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Expression, purification, and physicochemical characterization of the *N*-terminal active site of human angiotensin-I converting enzyme

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Abstract: We have cloned, over expressed, and purified one of the two catalytic domains (residues Ala^{361} to Gly^{468}, ACE-N) of human somatic angiotensin-I converting enzyme in *Escherichia coli.* This construct represents the *N*-catalytic domain including the two binding motifs and the 23 amino acid spacers as well as some amino acid residues before and after the motifs that might help in correct conformation. The overexpressed protein was exclusively localized to insoluble inclusion bodies. Inclusion bodies were solubilized in an 8-M urea buffer. Purification was carried out by differential centrifugation and gel filtration chromatography under denaturing conditions. About 12 mg of ACE-N peptide per liter of bacterial culture was obtained. The integrity of recombinant protein domain was confirmed by ESI/MS. Structural analysis using CD spectroscopy has shown that, in the presence of TFE, the ACE-N protein fragment has taken a conformation, which is consistent with the one found in testis ACE by X-ray crystallography. This purification procedure enables the production of an isotopically labeled protein fragment for structural studying in solution by NMR spectroscopy. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: angiotensin-I converting enzyme; ACE; Renin-angiotensin system; recombinant protein; zinc metallopeptidase

INTRODUCTION

Angiotensin-I converting enzyme (ACE) or kininase II is a type-I membrane–anchored dipeptidyl-carboxypeptidase distributed throughout the body [1–3]. The enzyme was originally named for its ability to convert angiotensin-I to angiotensin-II, which is a highly potent vasoconstrictor, [4] or to inactivate bradykinin, a vasodilatory peptide [5,6]. These two major activities render ACE [essential for blood pressure regulation and electrolyte homeostasis] through the renin–angiotensin–aldosterone system. ACE is a *gluzincin* dipeptidyl-carboxypeptidase I [7–9], of which thermolysin is considered the prototypical member [9].

There are two isoforms of ACE that are transcribed from the same gene in a tissue-specific manner. In somatic tissues, it exists as a glycoprotein composed of a single, large polypeptide chain of 1306 amino acids, whereas in sperm cells it exists as a lower-molecular-mass glycoform of 732 amino acids. The somatic form consists of two homologous domains (*N* and *C* domains), each one containing an active site with a conserved HEXXH zinc-binding motif [10], where the two histidines are zinc ligands, with a glutamate 24 residue downstream forming the third ligand [11]. The two domains differ in their substrate specificities, inhibitor and chloride activation profiles, and physiological functions [12]. There are two *N*-domain-specific substrates: the peptide *N*-acetyl-seryl-aspartyl-lysyl-proline, which regulates hematopoietic stem cells differentiation and proliferation; and the bradykinin-potentiating peptide angiotensin-(1–7) [13]. On the other hand, the active sites of both domains catalyze the hydrolysis of angiotensin-I and bradykinin with similar efficiency. Testis ACE is identical to the *C*-terminal half of somatic ACE, except for a unique 36-residue sequence constituting its *N*-terminus part [14].

The research for more selective inhibitors has been proved difficult owing to the lack of a three-dimensional structure of the enzyme. Although site-directed mutagenesis experiments and conformational studies through crystallography (testis ACE) have provided information about the structure and function of ACE [15–18], the rational design of domain-selective inhibitors requires more detailed information about the active site and the interaction of inhibitors with the various subsites, necessitating a high-resolution structure of the protein. Extensive conformational studies in solution via 3D NMR are necessary for the rational design of ACE inhibitors. Here we report, for the first time, the overexpression in bacteria, and purification of a 108 aa peptide, which corresponds to

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an extended domain of the human somatic *N*-terminal active site of ACE (Ala^{361}-Gly^{468}).

MATERIALS AND METHODS

Cloning of the *N*-Catalytic Domain of Somatic ACE into an Expression Vector

An expression vector, containing nucleotide sequences encoding the *N*-terminal catalytic domain of human somatic ACE (Ala^{361}-Gly^{468}), was constructed. The c DNA was generated by polymerase chain reaction (PCR) from a c DNA clone using two pairs of synthetic oligonucleotides as primers, each pair for each expression vector. The forward primer of the first pair contained additional nucleotides encoding a *Bam*HI restriction site, which introduce the insert in frame with the GST leader peptide of pGEX4T-1 expression vector. The reverse primer contained a stop codon and an *Eco*RI restriction site. The forward primer of the second pair contained additional nucleotides encoding an *Nde*I restriction site followed by the initiating ATG methionine. The reverse primer contained a stop codon and a *Bam*HI restriction site. PCR was performed, with either set of primers, for 35 cycles (95 °C, 1 min; 55 °C, 1 min; 72 °C, 30 s) using *Vent* DNA polymerase (New England Biolabs). The resulting PCR fragment, using the first set of primers, was subcloned into the *Bam*HI/*Eco*RI restriction sites of an *E. coli* expression vector, pGEX4T-1 (Amersham Pharmacia), and the resulting PCR fragment, using the second pair of primers, was subcloned into the *NdeI*/*Bam*HI restriction sites of an *E. coli* expression vector, pET3a (Novagen). The multiple cloning sites of both expression vectors are under the control of the strong T7 promoter, and protein expression occurs by the addition of IPTG. DNA sequencing was conducted to confirm the nucleotide sequence of the insert using the dideoxynucleotide chain termination method [19]. For expression of the recombinant protein, the resultant plasmids, pGEX4T-1-ACE-N and pET3a-ACE-N, were transformed into the *E. coli* strain ER2566 (New England Biolabs).

Protein Expression

The 108-residue *N*-catalytic domain of somatic ACE (ACE-N) was expressed in *E. coli* ER2566 cells transformed with the plasmids described above. Cells were cultured in baffled shake flasks at 37 °C in a Luria broth medium, supplemented with 100 μg/ml ampicillin. Protein expression was induced when the cells had grown to an OD_{600} value of 0.6–0.8 by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.3 mM 3 h after induction, cells were harvested by centrifugation at 2000 *g* for 20 min. The pelleted cells were washed twice with 20 ml of 20 mM tris, pH 8.0, 1 mM EDTA per liter 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% TritonX-100, 20 μM PhenylMethylSulfonylFluoride, and 1 μg/ml Leupeptin). After the addition of egg white lysozyme, at a final concentration of 10 μg/ml, the cell suspension was made 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 insoluble material was resuspended in 20 ml of buffer B (100 mM Tris pH 8.0, 200 mM NaCl, 8 M urea, 100 mM DTT). After incubation for 30 min at 4 °C, the insoluble material was removed by centrifugation at $14\,000 \times g$ for 1.5 h. The supernatant was clarified by passage through a 0.45-μm filter and loaded onto a HiLoad 16/60 Superdex 75 size exclusion column preequilibrated with 2 volumes of 6 M urea, 50 mM tris–HCl, and 150 mM NaCl, pH 8.0. The loaded column was washed with the same buffer. Eluted fractions (0.5 ml) were monitored by absorbency at 280 nm. Fractions containing denatured ACE (Ala^{361}-Gly^{468}) were pooled and reduced by the addition of 10 mM of DTT. The pooled sample was concentrated by centrifugation on a macrosep-3K-Omega – at the beginning – and on microsep-3K-Omega – at the end – centrifugal devices from Pall, to a volume of 0.5 ml. The sample was diluted 6 times by the addition of 10 mM of DTT, and the collection of the white insoluble material formed was carried out by centrifugation. The pellet was washed once with 10 mM of DTT and twice with ultra pure water and air-dried.

CD Spectroscopy

CD spectra were recorded at room temperature using a Jasco-J810 spectropolarimeter (Tokyo, Japan) equipped with a temperature-regulated sample chamber. Proteins were dissolved in 200 mM sodium chloride, 66% TFE, and 8 mM dithiothreitol (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 1.0 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.

Molecular Mass Determination

Molecular masses were determined by ESI-MS on an AQA Navigation Thermofinnigan.

RESULTS

Cloning and Expression of ACE-$N^{361-468}$

Our first attempt was to carry out an expression strategy using the pGEX4T-1 vector. Insertion of the *ACE-*$N^{361-468}$ gene at the downstream *BamHI* site resulted in translation of the insert fused to glutathione *S*-transferase (GST)-tag from *Schistosoma japonicum*. The affinity of GST with the glutathione allows affinity purification of the fusion precursor on a glutathione sepharose 4B column. The pGEX4T-1 vector contains a protease-recognition site for thrombin. This site can be used for the removal of the GST-tag from the desired protein.

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ACE-N$^{361-468}$ was efficiently overexpressed by the use of IPTG, in the form of inclusion bodies. The purification of the specific fused protein under denatured conditions is not possible because GST needs to be folded correctly to bind to gloutathione. This result prompted us to subclone the gene *ACE-N*$^{361-468}$ in pET-3a expression vector at the *NdeI-BamHI* restriction sites in order to achieve production of the protein with no tag. The sequence of the construct pET-3a-ACE-N was checked using the Sanger method [19] and found to be consistent with the one submitted by Soubrier and coworkers [10].

Preliminary experiments were performed to determine the optimal conditions for the ACE-N$^{361-468}$ expression under IPTG-inducible control of the T7 promoter of the pET-3a vector. Various conditions of temperature, IPTG concentration, time of induction, and optical density of the culture were tested. All the tests have shown that the ACE-N$^{361-468}$ has formed inclusion bodies. The yield was the same for all tested conditions (Table 1). Therefore, the conditions at which ACE$^{361-468}$ was expressed were, incubation at 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.

Table 1 Expression level of ACE-N at several conditions at 37 °C. The expression of peptide is not affected by these conditions. At lower temperatures, the expression is not affected (data not shown)

O.D.$_{550\ nm}$	IPTG conc. (mM)	Time of induction	Expression
0.4	0.25	1 h	+++
		2 h	+++
		3 h	+++
	0.5	1 h	+++
		2 h	+++
		3 h	+++
	0.75	1 h	+++
		2 h	+++
		3 h	+++
	1	1 h	+++
		2 h	+++
		3 h	+++
0.6	0.25	1 h	+++
		2 h	+++
		3 h	+++
	0.5	1 h	+++
		2 h	+++
		3 h	+++
	0.75	1 h	+++
		2 h	+++
		3 h	+++
	1	1 h	+++
		2 h	+++
		3 h	+++
0.8	0.25	1 h	+++
		2 h	+++
		3 h	+++
	0.5	1 h	+++
		2 h	+++
		3 h	+++
	0.75	1 h	+++
		2 h	+++
		3 h	+++
	1	1 h	+++
		2 h	+++
		3 h	+++
1	0.25	1 h	+++
		2 h	+++
		3 h	+++
	0.5	1 h	+++
		2 h	+++
		3 h	+++
	0.75	1 h	+++
		2 h	+++
		3 h	+++
	1	1 h	+++
		2 h	+++
		3 h	+++

Purification of ACE-N$^{361-468}$

The recombinant protein yielded approximately 12 mg/l of culture in a one-step chromatographic procedure. Denatured and reduced protein was loaded onto a *HiLoad 16/60 Superdex 75* column. Elution under denaturing conditions yielded two major peaks. To investigate which of the two peaks corresponds to ACE-N, samples from almost all fractions were collected and tested on SDS-PAGE. The electrophoresis has shown that the second peak contains ACE-N (Figure 1).

Two major bands are visible in Figure 1(b). The lower band corresponds to the monomer ACE-N peptide. The upper band is a result of the formation of the dimmer ACE-N peptide through a disulfide bond that bridged the cysteine residue present in the sequence, if the elution buffer lacks a reductive agent. This band is eliminated when DTT is added at a final concentration of 10 mM.

The fractions containing the ACE-N (29–36) were pooled together. The pooled fractions were concentrated by centrifugation using macrosep and microsep desalting filters. The concentrate was diluted 6 times and the resulting pellet collected by centrifugation. The sequence integrity of the recombinant protein was assessed by SDS-PAGE and ESI/MS techniques. The purified protein showed a single band of protein in Coomassie-stained SDS-PAGE (Figure 2). The molecular weight of the band was around 12 kDa, representing the molecular weight of ACE-N$^{361-468}$. The mass spectra of the purified ACE-N (Figure 3) demonstrated that it was successfully expressed and does not contain the *N*-terminal methionine (experimental MW = 12 419.4 ± 0.3 Da, theoretical MW = 12 419.2 Da).

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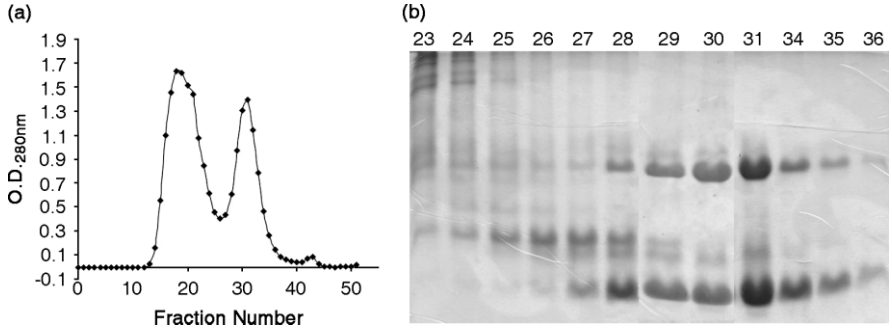

Figure 1 (a) Two major peaks are visible in the chromatogram of the gel filtration. (b) SDS-PAGE of fractions, 23–31,34,35,36, of gel filtration.

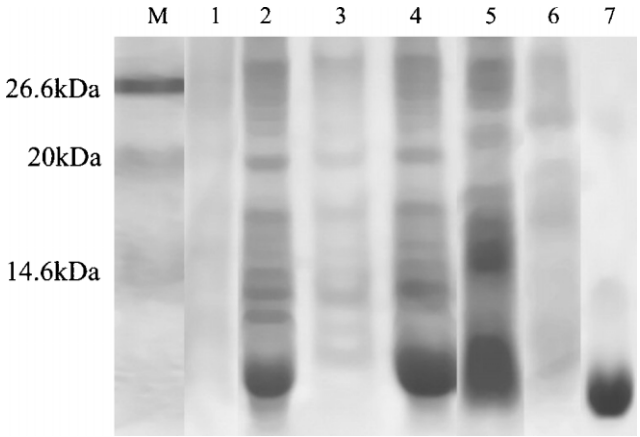

Figure 2 SDS-PAGE of the: (M) Marker, (1) uninduced cells, (2) induced cells, (3) supernatant of the centrifugation of the whole cell extract, (4) pellet of the centrifugation of the whole cell extract, (5) supernatant of the centrifugation of solubilized with 8 M urea inclusion bodies, (6) pellet of the centrifugation of solubilized with 8 M urea inclusion bodies, (7) purified ACE-N.

Solubilization – Secondary Structure Analysis

Solubilization of the ACE-N$^{361-468}$ peptide was achieved by diluting it, as described in the Materials and Methods section. Several conditions of refolding were tried out, without or with TFE, at several concentrations. These experiments had shown that the use of TFE is absolutely necessary for the solubilization of our protein.

The secondary structure of the isolated recombinant ACE-N$^{361-468}$ was assessed by CD analysis where the double ellipticity minimum was at approximately 208 and 222 nm, which is typical of an α-helical content (Figure 4(a)). The value for the α-helical content was 35.8%, calculated using the appropriate algorithms, when ACE-N was diluted in 66% TFE. This compares well with the proportion of α-helix found in the crystal structure of testis ACE, of the homologous domain [20], indicating that the backbone polypeptide chain of the purified protein had an almost identical conformation. Thus, the purification protocol did not disturb the overall structure of the protein. The value of α-helical content, when ACE-N was diluted in 100% TFE, was 41.1%, which is not much different from that found at 66% TFE. This evidence suggests that at 66% of TFE the molecule has already taken his conformation. In contrast, when the ACE-N protein was diluted in 33% TFE, the α-helical content was only 16.4%, while the coiled coil content was almost 50%. This result has shown that the ACE-N peptide did not adopt any conformation (Figure 4(b)), (Table 2).

Table 2 Analysis of CD spectra using the appropriate algorithms. The percentage of each structural motif is presented for each tested condition

	1 mg/ml peptide	2 mg/ml peptide		
	66% TFE	33% TFE	66%TFE	100% TFE
Helix	34.6%	16.6%	35.2%	41.5%
Antiparallel	7.6%	13.6%	7.5%	6.4%
Parallel	8.7%	16.9%	8.5%	7.1%
Beta-turn	16.5%	20.2%	16.4%	15.5%
Random Coil	32.4%	49.4%	32.0%	28.2%

DISCUSSION

This paper describes the expression of the *N*-catalytic domain (Ala^{361}-Gly^{468}) of human somatic ACE in *E. coli*. This construct, termed ACE-N$^{361-468}$, represents a truncated form of the enzyme without the carboxyterminal catalytic domain, which is almost identical with testis ACE.

We have successfully expressed and purified ACE-N$^{361-468}$ using the expression vector pET-3a in the *E. coli* ER2566 strain, without the presence of any tag. The reason for choosing this method was the insolubility of this domain. Our protein is a known zinc metallopeptidase [7–9], where the zinc ion binds to histidine residues. The His-tag has the ability to bind Zn ions. Therefore, the expression of our protein

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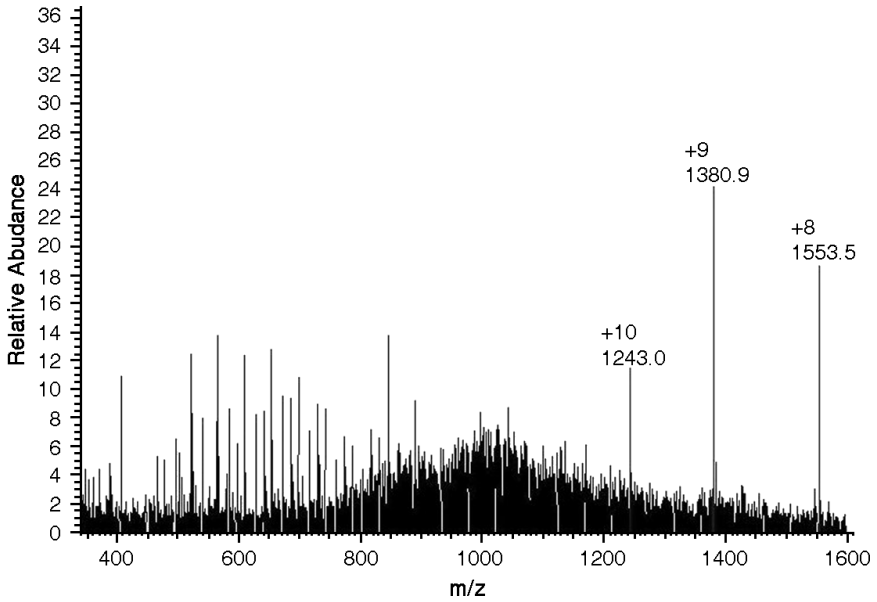

Figure 3 Mass spectra of the purified ACE-N. Calculation of MW using the three peaks M + 10, M + 9, and M + 8 with the appropriate algorithms verifies the correct expression of the ACE-N peptide.

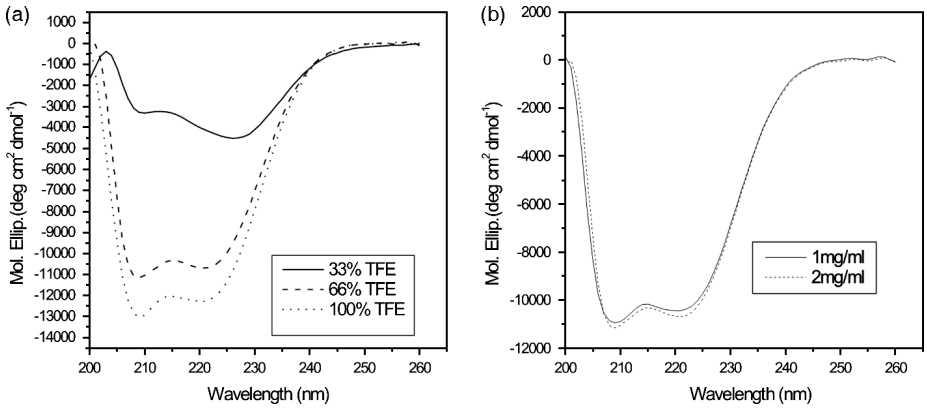

Figure 4 CD spectra of ACE-N peptide. (a) Several TFE concentrations were tested while the concentration of the peptide was 1 mg/ml. (b) The concentration of TFE was 66% while the peptidecs was 1 mg/ml and 2 mg/ml.

with the presence of His-tag is probably inappropriate because it might contribute to an incorrect folding of ACE-N$^{361-468}$.

Overall, the system proved to be advantageous in speed and facility of purification. This procedure showed good reproducibility for ACE-N$^{361-468}$ during purification. The *in vitro* folded recombinant protein had almost identical secondary and tertiary structural features compared to the homologous domain of testis ACE with a high content in α-helix secondary structure motif. Thus, we have developed an effective system for producing large amounts of pure ACE-N$^{361-468}$ and variants of the protein. This improvement was largely due to increased yields of expression.

Our method for purifying the angiotensin-I converting enzyme *N*-catalytic domain directly has significant advantages in terms of both the high yield of protein purified (12 mg/l of culture) and the amenability of a bacterial expression system to scale up for structural studies. Despite the fact that the structure of testis ACE has been resolved with the use of X-ray crystallography (in complex with some inhibitors) [20,21], the NMR spectroscopy study may be an alternative method for solving its structure in solution in the presence and in the absence of its substrate and its inhibitors. This kind of analysis will probably clearly present the exact catalytic mechanism of converting the rather inactive decapeptide angiotensin-I to the vasopressor octapeptide angiotensin-II. For this purpose, a bacterial expression system will be ideally suitable for the production of isotopically labeled protein. Determination of the tertiary structure of *N*- and *C*-catalytic domains of angiotensin-I converting enzyme would aid in the design of new selective inhibitors that might effectively control blood pressure.

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