# CONFORMATION OF SYSTEMIN, A POLYPEPTIDE ACTIVATOR OF PROTEINASE INHIBITOR SYNTHESIS IN PLANTS

Piotr MUCHA[a1], Agnieszka SZYK[a2], Piotr REKOWSKI[a3], Gotfryd KUPRYSZEWSKI[a4], Genowefa SLÓSAREK[b] and Jan BARCISZEWSKI[c,*]

[a] *Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland; e-mail: [1] fly@chemik.chem.univ.gda.pl, [2] chepc28@chemik.chem.univ.gda.pl, [3] rekowski@chemik.chem.univ.gda.pl, [4] gotfryd@chemik.chem.univ.gda.pl*

[b] *Department of Molecular Biophysics, Institute of Physics, A. Mickiewicz University, Umultowska 85, 61-614 Poznań, Poland; e-mail: leonardo@plpuam11.amu.edu.pl*

[c] *Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12, 61-704 Poznań, Poland; e-mail: jbarcisz@ibch.poznan.pl*

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An 18-amino acid polypeptide systemin was synthesized by the solid-phase method and its conformation was studied by circular dichroism spectroscopy. The peptide in solution is a mixture of random coil structure with β-sheet and β-turn motifs as has been previously suggested with NMR spectra. Free zone capillary electrophoresis analysis proved purity and chemical stability of systemin at different pH.

**Key words**: Systemin conformation; Circular dichroism; Free zone capillary electrophoresis; Peptide conformation; Peptides.

Systemin, an 18-amino acid polypeptide has been isolated from tomato and potato leaves[1–4]. It is the most powerful inducer of proteinase inhibitor genes from plants and is the only plant polypeptide hormone-like signaling molecule presently known[5]. Its primary structure shows a partial palindromic structure. The amino acid residues of systemin involved in this type of symmetry are shown in bold letters.

Ala[1]-Val-**Gln**-Ser-**Lys**-**Pro**-**Pro**-Ser-Lys-Arg[10]-Asp-**Pro**-**Pro**-**Lys**-Met-**Gln**-Thr-Asp[18]

Some years ago, systemin has been studied by NMR spectroscopy. The NMR measurements, performed in a phosphate buffer at neutral and acidic pH, showed strong overlap of proton resonance lines in the systemin spectra. To resolve them, the peptide mutants have also been studied. However, there were no evidence for the persistence of secondary or tertiary structure

elements[6]. Recently, some of us have obtained and analyzed proton NMR spectra for systemin dissolved in water at pH 3.2 (ref.[7]). A strong *cis-trans* isomerization effect was observed. A *cis* isomer of systemin showed a Z-like β-sheet structure[8] (Fig. 1). An increase of pH to 7.2 caused a clear multiplication of the isomeric forms of the peptide. From the analysis of the proton resonance lines, we have concluded that at least five different isomers of systemin were present in aqueous solution.

To better understand the molecular basis of a systemin biological activity, we have analyzed its conformation using circular dichroism (CD) spectroscopy.

## EXPERIMENTAL

### Synthesis and Purification

The protected 18-amino acid polypetide was synthesized manually on a Bio-Bead Sx-1 (Bio-Rad Laboratories, U.S.A.) cross-linked chloromethylated polystyrene resin (loading 0.68 mmol Cl/g) on a 0.5 mmol scale by solid-phase method, using Boc-type chemistry[6]. The resin was esterified with Boc-Asp(OChx)-OH using anhydrous potassium fluoride to a load of 0.36 mmol Boc-Asp(OChx)/g. The following side-chain protections were employed: benzyl (Ser, Thr), (2-chlorobenzyl)oxycarbonyl (Lys), cyclohexyl (Asp), tosyl (Arg). All protected amino acids (Boc-AA) were coupled as active *N*-hydroxybenzotriazole (HOBt) derivatives, generated *in situ*. Reaction was carried out for 1.5 h in dichloromethane (DCM) with the exception of Boc-Gln and Boc-Arg(Tos), which were allowed to react in dimethylformamide (DMF) for 2 h. Three-fold molar excess of protected amino acid was used. Coupling steps were controlled by the Kaiser or chloranil test. When the test was positive the coupling was repeated using 1.5-fold molar excess of Boc-AA, [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and diisopropylethylamine (DIEA) for 0.5 h in DMF (Boc-AA : BOP : DIEA, 1 : 1 : 2) (ref.[9]). Cleavage of the Boc-group was performed with 40% trifluoroacetic acid (TFA) in DCM in the presence of 1% thianizole. Neutralization was accomplished with 10% of triethylamine in DCM. The peptide was cleaved from the resin by the HF procedure[10]. The protected peptide resin (1 g) was reacted with HF (10 ml), anisole (1 ml) and dimethyl sulfide (1 ml) for 1 h at 0 °C. After evaporation of volatile components the residue was washed with a mixture of DCM–diethyl ether and diethyl ether.

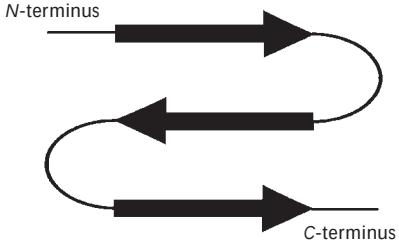


FIG. 1
A schematic representation of Z-like β-sheet structure

The peptide was extracted with 30% acetic acid (AcOH) and lyophilized. The crude product was desalted on a Sephadex G-25 column (2.8 × 105 cm) in 30% AcOH and chromatographed twice on a preparative reverse d-phase high performance liquid chromatography (RP HPLC), Vydac C-18 column (32 × 240 cm, 15–20 μm particle size) with 0–15% acetonitrile in 0.1% TFA linear gradient.

### Circular Dichroism (CD) Measurements

CD spectra were recorded on a Jasco J-20 spectropolarimeter with a 1.0 mm path length cell in the range of 190–250 nm. Experimental values were converted to mean residue molar ellipticities. Peptide sample (Table I) was prepared by appropriate dilution of stock solution to give concentration of 0.11 and 0.01 mol $l^{-1}$ in water, dilute hydrochloric acid and phosphate buffer, respectively, for pH dependence measurements.

### Capillary Electrophoresis (FZ-CE)

Free zone capillary electrophoresis (Beckman P/ACE system 2100) was used for purity investigation of systemin. Samples were separated in 0.1 M sodium phosphate buffer at pH 2.5 after 10, 21 and 30 h of incubation using an uncoated fused silica capillary (75 μm × 57 cm, 50 cm to the detector). Runs were made at 18 kV, 126 mA and 20 °C. Absorbance was measured at 214 nm. The sample volume was about 18 ml (2 pmol of the peptide) and was injected by high pressure in a 3 s time. All solutions and samples were filtered through 0.45 μm membrane filters.

## RESULTS AND DISCUSSION

α-Helices, β-turns and β-sheets are widely accepted as important peptide structural motifs that organize the three-dimensional structure of proteins. The amino acids composition has been extensively studied for their ability to form either helical or sheet structures. Various experiments have demon-

TABLE I
Analytical data on systemin

| Compound | FAB MS [M + $H^+$] Calcuated/Found | | $[\alpha]_D^{20}$, ° | FZ CE $T_m$, min | RP HPLC $R_T$, min | Amino acid analysis |
|---|---|---|---|---|---|---|
| SYSTEMIN $C_{85}H_{144}N_{26}O_{28}S$ | 2 010.4 | 2 010.2 | –52.6 | 11.26 | 13.86 | Ala 1.12 (1), Val 0.87 (1), Glu 1.86 (2), Ser 2.00 (2), Lys 3.00 (3), Pro 3.79 (4), Arg 1.14 (1), Asp 2.06 (2), Met 1.18 (1), Thr 0.91 (1) |

strated that the amino acids sequence plays a key role in determining the propensity for protein folding. One of the very powerful methods for structural analysis of peptides and proteins is CD spectroscopy. With this technique one can get insight into the tertiary structure changes of biologically important macromolecules. For some times we have been interested in tertiary structure of systemin – a polypeptide hormone activating the synthesis of proteinase inhibitors. Using NMR spectroscopy we have found that the 18-amino acid long peptide has Z-like β-sheet structure[7] (Fig. 1).

In this paper we analyze a conformation of systemin by CD spectroscopy and we have got another data, which support previous finding. The peptide has a peculiar primary structure. It contains two pairs of proline residues separated by four charged amino acids and has a palindromic structure. These properties suggested the tertiary structure of systemin to be unusual. The CD spectra of systemin obtained by chemical synthesis (Table I) were measured in water, dilute HCl solutions, phosphate buffers and trifluoroethanol (TFE) in order to evaluate an effect of pH and different solvent on its secondary structure (Fig. 2). They show a negative band around 200 nm and some changes occurred at pH 3. The CD spectra recorded in 0.01 M sodium phosphate buffer were very similar to those in water, except in the 207–212 nm region (Fig. 3).

A small pH effect, if any, is observed. However, some changes in the CD spectra of systemin in pure water at different pH are visible (Fig. 2). At pH 3 and 7, the minimum in the spectra appears, but most visible changes were

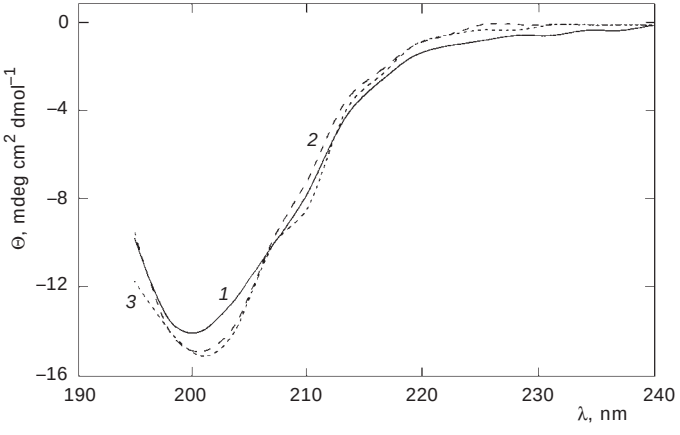


FIG. 2
Effect of pH on CD spectra of systemin in water; *1* pH 3, *2* pH 5, *3* pH 7

observed at 207–212 nm. They can be due to occurrence of *cis-trans* isomerization of systemin identified with NMR spectroscopy[7]. A slightly stronger effect is observed at pH 7. From NMR data[7] it follows that at pH 7 there should be at least five isomers present in the sample of systemin. Their presence is indicated by a very weak minimum at about 207–212 nm in the CD spectrum of systemin in water (Fig. 3).

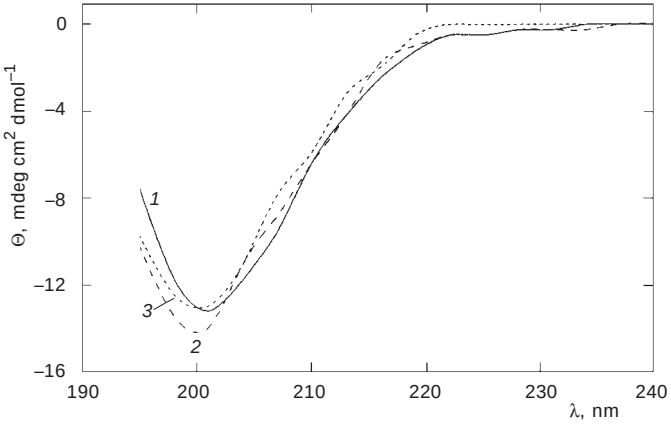


FIG. 3
Effect of pH on CD spectra of systemin in 0.01 M phosphate buffer; *1* pH 3, *2* pH 5, *3* pH 7

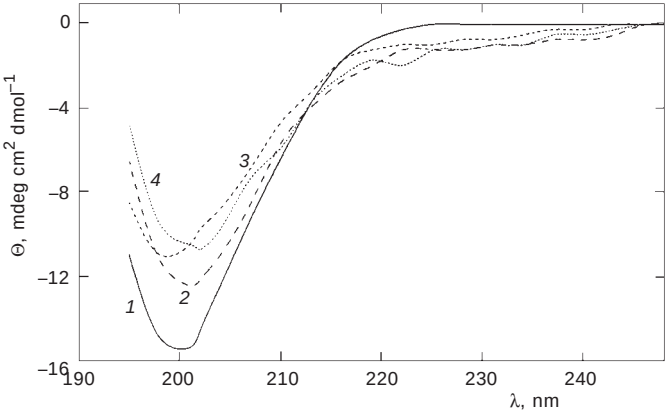


FIG. 4
Effect of TFE concentration on CD spectra of systemin. The observed decrease of $[Q]_{201}$ was about 32%. *1* $H_2O$, *2* 50% TFE, *3* 75% TFE, *4* TFE

Solvent perturbation experiments were performed in the presence of TFE, a well-known structure-perturbing agent. It is known that TFE simulates an environment which mimics a lipid membrane[10]. As one can see, increasing concentration of TFE causes only a very small shift of minimum at 201 nm to longer wavelengths in the CD spectra of systemin which is characteristic for a folded structure (Fig. 4). A decrease of about 32% in the ellipticity at 201 nm was observed. However, even in 100% TFE prevailing participation of non-helical structure was evident. With reference to a typical standard spectra of peptides, the systemin conformation can be a random coil, but there is no negative peak at 225 nm. This observation suggests that TFE stabilizes non-helical structures. Therefore, we think that our sample is a mixture of random coil with β-sheet and β-turn motifs. This conclusion is in agreement with NMR data[7]. A similar conclusion has been recently reached for streptococcal protein G, which has β-hairpin structure induced and stabilized by trifluoroethanol[11].

In summary, we conclude that systemin, a short peptide, showed not totally random coiled structure, but can adopt Z-like β-sheet structure with β-turns, responsible for its biological activity.

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