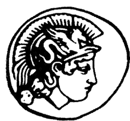

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Phytochelatin homologs induced in hairy roots of horseradish

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Abstract

When exposed to excess heavy metals, plants induce phytochelatins and related peptides (all designated as PCAs). Thus, when hairy roots of horseradish (*Armoracia rusticana*) were exposed for 3 days to cadmium (1 mM) along with reduced glutathione (2 mM), PCA induction occurred. Moreover, a new family of thiol peptides was detected as well as the previously known PCAs, as revealed by postcolumn-derivatization HPLC. Two were isolated and their structures were identified as (γ-Glu-Cys)$_n$-Gln (n = 3 and 4) by electrospray ionization-mass spectrometer spectra, this being confirmed by chemical synthesis of the peptides. These new analogs constitute the sixth PCA family identified to date. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Armoracia rusticana*; Cadmium; Cell culture; Cruciferae; Horseradish; Metallothionein; Phytochelatin

1. Introduction

When exposed to excess heavy metal ions, higher plants induce thiol peptides called phytochelatins (PCs) to protect against toxicity (Rauser, 1990), whose general structure is (γ-Glu-Cys)$_n$-Gly ($n \geq 2$). So far, PCs with n = 2–11 have been reported (Gekeler, Grill, Winnacker & Zenk, 1989).

Some analogous families of PCs have also been found. Homo-phytochelatins, which are (γ-Glu-Cys)$_n$-β-Ala (n = 2–7), are found in the family Fabaceae (Grill, Gekeler, Winnacker & Zenk, 1986). Hydroxymethyl-phytochelatins, namely (γ-Glu-Cys)$_n$-Ser (n = 2–4) are detected in rice (Klapheck, Chrost, Starke & Zimmermann, 1992). A family of peptides with a glutamyl residue at the C-terminus (n = 2–3) has also been reported in maize (Meuwly, Thibault, Schwan & Rauser, 1995). Moreover, desglycyl PCs (γ-Glu-Cys)$_n$ (n = 2–5) have been found in several species such as *Candida glabrata* (Mehra, Tarbet, Gray & Winge, 1988) and *Schizosaccharomyces pombe* (Mehra & Winge, 1988; Konya, Yoshimura, Yamazaki & Toda, 1990). Thus, five families of PC-related peptides have been reported to date (Rauser, 1995; Zenk, 1996). PCs and related peptides, all designated as PCAs, are a family of metallothioneins (Binz & Kägi, 1999).

Previously, we detected a series of desglycyl peptides in *Rubia tinctorum* which is a source of a red pigment (Kubota, Sato, Yamada & Maitani, 1995; Maitani, Kubota, Sato & Yamada, 1996). In the present study, we examined PCA-induction in hairy roots of horseradish which is a source of pungent components and identified a new family of PC-related peptides, (γ-Glu-Cys)$_3$-Gln and (γ-Glu-Cys)$_4$-Gln.

2. Results

Fig. 1 shows the postcolumn-derivatization HPLC chromatograms for the hairy roots of horseradish in the presence or absence of Cd. Many additional peaks were noted in the Cd-exposed hairy roots. By co-chromatography with Cd-exposed root cultures of *R. tinctorum* L. (Kubota et al., 1995), the peaks 1, 3, and 5 were assigned to PCs with n = 2, 3, and 4, respectively. In addition, some unknown peaks were observed in the Cd-exposed hairy roots, but these were not des-

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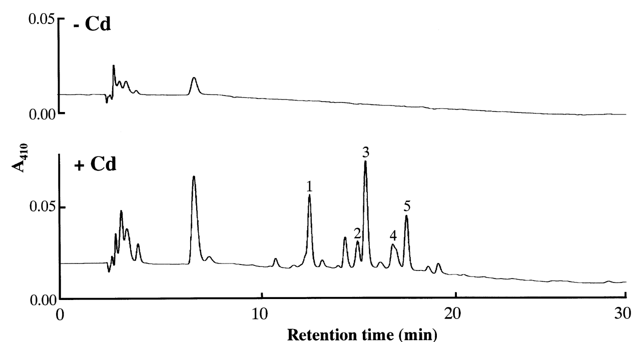

Fig. 1. HPLC chromatograms for control (upper) and Cd-exposed (lower) root cultures of horseradish generated by postcolumn derivatization. Hairy roots of horseradish were exposed to $CdCl_2$ (1 mM) with GSH (2 mM) for 3 days. The induced PCs and related peptides were analyzed as thiol-containing compounds as described in Section 4. Peaks 1, 3, and 5 were assigned to (γ-Glu-Cys)$_n$-Gly with n = 2, 3, and 4, respectively.

glycyl PCs (Kubota et al., 1995). The unknown peaks were also detected by Cd-treatment without reduced glutathione (GSH), but induction was at a lower level.

To clarify whether unknown peaks were PC-related peptides, peaks 2 and 4 were isolated and electrospray ionization-mass spectrometer (ESI-MS) spectra were measured (Fig. 2). The largest peak was detected at an m/z value of 843.2 in peak 2 and the largest and the second largest peaks at 1000.3 and 1075.3 in peak 4. Therefore, the peaks at m/z 843.2 and 1075.3 were presumed to be quasi-molecular ion peaks $[M + H]^+$, because the difference, 232, corresponds to a mass of γ-Glu-Cys. The peak at 1000.3 also may be quasi-molecular ion peaks of other compounds, since peak 4 is broad and so may contain two compounds.

The m/z values of 843.2 and 1075.3, however, did not correspond to the mass of the quasi-molecular ion (exact mass + 1.0) of PC-related peptides reported so far. The closest values were 844.2 and 1076.3 of (γ-Glu-Cys)$_3$-Glu and (γ-Glu-Cys)$_4$-Glu, respectively. Unexpectedly, the m/z values agreed completely with the exact mass of quasi-molecular ion of (γ-Glu-Cys)$_3$-Gln (843.2) and (γ-Glu-Cys)$_4$-Gln (1075.3).

To confirm the structures of peaks 2 and 4 including the presence of the γ-linkage, (γ-Glu-Cys)$_3$-Gln and (γ-Glu-Cys)$_4$-Gln were chemically synthesized and ESI-MS spectra were measured (Fig. 3). The tandem mass (MS-MS) spectra (Fig. 3, lower) quite closely resembled those observed previously for peaks 2 and 4 (Fig. 2, lower). The presence of fragment ions at 714.2 and 946.2 clearly demonstrates the presence of the Gln residue as understood from the fragmentation patterns shown in Fig. 3 (upper). Thus, peaks 2 and 4 were identified as (γ-Glu-Cys)$_3$-Gln and (γ-Glu-Cys)$_4$-Gln, respectively.

The synthetic (γ-Glu-Cys)$_3$-Gln and (γ-Glu-Cys)$_4$-Gln were added to the supernatant fraction of Cd-exposed hairy roots, and postcolumn-derivatization HPLC analysis of the sample was performed; synthetic (γ-Glu-Cys)$_3$-Gln and (γ-Glu-Cys)$_4$-Gln co-eluted with peaks 2 and 4, respectively (data not shown).

In Fig. 1, (γ-Glu-Cys)$_2$-Gln is not detectable. Under the present HPLC conditions, (γ-Glu-Cys)$_n$-Gln (n = 3 and 4) eluted faster than the corresponding PCs ((γ-Glu-Cys)$_n$-Gly), Fig. 1, whereas γ-Glu-Cys-Gln eluted slower than GSH (γ-Glu-Cys-Gly) (see below, Fig. 5). This may explain why (γ-Glu-Cys)$_2$-Gln cannot be seen in Fig. 1.

The retention times of peptides on an ODS column in a linear gradient elution mode are linearly related to the logarithm of the sum of the retention constant for the constituent amino acids (Sasagawa, Okuyama & Teller, 1982). Based on this fact, Gekeler et al. (1989) applied the formula to PCs and obtained a linear relationship between the retention time of PCs and the logarithm of the number of γ-Glu-Cys units when they were plotted.

To estimate the retention time of (γ-Glu-Cys)$_2$-Gln, the plot method was employed. Fig. 4 shows the plots for PCs ((γ-Glu-Cys)$_n$-Gly) (n = 1, 2, 3, and 4) and the new family of peptides ((γ-Glu-Cys)$_n$-Gln) (n = 1, 3 and 4). A good linear relationship was observed for both (γ-Glu-Cys)$_n$-Gly (r_2 = 0.9999) and (γ-Glu-Cys)$_n$-Gln (r_2 = 1.0000), and both lines intersected at the retention time of (γ-Glu-Cys)$_2$-Gly. This finding suggested that peak 1 in Fig. 1 is a mixture of (γ-Glu-Cys)$_2$-Gly and (γ-Glu-Cys)$_2$-Gln. In fact, (γ-Glu-Cys)$_2$-Gln was detected by ESI-MS (data not shown).

The induction of (γ-Glu-Cys)$_n$-Gln may suggest the presence of γ-Glu-Cys-Gln. To identify the peak of γ-Glu-Cys-Gln, chemically synthesized γ-Glu-Cys-Gln was added to the supernatant fraction of Cd-exposed hairy roots, and HPLC analysis was performed. The spiked γ-Glu-Cys-Gln overlapped a small peak adjacent to the peak of GSH (Fig. 5). The small peak was tentatively assigned to γ-Glu-Cys-Gln.

3. Discussion

On exposure to Cd, the hairy roots of horseradish induced a new group of SH-containing peptides as well as PCs. They are (γ-Glu-Cys)$_3$-Gln and (γ-Glu-Cys)$_4$-Gln. So far, five families of PC-related peptides have been reported, namely, (γ-Glu-Cys)$_n$-Gly, (γ-Glu-Cys)$_n$-β-Ala, (γ-Glu-Cys)$_n$-Ser, (γ-Glu-Cys)$_n$-Glu, and (γ-Glu-Cys)$_n$. PC-related peptides with Gln as the C-terminus were first identified in this study.

Meuwly, Thibault & Rauser (1993) reported similar peptides (γ-Glu-Cys)$_n$-Glu in maize. To confirm that our new peptides were not (γ-Glu-Cys)$_n$-Glu, we also synthesized (γ-Glu-Cys)$_3$-Glu chemically and this was co-chromatographed with the supernatant fraction of

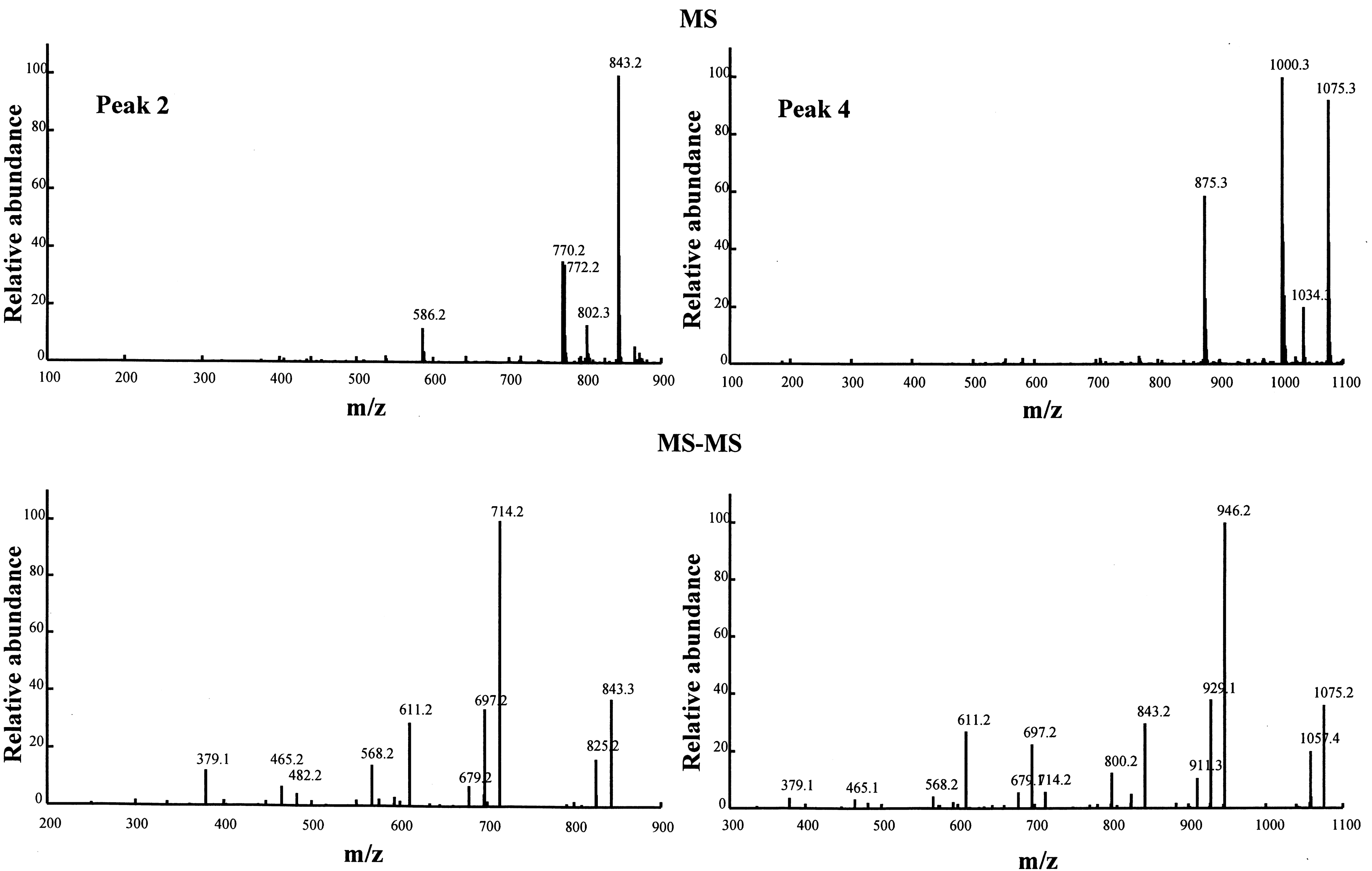

Fig. 2. MS spectra of isolated peaks 2 and 4 (upper) and MS-MS spectra of respective quasi-molecular ion peaks $[M + H]^+$ (lower).

Fig. 3. MS spectra of synthesized (γ-Glu-Cys)$_3$-Gln and (γ-Glu-Cys)$_4$-Gln (upper) and MS-MS spectra of respective quasi-molecular ion peaks $[M + H]^+$ (lower). Fragmentation patterns in MS-MS spectra are depicted in the respective upper parts.

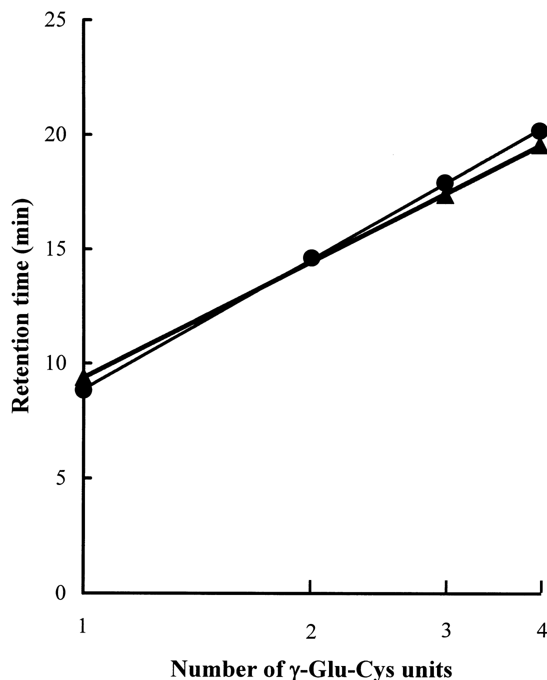

Fig. 4. The plots of the retention times in HPLC versus logarithm of the number of γ-Glu-Cys units for the families of (γ-Glu-Cys)$_n$-Gly and (γ-Glu-Cys)$_n$-Gln. ●, (γ-Glu-Cys)$_n$-Gly with n = 1–4; ▲, (γ-Glu-Cys)$_n$-Gln with n = 1, 3, and 4.

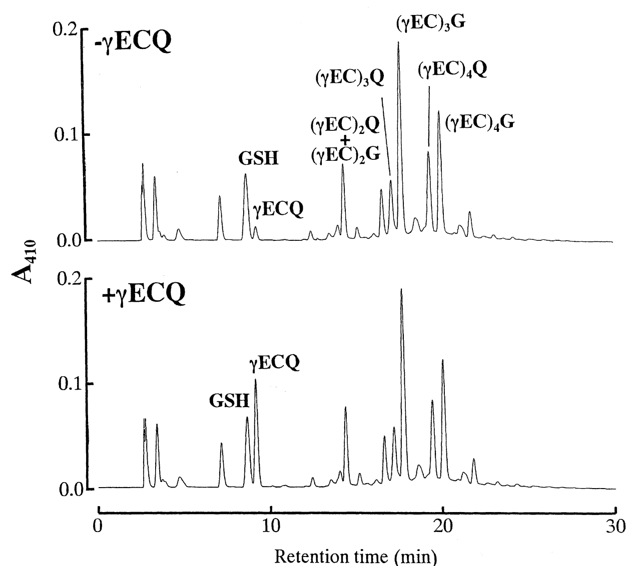

Fig. 5. HPLC chromatograms for the supernatant fraction of Cd-exposed hairy roots of horseradish before (upper) and after (lower) addition of synthesized γ-Glu-Cys-Gln (γECQ). (γEC)$_n$G and (γEC)$_n$Q mean (γ-Glu-Cys)$_n$-Gly and (γ-Glu-Cys)$_n$-Gln, respectively.

the Cd-exposed hairy roots. (γ-Glu-Cys)$_3$-Glu eluted at almost the same retention time as that of corresponding desglycyl PC ((γ-Glu-Cys)$_3$) (data not shown). Namely, (γ-Glu-Cys)$_n$-Glu eluted slower than the corresponding PC. On the other hand, (γ-Glu-Cys)$_n$-Gln elutes faster than the corresponding PC as shown in Fig. 1.

In the structural determination of PC-related peptides, amino acid analysis is frequently employed (Klapheck et al., 1992; Meuwly et al., 1995). However, Glu and Gln cannot be distinguished by the usual amino acid analysis. Therefore, it could not be employed in the present study.

It is considered that PCs are synthesized from GSH (Rüegsegger, Schmutz & Brunold, 1990; Tukendorf & Rauser, 1990). PC-related peptides may also be synthesized from the GSH-related peptides. In fact, γ-Glu-Cys-β-Ala (Gekeler et al., 1989), γ-Glu-Cys-Ser (Klapheck, Fliegner & Zimmer, 1994), and γ-Glu-Cys-Glu (Meuwly et al., 1993) were detected in the plants that induced (γ-Glu-Cys)$_n$-β-Ala, (γ-Glu-Cys)$_n$-Ser, and (γ-Glu-Cys)$_n$-Glu, respectively.

In this study, a small peak was detected at the retention time of γ-Glu-Cys-Gln. However, the complete identification could not be made because of its low quantity. Therefore, the presence of γ-Glu-Cys-Gln in the hairy roots of horseradish and, furthermore, in the intact horseradish plant itself remains to be established.

4. Experimental

4.1. Reagent

Fmoc-Gln(Trt)-Wang resin and Fmoc-Glu-α-*O*-*t*-Bu for peptide synthesis were purchased from Calbiochem–Novabiochem (La Jolla, CA). Fmoc-S-(*t*-Bu)-L-Cys, BOP (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate) reagent, dimethylformamide (DMF, grade for peptide analysis), *N*-methyl-morpholine, thioanisole, ethane-1,2-dithiol (EDT), cadmium chloride hemipentahydrate, and reduced glutathione (GSH) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Trifluoromethanesulfonic acid (TFM) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Kanto Chemical (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. Other chemicals used were either of reagent grade or of the highest grade commercially available.

4.2. Tissue culture

Hairy roots of *Armoracia rusticana* were established as reported (Sakamoto et al., 1992) according to a method described previously (Saitou, Kamada & Harada, 1991), and were subcultured every 3 weeks. After the last subculture, the hairy roots (0.25 g) were maintained for 7 days in 10 ml of Murashige–Skoog liquid medium in 50 ml Erlenmeyer flasks on a rotary shaker at 100 rpm at 25°C in the dark. Cadmium chloride (1 mM) and GSH (2 mM) were then added to

the medium. Hairy roots were maintained for 3 days, then washed with purified water, and stored at −80°C.

4.3. Postcolumn-derivatization HPLC analysis of PC-related peptides

PCAs were analyzed by postcolumn derivatization HPLC as reported previously (Kubota et al., 1995) by using 0.1% trifluoroacetic acid instead of 0.05% phosphoric acid.

4.4. Purification of isolated peptides

The hairy roots were homogenized with a Polytron tissue grinder (Kinematica GmgH, Littau, Switzerland) in 2 volumes of 10 mM Tris–HCl buffer solution (pH 7.4) containing 10 mM KCl and 1 M $MgCl_2$ under an N_2 atmosphere to prevent oxidation. The homogenates were centrifuged at $100{,}000 \times g$ for 60 min at 4°C. A 500-μl portion of the supernatant fraction was subjected to HPLC (LC-6A, Shimadzu, Kyoto, Japan) using a gel-filtration column (Asahipak GS520HQ, 7.6 mm i.d. × 300 mm, Showa Denko, Tokyo, Japan) with 10 mM Tris–HCl buffer (pH 7.4). The column was eluted with the 10 mM Tris–HCl buffer (pH 7.4) at a flow rate of 0.6 ml/min, and the Cd-containing fraction was collected; the collection was repeated about 50 times. The pooled solution was concentrated to about 500 μl. The solution was mixed with 500 μl of 0.1% $NaBH_4$ solution and centrifuged ($11{,}000 \times g$, 5 min). Each 100-μl portion of the supernatant fraction was acidified with 3.6 M HCl (20 μl) and the precipitates were separated by centrifugation ($11{,}000 \times g$, 5 min). The supernatant fraction (100 μl) was subjected to HPLC (LC-10AD × 2, Shimadzu, Kyoto, Japan) using a C_{18} column (Inertsil ODS-80A, 4.6 mm i.d. × 250 mm, GL Sciences, Tokyo, Japan). Fractions corresponding to respective thiol-containing peptides were collected as described previously (Kubota et al., 1995). The collection procedure was repeated 10 times, and pooled fractions were concentrated and freeze-dried.

4.5. Chemical synthesis of peptides

The authentic standard peptides were chemically synthesized by the Fmoc solid-phase method (Merrifield, 1997). Fmoc-Gln(Trt)-Wang resin was washed with DMF, and the Fmoc-group of the resin (0.5 g, 0.2 mmol) was removed with 10% piperidine/DMF solution. The Gln moiety was coupled with Fmoc-S-(*t*-Bu)-L-Cys (0.6 mEq) and Fmoc-Glu-α-*O*-*t*-Bu (0.6 mEq) successively for 2 h each, with the activation by BOP Reagent (0.6 mEq) and *N*-methyl-morpholine (1.8 mEq). Completion of the respective coupling reactions was confirmed by the ninhydrin-test reported by Kaiser, Colescott, Bossinger & Cook (1970). Removal of protecting groups and detachment from the support were performed by treating with a mixture of thioanisole and EDT, trifluoroacetic acid, and TFM successively. The peptides obtained were purified by HPLC, using a C_{18} column as described in "purification of isolated peptides".

4.6. ESI-MS (electrospray ionization-mass spectrometer) analysis

Each peptide (isolated or synthesized) was dissolved in 2.5% acetic acid in 10% aqueous methanol (v/v) and introduced into an ESI-MS (LCQ, Thermo Quest, San Jose, CA) by infusion.

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