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Folding in solution of the C-catalytic protein fragment of angiotensin-converting enzyme

Sotirios-Spyridon M. Vamvakas,[a,b] Leondios Leondiadis,[a]* George Pairas,[b] Evy Manessi-Zoupa,[c] Georgios A. Spyroulias[b] and Paul Cordopatis[b]* *

Angiotensin-converting enzyme (ACE) is a key molecule of the renin–angiotensin–aldosterone system which is responsible for the control of blood pressure. For over 30 years it has become the target for fighting off hypertension. Many inhibitors of the enzyme have been synthesized and used widely in medicine despite the lack of ACE structure. The last 5 years the crystal structure of ACE separate domains has been revealed, but in order to understand how the enzyme works it is necessary to study its structure in solution. We present here the cloning, overexpression in *Escherichia coli*, purification and structural study of the Ala_{959} to Ser_{1066} region (ACE_C) that corresponds to the C-catalytic domain of human somatic angiotensin-I-converting enzyme. ACE_C was purified under denatured conditions and the yield was 6 mg/l of culture. Circular dichroism (CD) spectroscopy indicated that 1,1,1-trifluoroethanol (TFE) is necessary for the correct folding of the protein fragment. The described procedure can be used for the production of an isotopically labelled $ACE_{959-1066}$ protein fragment in order to study its structure in solution by NMR spectroscopy. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: ACE; zinc metallopeptidase; recombinant protein; secondary structure; circular dichroism

Introduction

ACE is a key molecule of the renin–angiotensin–aldosterone system. Its major biological function is the conversion of the rather inactive decapeptide angiotensin-I to the highly potent vasoconstrictor octapeptide angiotensin-II. This enzyme is a *gluzincin* zinc peptidyl dipeptidase I. Another basic activity of the enzyme is the inactivation of bradykinin, a vasodilatory peptide [1,2].

In humans, ACE can be found in two isoforms. These isoforms are transcribed from the same gene in a tissue-specific manner. In brush-border epithelial and endothelial cells, somatic ACE is present as a glycoprotein composed of a single, large polypeptide chain of 1306 amino acids. It has two homologous domains (N and C domain) (Figure 1). Each domain contains an active site with a conserved *gluzincin* zinc-binding motif [3].

The two domains present differences in substrate specificities, inhibitors, chloride activation profiles and physiological functions [4]. Despite the fact that the active sites of both domains catalyse the hydrolysis of angiotensin-I and bradykinin with similar efficiency, C domain seems to be necessary and sufficient for controlling blood pressure and cardiovascular function, suggesting that the C domain is the dominant angiotensin-converting site [5,6].

Testicular ACE is present in sperm cells as a lower-molecular-mass glycoform of 732 amino acids and is identical to the C-terminal half of somatic ACE, except for a unique 36-residue sequence constituting its amino terminus [7] (Figure 1). It has also been shown that testicular ACE could play a role in sperm maturation and the binding of sperm to the oviduct epithelium [8].

In medicine ACE has become a challenging target for the regulation of arterial pressure. Since the discovery of the viper's venom agent, which causes complete inactivation of ACE and leads to death, many commercially available inhibitors have been synthesized, based on this agent's structure. These pharmaceutical agents, effectively regulate blood pressure, but occasionally, they present a considerable number of side effects. The most common side effects are cough, elevated blood potassium levels, low blood pressure, dizziness, headache, drowsiness, weakness, abnormal taste (metallic or salty taste) and rash [9]. The most serious, but rare, side effects of ACE inhibitors are kidney failure, allergic reactions, a decrease in white blood cells and swelling of tissues (angioedema) [10]. These problems would have been avoided if ACE inhibitors had not been synthesized without thorough knowledge of the ACE structure and its catalytic mechanism of action. The recent crystallographic study of the testicular form and the N-catalytic domain of the somatic form [11,12], provides significant information for ACE structure and will positively contribute to the design of novel-specific ACE inhibitors. The major disadvantage of crystallography, is the 'immobilization' of

* *Correspondence to: Leondios Leondiadis, Mass Spectrometry and Dioxin Analysis Lab, IRRP, National Center for Scientific Research "Demokritos", 27 Neapoleos St., GR-15310 Athens, Greece. E-mail: leondi@rrp.demokritos.gr*

** *Paul Cordopatis, Department of Pharmacy, University of Patras, GR-26500 Patras, Greece. E-mail: pacord@upatras.gr*

a *Mass Spectrometry and Dioxin Analysis Lab, IRRP, National Center for Scientific Research "Demokritos", 27 Neapoleos St., GR-15310 Athens, Greece*

b *Department of Pharmacy, University of Patras, GR-26500 Patras, Greece*

c *Department of Chemistry, University of Patras, GR-26500 Patras, Greece*

Abbreviations used: *ACE, angiotensin-converting enzyme; ACE_C, angiotensin-converting enzyme (Ala_{959}-Ser_{1066}); CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-beta-D-thiogalactopyranoside; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TFE, 1,1,1-trifluoroethanol; Tris, tris(hydroxymethyl)aminomethane.*

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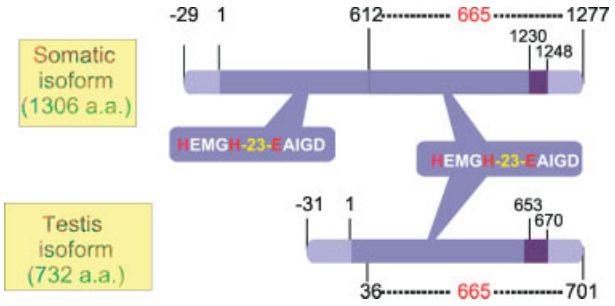

Figure 1. Schematic representation of the two ACE isoforms.

the molecule inside the crystal. So, conformational studies in solution have to be done in order to reveal the exact catalytic mechanism and the conformational transitions occurring during the binding of substrates and release of the products during the catalytic cycle. NMR spectroscopy has lately become a suitable method for solving a protein structure in solution as well as for revealing critical amino acids for the interaction between two protein molecules. Thus, the study of the structural features of ACE is necessary in the presence and in the absence of its inhibitors, which cannot be catalysed by the enzyme, in order to gather information about its catalytic mechanism. By considering the structural data from crystallography and NMR study, the designing of new selective and effective ACE inhibitors will be possible.

This study has two objectives. The first is the overexpression and purification of the sACE fragment Ala_{959}-Ser_{1066}. This fragment was chosen because its sequence contains the C-catalytic domain of sACE together with its N- and C- sequences which may be required for the substrate docking. The exact size of the fragment was also selected taking into consideration calculations of hydropathicity. The selected fragment showed a good profile of hydropathicity (data not shown) [13]. For this purpose a bacterial expression system was used because of its easy handling and for the production of an isotopically labelled protein fragment. The second objective of this study is the primary analysis of the overexpressed and purified fragment's secondary structure by CD spectroscopy. This technique was chosen because of its ability to calculate accurately the alpha-helix content of a protein, since it is known by the literature that the overexpressed ACE fragment has a high level of alpha-helix conformation.

Materials and Methods

Theoretical Calculation of Secondary Structure of ACE_C Protein Fragment

The PSIPRED protein structure prediction server performs a prediction of the secondary structure of a protein. This is a highly accurate method for protein secondary structure prediction. It is a simple and reliable method, incorporating two feed-forward neural networks which perform an analysis on output obtained from PSI-BLAST (Position-Specific Iterated–BLAST) [14,15]. Using a very stringent cross validation method to evaluate the method's performance [16] (http://cubic.bioc.columbia.edu/eva/sec/method/psipred.html), PSIPRED 2.0 is capable of achieving an average Q_3 [three-state per-residue accuracy (percentage of correctly predicted residues)] score of approximately 78%.

Isolation of $sACEAla_{959}$-Ser_{1066} (ACE_C) c-DNA

A c-DNA clone of somatic ACE (pECE/ACE) was used for the isolation of ACE_C c-DNA. *BglII* restriction enzyme was used for the digestion of the plasmid. The digestion resulted in the formation of three DNA fragments migrating as discrete bands in an agarose electrophoresis. The molecular masses of these bands were 4574, 1776 and 570 bp. The ACE_C coding fragment was included in the 4574 bp. This fragment was resolved from agarose gel using Nucleo-spin plasmid kit (Macherey-Nagel). The purified fragment was used as a template in a PCR in order to amplify the ACE_C c-DNA coding sequence. For the PCR, a pair of synthetic oligonucleotides were used as primers: the forward primer *NdeI/ACE_C* (GGAATTCCATATGGCCTCGGCCTGGGACTTC), contained additional nucleotides encoding a *Nde*I restriction site followed by the initiating ATG methionine and the reverse primer *BamHI/ACE_C* (CGGGATCCTCAGCTGAAGGGGATAAAGGCG) contained a stop codon and a *BamHI* restriction site. PCR was performed, for 35 cycles (95 °C, 1 min; 55 °C, 1 min; 72 °C, 30 s) using *Vent* DNA polymerase (New England Biolabs).

Introduction of ACE_C c-DNA into the Expression Vector

The resulting PCR fragment was purified and introduced into the *NdeI/Bam*HI restriction sites of the *E. coli* expression vector, pET3a (Novagen). The multiple cloning site of the expression vector is under the control of the strong T7 promoter and protein expression occurs by the addition of IPTG. DNA sequencing, using the dideoxynucleotide chain termination method [17], was conducted to confirm the nucleotide sequence of the insert. For expression of the recombinant protein, the resultant plasmid, pET3a-ACE_C, was used to transform first the *E. coli* strain ER2566 and then BL21 (DE3) (pLysS).

Protein Expression

For the expression of the ACE_C protein fragment, cells were cultured in baffled shake flasks at 37 °C in Luria broth medium supplemented with ampicillin to a final concentration of 100 μg/ml. When the cells had grown to an OD_{600} value of 0.6–0.8, protein expression was induced by the addition of IPTG to a final concentration of 0.3 mM. Cells were harvested 3 h post-induction by centrifugation at 4000 *g* for 20 min. The pelleted cells were washed twice with 20 ml of 20 mM Tris-Cl, pH 8.0, 1 mM EDTA/l of culture and stored frozen at −20 °C for up to several months.

Protein Purification

All steps were performed at 4 °C unless noted. Cells from 1 l of culture were thawed briefly at room temperature and completely resuspended in 60 ml of Buffer A (20 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.1% v/v TritonX-100, 20 μM (PMSF) and 10^{-6} g Leupeptin/ml). After the addition of white egg lysozyme at a final concentration of 10^{-5} g/ml, the cell suspension was let to stand on ice for 1 h. The mixture was sonicated on ice until a homogeneous suspension was formed. The resulting cell lysate was centrifuged at 15 000 *g* for 30 min. The supernatant (S1) was retained for analysis by SDS–PAGE. The pellet (P1) was resuspended in 20 ml of buffer B (100 mM Tris pH 8.0, 200 mM NaCl, 8 M urea, 10 mM DTT). After incubation for 30 min, the insoluble material was removed by centrifugation at 14 000 g for 1.5 h (P2). The supernatant (S2) was clarified by passage through a 0.45 μm filter and loaded onto a HiLoad 16/60 Superdex 75 size exclusion column pre-equilibrated

with 2 volumes of 6 M urea, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 10 mM DTT. The loaded column was washed with the same buffer. Eluted fractions (0.5 ml) were monitored by absorbance at 280 nm. Fractions containing denatured ACE_C were pooled. The pool was concentrated to a volume of 0.5 ml by centrifugation first on a macrosep-3K-Omega and then on microsep-3K-Omega centrifugal devices from Pall. The sample was diluted 6 times by the addition of 10 mM DTT and the insoluble material precipitating upon dilution was collected by centrifugation. The pellet was washed once with 10 mM DTT, twice with ultra pure water and air-dried.

Molecular Mass Determination

Electrospray ionization (ESI) mass spectral analysis was performed on an AQA Navigation ThermoFinnigan spectrometer. Test solution in 50% v/v aqueous acetonitrile was infused into the electrospray interface at a flow rate of 0.1 ml/min, using a Harvant Syringe pump. Negative or positive ion ESI spectra were acquired by adjusting the needle and cone voltages accordingly. Hot nitrogen gas (Dominic-Hunter UHPLCMS-10) was used for desolvation at 170 °C.

CD Spectroscopy

CD spectra were recorded at room temperature using a Jasco-J810 spectropolarimeter equipped with a thermostatted sample chamber. Proteins were dissolved in 200 mM sodium chloride, 8 mM DTT. A 0.1 cm optical path length quartz cell was used to record spectra of proteins in the far ultraviolet region (200–260 nm) at a protein concentration of 0.5 mg/ml. All CD spectra were acquired at a scan speed of 20 nm/min, 0.2 nm bandwidth and a response time of 1 s. Spectra were signal-averaged over eight scans. The amount of different secondary structure elements was calculated using the CD spectra deconvolution program CDNN [18].

Results

Theoretical Secondary Structure Calculation of ACE_C Protein Fragment

The theoretical calculation of secondary structure using the PSIPRED protein structure prediction server [14,15] revealed that ACE_C protein fragment has a content of alpha-helix conformation ~44% (Figure 2). The accepted value for the calculation of this content prediction confidence values was above 6 in a scale to 10. One thing that must been taken into account is that the PSIPRED prediction method does not have the ability to calculate with accuracy the b-turn and the parallel and antiparallel motif of beta-strand. In this study the calculated beta-structure percentage was ~3% while the one found by Natesh and co-workers was ~7%. The beta-strand predicted is in the same position as the one found by Natesh and co-workers. The second one is not predicted by the PSIPRED protein structure prediction server. This probably happened because the amino acids that form this beta-strand are very close to the N-terminus of the fragment [11]

Cloning and Expression of ACE_C

ACE_C was subcloned in pET-3a expression vector at the *NdeI–BamHI* restriction sites in order to achieve the production of the protein with no tag. The resulting construct was the pET-3a/ACE_C. The sequence of the construct was checked using the

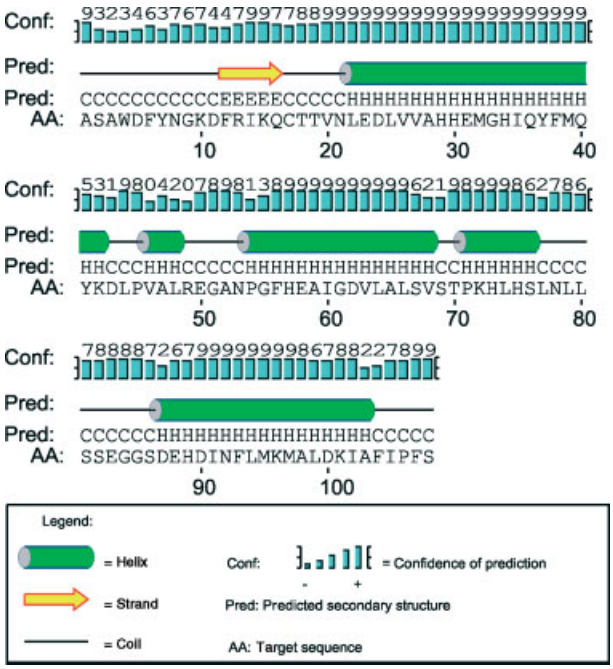

Figure 2. Theoretical calculation of ACE_C protein fragment's secondary structure using the PSIPRED protein structure prediction server. For the calculation of the α-helix content, only a.a. with confidence prediction value greater than 6 in a scale to 10 have been taken into account.

Sanger method [17] and was found to be consistent with the sequence submitted by Soubrier and co-workers [3].

In order to determine the optimal conditions for ACE_C expression under IPTG-inducible control of the T7 promoter of the pET-3a vector in ER2566 cells, preliminary experiments were conducted. No expression was achieved at several conditions of temperature, IPTG concentration, time of induction and optical density of the culture at which the IPTG was added.

This result may be explained by the possible toxicity of the gene product for bacterial cells, due to the existence of a basal expression of the gene product before the induction. In such situations a way of providing additional stability to target genes is to express them in host strains containing a compatible chloramphenicol-resistant plasmid that provides a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase [19,20]. T7 lysozyme is a bifunctional protein: it cuts a specific bond in the peptidoglycan layer of the *E. coli* cell wall [21], and it binds to T7 RNA polymerase, inhibiting transcription [22,23]. T7 lysozyme is provided to the cell by a clone of the T7 lysozyme gene in the *Bam*H I site of pACYC184 [24]. The cloned fragment (bp 10 665–11 296) of T7 lyzozyme DNA [25] also contains the φ3.8 promoter for T7 RNA polymerase immediately following the lysozyme gene. The plasmid having this fragment in the opposite orientation of the *tet* promoter of pACYC184 is referred to as pLysS. Cells carrying this plasmid accumulate low levels of lysozyme. pLysS causes a slight decrease in the growth rate of cells that carry it. The presence of pLysS increases the tolerance of λDE3 lysogens for plasmids with toxic inserts. Thus, unstable plasmids become stable, and plasmids that would not otherwise be established can be maintained and expressed. For very toxic genes, the combination of a T7*lac* promoter-containing vector and pLysS is preferable. Taking into account all the above-mentioned considerations, the use of BL21 (DE3) (pLysS) as host cells was chosen.

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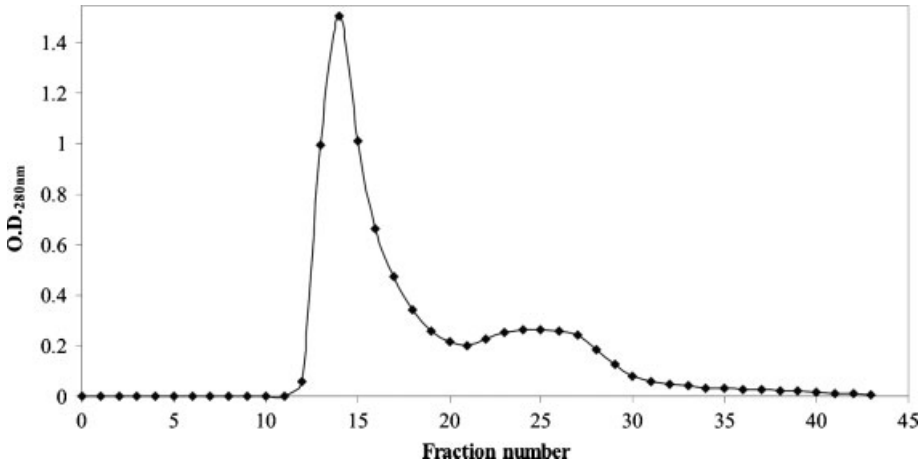

Figure 3. Gel filtration chromatogram. Chromatography took place at room temperature in denature conditions (Elution buffer contained 8 M urea). A major peak and a 'shoulder' are visible.

Several conditions of induction of transformed BL21 (DE3) (pLysS) cells with pET-3a/ACE_C plasmid were tested, showing that ACE_C was located in insoluble inclusion bodies (Figure 4). The yield of the overexpressed protein fragment at a temperature lower than 37 °C was minimal. The conditions at which ACE_C presented the best expression level were 37 °C until $O.D._{550\ nm}$ reached 0.8, and further incubation at 37 °C for 3 h, after the addition of IPTG at the final concentration of 0.25 mM.

Purification of ACE_C

The denatured and reduced protein fragment was loaded onto a *HiLoad 16/60 Superdex 75* column, and chromatography was carried out under denatured conditions. The chromatogram shows a major peak with 'shoulder' (asymmetry of major peak). The second peak (shoulder) corresponds to ACE_C (Figure 3). This was specified by SDS-PAGE (data not shown). Fractions containing the ACE_C were pooled together. The pool was concentrated by centrifugation using macrosep and microsep desalting filters. The concentrate was diluted 6 times and the resulting pellet collected by centrifugation. The recombinant protein yield was approximately 6 mg/l of culture.

The sequence integrity of the recombinant protein was assessed by SDS-PAGE and ESI/MS analysis. The purified protein showed a single band in Coomassie stained SDS–PAGE (Figure 4). The molecular weight of the band was around 12 kDa calculated by R_f (~0,77), corresponding to the molecular weight of ACE_C. The mass spectra of the purified ACE_C (Figure 5) contains three major peaks at *m/z* 1211.0, 1345.4 and 1513.5 corresponding to $[M+10]^{10+}$, $[M+9]^{9+}$ and $[M+8]^{8+}$, respectively. By algorithm deconvolution the calculated experimental molecular weight (MW) was 12 101.8± 0.3 Da which is in perfect agreement with the theoretical value calculated by the amino acid sequence 12 102 Da. This result demonstrates that the desired protein fragment was successfully expressed and purified and that its integrity and homogeneity was not affected by the expression and purification procedure.

Solubilization: Secondary Structure Analysis

In order to achieve complete solubilization and refolding of the ACE_C protein fragment, several conditions were tested in the presence and absence of TFE. It was concluded by these experiments that TFE is absolutely necessary for complete solubilization of ACE_C protein fragment, when its concentration in a solution is high (more than 2 mg/ml).

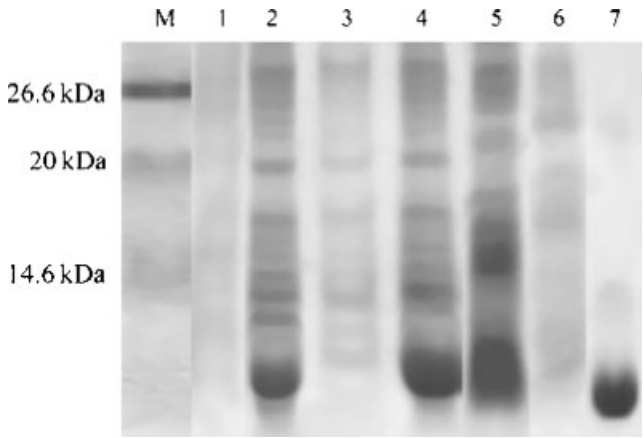

Figure 4. SDS-PAGE of the: (M) Marker, (1) uninduced cells, (2) induced cells, (3) supernatant of the centrifugation of the hole cell extract (S1), (4) pellet (P1) of the centrifugation of the hole cell extract, (5) supernatant (S2) of the centrifugation of solubilized with 8M urea inclusion bodies, (6) pellet (P2) of the centrifugation of solubilized with 8M urea inclusion bodies, (7) purified ACE_C.

The secondary structure of the recombinant ACE_C had been resolved by CD analysis. From the spectrum the double ellipticity minimum can be seen at approximately 208 and 222 nm, which is typical of alpha-helical content (Figure 6). The value for alpha-helical content was around 40%, calculated using appropriate algorithms, for ACE_C diluted in solutions with a TFE concentration above 70%. This is in agreement with the proportion of alpha-helix found in the crystal structure of testicular ACE regarding the investigated protein fragment [11], as well as the one calculated theoretically, indicating that the backbone polypeptide chain of the purified protein had an identical conformation with the native molecule. In addition Voronov and co-workers created a model of secondary structure of bovine ACE. The alpha-helix of the ACE_C protein fragment was also calculated a little less than 40% [26]. Thus the purification and refolding procedure leads to no loss of conformation of the recombinant peptide. The value of alpha-helical content, when ACE_C was diluted to the maximum

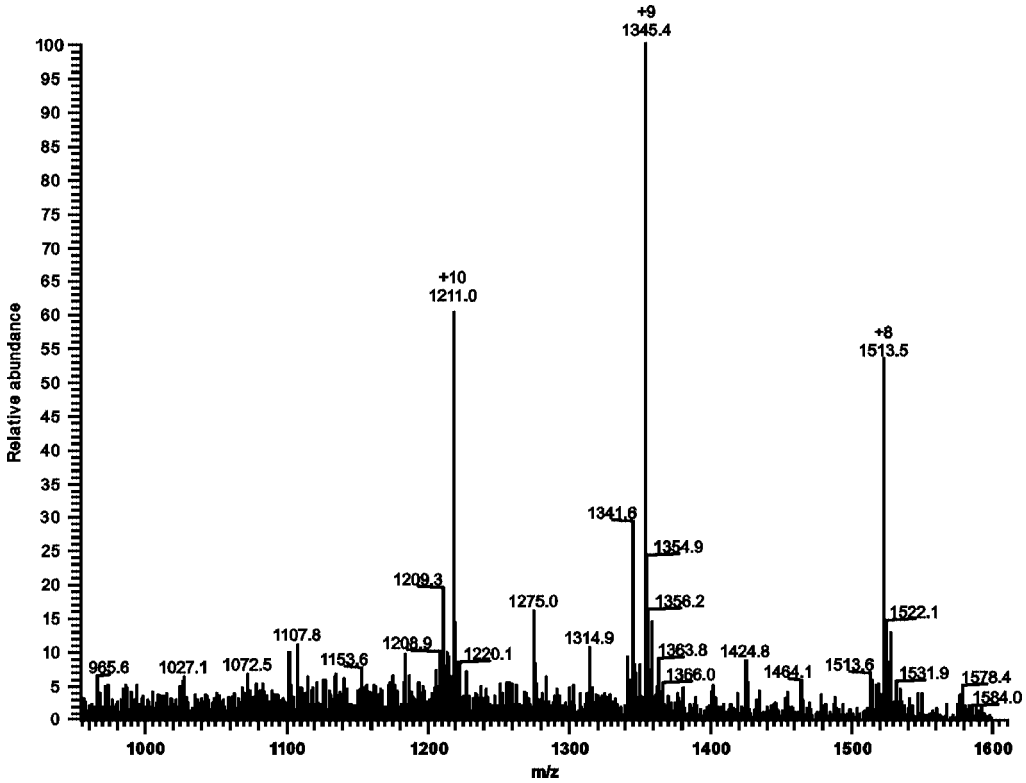

Figure 5. Mass spectra of the purified ACE_C. Calculation of MW using the three peaks M+10, M+9 and M+8 with the appropriate algorithms verifies the correct expression of the ACE_C peptide.

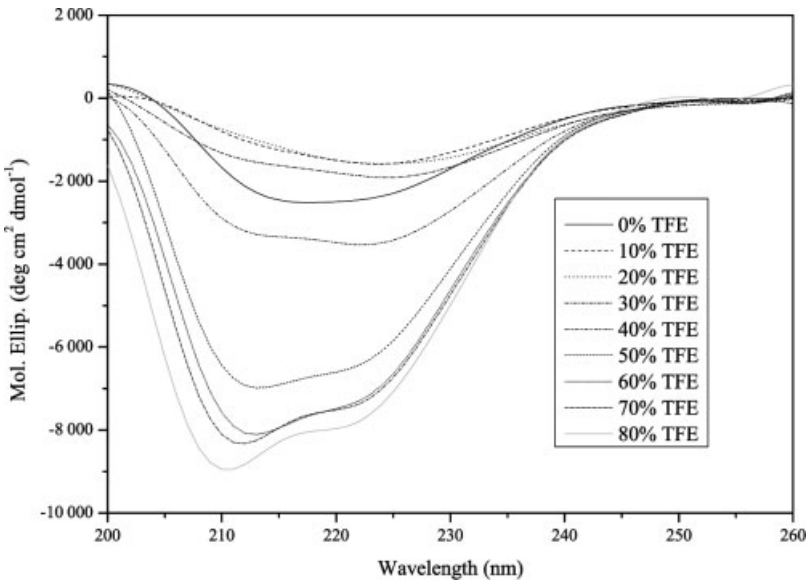

Figure 6. CD spectra of ACE_C protein fragment. Several TFE concentrations were tested while the concentration of the peptide was 1 mg/ml.

tested TFE concentration, was 41.1%, which is not much different from that found at 70% TFE. This evidence suggests that at 70% of TFE the molecule has already acquired its conformation. In contrast, when the ACE_C protein was diluted in solutions with lower concentrations of TFE, the alpha-helical content was much lower, while the coiled coil content increased up to of 40%. This finding implies that the ACE_C peptide did not have any conformation in the absence or in low concentration of TFE (Figure 4, Table 1).

The beta-strand percentage calculated by CD was higher than the one predicted by the PSIPRED prediction server and the one founded by Natesh and co-workers [11]. The maximum and the minimum values of the secondary structural features of the beta-strand and the coiled-coil are manifested at similar wavelengths. This could cause a serious difficulty in distinguishing between the 'beta-structure' characteristics and those of 'coiled-coil' even using the available software for the deconvolution of the CD spectrum. Therefore, the calculations of the 'beta-structure' and/or 'coiled

Journal of PeptideScience

Table 1. Analysis of CD spectra using the appropriate algorithms. The percentage of each structural motif is presented for each tested condition

	Peptide concentration = 1 mg/ml								
	0% TFE	10% TFE	20% TFE	30% TFE	40% TFE	50% TFE	60% TFE	70% TFE	80% TFE
Helix	14.8%	9.3%	9.4%	12.8%	15.6%	25.7%	35.0%	40.1%	41.1%
Antiparallel	12.1%	13.3%	13.4%	12.3%	12.1%	10.4%	9.4%	8.9%	9.2%
Parallel	12.1%	13.2%	13.4%	12.2%	12.0%	10.6%	9.6%	9.0%	9.1%
Beta-turn	20.4%	21.8%	21.8%	21.3%	20.2%	17.9%	17.3%	17.1%	16.7%
Random coil	40.6%	42.4%	42.0%	41.4%	40.1%	35.4%	28.7%	24.9%	23.9%

coil' might not be accurate [27–29]. Additionally, the necessary addition of DDT limits the bandwidth of the spectrum to above 200 nm. This limitation reduces the resolution of 'beta-structure' and 'coiled-coil' structural motifs.

Discussion

The first goal of this work was the overexpression in bacteria of the C-catalytic domain (Ala_{959} to Ser_{1066}) of human somatic angiotensin-I-converting enzyme. This protein fragment, termed ACE_C, represents a truncated form of the C-terminal domain of the somatic isotype of the enzyme.

A bacterial system for the overexpression of ACE_C protein fragment was developed despite the revealed toxicity of the gene product. The described method of overexpression and purification of the ACE C-catalytic domain has a good yield of purified protein (6 mg/l of culture). Overall, the system proved to be advantageous in speed and facility of purification. This procedure showed good reproducibility yield for ACE_C during purification. At this point it should be mentioned that ACE has specific glycosylated domains and sites that have been reported previously [30]. The ACE_C amino acid sequence does not include any of these. So, the inability for glycosylation of the bacterial expression system could not affect the structure of ACE_C.

The second goal of this research paper was the study of the secondary structure of the overexpressed protein fragment. The described procedure for the overexpression of the investigated protein fragment did not affect its final structure, as it had almost identical secondary structural features with those found in the crystal structure of testicular ACE by Natesh and co-workers [11] and with the one theoretically calculated in this study using the PSIPRED prediction server [14–16], for the corresponding region of sACE.

It is known [31] that TFE is an alpha-helix promoting and stabilization solvent and assist proteins to fold correctly. It reveals this ability only when the nature of the protein involves the formation of alpha-helix motifs. As shown by CD experiments the presence of TFE is absolutely necessary for the correct *in vitro* folding of the ACE_C protein fragment. This finding is completely logical considering on the one hand the calculated alpha-helix content of the ACE_C protein fragment and on the other hand the fact that only a fragment of a protein is expressed and not the whole protein. High concentration solutions of this fragment require the presence of TFE in order for it to achieve its complete solubilization. On the contrary at low concentration solutions of ACE_C the presence of TFE is not required for its solubilization but is necessary for correct folding.

ACE is a peptidyl dipeptidase that cleaves the C-terminal dipeptide from biologically inactive decapeptide angiotensin-I. This procedure results in the production of the vasoconstrictor octapeptide angiotensin-II. Additionally, ACE has the ability to cleave the c-terminal dipeptide from the vasodilator nonapeptide bradykinin. The enzymatic activity of the ACE_C fragment was tested. The fragment showed a negligible enzymatic activity. This does not necessarily mean that the structure of the enzyme is disturbed. The structure of testicular ACE by Natesh and co-workers [11] has revealed that the two chloride ions which are necessary for the catalytic activity have as ligands several amino acid residues and water molecules. These amino acid ligands are not included in the sequence of ACE_C. Additionally, for the enzymatic activity of the enzyme the glycosylated domains have been proved to be necessary [32]. These two facts, in combination with the necessity of TFE for the correct folding of ACE_C (TFE is an alchohol so it acts as a dehydrating agent), probably lead to the lack of significant components for the demonstration of catalytic activity. So, the non-existence of a catalytic activity is an expected result but it cannot support the fact that the structure of the produced protein fragment is disturbed. The interaction between ACE_C and its substrates may take place and because of the absence of the critical agents for the catalytic activity it might be unmodified. If this is the case (which is going to be elucidated in the future) the study of this interaction will probably be more reliable than any other using inactive substrate analogues or inhibitors.

Taking into consideration all the above-mentioned findings it can be concluded that an effective system for producing large amounts of pure ACE_C protein fragment and its variants has been developed.

The described procedure can be used for a thorough study of ACE_C protein fragmentt structure. This can be achieved by producing an isotopically labelled ACE_C protein fragment and studying its structure in solution by NMR spectroscopy as well as the interactions between this protein fragment and its substrates and/or its inhibitors. Thus information for the catalytic mechanism of the enzyme can be provided and used for the rational design of new selective and more effective inhibitors.

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