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Abstract

Eleven genes, including *prss59.1,* were selected as candidate ovulation-inducing genes on the basis of microarray analysis and RNA sequencing in our previous study. To address the role of *prss59.1*, the prss59.1 gene knock-out zebrafish strain is currently being established by genome editing. In this study, for further phenotypic analysis of *prss59.1*, biochemical characterization of Prss59.1 was conducted using recombinant protein.

A C-terminal histidine-tagged version of zebrafish Prss 59.1 was constructed. Although *E. coli*-produced recombinant Prss59.1 showed almost no activity, peptidase activities appeared after denaturation and renaturation. Zebrafish Prss59.1 showed the highest activity against Lys-MCA. The optimal temperature and pH of the activity toward Lys-MCA were 37°C and pH 8.0, respectively. The Km value was 0.17 mM. Thus, zebrafish Prss59.1 possesses the closed character of trypsin, as expected from the DNA sequence.

Keywords: prss59.1, zebrafish, trypsin, peptidase, recombinant protein, renature

1. Introduction

Prss59.1 (protease, serine, 59, tandem duplicate 1) is one of the genes that are highly upregulated in zebrafish genes during ovulation according to RNA sequencing analysis [1]. *Prss59.1* is a gene belonging to the trypsin family, and the protein digests peptides at the C-terminus of arginine and lysine residues [2]. Zebrafish Prss59.1 protein contains a conserved histidine active site and a serine active site consistent with that of the serine protease family [3]. Zebrafish possesses 10 paralogs of trypsin (*prss1*) genes in the genome (http://www.ensembl.org/Danio_rerio/Info/Index). Five paralogs, including *prss1* and *prss59.1,* are present on chromosome 16. *Prss59.1* and its duplicated gene *prss59.2* are located tandemly. *Prss59.1* is distributed in fishes, birds and reptiles.

Previously, we identified *prss59.1* as one of the genes that is highly upregulated before ovulation in zebrafish ovaries [1]. Establishing gene-knockout fish by the CRISPR/Cas9 system is in progress. However, information on the biochemical characteristics of zebrafish trypsins is lacking.

To investigate the role of *prss59.1* during ovulation, the recombinant zebrafish protein *prss59.1* was produced, and enzymatic characterization of the expressed protein was conducted in this study.

2. Materials and methods

Materials. The fluorogenic peptide substrates K-MCA, Glt-GR-MCA, Boc-AGPR-MCA, Bz-R-MCA, Boc-FSR-MCA, PFR-MCA, Boc-LRR-MCA, Boc-VLK-MCA, Boc-EKK-MCA, Suc-LLVY-MCA, F-MCA and Glt-AAF-MCA were purchased from Peptide Institute (Osaka, Japan).

Expression of the recombinant Prss59.1 protein. For the expression of Prss59.1 in

bacteria and production of polyclonal antibodies, the entire open reading frame of zebrafish *prss59.*1 was amplified by PCR with primers designed to introduce NdeI and XhoI sites at the 5' and 3'-ends, respectively. The PCR fragments were inserted into the pET27b expression vector (Novagen), which contained a penta-histidine tag at the C-terminus. The recombinant proteins were produced in *E. coli* BL21 (DE3) and purified by Ni-NTA agarose column (10 mL bed volume) chromatography with a linear gradient of imidazole. Fractions were checked by SDS-PAGE and Western blot analysis with anti-Histag antibody (BIOMOL, Aichi, Japan). Fractions containing Prss59.1 proteins were pooled and applied to a Q-Sepharose column (1 mL bed volume). Fractions containing Prss59.1 protein were pooled as purified enzyme fractions. Polyclonal antibodies specific for Prss59.1 were raised in a mouse against purified recombinant Prss59.1 following procedures described previously [4].

Renaturation of recombinant Prss59.1.

The denaturation and renaturation of purified recombinant Prss59.1 were conducted according to the method used for human pro-urokinase expressed in *E. coli* [5].

Enzyme assay.

The hydrolysis of peptidyl substrates was measured at 37 °C in 50 mM Tris-HCl, 100 mM NaCl, 10 mM $CaCl_2$ (pH-8.0), 1.82 μg/ml Prss59.1, and 100 μM peptidyl substrate in 100 μL of solution. The reaction was started by adding enzyme solution to the peptidyl substrate-containing solution. Fluorescence readings of released 7-amido-4-methyl-coumarin (AMC) were measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm

with a fluorescence spectrophotometer as described (Varioskan^{TM} LUX, Thermo Scientific, Waltham, USA) [6].

Western blot analysis. Protein samples were mixed with 2x SDS-PAGE sample buffer. The samples were analyzed by Western blotting as described [7].

3. Results and discussion

3.1. Expression and purification of the prss59.1 gene.

Prss59.1 is one of 10 trypsin-related paralogs in zebrafish. The predicted amino acid sequences of human trypsin (hprss1), zebrafish trypsin (zprss1) and zebrafish Prss59.1 (zprss59.1) were compared (Fig. 1A). The histidine active site and serine active site are conserved in zebrafish Prss59.1. Ten cysteine residues that are known to form 5 disulfide bonds are conserved across zebrafish Prss proteins [8], as is the Ca^{2+}-binding site [9]. The signal peptide sequence on the N-terminus of zebrafish prss proteins can be predicted from the sequence similarity with the sequence identified in human prss1 [10, 11]. These sequence conservations suggested that zebrafish Prss 59.1 shares a highly similar structure with human Prss1. However, a sulfated residue (Y151 of human Prss1 in the chymotrypsin numbering system) is present in zebrafish prss1 but absent in prss59.1 [12, 13]. Thus, it is clear that zebrafish prss59.1 evolved to have different structures and functions from prss1. A residue (R122 in human Prss1) that allows autolysis to convert a single peptide chain to a two-chain form was found not to be conserved in zebrafish prss1 and zebrafish prss59.1 [8]. A change in this amino acid (R122C) was shown to cause hereditary pancreatitis [14].

Recombinant Prss59.1 protein was expressed as a C-terminal histidine-tagged protein using the pET expression system (Fig. 1B). The purity of fractions obtained by affinity purification using Ni-NTA agarose column chromatography was not sufficient. Thus, the fractions were further purified by Q-Sepharose column chromatography. By these two steps of column chromatography, Prss59.1 protein was purified (Fig. 2A). An antibody against purified Prss59.1 was produced (Fig. 2A). Using various kinds of peptide substrates, the peptidase activity of Prss59.1 was examined (Fig. 2B). Fluorogenic peptide substrates were shown to be suitable substrates to analyze the peptide sequence specificity of proteases [15]. Trypsin specifically digests the C-terminus of the lysine or arginine residue of fluorogenic peptide substrates [11]. However, almost no activity was detected for purified recombinant zebrafish prss59.1 (Fig. 2B white bar). Thus, we tried to denature and renature the purified protein. By using lysine as a supporter during renaturation, we succeeded in obtaining enzyme activity. Prss59.1 showed substrate specificity as a trypsin-like peptidase (Fig. 2B black bar). The highest activity was obtained against the substrate for trypsin-like peptidase, K-MCA. The same magnitude of activity was detected against substrates possessing lysine or arginine at the C-terminus (GR, AGPR, R, FSR, PFR, LRR and VLK). Only faint activity was detected against chymotrypsin substrates with tyrosine or phenylalanine at the C-terminus (LLVY, F, AAF).

3.2. Enzymatic characterization of Prss59.1.

The temperature- and pH-dependence of Prss59.1 were examined using K-MCA as the substrate (Fig. 3A and B). Prss59.1 showed the highest activity at 37°C. The optimal pH among the tested values was 8.0. Then, we determined the Km value of Prss59.1 under this optimal condition using K-MCA. Km was determined to be 0.17 mM (Fig. 3C). The k_{cat} for

prss59.1 was determined to be 2.7 (S^{-1}). These values are comparable to reported values for trypsin determined by using fluorescent peptide substrates [11]. These results confirmed that zebrafish Prss59.1 is a trypsin-like peptidase, as expected from its cDNA sequence.

Biochemical characterization of zebrafish Prss59.1 was conducted for the first time using conventionally expressed recombinant protein in this study. Although the expressed protein did not show activity, we succeeded in renaturing the purified protein. This study provides evidence of the enzymatic activity of Prss59.1. We selected the *prss59.1 gene* as one of the most likely candidate ovulation-inducing genes [1]. We are currently establishing a mutant strain of prss59.1 by using the CRISPR/Cas9 system. The information about enzymatic characterization and the antibodies produced in this study will be useful for further analysis of the function of prss59.1.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. Protein sequence features of zebrafish Prss59.1. (A) An alignment of predicted amino acid sequences of zebrafish Prss59.1 (zprss59.1), zebrafish Prss1 (zprss1) and human Prss1 (hprss1) is shown. The histidine active site and serine active site are boxed. Ten conserved cysteine residues and 5 predicted disulfide bonds are indicated by circles and dashed lines, respectively. The Ca^{2+}-binding site composed of 4 amino acids (#) is indicated by a horizontal line. Residues R122 and Y151 in human Prss1 are indicated by arrows. (B) A

diagram of the produced recombinant Prss59.1. The predicted signal peptide sequence on the N-terminus and histidine-tag on the C-terminus are indicated by black boxes.

Fig. 2. Substrate specificity of expressed zebrafish Prss59.1. (A) SDS-PAGE analysis of purified recombinant Prss59.1. Protein bands were detected by CBBR staining (CBBR) or immunostaining with an anti-His-tag antibody (α-His-tag) or polyclonal antibodies against recombinant Prss59.1 (α-r-Prss59.1). An arrow indicates Prss59.1. (B) Substrate specificity of zebrafish Prss 59.1. Peptidase activities of Prss 59.1 before (white bars) and after (black bars) renaturation against various kinds of fluorogenic peptide substrates were measured. The values are the averages of triplicate assays.

Fig. 3. Effect of reaction temperature and pH on K-MCA hydrolysis activity and Km determination. (A) Effect of temperature. The reactions were carried out at the indicated temperature. (B) Effect of pH. The reactions were carried out at 37°C in a reaction mixture of pH 6.0-11.0. (C) Determination of Km value. The reactions were carried out against different concentrations of K-MCA. The values are the averages of triplicate assays.

A

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zprss59.1  MRSLVFLVLLGAAFAL----DDDKIVGGYECQPNSQPWQASLNSGYHFCGGSLVSEYWVV  56
zprss1     .KAFIL.A.FAV.Y.APLGD..........TK.GV.Y.V............I.NL..  60
hprss1     .NP.LI.TFVA..L.APF-D.........N.EE..V.Y.V............IN.Q..  59
           *  .  *  . .* *.. .********.*  *. *.*.************... ***
                          Ca2+
                          # #  # #
zprss59.1  SAAHCYKSRVEVRLGEHNIVINEGTEQFITSEKVIRNPNYDSWDLDSDIMLIKLSKPATL 116
zprss1     .....Q........DVT.......N......H.S.N.NT..N.V......SS.QI 120
hprss1     ..G.....IQ........EVL..N....NAA.I..H.Q..RKT.NN........SR.VI 119
           ** ******..******** . **.****...*.**.* *.. .*..*.******. * .
           histidine active site
zprss59.1  NKYVQPVALPNGCAADGTMCRVSGWGNTMSSTAD-SNKLQCLEIPILSDRDCNNSYPGMI 175
zprss1     .S..KT.S..SS..SS..S.LI.....MSA.GSNYPSR.M..NA.....ST.R.A...Q. 180
hprss1     .AR.STIS..TAPP.T..K.LI.....A..G..YPDE....DA.V..QAK.EA....K. 179
           * ** ...**    ... **-*.*****. .*...*.  *.** .*.**.  * ..*** *
           R122                                    Y151
zprss59.1  TDTMFCAGYLEGGKDSCQGDSGGPVVCNGELHGIVSWGYGCAEKNHPGVYGKVCMFSQWI 235
zprss1     SSN.....FM.....................NQ.Q.........QR.K....A...N.TT.. 240
hprss1     .SN...V.F.....................Q.Q.V....D...Q..K....T..YNYVK.. 239
           ...***.*..**********************..*.*.****.***..*.**** **...  **
                                serine active site

zprss59.1  ADTMRNN- 242
zprss1     RN..NS.- 247
hprss1     KN.IAA.S 247
            .*.  *
```

B

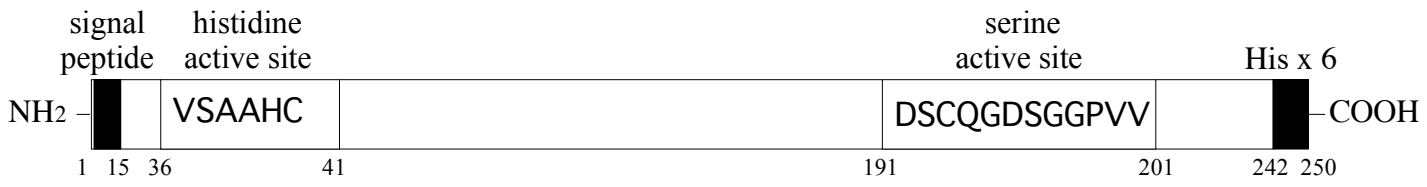

Figure. 1

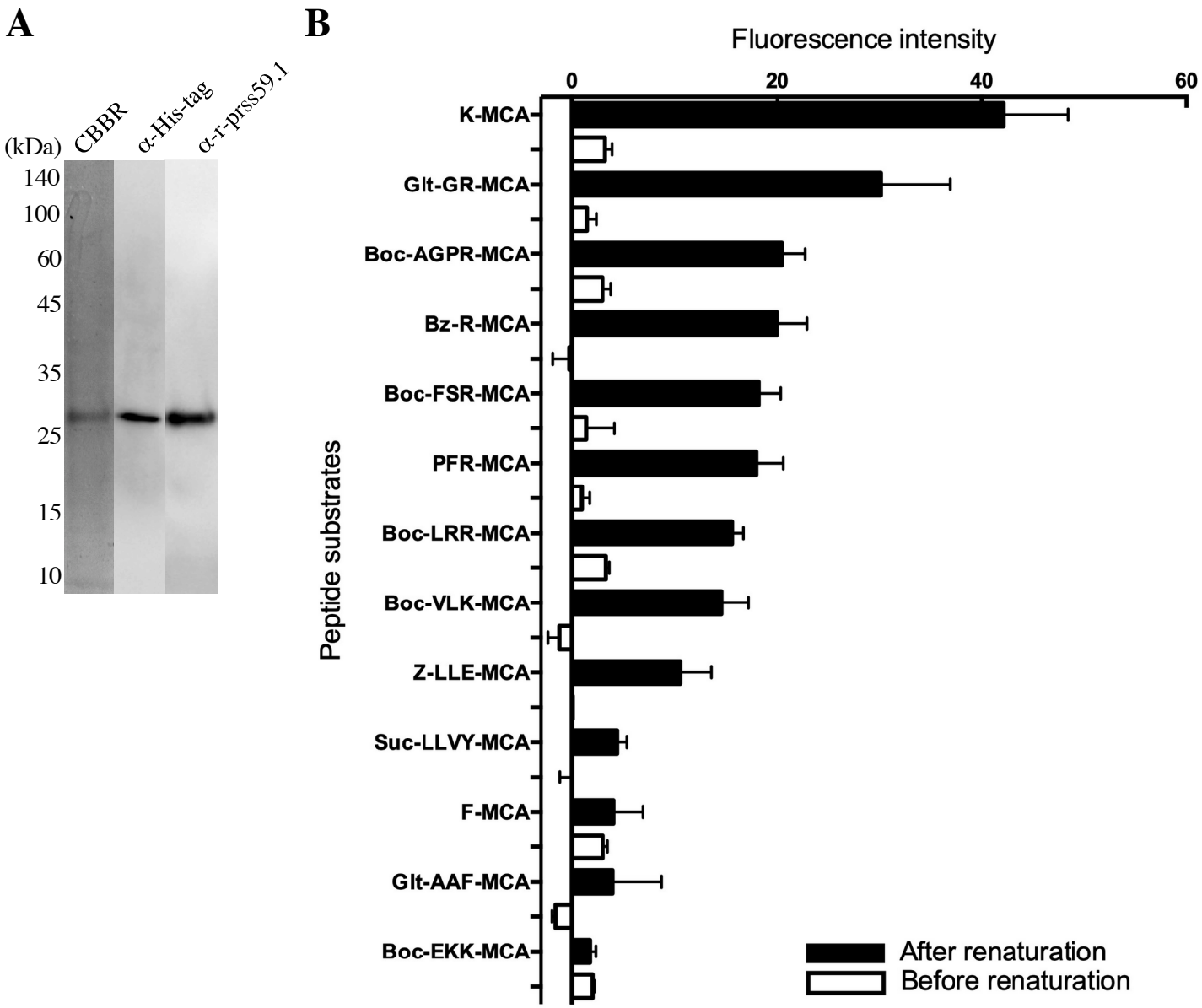
A
(kDa)
CBBR
α-His-tag
α-r-prss59.1
140
100
60
45
35
25
15
10
B
Fluorescence intensity
0
20
40
60
Peptide substrates
K-MCA
Glt-GR-MCA
Boc-AGPR-MCA
Bz-R-MCA
Boc-FSR-MCA
PFR-MCA
Boc-LRR-MCA
Boc-VLK-MCA
Z-LLE-MCA
Suc-LLVY-MCA
F-MCA
Glt-AAF-MCA
Boc-EKK-MCA
After renaturation
Before renaturation

Figure. 2

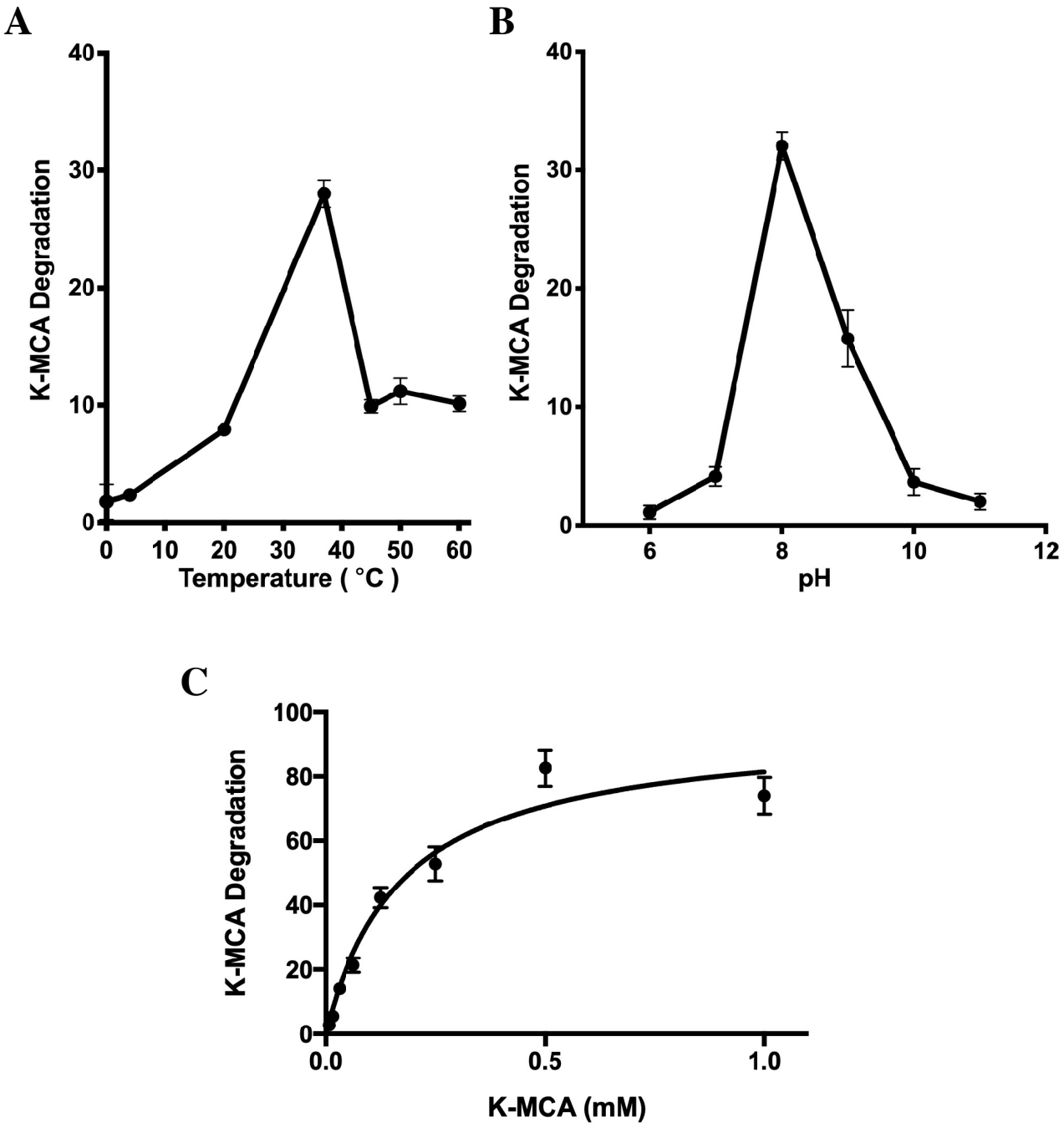
A
K-MCA Degradation
Temperature (°C)
B
K-MCA Degradation
pH
C
K-MCA Degradation
K-MCA (mM)

Figure. 3