Supplementary Information for publication

Interfacial biosilica coating of chitosan gel using fusion silicatein to fabricate robust hybrid material for biomolecular applications

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Figures

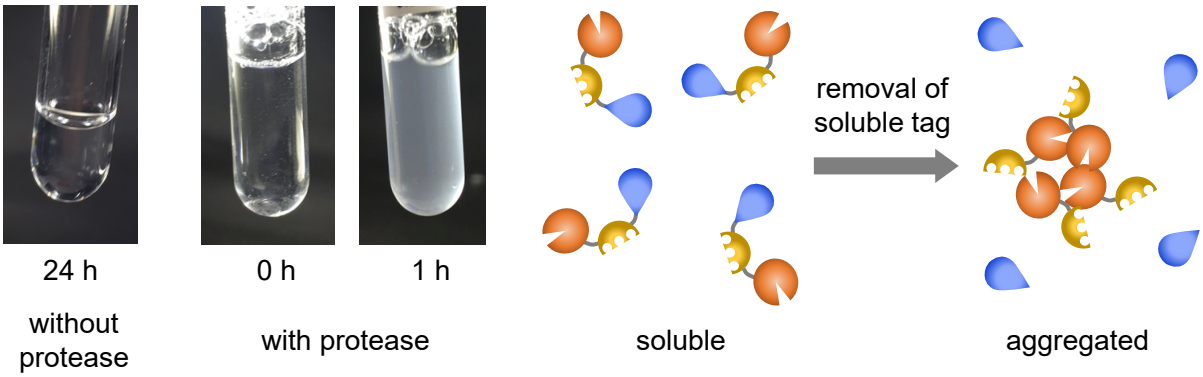

Fig. S1 Solubility of fusion silicatein in aqueous media.

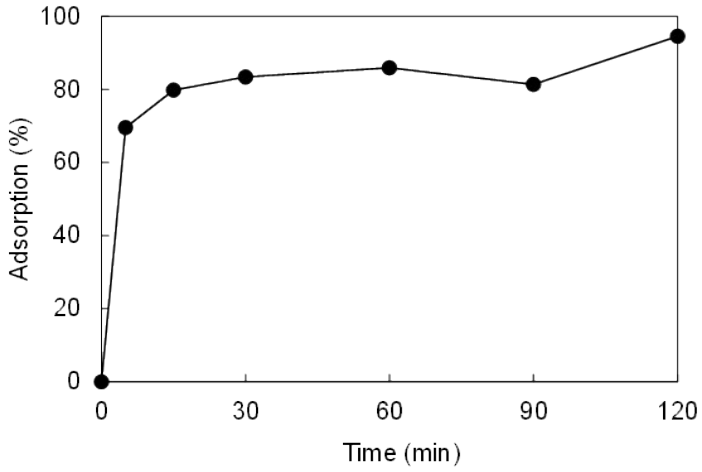

Fig. S2 Adsorption studies of fusion silicatein on chitosan gel.

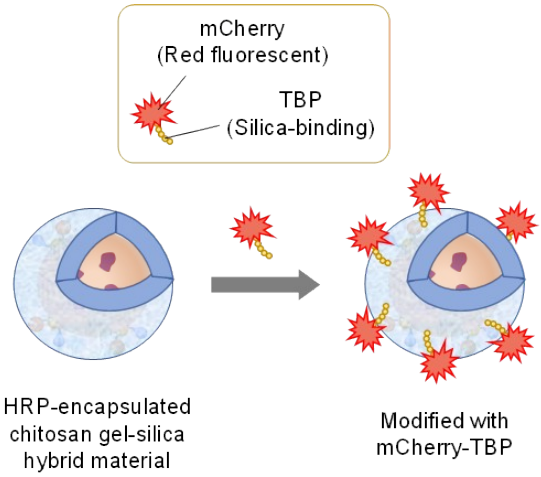

Fig. S3 Adsorption of mCherry-TBP on silica.

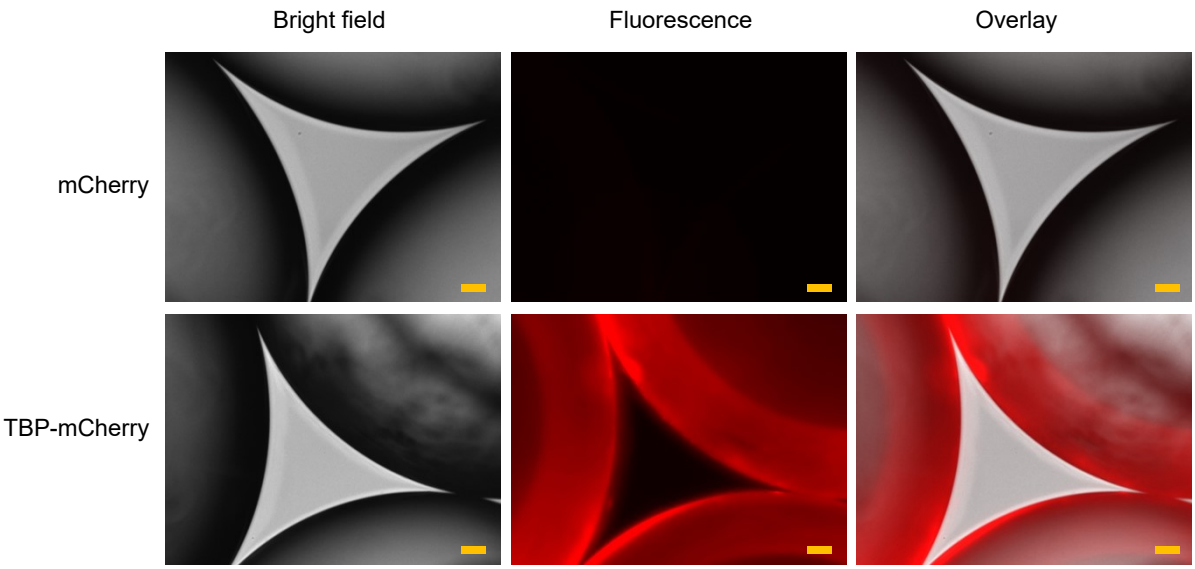

Fig. S4 Microscopic images of glass beads treated with TBP-mCherry and mCherry. Size bars indicate 100 µm.

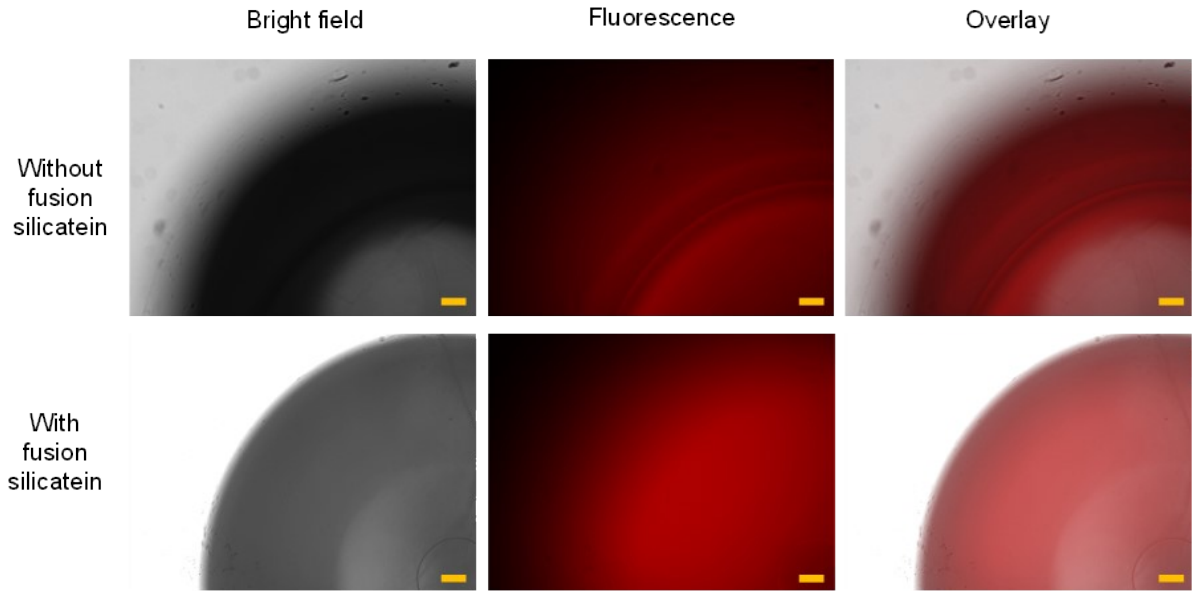

Fig. S5 Microscopic images of chitosan gel beads treated with TBP-mCherry. Size bars indicate 100 µm.

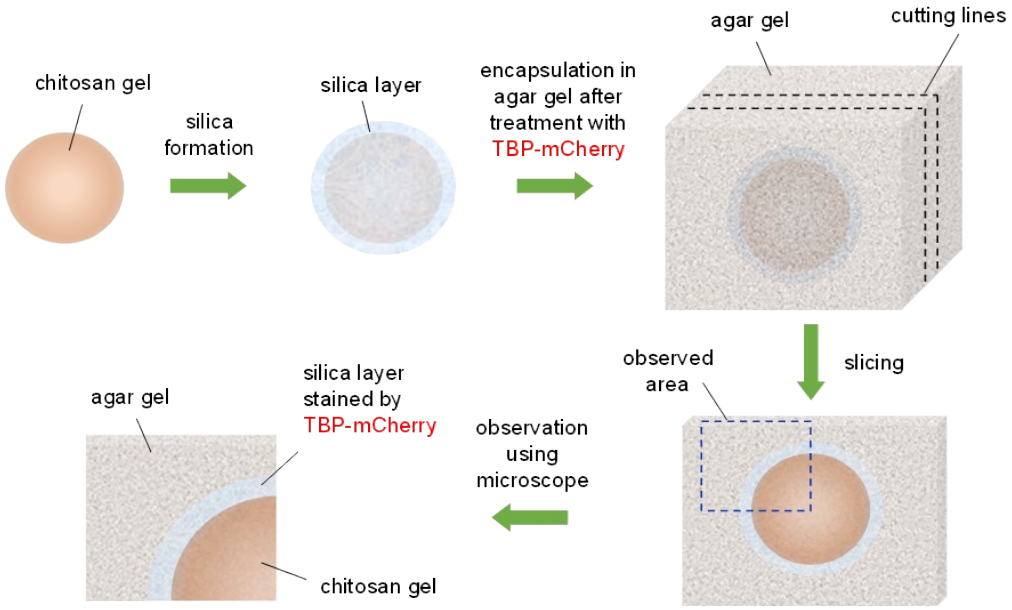

Fig. S6 Obtaining cross sections of chitosan gel beads for microscopic observation.

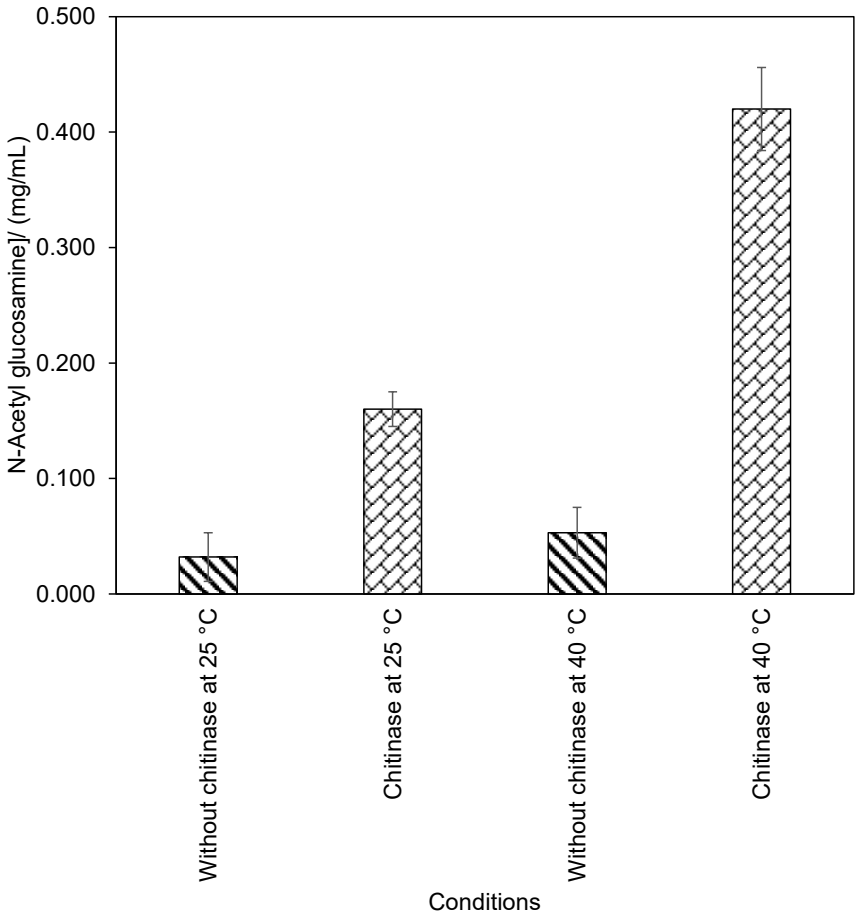

Fig. S7 Degradation of chitosan gel using chitinase.

Methods

Construction of the fusion genes to express of fusion silicatein (InaKC-ChBD-Sil) and purification

Construction of gene, expression and purification were performed as previously reported by our research group.[23] Using synthesized silicatein *α* cDNA from *S. domuncula* (accession number CAI46305), which encodes the mature enzyme (aa115-330), the InakKC gene from *P. syringae* (accession number AAB66891), and the ChBD gene from *B. circulans* WL-1 (accession number AAA81528, aa 655–697) from Eurofins Genomics with optimization for expression in *E. coli,* pCold_InaKC-ChBD-Sil vector to express fusion silicatein (InaKC-ChBD-Sil) was constructed. The transformation of the constructed vector into *E. coli* BL21(DE3) was performed, and the success of transformation was checked using colony PCR. *E. coli* BL21(DE3) cells transformed with the constructed vector to express the fusion silicatein were grown separately at 15 °C with shaking at 160 rpm for 24 h in LB by inducing protein expression using 1.0 mM IPTG. The bacterial cells were harvested by centrifugation at 4000 × g and 4 °C for 20 min. The resultant pellets were resuspended in sonication buffer (20 mM Tris/HCl, pH 8.0; 1 mM EDTA; and 0.5 M NaCl), and the cells were disrupted using an ultrasound disintegrator (VCX-130, Sonic & Materials Inc., USA). The samples were then centrifuged at 25, 000 × g and 4 °C for 20 min. The precipitated fractions were washed twice with lysis buffer containing a surfactant (20 mM Tris/HCl, pH 8.0; 1 mM EDTA; 0.5 M NaCl; and 4% TritonX-100) and washed twice with ultrapure water to remove membrane proteins. Resulted inclusion bodies were denatured and subjected to Ni-NTA column purification. Finally, fusion silicatein was obtained after refolding the denatured purified fusion silicatein.

Determination of enzymatic activity

InakC-ChBD-Sil (1.66 μM) and 100 mM tetraethyl orthosilicate (TEOS) as the substrate for the enzymes were incubated in Tris/HCl buffer at 25 °C while shaking at 185 rpm for 24 h. As the control silica polymerization activity was determined in the absence of proteins with the same conditions. The formed biosilica was recovered by centrifugation (13000 rpm, 10 min, 4 °C) and washed three times with ethanol and three times with ultra-pure water, followed by freeze-drying. Freeze-dried biosilica samples were hydrolyzed to silicic acid by 1 M NaOH for 30 min at 90 °C and neutralized with HCl. The concentration of silicic acid (hydrolyzed silica) was then determined by the molybdenum blue colourimetric method.

Expression of TBP-mCherry and mCherry

Gene encoding mCherry, optimized for expression in *E. coli*, was obtained from pmCherry vector (Clontech Laboratories, Inc., Takara Bio, vector number: PT3973-5). It was amplified with F1, forward primer composes of the sequence of TBP, and R1, reverse primer. The nucleotide sequence of primers used in the experiment has been mentioned in Table S1. The amplified gene was subcloned into pCold II expression vector at *Nde*I and *Xho*I restriction enzyme sites using In-Fusion (Takara Bio Inc., Japan) to express the protein named TBP-mCherry. To check the correctness of the constructed vector after multiplication in *E. coli* DH5α, DNA sequencing was conducted.

Table S1: Nucleotide sequence of primers used in this study.

Primer	Nucleotide sequence
F1	ATCATCATCATCATATGCGCAAACTGCCGGATGCGCCAG GCATGCATACCTGGGTGAGCAAGGGCGAGGAG
R1	ATTCGGATCCCTCGAGCAGGGTTGGATAAGAGGC

The transformed *E. coli* with the constructed vector was used to express TBP-mCherry at 15 °C by inducing the protein expression using 1.0 mM IPTG. After 24 h, bacterial cells were harvested by centrifugation (4000 × g, 20 min, 4 °C). Resultant pellets were resuspended in sonication buffer (20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.5 M NaCl), and cells were disrupted by using an ultrasound disintegrator (VCX-130, Sonic & Materials Inc., USA). Then the samples were centrifuged (25, 000 × g, 20 min, 4 °C). The supernatant was collected, and TBP-mCherry was purified by immobilized metal ion affinity chromatography on a 5 mL Ni-NTA column. The elution buffer of the target protein was then replaced with 50 mM Tris/HCl buffer (pH 9.0, 8 mM NaCl) by ultrafiltration (Amicon® Ultra-15 centrifuge filters, 10, 000 NMWL). Finally, the concentrations of refolded proteins were calculated using the Bradford assay (Bradford Protein Assay Kit, Takara Bio Inc., Japan).

Formation of Chitosan gel-silica beads

Optimization of chitosan percentage in gel beads First, chitosan gel concentration was optimized to form gel beads. For that 1%, 2% and 3% (m/v) of chitosan solutions were prepared by dissolving chitosan in 2% (v/v) acetic acid. Then the chitosan solution was added to 1 M NaOH solution drop by drop using a syringe connected with a needle. The formed gel beads in each case were collected and washed with 50 mM Tris/HCl buffer five times. Finally, the formed gel beads in each case were observed.

Immobilization of HRP in the chitosan gel matrix HRP was added into 2% (v/v) acetic acid solution containing 3% (m/v) chitosan to be the final concentration of 0.01 mg/mL. Then the acetic acid solution containing chitosan gel and HRP was added into 1 M NaOH solution drop by drop using a syringe connected with a needle. Immediately gel beads were recovered and washed with 50 mM Tris/HCl buffer five times.

Adsorption of fusion silicatein on chitosan gel To analyze the adsorption of the fusion proteins on chitin, chitosan gel beads (10 beads) were added to 50 mM Tris/HCl buffer solutions containing 10 μM of fusion silicatein, and the solutions were shaken for 1.5 h at 25 °C. During the shaking, 20 μL of the solutions without chitosan gel were periodically aliquoted to analyze the remaining concentration of the protein using Bradford assay. After 2 h, the chitosan gel was recovered and washed twice with 50 mM Tris/HCl buffer.

Silica formation on the chitosan gel Chitosan gel beads adsorbed fusion silicatein were subjected to silica formation using 100 mM TEOS as the precursor in the 50 mM Tris/HCl buffer at 25 °C while shaking at 185 rpm for 24 h. The treated chitosan gel beads were recovered and washed three times with ethanol and three times with ultra-pure water followed by freeze-drying for 2 days. Then the freeze-dried gel beads were observed using SEM-EDS. The amounts of silica formed on the chitosan gel beads with and without adsorption of fusion silicatein were quantified using the molybdenum blue colourimetric method after hydrolysing by 1 M NaOH. A similar experiment was conducted without adding the fusion silicatein as a control. For the detection of the activity of HRP, chitosan gel beads subjected to silica formation with and without adsorption of the fusion silicatein were recovered by washing with Tris/HCl buffer.

Detection of silica on chitosan gel using TBP-mCherry

First, to investigate the adsorption of TBP-mCherry and mCherry on glass beads, glass beads were treated with 2.5 μM of TBP-mCherry or mCherry at 25 °C for 3 h. Recovered glass beads were washed with distilled water to remove the unbound proteins on the glass beads. Finally, the glass beads were observed using a fluorescence microscope (KEYENCE Fluorescence Microscope, BZ-X800).

For the detection of silica formed on chitosan gel beads, three beads of chitosan gel subjected to silica formation with and without adsorption of the fusion silicatein were treated with 2.5 μM of TBP-mCherry at 25 °C for 3 h. Then the beads were recovered and washed with distilled water to remove the unbound TBP-mCherry. Finally, the gel beads were observed using the fluorescence microscope. To observe the cross-sections of chitosan get beads, gel beads slides were obtained by slicing using a shape blade after gel beads were immobilized in a 2% agar gel.

Scanning electron microscope analysis

After freeze-drying, the biosilica formed on the chitin material was examined using SEM (Acceleration voltage: 5 kV, JEOL JSM-IT200 InTouchScope™, Germany) without coating. Elements present on the chitin surfaces subjected to silica formation were analyzed using EDS (without coating; acceleration voltage: 10 kV for point EDS; JEOL JSM-IT200 InTouchScope™, Germany).

Determination of the activity of HRP

Hydrogen peroxide (1.7 mM) in 200 mM potassium phosphate buffer (pH 7), and phenol/aminoantipyrine (2.5 mM of 4-aminoantipyrine) solution were prepared. From freshly prepared above solutions, 467 μL of hydrogen peroxide (1.7 mM) and 500 μL of 2.5 mM phenol/aminoantipyrine were pipetted into a cuvette, and the solution was incubated in the spectrophotometer at 25 °C for 3-4 minutes to achieve temperature equilibration and establish a blank rate. Then, 33 μL of the HRP solution (1.2×10^{-4} mg/mL), or two chitosan gel beads with 33 μL of ultra-pure water was added to the cuvette, and an increase in absorbance value at 510 nm wavelength for 4-5 minutes was measured to determine the amount of 4-aminoantipyrine phenolic complex formed as the reaction shown in the Fig. S8. Equation S1 was used to calculate the activity of HRP where ΔAbs_{510} is the increase in absorbance value at

510 nm wavelength, and *6.58* is the absorption coefficient for 4-aminoantipyrine at 510 nm wavelength.

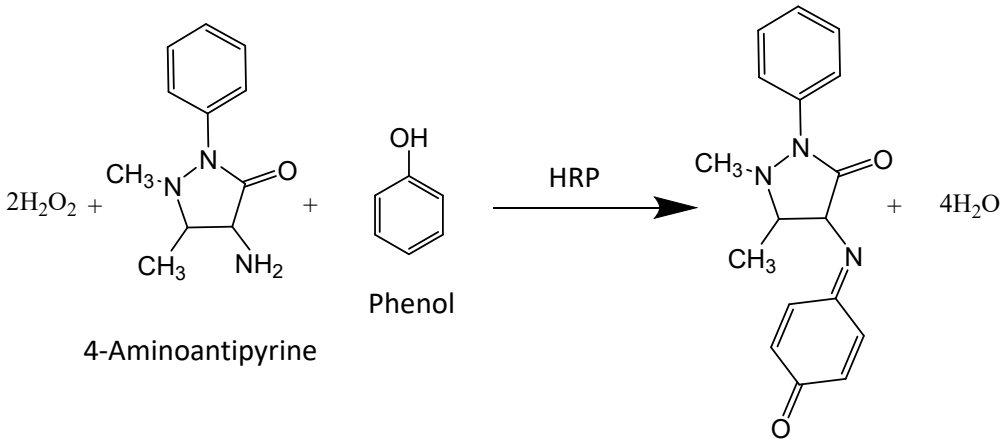

Fig. S8 Reaction catalysis by the HRP in the presence of 4-aminoantipyrine and phenol with hydrogen peroxide.

$$\text{Activity}\left(\frac{\text{U}}{\text{mg}}\right)=\frac{\frac{\Delta \text{Abs}_{510}}{\text{min}}}{6.58\times\frac{\text{amount of enzyme (mg)}}{\text{reaction volume (mL)}}} \qquad \text{(Eq. S1)}$$

The activity of HRP immobilized in chitosan gel beads that were subjected to silica formation with and without adsorption of the fusion silicatein was checked in 5 cycles (1 cycle: react with 4-aminoantipyrine and phenol presence of hydrogen peroxide for 1 h) by recovering them and adding them into cuvette containing freshly prepared hydrogen peroxide and phenol/aminoantipyrine solutions.

Degradation of chitosan gel

The degradation of chitosan gel was investigated using chitinase from *Pyrococcus furiosus* (Wako Pure Chemical Industries Ltd., Japan). Chitosan gel (0.1 g) was mixed with 0.8 mg/mL chitinase in pH 7.4 Tris/HCl buffer (total volume 2 mL). As the control chitosan gel was treated

with the Tris/HCl buffer without adding chitinase. Then the samples were incubated at 25 °C while shacking at 160 rpm for 2 days. The solutions were recovered by centrifuge at 13000 rpm for 15 min and used for the analysis of the concentration of N-acetyl glucosamine by 3, 5-dinitrosalicylic acid (DNS) assay.

DNS assay

The DNS reagent (50 mL) was prepared by mixing, 1.0 g of DNS and 0.8 g of NaOH (Wako Pure Chemical Industries Ltd., Japan) in ultrapure water at 80 °C followed by adding 3.0 g of sodium potassium tartrate (Wako Pure Chemical Industries Ltd., Japan). Then, 0.5 mL from the DNS reagent, and 0.5 mL from the solution that need to analyse the N-acetyl glucosamine concentration were mixed and heated for 10 min at 95 °C. Finally, the absorbance at 540 nm was measured after cooling down samples to room temperature. As the standard for the calibration curve, N-acetyl glucosamine (Wako Pure Chemical Industries Ltd., Japan) was used.