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Probing the Structure-Activity Relationship of a Novel Artificial Cellobiose Hydrolase

Xingxing He,[a] Fuyuan Zhang,[a] Lin Zhang,[a] Qiang Zhang,[a] Guozhen Fang [a], Jifeng Liu,*[a] Shuo Wang,*[a] Shuqiu Zhang[b]

a. Key Laboratory of Food Nutrition and Safety, Ministry of Education of China, Tianjin University of Science and Technology, Tianjin 300457, China.
b. Shandong Provincial Key Lab of Test Technology on Food Quality and Safety, Shandong Academy of Agricultural Sciences, Jinan 250100, China.

*Corresponding authors email: Jifeng Liu, China. Email: liujifeng111@gmail.com;
Shuo Wang, China. Email: s.wang@tust.edu.cn

Experimental methods

Materials. Peptides used in this work, purified by high performance liquid chromatography (HPLC) and characterized by MALDI-TOF-MS, were manufactured by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, P.R. China). Unless stated otherwise, all other chemicals used in this experiments were purchased from Sigma-Aldrich. Deionized water was obtained from a Milli-Q device (18.2 M Ω, Millipore, Molsheim, France) was used in all experiments.

Preparation of Self-Assembly Peptide Nanofibrils. Purified lyophilized peptides were first dissolved in dimethyl sulfoxide. Then water was poured into the above solution and ultrasonically treated for 90 s. 2 mM citrate-phosphate buffer (pH 3.0) was added into the peptide solution and to obtain a peptide hydrogel (5 mM) after storing for 24 h at room temperature. Before the activity was tested, the hydrogel was diluted 1:1 in 1mM citrate-phosphate buffer and vortexed vigorously to disrupt the gel-like self-assembled structure to obtain nanofibril solution at a final peptide concentration of 2.5 mM. Adherence to this protocol assured reproducibility between runs and provided a standard method to compare different peptides.

Thioflavin-T (ThT) Fluorescence. Fluorescence spectra were measured using an F-2500 fluorescence spectrophotometer (Hitachi, Japan). Nanofiber solutions of each peptide were incubated with thioflavin-T (ThT) for 10 min which the final concentration of peptide and ThT is 200 μM and 25 μM respectively. The emission of the dye, ThT, was recorded in the wavelength range of 450-600 nm by exciting at 440 nm with excitation and emission slit widths being kept at 5 nm. ThT solution without peptide was used as a blank group.

Circular Dichroism (CD) Spectroscopy. The CD spectra were collected on the Bio-logic ALX250 (France) at room temperature in citrate-phosphate buffer (1 mM, pH 3.0) at 400 μM concentration of peptides with a 1 cm quartz cell. The spectra were recorded from 190 to 260 nm with a scan speed of 30 nm/min, 1 nm step resolution, and 3 accumulations. Citrate-phosphate buffer was used as a baseline and an average of three times was measured for each sample to produce reliable ellipticity values.

Transmission Electron Microscopy (TEM) Characterization. The samples were prepared from 200 μM nanofiber solutions in citrate-phosphate buffer on a holey carbon-coated copper grid (Zhongxinkeji Technology Co. Ltd., Beijing, China). A 7 μL droplet of the PC solution was placed on the grid for approximately 30 min, followed by removal of excess solution with filter paper carefully. The grid was immediately added with 7 μL drops of freshly filtered 2 wt % phosphotungstic acid solution[1], and stained for 1.5 min. Then the grid was washed by deionized water three times and left covered under a petri dish to dry. TEM images were obtained using a JEOL JEM-2100 (Japan) operating at an acceleration voltage of 200 kV.

X-ray diffraction (XRD): The peptide array was characterized on a D/Max 2500 X-ray diffractometer (Rikagu, Japan) under the following conditions: scan speed, 4° min^{-1}; $Cu_{K\alpha}$ radiation, λ=1.5405 Å. All the samples were prepared on a glass substrate[2] and dried under vacuum.

Computational modeling. All the structures were built in Gaussian View 5 program according to the peptide sequence, and then the PM7 method was performed to optimize all the structures by OPAC package. Next, Gaussian 09 and Gaussian View 5[3]package were employed to conduct the molecule electrostatic potential (MEP) analysis of the peptides. The MEP were generated and mapped onto the Van der Waals surface.

Determination of the Catalytic Activity of Cellobiose Hydrolysis. Activity was ascertained by measuring the amount of glucose, the product released from cellobiose via the catalytic reaction of bio-mimetic catalyst, with enzymatic methods. Hydrolysis reactions were performed using 40 μL of 15 mM cellobiose solution and 60 μL of 2.5 mM nanofibril solutions in 1 mM citrate-phosphate buffer (pH 2.0-8.0) for 48 h at different temperature (20℃-65℃). We opted for a two enzyme-based assay over Miller's dinitrosalicylic acid-based method because

the former is relatively more sensitive, rapid, and consumes less reagents. The glucose in the 48 h reacted solutions was determined by using the following reaction[4]:

$$\text{D - glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} H_2O_2 + \text{Gluconate}$$

$$2H_2O_2 + \text{4 - Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Red pigment} + 4H_2O$$

The hydrogen peroxide formed red chromophores through quantitative oxidation condensation with phenol and 4-aminoantipyrine. The red chromophores have an absorption peak at 505 nm, so the glucose concentration was obtained by measuring the absorbance of the chromophore using a standard glucose with a microplate reader (BIO-RAD, Japan).

Hydrolysis properties of the PCs. 1 mM and 5 mM Ca^{2+} were pre-mixed with PC1 and PC5 for 24 h to form the Ca^{2+} - coordination fibers to evaluate the effect of Ca^{2+} on catalytic activities of peptides at 25℃ and pH 3.0, respectively. Meanwhile, different disaccharides were also used to study the specificity of PC5 under the same conditions of cellobiose. In addition, poly-acrylic acid, crosslinked by acrylic acid monomer with numbers of carboxyl groups, were used to see its activity on cellobiose as a comparison under the same conditions of PCs.

Cell Culture. PC12 cell line was maintained in DMEM/high glucose with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified atmosphere containing 5% CO_2 at 37℃.

Cell MTT Assay. Pheochromocytoma (PC12) cells were cultured in 96-well plates at an intensity of 2×10^4 cells per well (100 μL/well). After 24 h incubation at 37℃, the medium were replaced by 100 μL of peptide nanofibril solutions in Dulbecco's Modified Eagle Medium (DMEM) (Beit Haemek, Israel) at various concentrations (0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 1.5 mM). Each experiment was repeated 6 times. Then, 10 μL of 5 mg/mL thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich, USA) solution was added. After incubation at 37℃ for 4 h, DMSO (Sigma, USA) solution was added to each well to lyse the cells. The absorbance of supernatants was measured at 570 nm by using a microplate reader (BIO-RAD, Japan). Cell viability was calculated by the ratio of the absorbance of the nanofibrils treated cells to that of control cells.

Supporting Figures

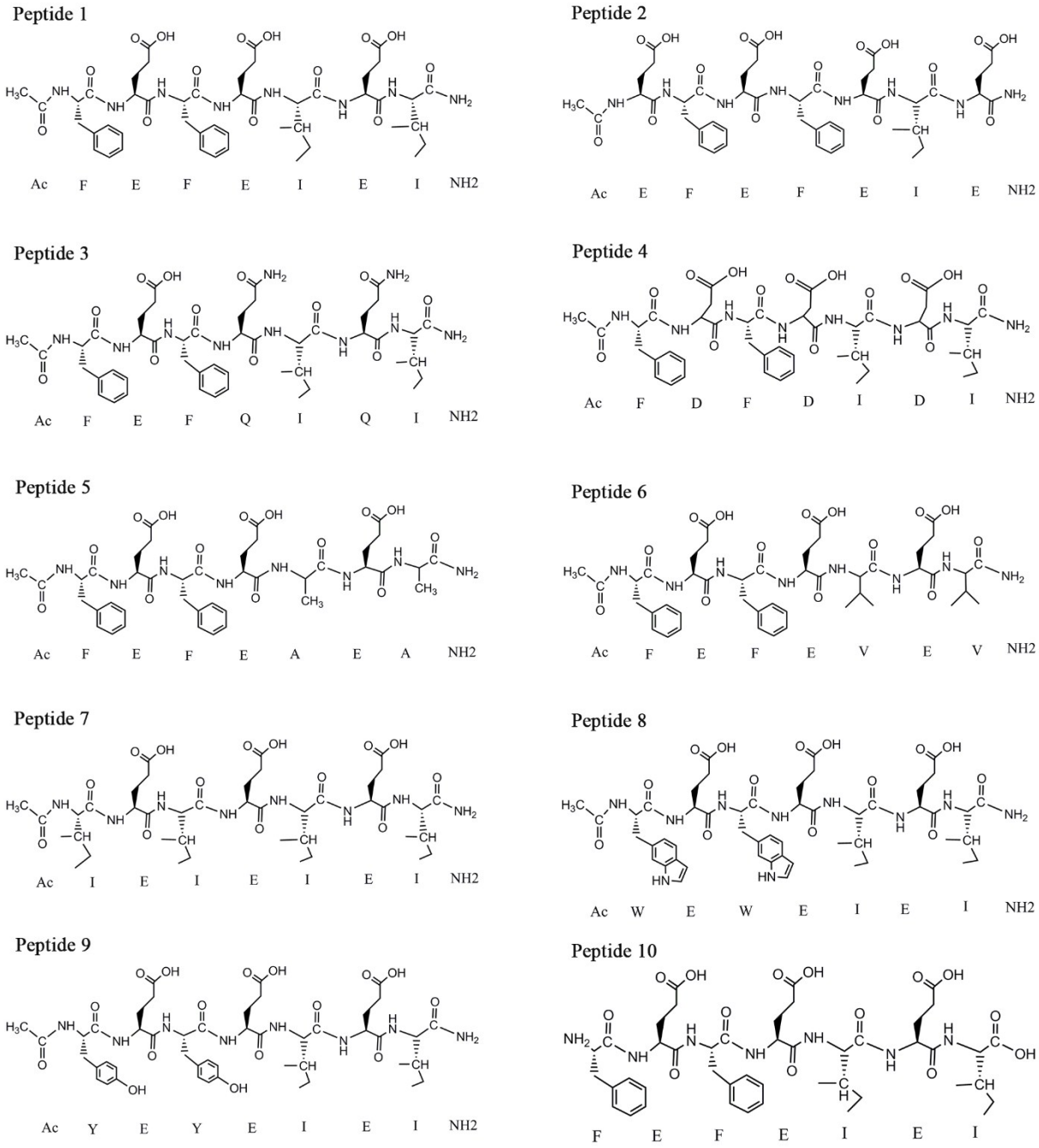

Fig. S1 Chemical structures of the peptides used to create hydrolysis models.

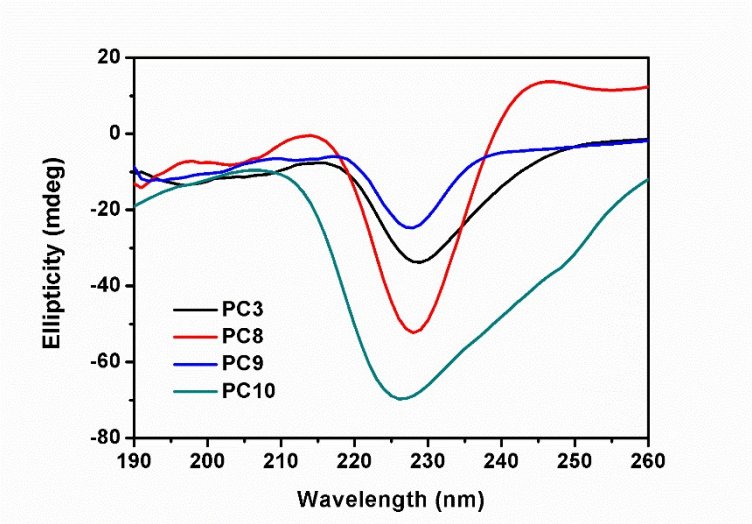

Fig. S2 Circular dichroism of inactive peptides (PC 3, 8, 9 and 10) in a dilute solution (400 μM). All samples showed a red shifted β-sheet-like CD spectrum at 25℃,pH 3.0.

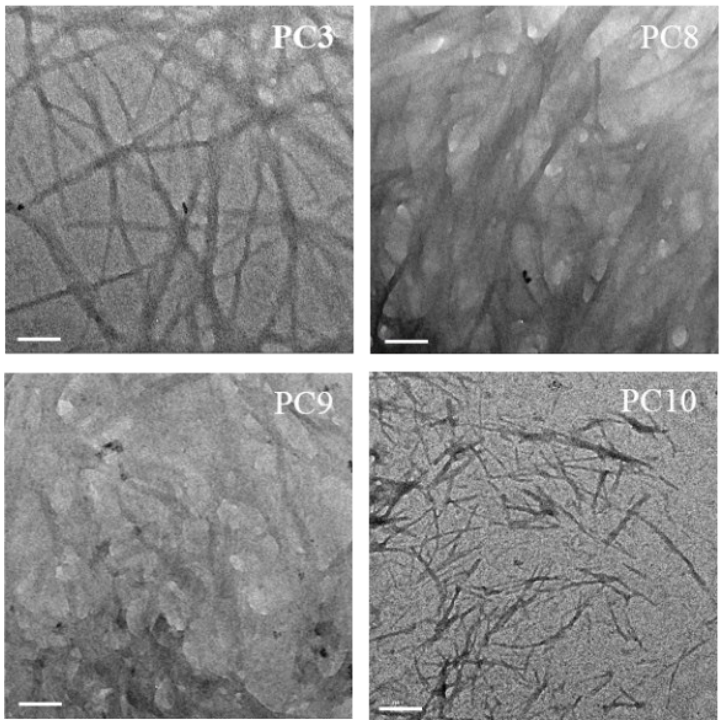

Fig. S3 Negatively stained TEM images of inactive peptides (PC 3, 8, 9 and 10). PC3 showed a crosslinked nanofibers formed as other active PCs. While PC8 and PC9 showed sol-gel films like images. The PC10 with unblocked N- and C-termini just produced short-protofibrils after incubation 24 h. Peptide concentration was 200 µM in all TEM experiments. Scale bar: 200 nm

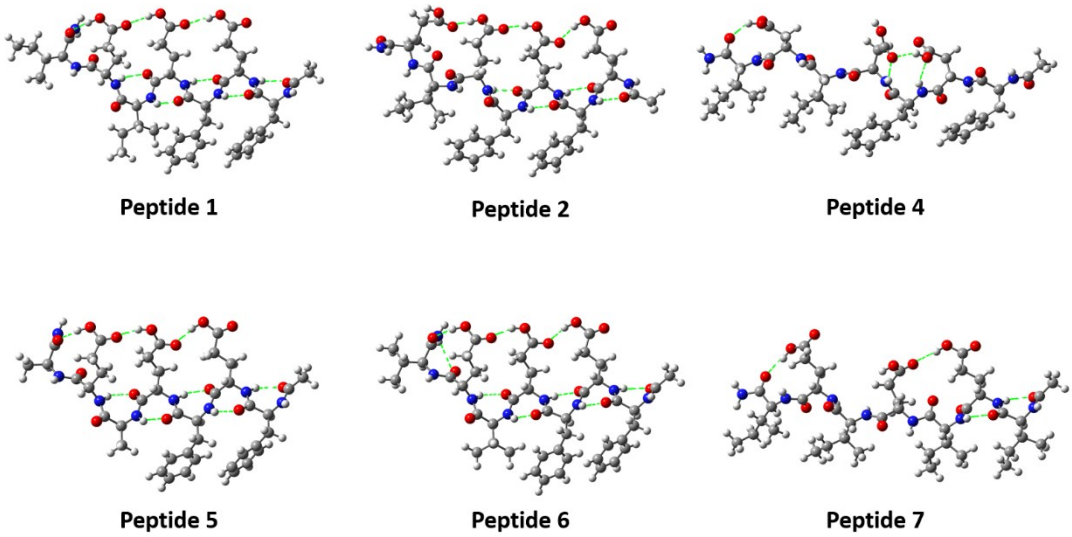

Fig. S4 The optimized molecular conformations of peptide1, 2, 4, 5, 6 and 7. Left is C-terminal and right is N-terminal in all peptides. The green dashed line represents the intramolecular hydrogen bond. Colors of atoms: white (hydrogen), gray (carbon), blue (nitrogen), red (oxygen).

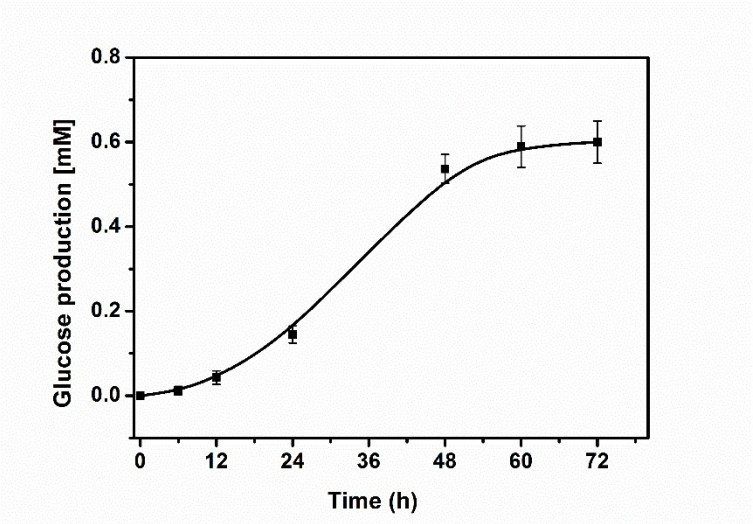

Fig. S5 Cellobiose hydrolyze by PC1 during 72 h at 25℃, pH 3.0. The values were means of replicates (n = 3) and their standard deviations. As show in the figure, we chose 48 h as experimental reaction time because of the significantly increased production of glucose.

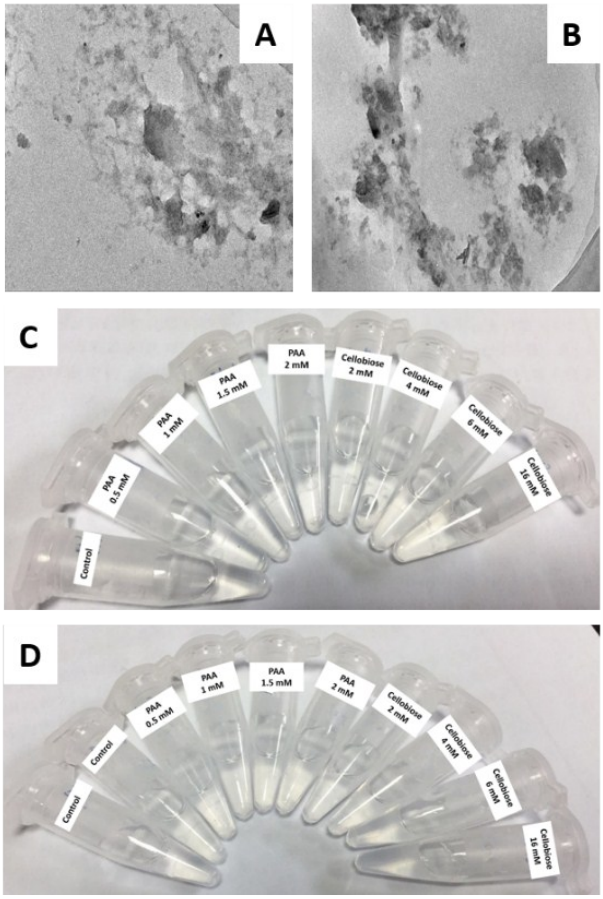

Fig. S6 TEM Images and hydrolytic activity of PAA. TEM Images of PAA of different molecular weight 1800 (A) and 3000 (B). PAA concentration was 200 μM in all TEM experiments. Scale bar: 200 nm; Results of PAA (C: Mw1800, D: Mw3000) on cellobiose hydrolysis with different concentration of PAA or substrate at 25℃, pH 3.0. Control groups: 6 mM cellobiose in 1 mM pH 3.0 citrate-phosphate buffer incubated for 48 h.

Reference:

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