An ACE2, SARS-CoV-2 spike protein binding protein, -like enzyme isolated from food-related microorganisms

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Abstract

Angiotensin-converting enzyme 2 (ACE2) is a binding target of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein. An ACE2-like enzyme, such as bacterial M32-carboxypeptidase (M32-CAP), is assumed to be a potential therapeutic candidate for coronavirus disease 2019 (COVID-19). Here, we screened bacteria with an ACE2-like enzyme activity from Japanese fermented food and dietary products using the fluorogenic substrate for rapid screening. The strain showing the highest activity, *Enterobacter* sp. 200527-13, produced an enzyme with the same hydrolytic activity as ACE2 on Angiotensin II (Ang II). The enzymatic analysis using the heterologously-expressed enzyme in *E. coli* revealed that the enzyme catalyzes the same reaction with that of ACE2, Ang II hydrolysis to Ang 1-7 and phenylalanine. The gene sequence information showed that the enzyme belongs to the M32-CAP family. These results suggested that the selected enzyme, M32-CAP (EntCP), from *Enterobacter* sp. 200527-13 was identified as an ACE2-like enzyme.

Keywords: ACE2, *Enterobacter* sp., M32-carboxypeptidase, SARS-CoV-2.

Introduction

Driven by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, coronavirus disease 2019 (COVID-19) has caused major problems for healthcare systems globally. On October 27, 2022, global infections were reported to be over 626 million with the number of deaths reaching 6 million (WHO, 2022). These numbers will continue to increase due to the rapid mutation rate of the virus, challenging researchers to find a specific vaccine or antiviral treatment (Lei et al., 2020). SARS-CoV-2 associates with the human angiotensin-converting enzyme 2 (ACE2) to enter into cells. ACE2 is a carboxypeptidase that attacks Angiotensin II (Ang II), degrading it into Angiotensin 1-7 (Ang 1-7) in the renin-angiotensin system (RAS) (Romero et al., 2015). Together with its homologs (the angiotensin-converting enzyme, ACE), ACE2 maintains homeostasis in cardiorenal activity and cell protection and proliferation. ACE2 is abundantly expressed in the lungs and intestinal epithelium of the esophagus, ileum, and colon epithelial cells (Salamanna et al., 2020). ACE activity produces Ang II by cleaving Ang I, with Ang II blood accumulation being associated with hypertension, accelerated thrombosis, and endothelial injury (Samavati et al, 2020). Therefore, a balance between ACE and ACE2 must be maintained.

SARS-CoV-2 has a surface-anchored spike (S) glycoprotein composed of the S1 domain responsible for human receptor binding through the receptor-binding domain (RBD) site and the S2 domain that mediates membrane fusion (Chen et al., 2021). The interaction of RBD with the human ACE2 drives ACE2 loss of function, leading to the accumulation of Ang II in the lung tissue and inflammatory lesions in the respiratory tree (alveolar wall thickening, edema, infiltration of inflammatory cells, and bleeding) (Verdecchia et al., 2020). Acute respiratory distress syndrome (ARDS) is commonly found in COVID-19 patients. Additionally, the accumulation of Ang II results in cardiac injury as a consequence of myocardial inflammation, oxidative stress, and myocyte apoptosis (Ravichandran et al., 2021). Hence, there is a need for a new approach to handle the accumulation of Ang II in patients with COVID-19 and those not.

The recombinant human ACE2 (rhACE2) protein is currently under clinical trials to treat patients with ARDS. However, rhACE2 may be unfavorable for drug development and the medical economy because it is glycosylated. Consequently, rhACE2 expression in mammalian or insect cell lines is time consuming and expensive. Another alternative for therapeutic development is the use of engineered bacterial proteins. One promising protein is the bacterial M32 family of carboxypeptidases (M32-CAP) containing the HEXXH motif within its active site. The structural analysis and crystal structure of three microbial strains—M32-CAP from *Thermus aquaticus* (TaqCP), *Pyrococcus furiosus* (PfuCP), and *Bacillus subtilis* (BsuCP)—have been reported in

61 the MEROPS database (http://merops.sanger.ac.uk/) (Minato et al., 2020; Arndt et al., 2002; Lee et al., 2009).
62 Among the three different microbes, only BsuCP is a protein structurally related to human ACE2 with the position
63 of the key amino acid constituting the catalytic site and substrate-binding region (Arg273/348, His345/234,
64 His505/408, and Tyr515/420 in ACE2/BsuCP), which are almost identical between the two proteins, suggesting
65 that BsuCP may have a similar substrate preference to ACE2. A study by Minato et al. (2020) found that M32-
66 CAP from *Paenibacillus* sp. B38 (B38-CAP) has ACE2-like activity in mammals under physiological conditions,
67 which could improve hypertension and heart failure. This work was continued by Yamaguchi et al. (2021) and
68 resulted in the relief of lung edema in SARS-CoV-2-infected hamsters. This uncovers a new path to improve the
69 prognosis of similarly infected humans.
70 Food-related microorganisms have been utilized for many years, the most impressive of which is in
71 health-inducing products, such as traditional fermented food. Food-fermenting microbes can improve nutritional
72 value and induce immune responses during pathogen infections (Surono, 2016; Muhialdin et al, 2021). A study
73 conducted by Lee et al. (2006) used *Lactobacillus casei* as a host vector that expresses the SARS-CoV spike on
74 the extracellular membrane surface, showing neutralizing antibody activity in mice on day 21 after immunization.
75 Hence, food-related microorganisms are promised to be potent candidates providing ACE2-like enzyme useful
76 for COVID-19 therapy, which can inhibit human ACE2 and SARS-CoV-2 binding.
77 Here, we show that M32-CAP can cleave human ACE2 substrates. This protein, defined as EntCP, is
78 found within the genome of the *Enterobacter* sp. isolated from garlic. EntCP shows hydrolytic activity toward the
79 fluorogenic substrate 2-(methylamino)benzoyl (Nma)-His-Pro-Lys[2,4-dinitrophenyl (Dnp)] and Ang II.
80
81 **Methods**
82 **Materials**
83 Angiotensin II was purchased from Bachem AG (Bubendorf, Switzerland); ACE2 was purchased from
84 Sino Biological, Inc. (Hong Kong, China); and Nma-His-Pro-Lys(Dnp) was purchased from the Peptide Institute,
85 Inc. (Osaka, Japan).
86
87 **ACE2-like enzyme bacteria screening**
88 Different types of Japanese fermented food and dietary products were collected, of which 12 were
89 commercially purchased from different convenience stores. We successfully isolated 61 bacteria from all of them
90 by growing them in a MRS medium (Becton, Dickinson and Company, New Jersey, United States of America).

The MRS medium is composed of 1 % (w/v) proteose peptone No. 3, 1 % (w/v) beef extract, 0.5 % (w/v) yeast extract, 2 % (w/v) dextrose, 0.1 % (w/v) polysorbate 80, 0.2 % (w/v) ammonium citrate, 0.5 % (w/v) sodium acetate, 0.01 % (w/v) magnesium sulfate, 0.005 % (w/v) manganese sulfate, and 0.2 % (w/v) dipotassium phosphate. We also added 16 additional bacterial isolates, including *Periweissella beninensis* JCM 18047, *Weissella minor* JCM 1168, *W. diestrammenae* JCM 18559, *W. kandleri* JCM 5817, *W. koreensis* JCM 11263, *W. oryzae* JCM 18191, *W. halotolerans* JCM 1114, *W. viridescens* JCM 1174, *W. hellenica* NBRC 15553, *W. cibaria* NBRC 106073, *W. soli* NBRC 106074, *W. confuse* NBRC 106469, *W. paramesenteroides* NBRC 109620, *W. thailandensis* NBRC 109621, *W. cryptocercid* NBRC 113066, and *W. muntiaci* NBRC 113537. Two bacterial strains, namely, *Bacillus subtilis* NBRC 3022 and *B. amyloliquefasciens* NBRC 13719, served as positive controls for M32-CAP gene content. All bacterial strains used in this study were grown in 500 mL MRS medium. Each bacterial cell was harvested via centrifugation at 12,000 × *g* (8,000 rpm) for 20 min at 4 °C using high-speed refrigerated centrifuge CR21N (Eppendorf Himac Technologies Co., Ltd., Ibaraki, Japan) equipped with R10A rotor. The supernatant was discarded, and the pellet was weighed to obtain a cell-free extract (CFE) and rehydrated using an assay buffer (pH 6.5) containing 50 mM 2-morpholinoethanesulfonic acid (MES), 300 mM NaCl, and 10 μM $ZnCl_2$ with volumes double that of the pellet weight. The pellet was placed in a suspension buffer fixed at 30–70 % volume of the multi-bead tube ST-0250 (Yasui Kikai, Osaka, Japan). YGB01 glass beads (Yasui Kikai) were added into a multi-bead tube containing cell suspension at a ratio of 1:1 (w/v), mixed by vortexing, and stored at 4 °C. A Multi-Beads Shocker instrument (Yasui Kikai) was used with the following setup: 2,500 rpm speed, 60 s on time, 60 s off time, and 3–6 cycles. The sample was then centrifuged at 20,400 × *g* (15,000 rpm) at 4 °C for 5 min using high speed refrigerated micro centrifuge MX-307 (Tomy Seiko Co., Ltd., Tokyo, Japan) to collect CFE for further investigation. Rapid ACE2-like enzyme screening was performed using Nma-His-Pro-Lys(Dnp). All CFE was concentrated by centrifugation at 5,280 rpm at 4 °C for 10 min by an ultrafiltration membrane 10,000 MWCO. Concentrated CFE was then dissolved in assay buffer at a ratio of 1:3 (v/v), and CFE hydrolysis activity was determined in dark microtiter plates at room temperature. The reaction mixture contained 40 μL assay buffer and 10 μL Nma-His-Pro-Lys(Dnp) (final concentration: 0.1 mM). The reaction was initiated by adding 150 μL CFE or 250 μg/mL ACE2 (final concentration: 1.3 μg/mL) as a control, so that the total reaction volume was 200 μL. The emission and excitation wavelength of the fluorescence were 440 nm and 340 nm, respectively. Fluorescence intensity was recorded every 15 min for 4 h of incubation.

Determination of bacterial CFE activity toward Ang II

Bacterial CFE exhibiting hydrolytic activity against Nma-His-Pro-Lys(Dnp) were further evaluated using Ang II as a native substrate of human ACE2. To determine the hydrolysis products produced by this enzyme within the CFE, peak detection and elution from the HPLC column were used. The reaction was performed at room temperature in a 100-µL total volume mixture containing 40 µL assay buffer, 10 µL of 2 mM Ang II (final concentration: 0.1 mM), and 50 µL CFE or 250 µg/mL ACE2. The mixture was incubated for 5 and 30 min, and the reaction was quenched by the addition of 0.5 M EDTA. A reaction sample (10 µL) was injected into the HPLC system. Substrate and peptides produced were resolved by a YMC-Pack ODS-A 4.6 × 300-mm (AA12S05-3046WT) column using 10 % acetonitrile (v/v) with 0.1 % acetic acid (v/v) and 40 % acetonitrile (v/v) with 0.1 % acetic acid (v/v) as the mobile phases A and B, respectively. The gradient elution scheme was performed as follows: 0–10 min, 100 % of mobile phase A; 10–25 min, linear gradient from 0–100 % of mobile phase B; 25–35 min, 100 % of mobile phase B; 35–40 min, linear gradient from 100 % of mobile phase A; and a 0.8 mL/min flow rate. Both the substrate and peptide products were detected by measuring absorbance at 215 nm.

The hydrolytic activity of bacterial CFE against Ang II was also determined using LC-MS to detect any amino acid released during the enzymatic reaction based on its molecular weight. The LC-MS system was equipped with the same column and elution buffer. The gradient elution scheme was performed as follows: 0–40 min, 100 % of mobile phase A; 40–100 min, linear gradient from 0–100 % of mobile phase B; 100–140 min, 100 % of mobile phase B; 140–160 min, linear gradient from 100 % of mobile phase A; and 0.3 mL/min flow rate. Detection was performed using negative-mode electrospray ionization (ESI). The MS conditions were as follows: interface temperature, 350 °C; DL temperature, 250 °C; nebulizing gas flow 1.5 L/min; heat block temperature, 200 °C; and drying gas flow, 10 L/min.

Bacterial CFE protein purification

Protein purification was conducted to isolate the expected protein of interest within bacterial CFE using the ÄKTA explorer fast protein liquid chromatography system (Cytiva, Tokyo, Japan). The first purification step was done using HiPrep™ Q XL 16/10 column (Cytiva, Tokyo, Japan). The binding buffer was prepared by mixing 20 mM MES in ultrapure water at pH 6.5, whereas the elution buffer consisted of 1 M NaCl and 20 mM MES in ultrapure water at pH 6.5. A fraction of 190 µL was collected and assayed for Nma-His-Pro-Lys(Dnp) hydrolytic activity. The highest activity of the protein that exhibited ACE2-like enzyme activity was pooled and subjected to a second purification step using a Superdex™ 200 Increase 10/300 GL column (Cytiva). Equilibration and elution were performed at room temperature using 150 mM MES in ultrapure water at a pH of 6.5. Approximately 50 µL

of each fraction was rapidly screened using Nma-His-Pro-Lys(Dnp). The fractions exhibiting ACE2-like enzyme activity were analyzed by sodium dodecyl–sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a subsequent transfer to a PVDF membrane (for electroblotting using HorizeBLOT). The transferred protein was loaded into the protein sequencer to determine the amino acid sequences through Edman degradation. The amino acid sequences were read and searched in the NCBI database using BLASTP.

Draft genome sequence of the selected strain 200527-13

The genome was purified from the selected strain 200527-13 by DNeasy® Blood & Tissue Kit (Qiagen, Venlo, Netherlands). Next-generation sequencing of the strain was performed in the Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). The assembled genome was annotated using the DDBJ Fast Annotation and Submission Tool (DFAST). The accession numbers are BSCM01000001–BSCM01000033.

Recombinant ACE2-like enzyme

The bacterial isolate, expected to have ACE2-like enzyme gene content based on protein sequencing results, underwent genomic DNA extraction. The DNA of gene interest within the bacterial genomic DNA was isolated by PCR for cloning purposes. Double digestion of the pET21b plasmid was carried out using a mixture (50 µL) of 10 U Nde I (Takara), 10 U Xho I, pET21b DNA ≤ 1 µg, and 1 × H buffer. The reaction mixture was then incubated for 1 h at 37 °C. The forward and reverse primers used to amplify EntCP gene were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). The forward primer sequence consisted of GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG AGC GAA AAC AGC CAC TA. The reverse primer used in this study was CGG ATC TCA GTG GTG GTG GTG GTG GTG CTC CAG ATA ACG GGA CGT CAA AT. EntCP was amplified by PCR using primers and Tks Gflex DNA Polymerase (Takara Bio Inc., Shiga, Japan). The digested pET21b plasmid and PCR products were purified by electrophoresis using agarose gel and FastGene Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo, Japan). The purified PCR product was inserted into pET21b by incubating the reaction mixture of 4 µL purified digested pET21b, 4 µL purified PCR product, and 2 µL 5 × In-Fusion HD Enzyme Premix (Takara Bio Inc., Shiga, Japan) for 15 min at 50 °C. The resulting plasmid, pET21b-EntCP, was transformed into *Escherichia coli* DH5α competent cells as a cloning host before being transformed into the expression host, *E. coli* Rosetta 2 (DE3), which supplies tRNA for rare codons. Transformants were selected by growing them on a Luria-Bertani medium containing ampicillin (50 µm/mL) and chloramphenicol (25 µg/mL). EntCP gene expression within transformant cells was induced by adding isopropyl

β-D-thiogalactopyranoside (final concentration of 1 mM) to the culture media. The cells were harvested by centrifugation and disrupted to obtain CFE. Hydrolytic activity was confirmed using Nma-His-Pro-Lys(Dnp), as previously mentioned. Protein expression was visualized using SDS-PAGE before purification. Protein purification was performed using the HisTrap™ column (Cytiva) for affinity chromatography. The CFE was equilibrated with 50 mM imidazole and 20 mM MES in 500 mL ultrapure water at pH 6.5. Proteins were eluted using 250 mM imidazole and 20 mM MES in ultrapure water at pH 6.5. The purified protein fraction was then subjected to LC-MS analysis.

Accession numbers

The accession number of the ACE2-like enzyme (EntCP) from the *Enterobacter* sp. 200527-13 was registered as LC739013. Accession numbers of genomes of the *Enterobacter* sp. 200527-13 were registered as BSCM01000001-BSCM01000033.

Results

Screening of ACE2-like enzyme in food-related microorganisms

Nma-His-Pro-Lys(Dnp) was used for rapid screening to find an ACE2-like enzyme, as previously described by Takahashi et al. (2015). Among all bacterial cells tested, only five isolates (200527-1, 200527-5, 200527-14, 200527-13, and 191211-2) exhibited Nma-His-Pro-Lys(Dnp)-hydrolyzing activity (Figure 1). Among these five, the isolate 200527-13 (isolated from garlic) showed the highest hydrolysis activity, as shown in Figure 1. The CFE of both *B. cereus* NBRC 3022 and *B. amyloliquefasciens* NBRC 13719 as a positive control showed much lower hydrolytic activity than the others. To confirm the presence of an ACE2-like enzyme within the strain 200527-13, its CFE was challenged with the 2 mM Ang II peptide as a native human ACE2 substrate. The reaction products generated after incubating Ang II with the CFE of the strain 200527-13 were compared to those generated by the three different positive controls. After 5 h of incubation of 2 mM Ang II with 250 μg/mL ACE2, the peak of Ang 1-7 appeared, and the peak of Ang II disappeared (Figure S1(A)). On the other hand, Ang 1-7 was not detected in the reaction with the CFE of either *B. cereus* or *B. amyloliquefasciens*, although no Ang II peak was detected (Figure S1(A)). The CFE of the strain 200527-13 can digest the Ang II, however, no Ang 1-7 peptide peak was observed (Figure S1(B)). To investigate the mode of action of bacterial enzymes showing different results with that of human ACE2, LC-MS analysis and successive enzyme purification were performed.

Identification of an ACE2-like enzyme in the strain 200527-13

The hydrolysis of Ang II peptide (DRVYIHPF, *m/z* 1046.5 on positive mode ($[M + H]^+$) LC-MS analysis) by human ACE2 resulted in generations of Ang 1-7 (DRVYIHP, *m/z* 899.5) and phenylalanine (F, *m/z* 166.1) (Figure S2(A)). The CFE of *B. subtilis* NBRC 3022 also showed hydrolytic activity similar to that of human ACE2, as reported by Minato et al. (2020) (Figure S2(B)). However, with the CFE of the strain 200527-13 no accumulation of Ang 1-7 was observed; indicating that the CFE contained Ang 1-7 degrading enzymes together with an ACE2-like enzyme. Then, we proceeded to enzyme purification from the CFE of the strain 200527-13.

Through two column chromatography steps using HiPrep™ Q XL 16/10 column and Superdex 200 Increase 10/300 GL column with the activity assay using the fluorogenic substrate, the protein was purified by approximately 1,000-fold with 6 % yield from the CFE (Table 1) . Based on the comparison of protein profile on SDS-PAGE and the activity profile of each fraction obtained by column chromatography, the protein with a molecular weight of 50 kDa was assumed to be the target protein (Figure 2(A-B)). Therefore, the 50 kDa protein in the fraction No. 12 was applied to Edman degradation for protein sequencing. We detected a NH_2-terminal amino acid sequence of SENSXYQQLTXTXQRLSXX. By comparing the amino acid sequence to the database BlastP NCBI, the protein was found to show 82.4 % identity with M32-CAP from the *Enterobacter* sp. The whole gene sequence of the purified enzyme, EntCP (accession number: LC739013), was obtained from the genome data of the *Enterobacter* sp. 200527-13 (BSCM01000001-BSCM01000033).

Classification of EntCP in comparison with proteins in the M32-CAP family

To verify the protein family that EntCP belongs to, we conducted sequence alignment analysis against other proteins. Through multiple sequence alignment using the MUSCLE 3.8 tool, EntCP was noted to have the signature zinc-binding motif HEXXH of the M32-CAP family (in solid line square) in Figure 3(A)). This motif is conserved among selected proteins, including carboxypeptidase from *Paenibacillus* sp. B38 (B38-CAP, NCBI LC406946), *Fervidobacterium islandicum* (FisCP, PDB 5E3X, and NCBI WP_033190981.1), *Pyrococcus furiosus* (PfuCP, PDB 1K9X, and NCBI WP_011011573.1), *Bacillus subtilis* (BsuCP, PDB 3HQ2, and NCBI WP_004398513.1), *Deinococcus radiodurans* (DnrCP, PDB 5GIV, and WP_010889049.1), and *Thermus thermophilus* (TthCP, PDB 3HOA, and NCBI WP_011174078.1), while the DsbA (PDB 1BED, and NCBI WP_002050636.1) from *Vibrio cholerae* serves as an outgroup. In addition, EntCP revealed to have five other conserved motifs, including HPF, DXRXT, HESQ, IRXXAD, and GXXQDXHW (in dashed line square) that further define its protein family. Regarding the phylogenetic tree, EntCP appears in a separate branch from the

other homologous proteins (Figure 3(B)). EntCP has 75 % and 74 % similarity to B38-CAP and BsuCP, respectively.

Hydrolysis activity of recombinant EntCP

Recombinant EntCP was successfully expressed in *E. coli* Rosetta 2 (DE3) (Figure 4(A)). The recombinant protein was purified from the crude CFE (Figure 4(B)). The purified recombinant EntCP showed the activity against the fluorogenic substrate with a value of 495×10^3 relative fluorescence units (RFU)·sec^{-1}·L·mg^{-1} protein. Ang 1-7 and phenylalanine were also produced during the incubation of the purified recombinant EntCP with Ang II, as revealed by LC-MS analysis (Figure 4(C)).

Discussion

The food-related microorganisms used here were chosen because of their greater ability to promote and maintain our health. This not only reduces intestinal inflammatory disease but also enhances the function of the innate immune system, specifically natural killer cells (NKC), which act as the first line of defense against bacterial or viral infections and tumor formation (Muhialdin et al., 2021). An ACE2-like enzyme within food-related microorganisms was screened based on the ability of each CFE to cleave Nma-His-Pro-Lys(Dnp). Dnp bound to the amino acid lysine serves as a fluorescence quencher for Nma. Once ACE2 recognizes and cleaves lysine-Dnp within the carboxy-terminal site, the fluorophore is released and detected by a spectrofluorometer, as illustrated in Figure 5(A-B) (Takahashi et al., 2015). Therefore, Nma-His-Pro-Lys(Dnp) was used as a prompt screening method here to determine the ACE2-like enzyme activity among several bacterial isolates. This was then confirmed by incubating the bacterial CFE candidate with Ang II. Among all the bacteria tested, the highest fluorogenic activity was found with the CFE of the strain 200527-13. The strain was considered to contain more than one protein involved in Ang II degradation. While, this study only focused on proteins that exhibit proteolytic activity similar to that of human ACE2. Through purification, we succeeded in isolating M32-CAP from the *Enterobacter* sp. and named it EntCP. This protein is quite similar to the one used in the study conducted by Minato et al. (2020), who described B38-CAP isolated from the *Paenibacillus* sp. B38 and BsuCP from *Bacillus subtilis* (Lee et al., 2009) by having the conserved motif shown in Figure 3(A). Our ACE2-like enzyme was isolated from the opportunistic pathogen *Enterobacter* sp. However, the *Enterobacter* sp. has also been exploited to produce fermented wheat flour, which improves the nutritional value of food (Manguntungi et al., 2020; Nagano et al., 2003).

Recombinant EntCP, with a molecular weight of 50 kDa, showed a branch close to BsuCP in the phylogenetic tree. Other M32-CAP including B38-CAP and ChtCP, have similar molecular weights (Minato et al., 2020; Fernandes et al., 2021). BsuCP was the first M32-CAP that was structurally similar to human ACE2, as elucidated by performing a superimposition. By having a similar structure, the positions of the substrate- and metal-binding residues match each other (Minato et al., 2020). Arg273, situated within the ACE2 active site, plays an important role in the formation of a salt bridge with a C-terminal substrate, while His505, His345, and Tyr515 are involved in the binding or activity of metal ions (Biswas et al., 2020). These residues are equivalent to Arg348, His408, His234, and Tyr420 in BsuCP (Lee et al., 2009). Sequence alignment revealed that all residues were conserved within EntCP at Arg346, His407, His234, and Tyr455. We also found the conservation of a signature metal-binding motif, namely, HEXXH, and another motif that is responsible for substrate binding or metal ion binding (Lee et al., 2006). Based on the conservation of key residues that constitute the protein structure of both EntCP and human ACE2, we suggest EntCP derived from food-related microorganisms as an alternative tool to develop human ACE2 "receptor mimics" inhibiting SARS-CoV-2 infection. In addition, this protein is promising in drug development to treat Ang II-related diseases.

Conclusions

Based on the hydrolysis activity against fluorogenic substrate and Ang II, the food-related bacterial strain, *Enterobacter* sp. 200527-13, was considered to contain more than one active enzyme involved in Ang II degradation. One of the most effective enzymes found was defined as EntCP, which has a molecular weight of 50 kDa (similar to those of BsuCP, B38-CAP, and ChtCP) and catalyzed the same hydrolytic reaction with ACE2 on Ang II, generating Ang 1-7 and phenylalanine. By having a similar key residue and protein structure with human ACE2, EntCP appears to be a potential therapeutic candidate against SARS-CoV-2 infection. It is predicted to not only block viral entry but also decrease Ang II levels in patients to attenuate the effects of disease progression.

Funding

This study was partially funded by the New Energy and Industrial Technology Development Organization (NEDO) (to J.O.) and the Ministry of Health and Welfare of Japan and Public/Private R&D Investment Strategic Expansion PrograM: PRISM (to J.O.).

Data availability

The nucleotide sequence data of EntCP is available with accession number LC739013. The genome data of the *Enterobacter* sp. 200527-13 is available with accession number BSCM01000001-BSCM01000033.

Author contribution

MT and JO conceived of and designed the study. INL and TM conducted experiments. INL, MT, and JO analyzed the data. INL, MT, and JO wrote the manuscript. All authors have read and approved the manuscript.

Disclosure statement

All authors declare that they have no conflicts of interest.

References

Arndt J, Hao B, Ramakrishnan V, et al. Crystal structure of a novel carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus. Structure* 2002;**10**(2):215–224. doi:10.1016/s0969-2126(02)00698-6

Biswas A, Bhattacharjee U, Chakrabarti AK, et al. Emergence of Novel Coronavirus and COVID-19: whether to stay or die out? *Crit Rev Microbiol* 2020;1–12. doi:10.1080/1040841X.2020.1739001

Chen F, Zhang Y, Li X, et al. The Impact of ACE2 Polymorphisms on COVID-19 Disease: Susceptibility, Severity, and Therapy. *Front Cell Infect Microbiol* 2021;**11**:753721. doi:10.3389/fcimb.2021.753721

Fernandes GC, Sierra EGM, Brear P, et al. From data mining of *Chitinophaga* sp. genome to enzyme discovery of a hyperthermophilic metallocarboxypeptidase. *Microorganisms* 2021;**9**(2):393 doi.org/10.3390/microorganisms9020393

Lee JS, Poo H, Han DP, et al. Mucosal immunization with surface-displayed severe acute respiratory syndrome coronavirus spike protein on *Lactobacillus casei* induces neutralizing antibodies in mice. *J Virol* 2006;**80**:2079–4087. doi:10.1128/JVI.80.8.4079–4087.2006

Lee M, Isaza C, White JD, et al. Insight into the substrate length restriction of M32 carboxypeptidases: Characterization of two distinct subfamilies. *Proteins* 2009;77(3):647–657. doi:10.1002/prot.22478

Lei C, Qian K, Li T, et al. Neutralization of SARS-CoV-2 spike pseudotyped virus by recombinant ACE-Ig. *Nat commun* 2020;**11**:2070. doi:10.1038/s41467-020-16048-4

Manguntungi B, Saputri DS, Afgani CA, et al. Biodiversity of Enterobacteriaceae on masin (fermented sauce) from Sumbawa, West Nusa Tenggara, Indonesia. *Biodiversitas* 2020;**21**(3):1001–1006. doi:10.13057/biodiv/d210320

Minato T, Nirasawa S, Sato T, et al. B38-CAP is a bacteria-derived ACE2-like enzyme that suppresses hypertension and cardiac dysfunction. *Nat commun* 2020;**11**:1058. doi:10.1038/s41467-020-14867-z

Muhialdin BJ, Zawawi N, Razis AFA, et al. Antiviral activity of fermented foods and their probiotics bacteria towards respiratory and alimentary tracts viruses. *Food control* 2021;**127**:108140. doi:10.1016/j.foodcont.2021.108140

Nagano H, Kasuya S, Shioji Z, Tamura A, Omori M, Iibuchi S, Arai M. Identification of microorganisms in traditional asian foods made with fermented wheat flour and their hypoallergenization. *Food Sci Technol Res* 2003;**9**(1),7–10. doi:10.3136/fstr.9.7

Ravichandran B, Grimm D, Kruger M, et al. SARS-CoV-2 and hypertension. *Physiol Rep* 2021;**9**:e14800. doi:10.14814/phy2.14800.

Romero CA, Orias M, Weir MR. Novel RAAS agonists and antagonists clinical applications and controversies. *Nat Rev Endocrinol* 2015;**11**,242–252. doi: 10.1038/nrendo.2015.6.

Salamanna F, Maglio M, Landini MP, et al. Body localization of ACE-2: On the trail of the keyhole of SARS-CoV-2. *Front Med* 2020;7. doi:10.3389/fmed.2020.594495.

Samavati L, Uhal BD ACE2, much more than just a receptor for SARS-CoV-2. *Front Cell Infect Microbiol* 2020;**10**:317. doi:10.3389/fcimb.2020.00317.

Surono IS. Ethnic Fermented Foods and Beverages of Indonesia. In: Tamang, J. (ed) Ethnic Fermented Foods and Alcoholic Beverages of Asia: Springer, 2016;341–382 doi:10.1007/978-81-322-2800-4_14.

Takahashi S, Yoshiya T, Yoshizawa-Kumagaye K, et al. Nicotinamine is a novel angiotensin-converting enzyme 2 inhibitor in soybean. *Biomed Res J* 2015;**36**(3):219–224. doi:10.2220/biomedres.36.219

Verdecchia P, Cavallini C, Spanevello A, et al. The pivotal link between ACE2 deficiency and SARS-CoV-2 infection. *Eur J Inter Med* 2020;**76**:14–20. doi:10.1016/j.ejim.2020.04.037.

WHO. WHO Coronavirus Disease (COVID19) Dashboard https://covid19.who.int/ (27 October 2022, date last accessed).

Yamaguchi T, Hoshizaki M, Minato T, et al. ACE2-like carboxypeptidase B38-CAP protects from SARS-CoV-2-induced lung injury. *Nat Commun* 2021;**12**:6791. doi:10.1038/s41467-021-27097-8

Figure legends

Figure 1. Screening results of hydrolysis activity of bacterial CFE against Nma-His-Pro-Lys(Dnp).

NBRC 3022 (*Bacillus subtilis*) and NBRC 13719 (*Bacillus amyloliquefasciens*) are positive controls. Each $\Delta RFU \cdot sec^{-1}$ during 4500 s and 15300 s was listed below. 200527-1, 0.021; 200527-5, 0.022; 200527-14, 0.054; 200527-13, 0.21; 191211-2, 0.038; NBRC 3022, 0.026; NBRC 13719, trace.

Figure 2. Enzyme purification from the CFE of ther strain 200527-13. SDS-PAGE analysis of the each fraction obtained by Superdex 200 Increase 10/300 GL column chromatography (A) and the hydrolysis activity of the each fraction toward the fluorogenic substrate (B).

Figure 3. Sequence analysis of EntCP. (A) Sequence alignment of EntCP and selected sequences of the M32-CAP family proteins retrieved from the PDB database. The conserved motif HEXXH is in solid line square, while others are in dashed line square. (B) Phylogenetic tree analysis by the maximum likelihood method. B38-CAP, BsuCP, PfuCP, FisCP, DnrCP, and TthCP are carboxypeptidase from *Paenibacillus* sp. B38, *Bacillus subtilis*, *Pyrococcus furiosus*, *Fervidobacterium islandicum*, *Deinococcus radiodurans*, and *Thermus thermophilus*, respectively. DsbA, disulfide oxidoreductase from *Vibrio cholerae*, serves as an outgroup.

Figure 4. Expression, purification, and characterization of the recombinant EntCP. (A) SDS-PAGE results of CFE of *E. coli* harboring empty plasmid or plasmid with EntCP gene. The recombinant EntCP is indicated by the arrows). (B) SDS-PAGE results of each fraction obtained by HisTrap™ column chromatography. (C) Ang II cleavage product of human ACE2 and purified recombinant EntCP detected by positive mode of the LC-MS system. Monoisotopic molecular weights of Ang II, Ang 1-7, and phenylalanine is 1045.5, 898.5, and 165.1, respectively.

Figure 5. ACE2 and EntCP catalyzing reactions . (A) Nma-His-Pro-Lys(Dnp) or (B) Ang II as the substrate.

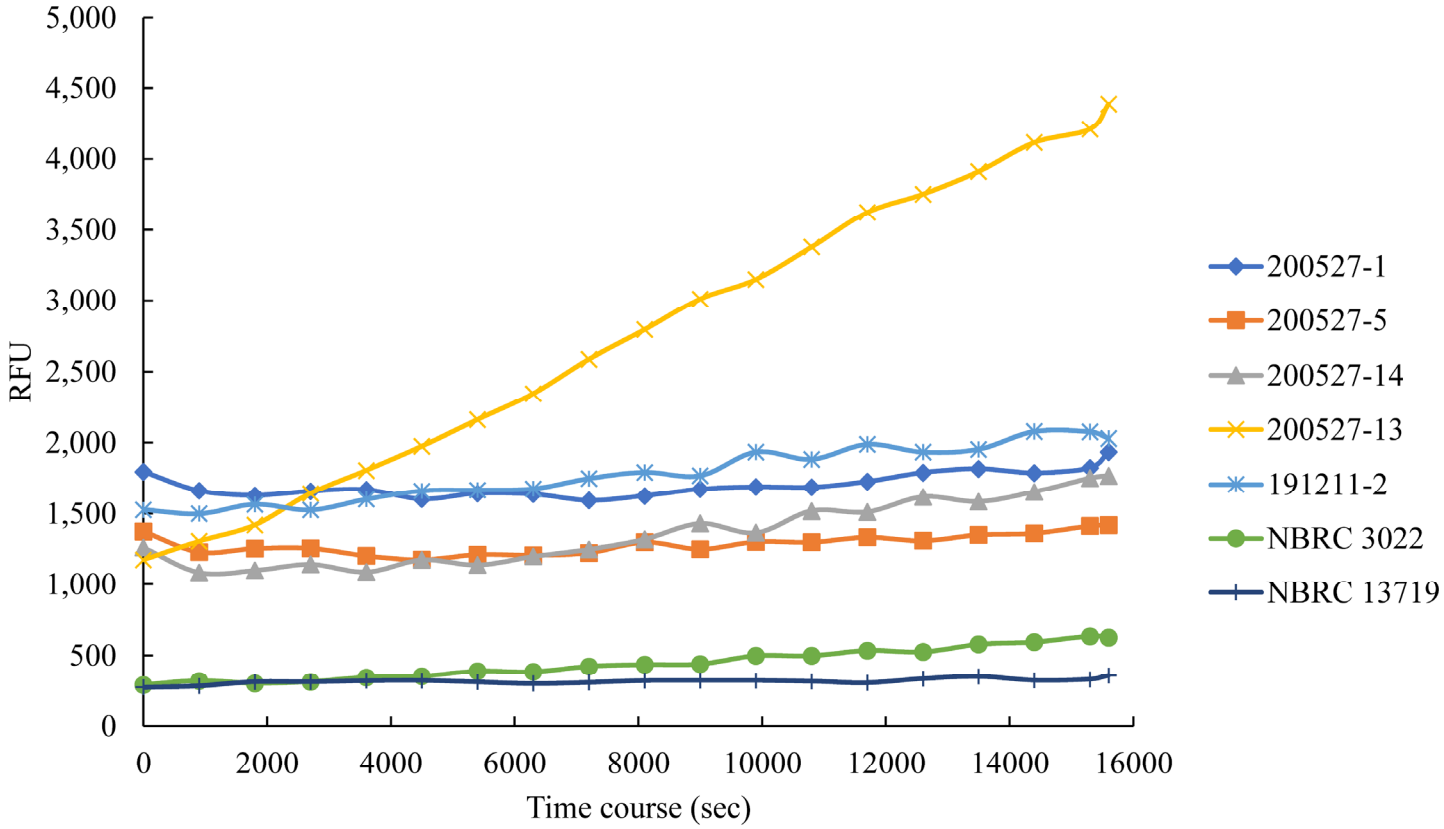
5,000
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
RFU
0
2000
4000
6000
8000
10000
12000
14000
16000
Time course (sec)
200527-1
200527-5
200527-14
200527-13
191211-2
NBRC 3022
NBRC 13719

Figure 1

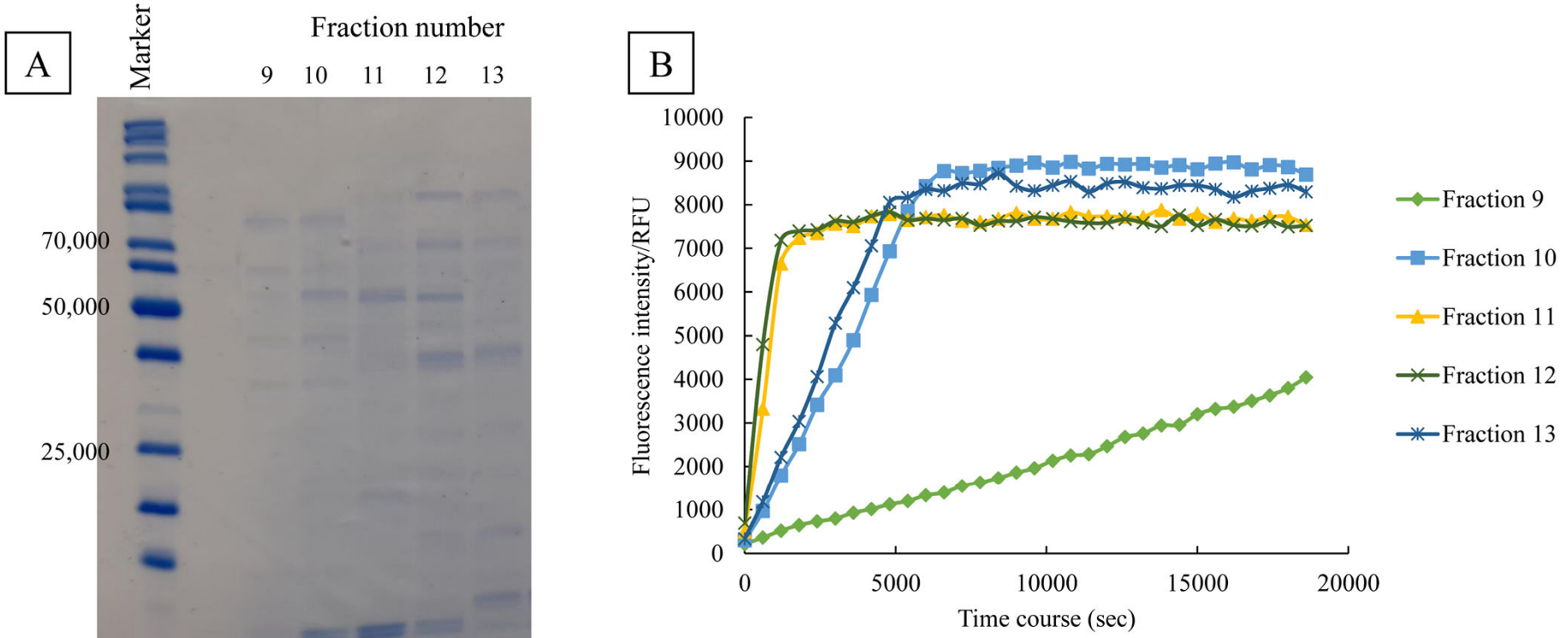
A
Marker
Fraction number
9
10
11
12
13
70,000
50,000
25,000
B
Fluorescence intensity/RFU
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5000
10000
15000
20000
Time course (sec)
Fraction 9
Fraction 10
Fraction 11
Fraction 12
Fraction 13

Figure 2

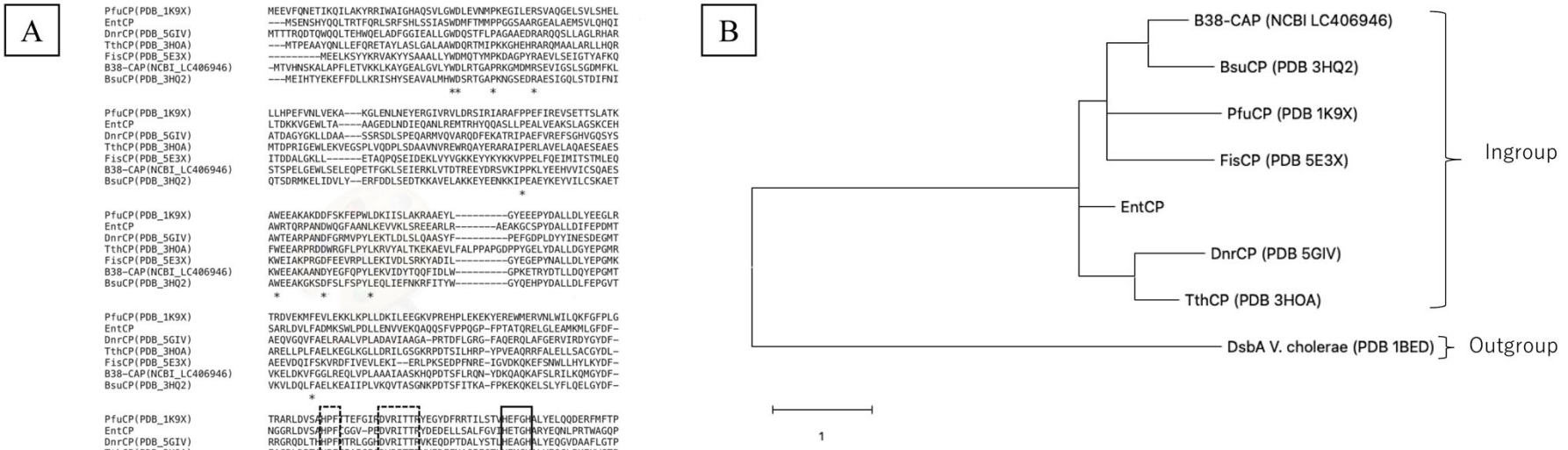
A
B
B38-CAP (NCBI LC406946)
BsuCP (PDB 3HQ2)
PfuCP (PDB 1K9X)
FisCP (PDB 5E3X)
EntCP
DnrCP (PDB 5GIV)
TthCP (PDB 3HOA)
DsbA V. cholerae (PDB 1BED)
Ingroup
Outgroup
1

Figure 3

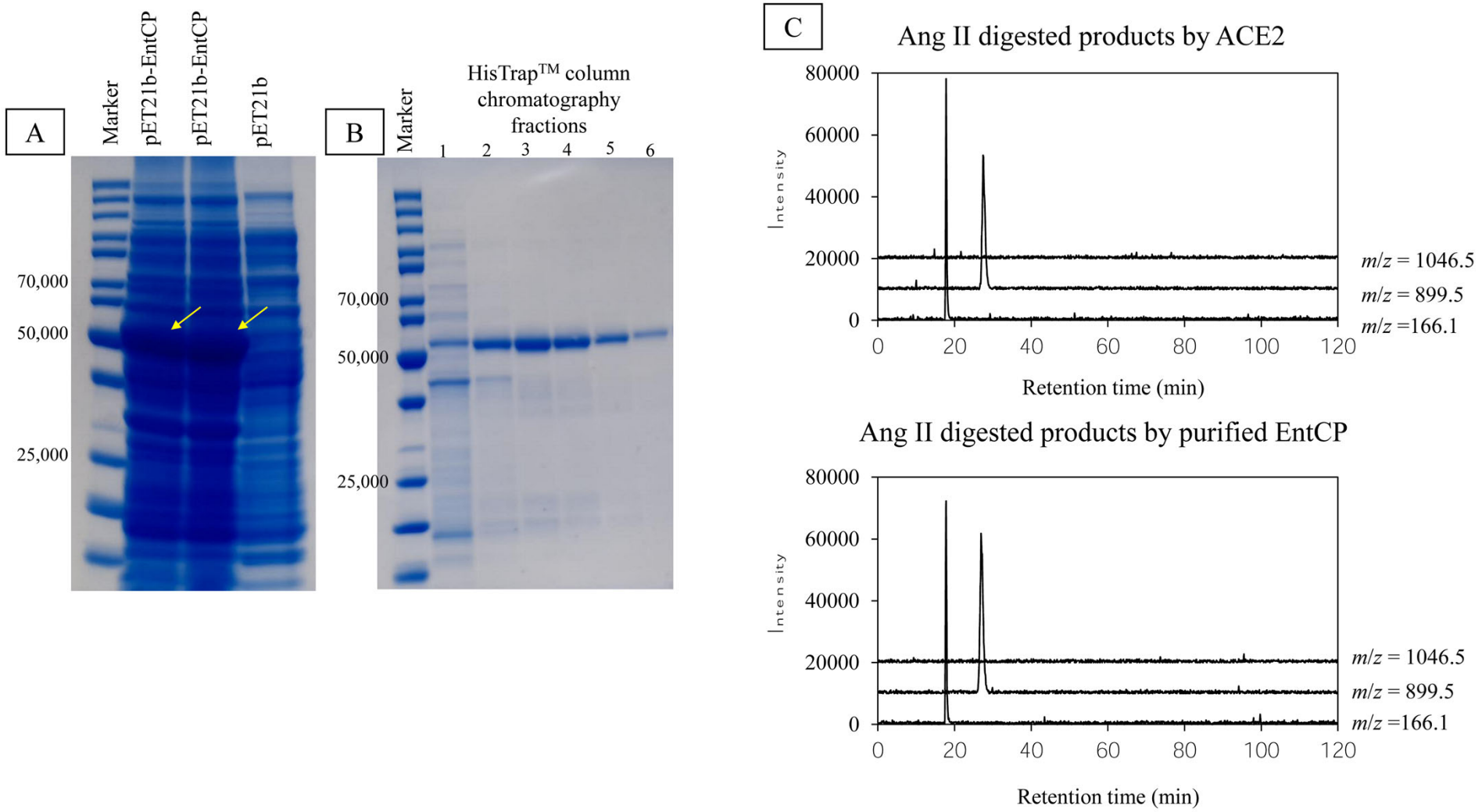
A
Marker
pET21b-EntCP
pET21b-EntCP
pET21b
70,000
50,000
25,000
B
Marker
HisTrap™ column chromatography fractions
1
2
3
4
5
6
70,000
50,000
25,000
C
Ang II digested products by ACE2
Intensity
80000
60000
40000
20000
0
0
20
40
60
80
100
120
Retention time (min)
m/z = 1046.5
m/z = 899.5
m/z =166.1
Ang II digested products by purified EntCP
Intensity
80000
60000
40000
20000
0
0
20
40
60
80
100
120
Retention time (min)
m/z = 1046.5
m/z = 899.5
m/z =166.1

Figure 4

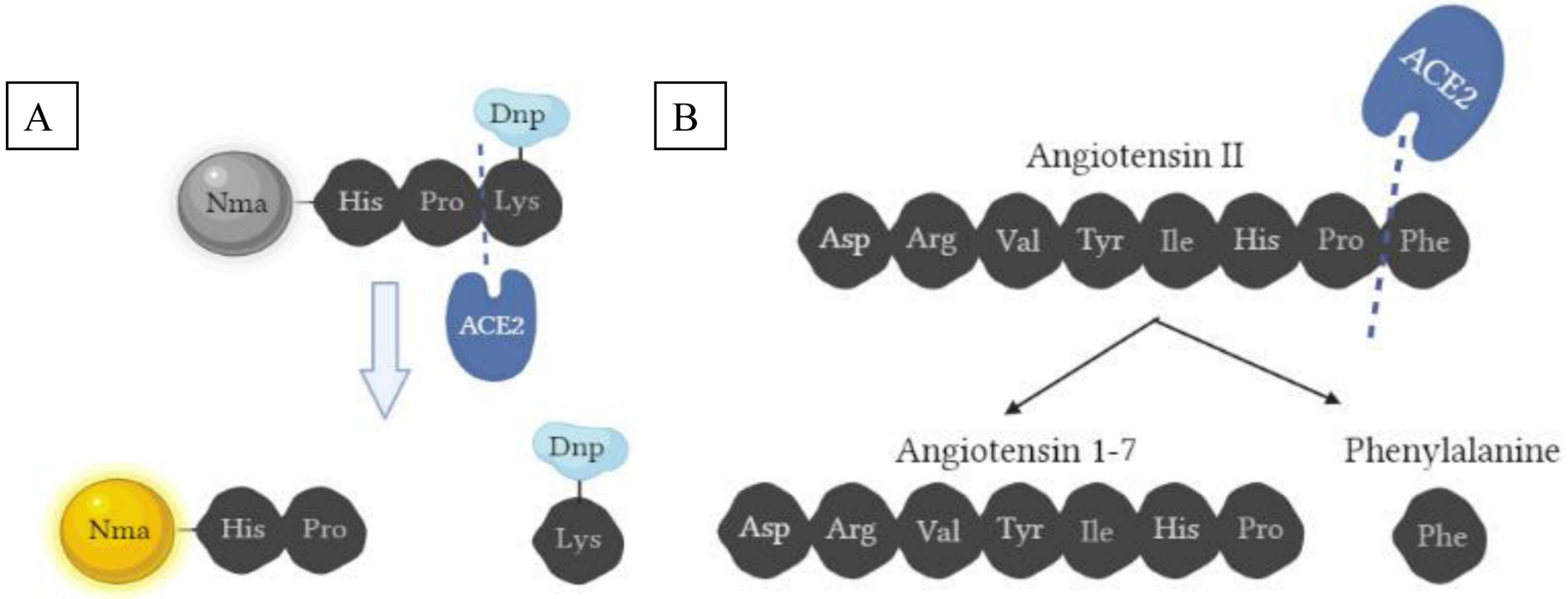
A
Dnp
Nma
His
Pro
Lys
ACE2
Nma
His
Pro
Dnp
Lys
B
ACE2
Angiotensin II
Asp
Arg
Val
Tyr
Ile
His
Pro
Phe
Angiotensin 1-7
Phenylalanine
Asp
Arg
Val
Tyr
Ile
His
Pro
Phe

Figure 5

Table 1. Quantification of specific activity during protein purification

Step	Vol (ml)	Total protein (mg)	Specific activity (RFU·sec^{-1}·L·mg^{-1} protein)	Purification fold	Yield (%)
Cell free extracts	100	110	3300	1	100
HiPrep Q XL 16/10	10	6.2	8200	2.5	14
Superdex 200 Increase 10/300 GL	1	0.38	79000	24	1.7

Supplementary data

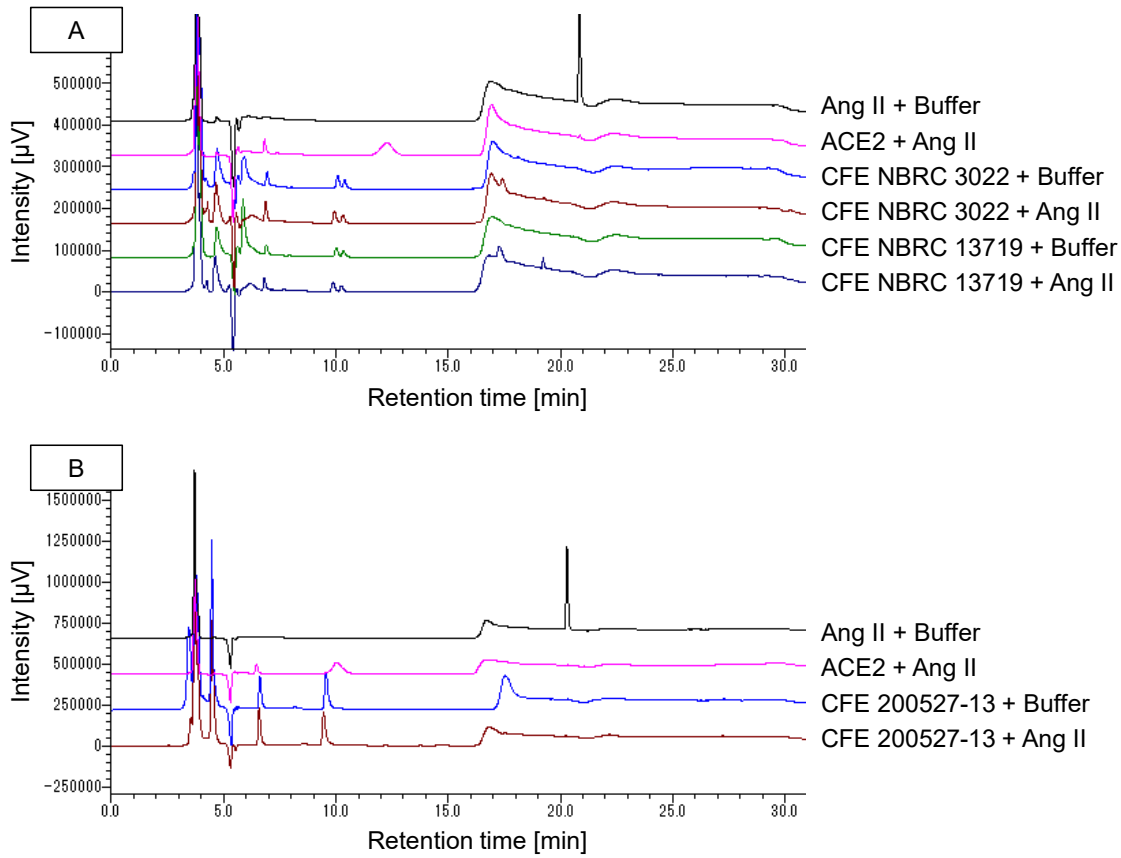

Figure S1. HPLC analysis of Angiotensin II (Ang II) hydrolysis products. (A) by three different positive control samples, (B) by the CFE of the strain 200527-13. Control samples of the CFE from NBRC 3022: *Bacillus subtilis* and NBRC 13719: *Bacillus amyloliquefasciens.* Retention times of Ang II and Ang1-7 were 21.0 min (20.5 min (B)) and 12 min (10.0 min (B)), respectively, in the results of 'Ang II + Buffer' and 'ACE2 + Ang II'.

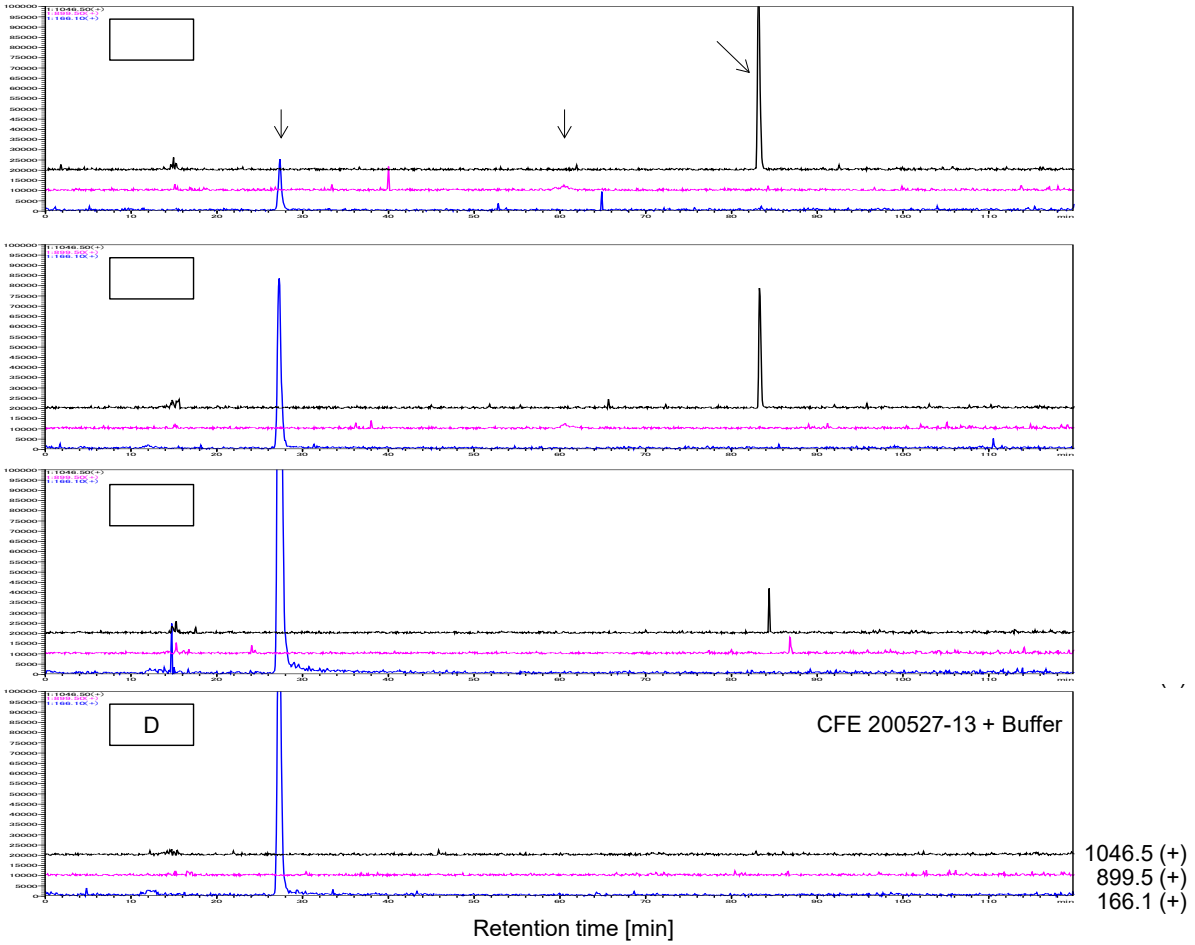

Figure S2. LC-MS analysis of Angiotensin II (Ang II) hydrolysis products. (A) by human ACE2, (B) by the CFE of *Bacillus subtilis* NBRC 3022, and (C) by the CFE of the strain 200527-13. The analysis of the CFE of the strain 200527-13 without Ang II (D) indicated endogenous phenylalanine in the CFE of the strain 200527-13.