Chemistry of Natural Compounds, Vol. 40, No. 4, 2004

CONFORMATION ANALYSIS OF ILE-ALA-VAL-PRO PEPTIDE AND ITS DERIVATIVES BY CIRCULAR DICHROISM

V. V. Pak,[1] M. S. Koo,[2] D. Y.Kwon,[2] and T. D.Kasimova[1]

UDC 547.964.4:542.95

Ile-Ala-Val-Pro as a hypocholesterolemic peptide was isolated from soybean protein. We have synthesized four peptides, Ile–Ala–Val–Pro–Gly–Glu–Val–Ala, Leu–Ile–Ala–Val–Pro–Gly–Glu–Val–Ala, Ile–Ala–Val–Pro–Thr–Gly–Val–Ala, Leu–Ile–Ala–Val–Pro–Thr–Gly–Val–Ala, with a conserved Ile–Ala–Val–Pro amino acid sequence, for circular dichroism investigations. These four peptide sequences were also found in the amino acid sequence in soybean protein, which was defined from the genomic sequence. Additionally for a detailed analysis of conformation features of these peptides, the Ile–Ala–Val–Pro and Leu–Ile–Ala–Val–Pro were also synthesized. All peptides were prepared using standard fluorenylmethyloxycarbonyl methodology and the peptide yields ranged from 90 to 95% of the theoretical yields with purity after purification above 99%.

Key words: circular dichroism, β-turn, Ile–Ala–Val–Pro peptide, hypocholesterolemic peptide.

The design of short peptide sequences having a β-turn structure and adopting a well-defined conformation in solution is an important element in the analysis and development of synthetic proteins. β-Turns are ubiquitous structural elements in peptide and protein structures [1]. β-Turns were first identified by [2] during an attempt to systematically delineate intramolecular hydrogen-bonded structures in peptide chains. The subsequent identification of these features in experimentally determined protein crystal structures [3] and their frequent occurrence in cyclic peptide structures [4] provided the impetus for a very large number of investigations on the stereochemistry of β-turns [5, 6]. The two most frequently occurring β-turns in peptide and protein structures are the type I and type II turns, which are distinguished on the basis of the backbone dihedral angles adopted by the two corner residues designated as $i + 1$ and $i + 2$ (type I, $\varphi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\varphi_{i+1} = -90°$ and $\psi_{i+2} = 0°$; and type II, $\varphi_{i+1} = -60°$, $\psi_{i+1} = 120°$, $\varphi_{i+2} = 80°$ and $\psi_{i+2} = 0°$). Both types of turns are stabilized by an intramolecular 4–1 hydrogen bond. In an examination of secondary structures [7] a total of 135 proteins from the original data set had homologous entries and these protein structures were used to generate the data set of 1120 isolated β-turns. The overwhelming majority (83%) of β-turns fall into the type I and type II categories, while a smaller number (17%) correspond to the "enantiomeric" structures (type I′ and type II′). In these β-turns, Pro (20%), Lys (16%) and Ser (12%) were most often found at the i + 1 position. Glycine was found to occur overwhelmingly at position i + 2 (50%), while Ser (12%) and Asn (10%) were among the most frequent residues.

The first conformation information on peptides and proteins is provided by circular dichroism (CD) analysis. However, the interpretation of CD spectra for molecules containing more than three or four amide groups meets both theoretical and practical difficulties because the intensity of β-turn spectra is low relative to that of the α-helix or β-sheet. So, the basis for CD studies in solutions is the investigation of small peptides with well-characterized steric structure and limited conformational flexibility such as cyclic peptides, which provide the largest part of experimental data. Linear peptides up to ten residues forms a small part of β-turn CD studies because they are increasingly flexible molecules. To adopt a single or predominant conformation, these peptides require special conditions (solid state, non-polar solvent, low temperature, etc.). Thus, the detection of a given conformation in solution provides evidence for the increased stability of this particular structure relative to other conformers or conformational regions.

1) S. Yu. Yunusov Institute of the Chemistry of Plant Substances, 700170 Tashkent, Uzbekistan, 2) Korean Food Research Institute, Songnam, Kyongki-do, 463-746, Korea. Published in Khimiya Prirodnykh Soedinenii, No. 4, pp. 324-329, July-August, 2004. Original article submitted March 1, 2004.

0009-3130/04/4004-0398 ©2004 Springer Science+Business Media, Inc.

TABLE 1. Retention Time and Purity of Synthesized Peptides after Their Synthesis and Purification

Peptide	Retention time, min	Purity after synthesis, %
IAVPGEVA	8.45	93.2
LIAVPGEVA	10.78	92.4
IAVPTGVA	8.59	90.8
LIAVPTGVA	10.85	92.3
IAVP	9.10	91.8
LIAVP	10.76	90.1

Purity after purification, % - >99.

TABLE 2. Comparison of Theoretical Molecular and Determined Molecular Weights by LC-MS

Peptide	Molecular weight	
	Theoretical	Determined by LC-MS
IAVPGEVA	754.8	755.5
LIAVPGEVA	868.0	868.8
IAVPTGVA	726.8	727.6
LIAVPTGVA	840.0	840.1
IAVP	398.5	399.4
LIAVP	511.6	512.5

Having taken into account that the turns have frequently been suggested as the bioactive conformation and Ile–Ala–Val–Pro (IAVP) was isolated as a hypocholesterolemic peptide from soybean protein, we have tried to elucidate the possibility of obtaining the predominant conformation of the β-turn for linear peptides on the base of Ile–Ala–Val–Pro–Gly–Glu–Val–Ala (IAVPGEVA), Leu–Ile–Ala–Val–Pro–Gly–Glu–Val–Ala (LIAVPGEVA), Ile–Ala–Val–Pro–Thr–Gly–Val–Ala (IAVPTGVA), and Leu–Ile–Ala–Val–Pro–Thr–Gly–Val–Ala (LIAVPTGVA) peptides in solution, proposing the propensity to adopt the β-turn conformations because they: 1) contain predominantly a hydrophobic amino acid [8, 9]; 2) consist of two parts, the first of which is relatively rigid because it comprises only a hydrophobic amino acid (IAVP, LIAVP) and the second part which is a relatively flexible portion because it includes Glu- and Thr-amino acid residues for IAVPGEVA, LIAVPGEVA and IAVPTGVA, LIAVPTGVA , respectively; 3) the Pro–Gly sequence, which was earlier shown to have a high β-turn propensity [10]; 4) the Pro–Thr sequence in dipeptide models appears to refer to the β-turn I conformation [11]; 5) the IAVP, LIAVP amino acid sequences of the given peptides are able to promote adopting the β-turn conformation both in the relatively rigid part and in the relatively flexible portion by an intramolecular hydrogen bond.

For this purpose we have synthesized four peptides (IAVPGEVA, LIAVPGEVA, IAVPTGVA, LIAVPTGVA) and, additionally for the detailed analysis of these structures, we have also synthesized only the hydrophobic part (IAVP, LIAVP) of the above peptides.

After cleavage, the synthesized peptides were purified by HPLC using a semi-preparative C_{18} column which gives a good performance for these relatively hydrophobic peptides despite the fact that the synthetic peptides were small molecules. The retention time and purity of each peptide in these optimized conditions are presented in Table 1.

Mass spectrometry is a convenient method for identifying a peptide or protein. The electrospray mass spectrometry data show that the peptides were synthesized correctly.

The theoretical molecular weights and determined molecular weights of each peptide are shown in Table 2.

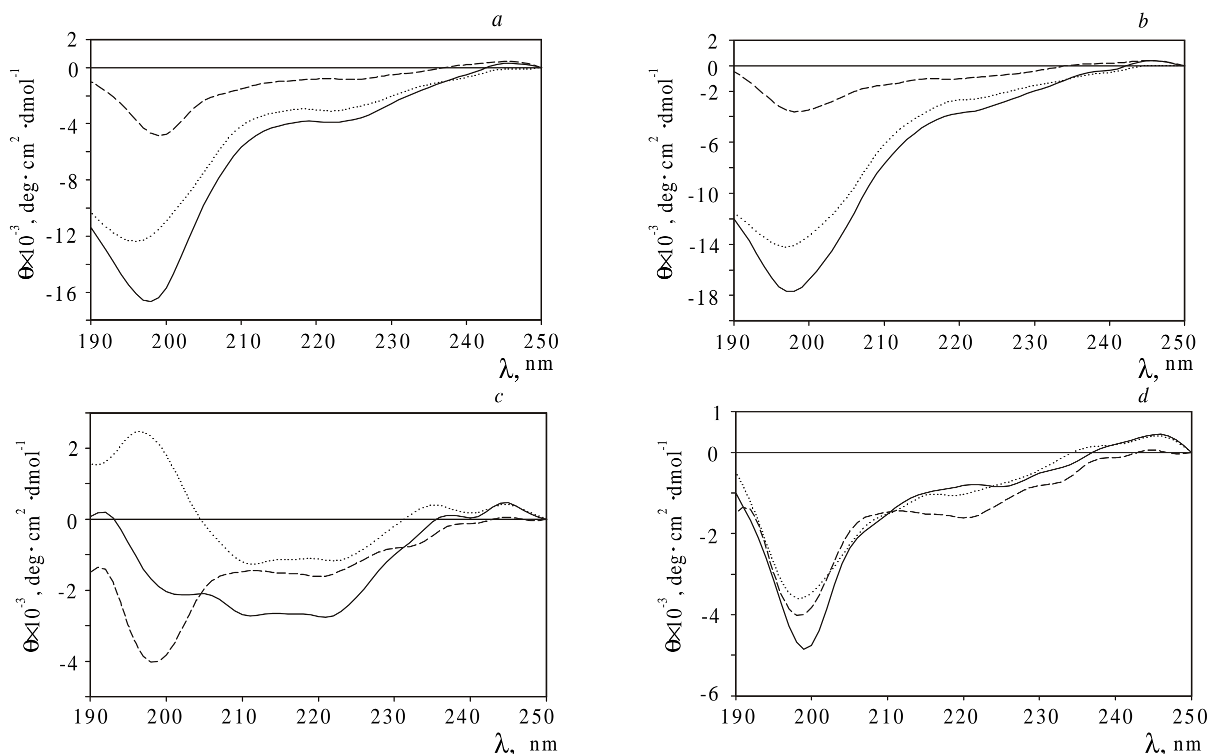

Fig. 1. Leu-residue contribution to the CD spectra of LIAVPGEVA, LIAVPTGVA, and LIAVP peptides. (a). (1) – LIAVPGEVA, (2) – IAVPGEVA, (3) – resulting spectra from LIAVPGEVA minus IAVPGEVA; (b). (1) – LIAVPTGVA, (2) – IAVPTGVA, (3) – resulting spectra from LIAVPTGVA minus IAVPTGVA; (c). (1) – LIAVP, (2) – IAVP, (3) – resulting spectra from LIAVP minus IAVP; (d). (1) – resulting spectra from LIAVPGEVA minus IAVPGEVA, (2) – resulting spectra from LIAVPTGVA minus IAVPTGVA, (3) – resulting spectra LIAVP minus IAVP.

All peptides were soluble in aqueous solution and no concentration dependence was found. CD analysis in the range from 50 to 500 mM. CD analysis was done at 200 mM for each peptides. The CD spectra for IAVPGEVA, LIAVPGEVA, IAVPTGVA, and LIAVPTGVA peptides are close to that of a random coil, with a deep minimum at 198 nm (for IAVPGEVA, LIAVPGEVA) and 196 nm (for IAVPTGVA, LIAVPTGVA) and no positive ellipticity below 200 nm; also the CD spectrum is very similar in the 210–230 nm region to those from IAVPGEVA, LIAVPGEVA, IAVPTGVA, and LIAVPTGVA peptides, especially the weak negative ellipticity at 222 nm {Fig. 1, *a*, *b* (solid and dotted curves)}. Further, the CD spectrums were not significantly altered by changing the pH from 1 to 7 for the above peptides.

In the case of IAVP there are some negative ellipticities at 209 nm and 222 nm and a positive ellipticity at 198 nm, indicating that the torsion angles (φ, ψ) are close to those in the α-helix structure or the kinds of turn conformation (Fig. 1, *c* (dotted curve)). In the CD spectrum of LIAVP (Fig. 1, *c* (solid curve)) there is an additional negative minimum around 200 nm compared with IAVP. We took into account that this is the contribution of a Leu-residue in this region which appears in LIAVP.

To determine the Leu-residue contribution we have subtracted the spectrum of random-coil IAVPGEVA and IAVPTGVA peptides from that of LIAVPGEVA and LIAVPTGVA peptides, respectively (Fig. 1, *a*, *b*). The analogous procedure was done for IAVP and LIAVP peptides (Fig. 1, *c*). The resulting spectra showed a similar profile with negative ellipticity at 198 nm, thus confirming the same contribution to the conformations of LIAVPGEVA, LIAVPTGVA, and LIAVP peptides.

Comparing the CD spectra of IAVP and LIAVP peptides and the resulting Leu-residue contribution reveals the identical conformation in the IAVP amino acids sequence, and we propose that the main contribution to the unordered structure of IAVPGEVA, LIAVPGEVA and IAVPTGVA, LIAVPTGVA is determined by GEVA and TGVA fragments of these peptides.

For this purpose we have subtracted the spectrum of IAVP and LIAVP from IAVPGEVA, IAVPTGVA and LIAVPGEVA, LIAVPTGVA, respectively. The resulting spectra show analogous contours with a deep negative minimum at 198 nm thus confirmed our suppositions.

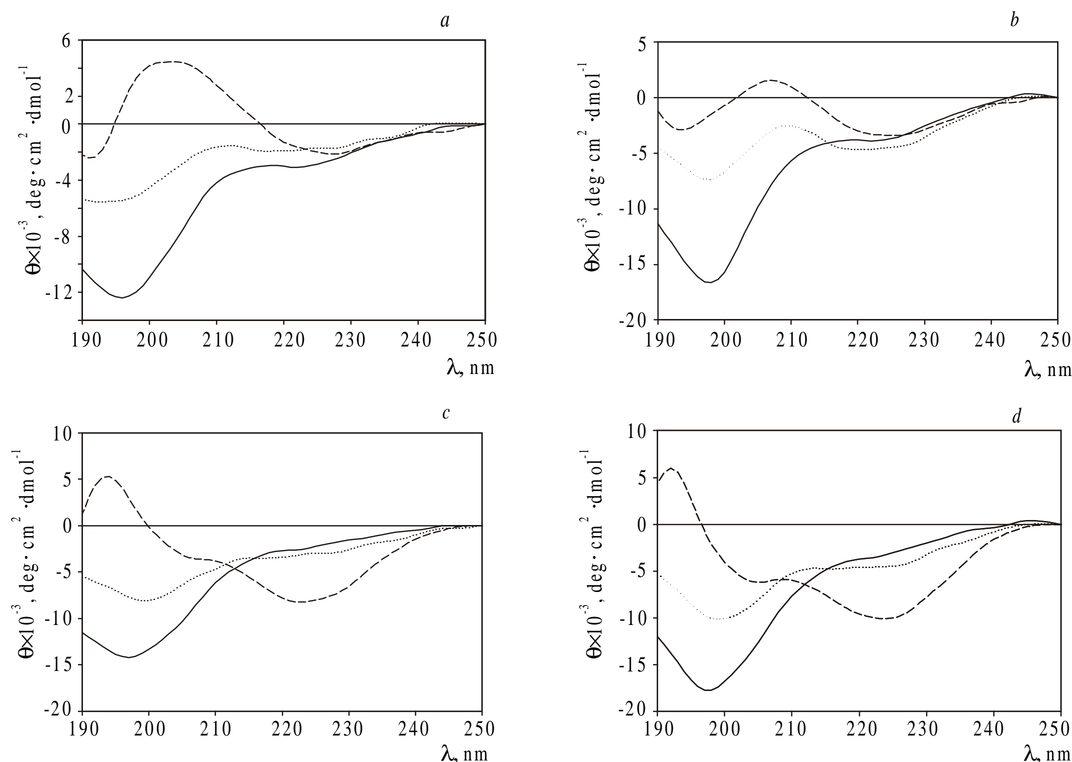

Fig. 2. Conformational change of IAVPGEVA (*a*), LIAVPGEVA (*b*), IAVPTGVA (*c*), and LIAVPTGVA (*d*) peptides induced by TFE. Percentage TFE/water (v/v) used are: (1) – 0%; (2) – 30%; (3) – 90%.

The circular dichroism spectra of IAVPGEVA, IAVPTGVA, LIAVPGEVA, and LIAVPTGVA peptides in water-TFE mixture are reported in Fig. 2.

We have observed the change in CD spectrum profile under the following concentrations of TFE (10, 20, 30, 50, 60, 70, 80, 90%) in water. Increasing the concentration of TFE changes the profile for IAVPGEVA, LIAVPGEVA to a predominant β-turn II and for IAVPTGVA, LIAVPTGVA peptides to a predominant β-turn I conformation. The CD spectrum of IAVPGEVA and LIAVPGEVA at a TFE concentration of 90% in a water mixture shows the characteristics of a β-turn II negative band between 220 and 230 nm, a positive band between 200 and 210 nm, and a negative band predicted between 180 and 190 nm [20], although the exact position of the last negative minimum for IAVPGEVA and LIAVPGEVA peptides is different (between 190–200 nm).

In the case of IAVPTGVA and LIAVPTGVA peptides the CD spectra show an α-helix-like profile with the characteristics of a β-turn I double negative minimum (208–210 nm and 222 nm) and a positive band around 198 nm [21]. A gradual increase in both the β-turn II (IAVPGEVA, LIAVPGEVA) and β-turn I (IAVPTGVA, LIAVPTGVA) content was observed with increasing TFE in the mixture to a value as high as 90% TFE/water v/v.

Reversed-phase liquid chromatography (RP-HPLC) has been particularly useful as a physicochemical model of biological systems, where studies have generally centered on correlating the retention behavior of proteins [22] or peptides [23] during RP-HPLC with their conformational stability. The assumption in such RP-HPLC studies is that the hydrophobic interactions between peptides and proteins with the nonpolar stationary phase characteristic of RP-HPLC [24–26] reflect the hydrophobicity and interactions between nonpolar residues that are the major driving forces for protein research. Chromatographic retention of small peptides highly correlates with the summing of the hydrophobic contribution of the respective amino acid residues.

All peptides were analyzed in the same condition on an analytical C_{18} reversed-phase column using an acetonitrile gradient in aqueous 0.1% (v/v) trifluoroacetic acid (TFA) buffer, pH 2.1. The elution profile was determined by monitoring UV absorbance at 220 nm.

Based on the retention times shown in Table 1 we note that LIAVPGEVA, LIAVPTGVA, and LIAVP peptides demonstrate very close retention times (10.78, 10.85, 10.76, respectively) and consequently analogous hydrophobic interactions between the LIAVP fragment of these peptides with the nonpolar stationary reversed phase. Thus, the above amino acid

sequence plays the main role in retention behavior and as a result LIAVPGEVA and LIAVPTGVA peptides have a similar conformation features.

Also, IAVPGEVA and IAVPTGVA peptides show close values (8.45 and 8.59) of the retention time, but the retention time for IAVP (9.10) lies between the retention times of LIAVPGEVA, LIAVPTGVA, LIAVP and IAVPGEVA, IAVPTGVA peptides. Consequently only a portion of the IAVP fragment participates in hydrophobic interaction on the stationary reversed phase in the case of IAVPGEVA and IAVPTGVA peptides despite the fact that they also present similar conformation structures.

Thus data on the retention times show that the LIAVP fragment in the case of LIAVPGEVA and LIAVPTGVA peptides has a close conformation structure as in case of the IAVP amino acid sequence for IAVPGEVA and IAVPTGVA peptides.

In aqueous solution, in the case of IAVPGEVA and LIAVPGEVA peptides, as was shown above, the main contribution to the unordered structure was the EGVA fragment. Perhaps solvation of the amide group plays the main role in this, including the Glu residue, which has two side-chain oxygen atoms able to accept hydrogen bonds in aqueous solution because these have a negative charge. Additionally, the side chain of Glu has a particularly wide range of rotation [7]; consequently, it has a high propensity to change the conformation of the main chain.

In the case of IAVPTGVA and LIAVPTGVA peptides, we can propose an analogous relation between the hydroxyl group of the side chain of Thr-residue and the conformation changes of the main chain despite the fact that the Thr side chain is shorter than the Glu side chain and it has a less polar group.

CD analysis shows that increasing the concentration of TFE changes the profile for IAVPGEVA and LIAVPGEVA to a predominant β-turn II and for IAVPTGVA and LIAVPTGVA peptides to a predominant β-turn I. Perhaps β-turn formation is enhanced in TFE solution through destabilizing solvation of amide groups in aqueous solution, thereby promoting intra-molecular hydrogen bonding.

The data of CD and RP-HPLC analyses show that LIAVP and IAVP fragments have definite conformation stability for the respective peptides. Additionally, these fragments contain such amino acid sequences that adopt a conformation similar to the fist turn of the helix. Also, we have determined the contributions of the leucine amino acid residue in the structure and revealed similar characteristics of it for LIAVPGEVA, LIAVPTGVA and LIAVP peptides. As mentioned above, the GEVA and TGVA fragments are sensitive to changes of the aqueous environment, thus showing the flexible portion of the respective peptides. Thus, on the basis of CD and RP-HPLC analysis, the last mentioned fragments adopt a similar conformation in TFE-water solution as LIAVP and IAVP fragments with a turn in the position of Pro–Gly and Pro–Thr for IAVPGEVA, LIAVPGEVA and IAVPTGVA, LIAVPTGVA peptides, respectively, because Pro–Gly and Pro–Thr sequences have β-turn propensity. Also, we propose that the side-chain of glutamic acid additionally forms a hydrogen bond involving the NH group of IAVPGEVA and LIAVPGEVA peptides. In the case of IAVPTGVA and LIAVPTGVA peptides an analogous interaction between the threonine side chain and the NH group of the respective peptides will also be essential. Thus, additional formation of hydrogen bonds can lead to greater conformation stability of the given peptides in TFE-water solution. We have examined the conformation stability for each peptide by CD analysis in 90:10% TFE-water mixture for a week and have not found any difference in peptide structure. Thus, the β-turn I, II conformations in the studied peptides are stable under the above conditions. On the basis of the data obtained, we can conclude that the suggested approach to the design of linear peptides allows obtaining a predominant β-turn conformation in solution.

EXPERIMENTAL

Materials. Fmoc alanine preloaded HMP resin (HMP resin – 4-hydroxymethylphenoxyacetyl-4′-methylbenzyhydryl-amine resin) – (Fmoc–Ala–HMP Resin, substituted at 0.68 mmol/g) and proline preloaded trityl resin (H-Pro-2-ClTrt resin, substituted at 0.69 meq/g), Fmoc-Glu(OtBu)-OH (N-α-Fmoc-L-glutamic acid γ-t-butyl ester), Fmoc-Thr(tBu)-OH (N-α-Fmoc-O-t-butyl-L-threonine), Fmoc-Val-OH (N-β-Fmoc-*L*-valine), Fmoc-Ala-OH (N-α-Fmoc-*L*-alanine), Fmoc-Gly-OH (N-α-Fmoc-glycine), Fmoc-Ile-OH (N-α-Fmoc-*L*-isoleucine), Fmoc-Leu-OH (N-α-Fmoc-*L*-leucine), Fmoc-Pro-OH (N-α-Fmoc-proline) were purchased from AnaSpec (San Jose, CA, USA). Chemicals for peptide synthesis: diisopropylethylamine (DIEA), methanol, dichloromethane (DCM), N-methylpyrrolidone (NMP), 0.1 M 4-dimethylaminopyridine (DMAP)/dimethylformamide (DMF), 1.0 M dicyclohexylcarbodiimide (DCC)/NMP, solid 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium (HBTU), 0.45 M 1-

hydroxybenzotriazole (HOBt) in DMF, 2 M DIEA/NMP, piperidine, 1-HOBt/DMF, and 0.5 M HOBt in NMP were products of Perkin Elmer (Foster City, CA, USA). Chemicals for cleavage and trifluoroacatic acid (TFA) were obtained from Sigma Chemical Co., (St. Louis, MO, USA).

Acetonitrile and methanol for RP-HPLC were products of Burdict & Jackson (Muskegon, MI, USA). All other chemicals or solvents were of reagent grade or HPLC grade.

Methods. Synthesis of peptides was performed using standard fluorenylmethyloxycarbonyl (Fmoc) methodology [12, 13] on an automated synthesizer and an Applied Biosystem Peptide Synthesizer (Model 433A, Perkin Elmer). Peptide syntheses starting with the proline preloaded resin (IAVP, LIAVP) or Fmoc-alanine preloaded HMG resin (IAVPGEVA, LIAVPGEVA, IAVPTGVA, LIAVPTGVA) were carried out using the FasMoc strategy.

Cleavage Protocol. After the peptide synthesis, the Fmoc group from the final peptide-resin was automatically removed under nitrogen by the peptide synthesizer. Then the peptide was cleaved from the resin by a mild TFA cleavage method [14]. The cleavage mixture contained 9.5 ml TFA and 0.5 ml of deionized H_2O for 1.5–2 g of peptide-resin. The peptide yields ranged from 90 to 95% of the theoretical yields.

Analysis and Purification of Peptides. The synthetic peptides were analyzed and purified using a reversed-phase high pressure liquid chromatography (HP-HPLC) system (Waters, USA), an automated gradient controller, a tunable absorbance detector (Waters 484) and a data module (Waters 745B) using a Vydac 218TR54 analytical column (C_{18}, 5 mm, 4.6 mm ID ´ 250 mmL), and a Vydac 218TR510 semi-preparative C_{18} column (C_{18}, 5 mm, 10 mm ID ´250 mmL) with the same conditions: mobile phase 0.1% TFA in water: 0.1% TFA in acetonitrile. Gradient condition 10:90, 50:50. Time 0–25 min.
The peak fraction during purification of the peptides was collected manually and vacuum evaporated with an automatic SpeedVac concentrator (Savant AS 260, Farmingdale, NY, USA).

Electrospray Ionization Mass Spectrometry. Mass spectral analyses were performed using an electrospray mass spectrometer (Platform II, Manchester, UK). Purified peptide dissolved in 0.1% of formic solution in water/acetonitrile (1:1, v/v) was introduced using a microliter syringe.

Circular Dichroism Spectroscopy. Spectra were recorded as previously described [15–17] using a JASCO model J710 spectropolarimeter which had been stabilized for at least 30 min with a nitrogen flow rate of 5 l/min, and which was frequently calibrated using a (+)-10-camphor-sulfonic acid (CSA) sample provided by JASCO. For the far UF range, we assume that the CSA minimum corresponds to $[q]_{192.5} = -15600$ [23, 24]. The accumulated (4-6 scans) average spectra were trimmed at a photomultiplier voltage of 500 V and smoothed using reverse Fourier transform in the JASCO software prior to subtracting a similarly smoothed solvent baseline. All subsequent CD spectral values for peptides are expressed in units of molar ellipticity (deg cm^2/ dmol), and solvent compositions as vol. % values.

ACKNOWLEDGEMENT

This work was supported in part by the Project of Function Food and Development (2003) of the Korean Institute of Science and Technology Evaluation and Planning (KISTER).

REFERENCES

1. G. D. Rose, L. M. Gierasch, and J. A. Smith, *Adv. Protein Chem.,* **37**, 1 (1985).
2. C. M. Venkatachalam, *Biopolymers,* **6**, 1425 (1968).
3. P. Lewis, F. Momany, and H. Scheraga, *Biochim. Biophys. Acta,* **303**, 211 (1973).
4. J. A. Smith and L. G. Pease, *CRC Crit. Rev. Biochem*., **8**, 315 (1980).
5. R. Chandrasekaran, A. V. Lakshminarayanan, U. V. Pandya, and G. N. Ramachandran, *Biochim. Biophys. Acta,* **303**, 14 (1973).
6. C. Amakrishnan, N. Srinivasan, and D. V. Nataraj, *Int. J. Pept. Protein Res*., **48**, 420 (1996).
7. K. Gunasekaran, L. Gomathi, C. Ramakrishman, J. Chandrasekhar, and P. Balaram, *J. Mol. Biol*, **284**, 1505 (1998).
8. U. Azzena and P. L. Luisi, *Biopolymers*, **25**, 555 (1986).
9. S. K. Awasthi, S. Raghothama, and P. A. Balaram, *Biochem. Biophys. Res. Commun.*, **216**, 375 (1995).

10. B. N. Rao, A. Kumar, H. Balaram, A. Ravi, and P. Balaram, *J. Am. Chem. Soc.,* **105**, 7423 (1983).
11. A. Aubry and M. Marraud, *Acta Crystallogr.*, **41**, 65 (1985).
12. L. A. Caprino and G. Y. Han, *J. Org. Chem.*, **37**, 3404 (1972).
13. C. Chang and J. Meienhofer, *Int. J. Pept. Protein Res.,* **11**, 246 (1978).
14. *Perkin Elmer Cooperation, Introduction to Cleavage Techniques*, 2nd ed. Foster City, CA, USA (1995).
15. N. H. Andersen, B.Cao, A. Rodriques-Romero, and B. Arreguin, *Biochemistry*, **32**, 1407 (1993).
16. N. H. Andersen, S. M. Harris, V. G. Lee, E. Lie, S. Moreland, and S. J. T. Hunt, *BioOrg. Med. Chem. Lett.,* **3**, 113 (1995).
17. N. H. Andersen and R. B. Palmer, *BioOrg. Med. Chem. Lett*., **4**, 817 (1994).
18. J. T. Yang, C.-S. C .Wu, and H. M. Martinez, *Methods Enzymol.*, **130**, 208 (1986).
19. W. C. Johnson Jr., *Protein Struct. Funct. Genet.,* **7**, 205 (1991).
20. R. W. Woody, Studies of theoretical circular dichroism of polypeptides: Contributions of β-turns, in: *Peptides, Polypeptides, and Proteins*, E. R. Blout, F. A. Bovey, N. Lotan, and M. Goodman, eds., Wiley, New York, 1974, pp. 338–350.
21. R. W. Woody, in: *The Peptides*, V. J. Hruby, ed., Academic Press, New York, 1985, Vol. **7**, pp. 15–114.
22. L. M. Gierasch, C. M. Deber, V. Madison, C. H. Niu, and E. R. Blout, *Biopolymers*, **31**, 835 (1981).
23. R. H. Ingraham, S. Y. M. Lau, A. K. Taneja, and R. S. Hodges, *J. Chromatogr.*, **327**, 77 (1985).
24. E. Lazoura, I. Maidonis, E. Bayer, and M. T. W. Hearn, *Biophys*., **72**, 238 (1997).
25. C. T. Mant and R. S. Hodges (Eds.), *High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation*, CRC Press, Boca Raton, FL, USA, 1991.
26. M. T. W. Hearn (Ed.), *HPLC of Proteins, Peptides, and Polynucleotides: Contemporary Topics and Applications*, VCH, New York, 1991.
27. C. T. Mant, R. S. Hodges, in: K. M. Gooding, and F. E. Regnier, eds., *HPLC of Biological Macromolecules*, Marcel Dekker, New York, 2002, pp. 433–511.