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A new approach for purification of the catalytic site of the angiotensin-conversion enzyme, N-domain, mediated by the ELP-Intein system

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ABSTRACT

Angiotensin-converting enzyme I (ACE) is a key part of the renin-angiotensin system. Its main function is to regulate blood pressure and the balance of salts in the body. Somatic ACE has two domains, N-C-, each of which has a catalytic site that exhibits 60%sequence identity. The N-domain has a specific action in the hydrolysis of beta-amyloid bodies and angiotensin (1–7), which activates the MAS receptor and triggers anti-thrombotic and anti-inflammatory actions. Our goal was to obtain the catalytic site Ala^{361} to Gly^{468} of the N domain region, $csACE_N$, without needing purification by chromatography. We employed a method that uses an Elastin-like Polypeptide (ELP) and Intein sequences linked to the peptide of interest. The more differential for obtaining the pure peptide was the cultivation temperatures in the synthesis of $ELPcsACE_N$ at 37 °C, with a significant increase in expression. In the purification by ELP precipitation, we recorded the highest efficiency in the concentrations of 0.57 M and 0.8 M of ammonium sulfate buffer. Intein autocleavage study allows removal of the ELP sequence at acidic pH, with the buffers MES and Tris-HCl The present study defined the best conditions for obtaining pure $csACE_N$ that the literature has not yet described for peptides. Obtaining pure $csACE_N$ aims at future studies for therapeutic use in hypertension, Alzheimer's, and oncology.

1. Introduction

The risk of public health problems that affect the world are cardiovascular, pulmonary, and dementia diseases (WHO, 2020). The common denominator of these diseases is the renin-angiotensin system (RAS). In this system, the angiotensin-converting enzyme 1 (ACE1) participates in blood pressure regulation, brain protection, cell proliferation, hematopoietic stem cell formation, and other regulatory processes (Guang, Phillips, Jiang, & Milani, 2012). ACE1 is a membrane-bound and zinc-dependent dipeptidase, this catalyzes the conversion of the potent vasopressor angiotensin I (Ang I) to angiotensin II (Ang II) and inactivates Bradykinin (Anthony et al., 2010).

There are two isoforms of human ACE1 in the body, the somatic ACE (sACE) present in somatic cells and the testicular ACE (tACE) present in male germinal cells (Guang et al., 2012). The sACE isoform has two domains, N- and C-, which share 60% similar sequences. A more detailed analysis shows that these regions are composed of HEMGH and EAIGD sequences that bind zinc ions to facilitate catalytic activity (Fig. 1A) (Anthony et al., 2010).

The two domains differ in chloride-ion activation profiles, hydrolysis rates of the peptide such as Ang I and Bradykinin, and sensitivities to various inhibitors (Guang et al., 2012). The N domain is typically associated with the metabolism and hydrolysis of specific biologically active peptides, as a regulator of cardiac fibroblast proliferation and collagen synthesis; hydrolysis of goralatide, which inhibits hematopoietic stem cell proliferation. The hydrolysis that is still under study is the hydrolysis of amyloid beta-peptide (Aβ), characterized by Aβ aggregation and Alzheimer's disease; and angiotensin (1–7), which activates the MAS receptor triggering antithrombotic and anti-inflammatory actions (Elisseeva & Kugaevskaya, 2009; Guang et al., 2012; Lubbe, Cozier, Oosthuizen, Acharya, & Sturrock, 2020).

The use of peptides for drugs or bioactive agents to control

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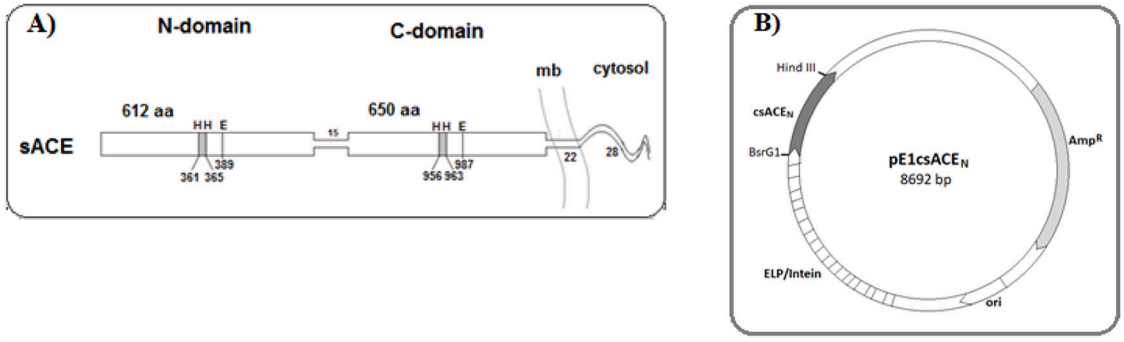
hypertension can fatally inhibit the action of the N-domain and its protective brain and oncolytic action. They can also increase blood thrombosis and inflammation in acute diseases. Synthetic processes for obtaining peptides require reagents in low pH precipitation, such as trifluoroacetic acid (TFA) or trifluoroethanol (TFE), resulting in loss of peptide activity. (Spyranti et al., 2010; Vamvakas, Leondiadis, Pairas, Manes, & Cordopatis, 2007; Vamvakas, Leondiadis, Pairas, Manes, & Cordopatis, 2009).

A bacterial system is a good option for producing active peptides, especially if combined with an innovative strategy. Elastin-like Polypeptide (ELP) linked to Intein sequences is an efficient system for the purification of recombinant protein without the use of chromatography



C)

Homo sapiens angiotensin I converting enzyme (ACE), transcript variant 1, mRNA
Sequence ID: ref|NM_000789.3|Length: 4969Number of Matches: 2
Related Information. Map Viewer-aligned genomic context
Range 1: 1147 to 1437 GenBankGraphics Next Match Previous Match First Match
Alignment statistics for match #1 **Identities** 289/291(99%)
Features:

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Query  7     CTTCAGGATCAAGCAGTGCACACGGGTCACGATGGACCAGCTCTCCACAGTGCACCATGA  65
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1147  CTTCAGGATCAAGCAGTGCACACGGGTCACGATGGACCAGCTCTCCACAGTGCACCATGA  1206

Query  66    GATGGGCCATATACAGTACTACCTGCAGTACAAGGATCTGCCTGTCTCCCTGCGTCGGGG  125
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Query  126   GGCCAACCCCGGCTTCCATGAGGCCATTGGGGACGTGCTGGCGCTCTCGGTCTCCACTCC  185
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Query  186   TGAACATCTGCACAAAATCGGCCTGCTGGACCGTGTCACCAATGACACGGAAAGTGACAT  245
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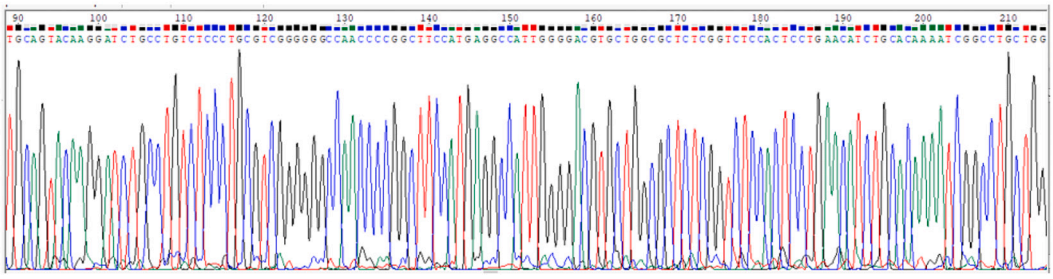

Fig. 1. A) Schematic of the primary structural domains of somatic ACE (sACE). The cytoplasmatic region spans 28 aa, the C-terminal membrane anchor consists of 22 aa, and the locations of the active-site zinc-binding motifs are indicated by HEXXH and E (modified from various authors, and the sequences numbers were obtained from Acharya, Sturrock, Riordan, and Ehlers (2003). Abbreviatures: cytosol, cytoplasm; mb, membrane; aa, amino acid; H, histidine; E, glutamate; X, any amino acid. B) Esquematic representation of pE1csACE$_N$ vector. Abbreviations: Amp^R, ampicillin resistance gene; ori, replication origin; ELP/Intein, cDNA sequence of Elastin Polypeptide/Intein; pE1csACE$_N$, pE1 vector with csACE$_N$ cDNA sequence; BsrG1 and Hind III restriction enzymes. (https://www.addgene.org/browse/sequence/141742/).
C) Analysis of csACE$_N$ cDNA by sequencing. Programs used Blast (BLAST: Basic Local Alignment Search Tool (nih.gov)) and Chromas. (http://technelysium.com.au/wp/chromas/)

(Lahiry, Fan, Stimple, Raith, & Wood, 2018; Shi, Meng, & Wood, 2013). Inverse Transition Cycling (ITC) is a process that inverting the sample temperature and adding specific salt separates the ELP from bacterial contaminants. Changing the pH of the sample buffer has allowed autocleavage of the Intein sequence to release the fused target peptide (Lahiry et al., 2018). The ELP is separated from the protein of interest by new ITC precipitation and pure form (Coolbaugh, Shakalli Tang, & Wood, 2017; Fan, Miozzi, Stimple, Han, & Wood, 2018; Hassouneh, Christensen, & Chilkoti, 2010).

Our objective was to study the best expression conditions of the catalytic site Ala^{361} to Gli^{468} of human sACE linked to ELP / Intein; purification of this by precipitation of the ELP sequence; and Intein autocleavage to obtain the pure catalytic site. Not many studies have used this strategy for peptide sequences so far, and they have only used proteins length than 50 kDa.

2. Material and methods

2.1. Design and construction of the pE1csACE$_N$ vector

Fig. 1B shows the strategy used to construct the pE1csACE$_N$ vector, the cDNA for csACE$_N$, with the 380 bp sequence, was inserted into the pE1 vector (kindly provided by Dr. David W. Wood, Chemical and Biomolecular Engineering, The Ohio State University, EUA). The cDNA amplification used the forward primer containing the last four bases of the C-terminal of Intein (CAAC), which provides efficient cloning, as described by Coolbaugh et al. (2017). In cloning, this construct used the restriction enzymes BsrG1 and Hind III. Thus, the cDNA sequence for the catalytic site followed the sequence for the ELP/Intein, giving rise to the recombinant protein ELPcsACE$_N$ (Fig. 1B).

2.2. Expression conditions of pE1csACE$_N$ vector

The transformed *E. coli* BL21(DE3) strain (Invitrogen, USA) containing the pE1csACE$_N$ vector was grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with ampicillin (100 μg/mL) at 37 °C, while shaking at 180 rpm overnight. This inoculum was diluted 1:200 into Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 2.31 g/L KH_2PO_4, 12.54 g/L K_2HPO_4) prewarmed at 37 °C expression media, supplemented with ampicillin (100 μg/mL). This culture was shaken at 140 rpm until the optical density of the culture reached 0.4–0.8 A_{600}. From this absorbance, the expression of ELPcsACE$_N$ used two protocols:

a) A culture was incubated at 20 °C in a shaking water bath at 140 RPM for 20 min. After incubation, the culture received 0.1 mM isopropyl-β-D-thiogalactoside (IPTG, Sigma, São Paulo, Brazil) for expression induction and cultured at 16 °C at 140 rpm for 20–24 h. After expression, culture was chilled at 4 °C for 30 min, and this was harvested by centrifugation at 5000 *g* for 10 min. The cell pellet was resuspended in 10% of the volume of the original culture of TE buffer (20 mM Tris-HCl, 2 mM EDTA, pH 8.5) (Coolbaugh et al., 2017).
b) Another culture was induced with 0.1 mM IPTG and cultivated at 37 °C at 150 rpm for 20–24 h. After expression, culture was harvested by centrifugation at 5000 *g* for 10 min at 20 °C. The cell pellet was resuspended in ice-cold TE buffer, in a volume calculated as the final culture volume x A_{600} measurement at the end of the expression/the ratio of 12.5 (Pereira et al., 2014).

Both cultures had negative controls without IPTG. All samples were stored at −20 °C (Pereira et al., 2014).

2.3. Isolation of the ELPcsACE$_N$

Culture pellets from both protocols were resuspended in TE buffer, sonicated five times for 30 s on the ice, and centrifuged at 9500 *g* for 10 min at 4 °C. The supernatant filtered (0.22 μm) was stored on ice. In all samples and all steps of processes was added 0.5 mM PMSF (**Phenylmethanesulfonylfluoride fluoride,** Sigma, São Paulo, Brazil).

ELPcsACE$_N$ was isolated by Inverse Transition Cycling (ITC), with the protocol described by Coolbaugh et al. (2017), in which two precipitations by ITC used ammonium sulfate buffer with a final concentration of 0.8 M, the first incubation for 20 min and the second for 10 min and all incubated at 150 rpm at 20 °C. Then, all samples were centrifuged at 9500 *g* for 20 min at 20 °C.

Another protocol used in the first ITC precipitation was 0.57 M, following the incubation and centrifugation conditions described above.

2.4. ELP/Intein cleavage by acid pH

The self-cleavage of the Intein sequence used four different buffers with concentrations of 20 mM: sodium phosphate pH 6.0, sodium cacodylate pH 6.2, MES pH 6.3, and Tris-HCl pH 6.8 (Sigma, São Paulo, Brazil). Each of the four samples was incubated for 20–24 h at 20 °C and 150 rpm. After incubation, ELP/Intein was precipitated by adding 0.8 M ammonium sulfate buffer and incubated for 20 min at 20 °C and 150 rpm. Then, all samples were centrifuged at 9500 *g* for 10 min at 20 °C, and supernatants with pure csACE$_N$ were stored at −20 °C. Analyzes of the pellets were by resuspension in ice-cold TE buffer.

2.5. SDS-PAGE and dot blotting analyses

The study analyzed induced and non-induced cultures by SDS-PAGE and Dot blotting and samples from each step of the purification process. Samples were analyzed under reduced conditions by denaturing 12% polyacrylamide gel and staining with Coomassie Blue G250 (Sigma, São Paulo, Brazil) (Carvalho, Ricci, & Affonso, 2014).

For Dot blotting, samples were transferred to a nitrocellulose membrane by vacuum suction. Then, the membrane was incubated with monoclonal anti-ACE, mouse antiserum (ab75762, Abcam, Nova Analítica, SP, Br) at a 1:800 dilution (Carvalho et al., 2014). The membrane was washed with PBS + M buffer (137 mM NaCl, 2.6 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, pH 8.0 + 5% Milk.). Next, it was added polyclonal horseradish-peroxidase-conjugated anti-mouse IgG, goat antiserum, at a 1:2000 dilution (ab20571, Abcam, Nova Analítica, SP, Br). Lastly, the membrane was washed with PBS.

Images were obtained by chemiluminescence (ECL- Advance Western Blotting Detection Kit, GE, Thermofisher, USA) using the UVITEC photo documenter System (Cambridge, United Kingdom).

3. Results

3.1. Construction of the pE1csACE$_N$ vector

The csACE$_N$ cDNA used in the construction came from the work of Sampaio and Sant'Ana (2015), in which the authors amplified from human sACE cDNA. The specific primers amplified the cDNA from the catalytic site, as described by Coolbaugh et al. (2017), for cloning in the pE1 vector. We used sequencing to confirm this cloning product, pE1csACE$_N$ vector (Fig. 1C).

Sequencing analysis showed a shift from C to T base (C, cytosine; T, thiamine), Fig. 1C, shaded bases. However, the original amino acid Proline (cc_) remains the same.

3.2. Expression of the ELPcsACE$_N$ protein

Bacteria transformed with the pE1csACE$_N$ vector produced 16 clones. Analyzes five clones evaluated for ELPcsACE$_N$ expression in the conditions a), Mat. Meths 2.2, for 23 h at 16 °C. The experiments for the protein of interest used the clone with the highest expression.

Analyzes of ELPcsACE$_N$ synthesis with the two protocols:

In protocol a), the expression followed the protocol for the ELP tag

described by Coolbaugh et al. (2017), and the synthesis was low (Fig. 2A, lanes 3, 4, and 5). In these lanes, the intensity of ELPcsACE$_N$ of ~78 kDa is weak relative to bacterial proteins.

In protocol b), the culture activation temperature was 37 °C resulting in the amount of ELPsACE$_N$ being similar to bacterial protein bands (Fig. 2B, lanes 1 to 3).

Dot blotting analysis confirmed that ELPcsACE$_N$ expression was higher with protocol b), with activation at 37 °C (Fig. 2C).

3.3. Isolation of ELPcsACE$_N$ by ITC method

All further experiments followed protocol b) since the cultivation at 37 °C expressed more protein of interest. The analysis by dot blotting of the discarded supernatant samples, in Fig. 2D, dots 2 to 5, in the ITC isolation process. In the first wash, the more loss observed was with the concentration of 0.8 M (dot 3). The loss in the second wash of 0.8 M was similar in the two samples, 4 and 5 dots, which originated from samples 2 and 3, respectively. Dot 1, Fig. 2D, was the filtered supernatant of the sonicated culture that exceeded the technique's sensitivity.

3.4. Intein autocleavage at acid pH

To the obtention of pure csACE$_N$, were evaluated four buffers with four different pHs, 6.0, 6.2, 6.3, and 6.8. Fig. 3A shows the analysis of the autocleavage product and resuspended pellets. The pure catalytic site (lanes 1, 3, 5, and 7) was present in all final samples.

The immunological characterization of pure csACE$_N$, by dot blotting, is in Fig. 3B. The catalytic site was present in all final samples. However, in samples with Tris-HCl and MES buffers, the autocleavage of the Intein sequence was more efficient (Fig. 3B, dots 2 and 4, respectively).

4. Discussion

The value of peptides as therapeutic agents is a reality today, whether as anti-inflammatories, antimicrobials, in neurological disorders, anti-hypertensives, and vaccines; in a number as expressive as the dysfunctions that affect the human body (Keservani, Sharma, & Jarouliya, 2015). This broad applicability is due to the peculiarity of the structure of the peptides, as they represent a unique class of pharmaceutical compounds. These small molecules can be targeted to specific tissues or organs by intrinsic compatibility (lock-and-key) or linked to nanoparticles. (Lau & Dunn, 2018). In our laboratory, we have already expressed the catalytic sites of c-Jun (transcription factor AP1) and ACE1 (catalytic site of the C domain) using the pET23 vector. We obtained low yields in the purification process by affinity and molecular exclusion chromatography. (Elias et al., 2017; Silva, 2014). In the present study, a new strategy for the purification process used the ELP sequence linked to the peptide sequence of interest and the Intein autocleavage to obtain the pure peptide. There are no studies in the literature using this strategy to obtain peptides to our knowledge.

Studies on protein expression with ELP tag have used low temperature in culture during protein synthesis not to aggregate ELP molecules (Coolbaugh et al., 2017; Rauscher & Pomès, 2017; Wu, Mee, Califano, Banki, & Wood, 2006). The direct consequences of temperature reduction include partial elimination of heat shock proteases (induced under conditions of overexpression); inhibition of replication, transcription, and translation; and decrease in the growth rate of *E. coli* (Sørensen & Mortensen, 2005).

The cultivation at low temperatures expresses ELP in its soluble form, but the production can be below (Zhou et al., 2019). In the case of ELPsACE$_N$ expression, expression at a reduced temperature of 16 °C was low compared to protein expression at 37 °C. (Fig. 2 A and B). Dot blot analysis confirmed these results (Fig. 2C).

The increase in temperature can provide ELP aggregation since they are repetitive polypeptide sequences with hydrophobic domains that have a thermodynamic reverse phase transition. At high temperatures, the intermolecular contacts between non-polar regions result in beta structure and aggregation (Zhou et al., 2019). With the possibility of forming aggregates and inclusion bodies, the bacterial culture was lysed in an ice-cold buffer and then centrifuged at 4 °C. This low temperature

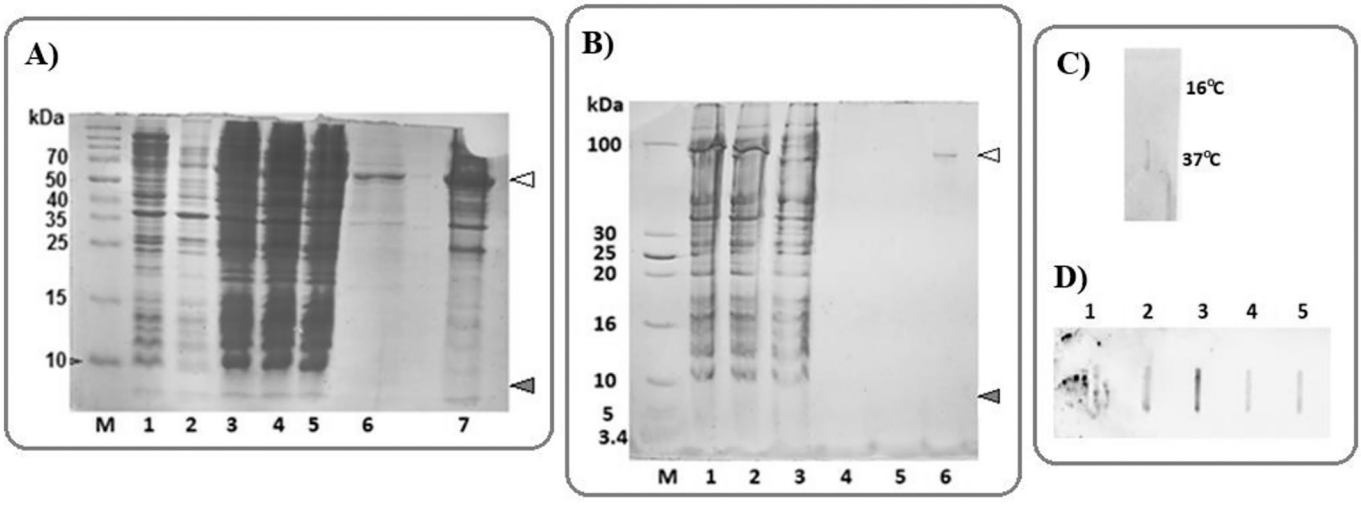

Fig. 2. Analysis of ELPcsACE$_N$ samples that were expressed in cultures of *E. coli* transformed with vector pE1csACE$_N$, by SDS-PAGE and Dot blotting (◁, ELPcsACE$_N$; and ◀, csACE$_N$).

A) Protocol a): Lane M, protein molecular weight; lane 1, culture without activation; lane 2, pellet resuspended of the sonicated sample after centrifugation (discarded); lane 3, activated culture; lane 4, supernatant after sonication; lane 5, sample 4 filtered; lane 6, final sample after self-cleavage; and lane 7, pellet discarded with ELP tag. All samples have a volume of 10 uL, with the exception sample 6 with 18 uL.

B) Protocol b). Lane M, protein molecular weight; lane 1, activated culture and sonicated; lane 2, supernatant of the sonicated sample after centrifugation; lane 3, sample 2 filtered; lane 4, sample of the first wash of the ITC process (discarded); lane 5, final sample after cleavage; and lane 6, pellet discarded with ELP tag. All samples have a volume of 10 uL.

C) Dot blotting analysis ELPcsACE$_N$ of protein expression in bacteria cultures grown at 16 °C and 37 °C. Samples volume 20 uL.

D) Analysis of ELPcsACE$_N$ samples of the supernatants of the ITC process washes. Dot 1, ELPcsACE$_N$ sample filtered before washing; dots 2 and 3, first wash with 0.57 M and 0.8 M of ammonium sulfate discarded, respectively; dots 4 and 5, second wash with 0.8 M of ammonium sulfate discarded.

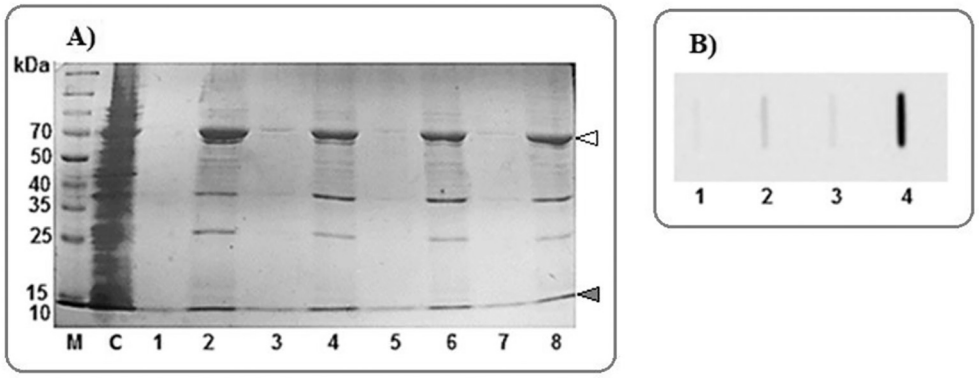

Fig. 3. Analysis of csACE$_N$ samples cleaved with four different buffers and pHs (◁, ELPcsACE$_N$; and ◀, csACE$_N$).
A) SDS-PAGE: Lane M, protein molecular weight; lane C, activated culture; lanes 1, 3, 5 and 7 pure csACE$_N$ with cleaving in 20 mM buffers: sodium phosphate pH 6.0, sodium cacodylate pH 6.2, MES pH 6.3 and Tris-HCl pH 6.8, respectively; lanes 2, 4, 6 and 8, pellet resuspended after cleavage and centrifugation in the respective sequence of the four buffers described for the purified samples.
B) Dot blotting of pure csACE$_N$ samples in cleavage buffers: dots 1–4, sodium cacodylate pH 6.2, Tris-HCl pH 6.8, sodium phosphate pH 6.0, MES pH 6.3, respectively.

allows the ELP to remain soluble, as the water molecules help maintain their soluble form (no defined structural conformation) (Christensen, Hassouneh, Trabbic-Carlson, & Chilkoti, 2013). Sonication under icy conditions resulted in soluble ELPcsACE$_N$ and almost no loss in the pellet.

The other change in the ITC process was a reduction in sample concentration. The volume used to resuspend the bacterial pellet in the protocol a) was 10% of the final culture volume, while in protocol b), there was an increase of 3.2-fold (32% of the final culture volume). The volume calculations using the absorbance values of the bacterial culture allow comparative analysis between samples inter and intra-experiments.

In the ITC precipitation process, a more dilute ELPcsACE$_N$ protein sample increases its aggregation and precipitation efficiency. This process is due to the basic principle of precipitation of proteins with salts, where the exposure of hydrophobic regions on the surface of the protein, by the removal of water molecules, allows the attraction between neighboring proteins; and a decrease in their solubility with consequent aggregation and precipitation (Doran, 2013). In the samples shown in Fig. 2, A, and B, there are significant differences in the initial concentration used for each culture dilution (lanes 3 and 1, respectively).

In protocol b), the samples were resuspended in standardized volume and concentration, which allowed for comparison between independent experiments. The addition of salt to reduce the temperature, in the ITC precipitation process, used ammonium sulfate and sodium chloride. The ELPcsACE$_N$ precipitation results using 2 M NaCl, as described by Hassouneh et al. (2010), were inefficient in the ITC process (data not shown). The precipitation with ammonium sulfates presented positive results in the two concentrations evaluated, in the first precipitation 0.57 M and the second 0.8 M.

The concern in choosing the cleavage buffer was related to pH. The pI for csACE$_N$ is 6.65, and the pI for ELPcsACE$_N$ is 6.87, both acidic pHs just like the pH for Intein self-cleavage. Inteins are proteins that occur naturally in organisms and are capable of post-translational self-excision - a process called protein splicing (Topilina & Mills, 2014; Zhou et al., 2019). The pH range used for cleavage was 6.0–6.8, and the best cleavage efficiencies were MES buffer with pH 6.3 and Tris-HCl buffer with pH 6.8. The Intein cleavage was efficient not only because of the adequate pH but also because of the cleanliness of the used sample, free from bacterial contaminants. The final sample of csACE$_N$ peptide analyzed by SDS-PAGE was pure (Fig. 3A, lanes 2 and 3), and the results obtained by dot blotting confirmed its immunological identity (Fig. 3B).

Here, expression studies of the csACE$_N$ catalytic site, a peptide of just over 8 kDa, were performed with an increase in culture temperature during peptide synthesis, change in the buffer concentration in the ITC and a pH test in the auto-cleavage of the sequence of Intein, obtaining promising results. Overall, the strategy for obtaining pure csACE$_N$ peptide proved to be technically accessible, fast, economical, and without the need for specialized equipment or reagents. This study defined the best conditions for obtaining pure csACE$_N$, a peptide not yet described by the technique, and with an appropriate concentration for future works of activity and structural characterization.

In conclusion, this work aimed to obtain the csACE$_N$ catalytic site, Ala^{361} - Gli^{468}, N-domain region of human ACE1. The best ITC precipitation conditions by ELPcsACE$_N$ and the Intein autocleavage to remove the ELP marker were defined, enabling this strategy in research on the use of therapeutic peptides for hypertension, Alzheimer's, and oncology.

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Declaration of Competing Interest

The authors declare no competing interests.

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