most notable feature of PSK-α and PSK-β is the presence of tyrosine residues with the phenolic hydroxyl esterified with sulfuric acid. The sulfated peptides have also been found in vertebrate and invertebrate tissues (6-9), and the sulfation of the tyrosine residue in peptides has been recognized as a general posttranslational modification (10). Biological evaluation of the unsulfated analogs of an intestinal hormone, cholecystokinin (CCK-8) and an insect neuropeptide, leucosulfakinin, showed that these peptides require the sulfate ester on the tyrosine residue for the expression of their biological activities (9, 11). The sulfate ester on Tyr^1 and Tyr^3 of PSK-α also has been demonstrated to be indispensable because no activity was found in the unsulfated analog of PSK-α (1). Thus, the sulfate groups of PSK-α would play a vital role in binding the putative receptor(s) and inducing a molecular change in the receptor(s) that initiates physiological events inside the target cells.

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Abbreviations: Bzl, benzyl; EDT, ethanedithiol; Fmoc, 9-fluorenylmethoxycarbonyl; DMF, *N*,*N*-dimethylformamide; DMF-SO_3, *N*,*N*-dimethylformamide-sulfur trioxide; PSK, phytosulfokine; *t*-Bu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, trimethylphenyl.

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For analysis of structural requirements of the putative PSK-α receptor, we chemically synthesized various PSK-α analogs by the solid phase peptide synthesis, followed by the direct sulfation to the peptide-resin using DMF-SO_3 complex. The mitogenic activities of PSK-α analogs obtained were measured by the bioassay system described previously and were compared with ED_{50} values (1). Structure-activity relationships of twelve PSK-α analogs demonstrated that the tripeptide fragment H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-OH is the active core of PSK-α. This information would be the basis for studies focused on identification of the receptor(s) for PSK-α.

MATERIALS AND METHODS

Peptide synthesis by solid phase methodology. Fmoc-Tyr(*t*-Bu), Fmoc-Ile, Fmoc-Thr(*t*-Bu), Fmoc-Gln, Fmoc-Gly, Boc-Ile, and Boc-Tyr(OH) were purchased from Peptide Institute (Osaka, Japan). Boc-Tyr(*t*-Bu) was purchased from Advanced Chemtech (Louisville, KY). Fmoc-Tyr(OH) was synthesized by coupling Tyr and 9-fluorenylmethyl *N*-succinimidyl carbonate (Fmoc-Osu) (12). Preloaded 4-hydroxymethyl-phenoxymethyl (HMP) resins, Fmoc-Gln(Trt) resin, Fmoc-Thr(*t*-Bu) resin, Fmoc-Gly resin, and Fmoc-Cys(Trt) resin were purchased from Applied Biosystems (Foster City, CA). The partially protected peptide-resins were synthesized by the *Fastmoc* chemistry with a peptide synthesizer (Applied Biosystems Model 433A) according to the manifacturer's protocol. The *N*-terminal amino acids were successively introduced as Boc amino acids by the same procedure. Tyr residues which would be sulfated were introduced in the peptide chain as -Tyr(OH)- instead of -Tyr(*t*-Bu)-. The partially protected peptide-resins (50 μmol) in DMF-pyridine (4:1, 1.5 ml) were succesively sulfated by DMF-SO_3 (225 mg, 30 equiv.) at room temperature for 16 h. The sulfated peptide-resin was collected by filtration, washed with distilled water, and cleaved in 5.0 ml of 95% aqueous trifluoroacetic acid (TFA) in the presence of 2.5% of ethanedithiol (EDT) at room temperature for 30 min. The cleaved and deprotected peptides were precipitated with ice cold ether (30 ml), dissolved in 10% ammonium hydroxide (2.0 ml), and purified by HPLC on a Develosil ODS-10 column (20 $\times$ 250 mm, Nomura chemical, Seto, Japan) by an isocratic elution of 8% acetonitrile containing 0.1% ammonium acetate at a flow rate of 20 ml/min.

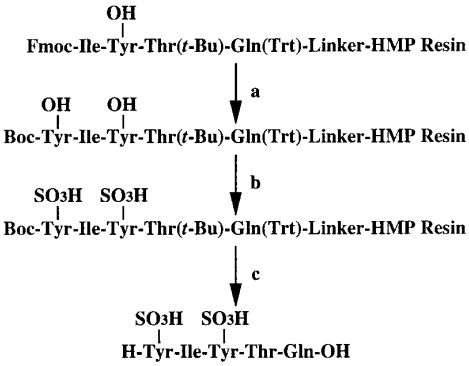
Peptide synthesis by liquid phase methodology. [1-3]PSK-α was synthesized by conventional liquid phase methodology as follows. Fmoc-Tyr(*t*-Bu)-OBzl prepared as previously reported (13) was coupled with Fmoc-Ile and with Fmoc-Tyr(*t*-Bu) successively by diethylphosphorocyanidate (DEPC) (14). The crude products were treated with 95% aqueous TFA to deprotect the *t*-Bu groups and purified by silica gel column chromatography using chroloform-acetone (9:1) as an eluent. Fmoc-Tyr(OH)-Ile-Tyr(OH)-OBzl (35 mg, 0.1 mmol) thus obtained was sulfated by the same procedure described above for 12 h. The reaction mixture was concentrated, neutralized with 10% ammonium hydroxide, and extracted with n-butanol. The organic layer was washed with water and evaporated *in vacuo* to afford the colorless amorphous products. The Bzl group was deprotected by catalytic hydrogenation (15) and the Fmoc group was further deprotected by 50% piperidine in DMF for 1 h. The crude material thus obtained was purified by HPLC on a Develosil ODS-10 column under the same conditions as described above. Similarly, [1-2]PSK-α was prepared by the sulfation and deprotection of Fmoc-Tyr(OH)-Ile-OBzl.

Peptide sequence analysis and fast atom bombardment mass spectrometry. One nmol of the synthesized peptide was dissolved in 15 μl of distilled water and sequenced with a gas-phase sequencer (Applied Biosystems model 476A). Fast atom bombardment mass spectra (FAB-MS) were obtained by adding 10 μg of peptide in distilled water (1.0 μl) to glycerol (1.0 μl) on a stainless probe, followed by bombardment with 6 kv Xe fast atom on a mass spectrometer (JEOL model DX-705L, Tokyo, Japan).

Bioassay. The mitogenic activities of PSK-α analogs were measured in triplicate by the bioassay system described previously (1) and were expressed as the concentrations required for 50% cell division on the 6th day of culture (ED_{50}).

RESULTS AND DISCUSSION

The synthesis of Boc-Tyr(OH)-Ile-Tyr(OH)-Thr(*t*-Bu)-Gln(Trt)-Linker-Resin was accomplished by the solid phase procedure with Fmoc chemistry (Scheme 1). The unprotected phenolic hydroxy groups of Tyr incorporated in the peptide chain did not cause side reactions in the coupling procedure. The partially protected peptide constructed on the resin was successively sulfated with 30 equivalent of DMF-SO_3 in a weakly basic condition. After the removal of unreacted DMF-SO_3 by filtration, final deprotection and cleavage from the resin was simultaneously conducted with 95% aqueous TFA in the presence of EDT at room temperature for 30 min. The sulfate group of Tyr is acid labile, but has been reported to survive under a brief treatment of TFA (16). Deprotected peptides were purified by HPLC on reverse phase column



SCHEME 1. The outline of the synthetic approach to PSK-α. (a) Successive coupling of the Boc amino acid with the peptide chain constructed on the HMP resin using peptide synthesizer. (b) Sulfation of Tyr with 20 eqiv. of DMF-SO_3 in DMF-pyridine (4:1). (c) Final deprotection and cleavage from the resin conducted with 95% aqueous TFA in the presence of 2.5% of EDT.

to afford analytically pure PSK-α in a total yield of 21.9% (Fig. 1). The presence of Tyr(SO_3H) was confirmed by FAB-MS experiments. Two advantages of this synthetic procedure are that the partially protected peptide chain can be constructed automatically within 6 h and the sulfated PSK-α is obtained by one-step deprotection and cleavage in the acidic condition.

By a similar procedure, 9 analogs of PSK-α including monosulfated, terminal truncated, amino acid substituted, and glycine spacer containing peptides were synthesized. The partially protected peptides for the synthesis of monosulfated PSK-α analogs ([Tyr^1(OH)]PSK-α and [Tyr^3(OH)]PSK-α) were prepared respectively by the incorporation of Boc-Tyr(*t*-Bu) instead of Boc-Tyr(OH), and Fmoc-Tyr(*t*-Bu) instead of Fmoc-Tyr(OH). The *N*-terminal tripeptide ([1-3]PSK-α) and the dipeptide ([1-2]PSK-α) were synthesized by the sulfation of the corresponding Fmoc and Bzl protected peptides prepared by the liquid phase synthesis using DEPC as a coupling reagent.

The mitogenic activities of these PSK-α analogs were measured in triplicate by the bioassay

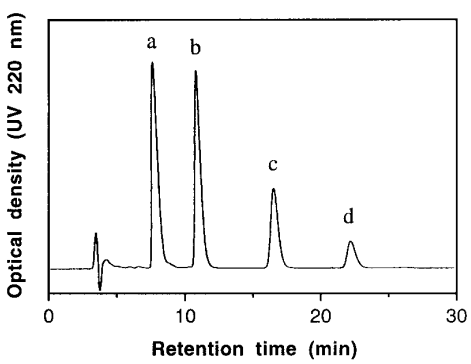


FIG. 1. HPLC purification of cleaved peptides. The cleaved crude products were chromatographed on a Develosil ODS-10 column (20 × 250 mm) by an isocratic elution of 8% acetonitrile containing 0.1% ammonium acetate at a flow rate of 20 ml/min with monitoring the absorbance at UV 220 nm. The peak eluted at 7.9 min (a) was identified as PSK-α, and the two peaks eluted at 11.1 min (b) and 16.7 min (c) were identified as monosulfates from the FAB-MS experiments. Unsulfated peptide was eluted at 22.4 min (d).

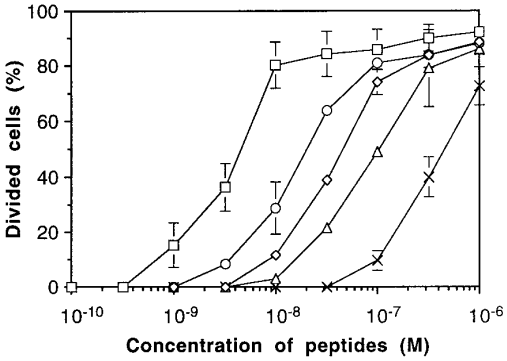


FIG. 2. Dose-response curve for mitogenic activities of PSK-α (open square), PSK-β (open diamond), [1-3]PSK-α (open circle), [*C*-$(Gly)_3$]PSK-α (open triangle), and [*N*-$(Gly)_3$]PSK-α (cross). Mesophyll cells of asparagus were incubated under various concentrations of each PSK-α analog. Mitogenic activities were determined on the 6th day of culture.

system described previously and were compared with ED_{50} values (Fig. 2). PSK-α gave a threshold dose of 1 nM and the ED_{50} was 4 nM (Table 1). The dose response curve of synthesized PSK-α showed good agreement with that of the natural product. To estimate precisely the active core of PSK-α, the *C*-terminal and *N*-terminal truncated peptides with different chain lengths were synthesized and mitogenic activities were tested. The *N*-terminal tetrapeptide (PSK-β) and tripeptide ([1-3]PSK-α) retained relatively high activities (8% and 20%, respectively). The *N*-terminal dipeptide ([1-2]PSK-α) and the *C*-terminal tetrapeptide ([2-5]PSK-α), however, showed no activity even at a concentration of 1.0×10^{-6} M. Moreover, they did not block the mitogenic activity of PSK-α in a competition assay (data not shown). Thus, the most critical sequence within PSK-α for mitogenic activity was limited to the *N*-terminal tetrapeptide, H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-OH. The relative flexibility to sequence

TABLE 1
Mitogenic Activities of PSK Analogs

Name	PSK analogs	Yield (%)[a]	ED_{50} (nM)	Relative activity
PSK-α	Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Gln	21.9	4	100
PSK-β	Tyr(SO3H)-Ile-Tyr(SO3H)-Thr	12.9	50	8
[1-3]PSK-α	Tyr(SO3H)-Ile-Tyr(SO3H)	(41.2)[b]	20	20
[1-2]PSK-α	Tyr(SO3H)-Ile	(92.5)[b]	>1000	<0.1
[2-5]PSK-α	Ile-Tyr(SO3H)-Thr-Gln	51.7	>1000	<0.1
[C-$(Gly)_3$]PSK-α	Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Gln-Gly-Gly-Gly	34.7	100	4
[N-$(Gly)_3$]PSK-α	Gly-Gly-Gly-Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Gln	20.4	500	0.8
[C-$(Gly)_3$-Cys]PSK-α	Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Gln-Gly-Gly-Gly-Cys	11.2	40	10
[Tyr^3(OH)]PSK-α	Tyr(SO3H)-Ile-Tyr(OH)-Thr-Gln	20.7	100	4
[Tyr^1(OH)]PSK-α	Tyr(OH)-Ile-Tyr(SO3H)-Thr-Gln	32.8	700	0.6
[Tyr(OH)]PSK-α	Tyr(OH)-Ile-Tyr(OH)-Thr-Gln	77.6	>1000	<0.1
[Val^2]PSK-α	Tyr(SO3H)-Val-Tyr(SO3H)-Thr-Gln	27.7	100	4
[Ser^4]PSK-α	Tyr(SO3H)-Ile-Tyr(SO3H)-Ser-Gln	24.5	200	2

Note. Mesophyll cells of asparagus were incubated in the presence of each PSK-α analog. Mitogenic activities were determined on the 6th day of culture and ED_{50} value was defined as the concentration of the compound required for 50% cell division.

[a] The total yield of product based on the preloaded resin.

[b] The total yield of product based on the corresponding Fmoc and Bzl protected peptides prepared by liquid phase methodology.

manipulation in the *C*-terminal portion of PSK-α was further confirmed by the spacer insertion experiments. The octapeptide which had a glycine spacer in the *C*-terminal ([*C*-$(Gly)_3$]PSK-α) retained 4% of the activity, whereas the *N*-terminal elongated peptide ([*N*-$(Gly)_3$]PSK-α) showed only 0.8% of PSK-α activity. In addition, nonapeptide in which Cys residue was incorporated through Gly spacer in the *C*-terminal region ([*C*-$(Gly)_3$-Cys]PSK-α) exhibitted 10% of the activity with no changes in the configuration of the dose-response curve. Because the reactive thiol group of Cys residue has been utilized in a wide variety of synthesis of peptide conjugates, [*C*-$(Gly)_3$-Cys]PSK-α would be available for the starting materials to construct the biologically active probe for the investigation of the putative recepor(s). We also compared the biological activity of two analogs by replacing internal sequences of PSK-α with other amino acids. Substitution of Ile with Val ([Val^2]PSK-α), both hydrophobic in character, resulted in a 20-fold decrease in ED_{50} value. Similar decrease in activity was observed by replacing Thr with Ser ([Ser^4]PSK-α) indicating that these two amino acid residues do not simply serve as a spacer function and are involved in increasing the mitogenic activity of PSK-α.

As mentioned in a previous paper, the sulfate groups of PSK-α are indispensable for the expression for its activity (1). It has also been reported that two sulfated peptides, CCK-8 and leucosulfakinin, require the sulfate ester on the Tyr residue for the expression of full biological activities (9, 11). We synthesized two monosulfated PSK-α analogs and compared their activities with those of the parent PSK-α. The Tyr^1 sulfated peptide ([Tyr^3(OH)]PSK-α) retained a threshold dose of 30 nM and an ED_{50} of 100 nM (4%), being one order magnitude lower activity than that of PSK-α. The Tyr^3 sulfated peptide ([Tyr^1(OH)]PSK-α), however, gave a threshold dose of 100 nM and an ED_{50} of 700 nM (0.6%). The unsulfated peptide ([Tyr(OH)]-PSK-α) showed no activity up to 100 nM. These results indicate that the sulfate group of Tyr^1 is more important for the expression for its activity than that of Tyr^3, although the activities of both monosulfated peptides were much lower than that of PSK-α.

In conclusion, we showed that sulfated peptides could be prepared by the solid phase synthesis using peptide synthesizer. The advantage of this method is that the desired peptides can be synthesized quickly and easily in a relatively high yield (11-52%). This approach would be applicable to the synthesis of other sulfated peptides. Biological evaluation of PSK-α analogs prepared by this procedure gave structure-activity relationships of PSKs. Unless even one of the Tyr(SO_3H) residue is eliminated, the dose response curve of the PSK-α analogs would show similar slope and maximal response relative to the PSK-α, although their ED_{50} values varied from each other (Fig. 2). Changes in the maximal response are thought to be reflected by the structural changes in the message portion of the peptide molecule that is responsible for transduction to the physiological response (17). Thus, the critical portion for the transduction of the cell proliferation is not distributed over the molecule, and is limited to the *N*-terminal tripeptide sequence including two sulfate esters on Tyr residues.

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