SDC, Materials and Methods

Preparation of recombinant CP (Main Body text p=17)

The recombinant CP was prepared as described below. A plasmid, pHT43-CP, which expressed recombinant CP, was constructed as follows: A DNA fragment encoding CP without its signal peptide(Asn^{28}-Trp^{526}) was amplified from Ch genomic DNA by polymerase chain reaction(PCR) using a set of primer pair 5'-TTATAGATCTAATCCAGTAACTAAATCCAAGGATAATAAC-3' and 5'-TAATATCCCGGGCTATTACCATTGGTAATGATTAACTCC-3'. The PCR product was digested with *Bgl* II and *Sma* I, and then inserted into pHT43 digested with *Bam*H I and *Sma* I. *Bacillus subtilis* KN2(1) was transformed with each plasmid, pHT43-CP and pHT43-NP, by electroporation. Transformants harboring the expression of plasmid were selected on Luria Broth(LB) agar plates containing 20 μg/ml of chloramphenicol. *Bacillus subtilis* KN2 carrying the expression plasmid(pHT43-CP) was inoculated into 170 mL of 2 x animal-derived component free medium[2% Polypepton N(NIHON PHARMACEUTICAL Co., Ltd, Tokyo, Japan), 1% yeast extract, 1% NaCl, adjusted pH

7.5 with NaOH], containing 20 μg/ml chloramphenicol in a 500 mL Erlenmeyer flask with baffles and pre-cultured at 30˚C for 18 h. The bacteria were transferred into 1.5 L of 0.5 x animal-derived component free medium(0.5% Polypepton N, 0.5% yeast extract, 0.5% NaCl; pH 7.5), containing 10 mg/ml chloramphenicol in a 3 L Erlenmeyer flask with baffles, and aerobically cultured at 37˚C. After 3 h, Isopropyl β -D-1-thiogalactopyranoside(IPTG) and calcium acetate were added to the culture to a final concentration of 2 mM and 100 mM, respectively, and the culture was continued at 37˚C for 24 h. The cultured flasks were cooled at 4˚C for 3 days, and the culture broth was harvested by centrifugation at 10,800 *g* and 4˚C for 30 min. All purification procedures as follows were performed at 4˚C. Ammonium sulfate was added to the culture broth to 80 % saturation and stirred at 4˚C for 18 h. The mixture was centrifuged at 10,800 *g* and 4˚C for 30 min. The precipitate was dissolved with 10 mM Tris(hydroxymethyl)-aminomethane (tris)-HCl buffer(pH 8.0), containing 1 mM $CaCl_2$, and then was dialyzed extensively against the same buffer. The dialyzed suspension was centrifuged at 10,800 *g* and 4˚C for 30 min. Dithiothreitol(DTT) and NaCl were added to the supernatant to a final concentration of 5 mM and 0.5 M, respectively and then stirred at 4˚C for 2 h. The

activated enzyme solution was loaded onto a Benzamidine-Sepharose 6B column(*f*2 x 3.2 cm; GE Healthcare Bio-Sciences KK, Tokyo, Japan) equilibrated with 50 mM Tris-HCl buffer(pH 8.0), containing 1 mM $CaCl_2$, 0.5 M NaCl and 5 mM DTT. The column was washed extensively with 50 mM Tris-HCl buffer(pH 8.0), containing 1 mM $CaCl_2$ and 0.5 M NaCl. The enzyme was eluted with 50 mM Tris-HCl buffer(pH 8.0), containing 1 mM $CaCl_2$ and 0.5 M L-Arg-HCl. The eluted fractions were collected, dialyzed against HBSS(life technologies, NY, USA) and desalted by dialysis against water. The desalted fraction was lyophilized and stocked at -20˚C. Enzymatic activity of CP was assayed with Bz-L-Arg-*p*NA. Six hundred fifty micro litter of 72 μM substrate solution in 49.6 mM HEPES-NaOH buffer(pH 7.6) containing 1 mM $CaCl_2$ was pre-incubated at 37˚C. The enzyme reaction was started by addition of 20 μl of the enzyme solution. The color of *p*-nitroaniline produced was monitored at 405 nm and 37˚C(cell-length = 1 cm and band-pass = 2 nm) using a Hitachi UV-Visible, Scanning Spectrophotometer(model U-3010). The enzymatic activity was calculated using a molar extinction coefficient of *p*-nitroaniline at 405 nm, $e_{405\,nm} = 11{,}500\ M^{-1}\ cm^{-1}$. One unit of CP activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitroaniline in 1 min under the

above described condition.

Preparation of recombinant ChNP (Main Body text p=17)

ChNP was cloned and recombinant ChNP was prepared with almost the same method as that used for CP described above(*Manuscript in preparation*). The enzymatic activity of ChNP was assayed using azocasein. Eighty micro litter of 1.25% azocasein with 100 mM Tris-HCl buffer(pH 7.5) containing 1 mM $CaCl_2$ were pre-incubated at 37˚C. The enzyme reaction was started by the addition of 20 µl of the enzyme solution. After incubation at 37˚C for 30 min precisely, the enzyme reaction was terminated by addition of 100 µl of 10 % trichloroacetic acid and then chilled on ice. After being left on ice for 10 min, the mixture was then centrifuged at 15,000 *g* and 4˚C for 10min. An equal volume of 0.75 M NaOH was added to the supernatant, and the color of free azo color was monitored at 440 nm(cell-length = 1 cm and band-pass = 4 nm) using a Hitachi ratio beam spectrophotometer(model U-1900). One unit of ChNP activity was defined as the amount of enzyme releasing 1 absorption unit at 440 nm of colored substrate in 1 min under the above described conditions.

Determination of the amount of TL and ChNP (Main Body text p=17)

The standard amount of TL(0.075 mg) was adjusted to equal that of the crude collagenase from Ch using azocasein as a substrate. To clarify the effectiveness of the two types of neutral proteases(TL and ChNP), the marginal dose of TL was sought by performing rat islet isolation using several amounts of TL(0.075, 0.015, 0.0075 or 0.0038 mg) together with the same amount of collagenase. The islet yield in each group was 2,174.8±122.3, 1,523.0±137.7, 1,039.8±128.2 and 433.8±73.6 IEQs, respectively(n=8/group). Given that a substantial number of islets were needed to perform functional assays, the suitable dose of TL was considered to be 0.015 mg in the present study. Accordingly, the dose of ChNP was also adjusted to the same amount of protein as TL(0.015 mg).

Measurement of tryptic activity and chymotryptic activity (Main Body text p=19)

The tryptic activity was assayed using N-CBZ-Val-Gly-Arg-*p*NA(2), and the chymotryptic activity was assayed using Suc-Ala-Ala-Pro-Phe-*p*NA(3). The assay using

the *p*NA-substrate was performed according to the method described by Grant et al.(4). Each substrate was dissolved in an assay buffer(50 mM Tris-HCl buffer, pH 8.0, 20 mM $CaCl_2$) at a concentration of 100 μM. Next, 490 μL of the substrate solution was pre-incubated at 37˚C, and the enzyme reaction was started by the addition of 10 μl of the enzyme sample. The color of liberated *p*-nitroaniline was monitored at 405 nm using a Hitachi U-3010 UV-Visible Scanning Spectrophotometer(Hitachi High-Technologies Co., Tokyo, Japan). The enzymatic activities were calculated using a molar extinction coefficient of ε_{405} = 11,500 M^{-1} cm^{-1} for *p*-nitroaniline. One unit of enzymatic activity toward each *p*NA-substrate was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitroaniline in 1 min at 37˚C, pH 8.0.

References

1. Nakamura A, Koide Y, Kawamura F, Horinouchi S, Uozumi T, Beppu T. Construction of a Bacillus subtilis strain deficient in three proteases. Agric Biol Chem 1990; 54 (5): 1307.

2. Saito T, Ishihara K, Kato T, Okuda K. Cloning, expression, and sequencing of a

protease gene from Bacteroides forsythus ATCC 43037 in Escherichia coli. Infect Immun 1997; 65 (11): 4888.

3. DelMar EG, Largman C, Brodrick JW, Geokas MC. A sensitive new substrate for chymotrypsin. Anal Biochem 1979; 99 (2): 316.

4. Grant GA, Eisen AZ, Bradshaw RA. Proteolytic Enzymes. Part C. Methods in Enzymology 1981; 80: 722.

Figure S1

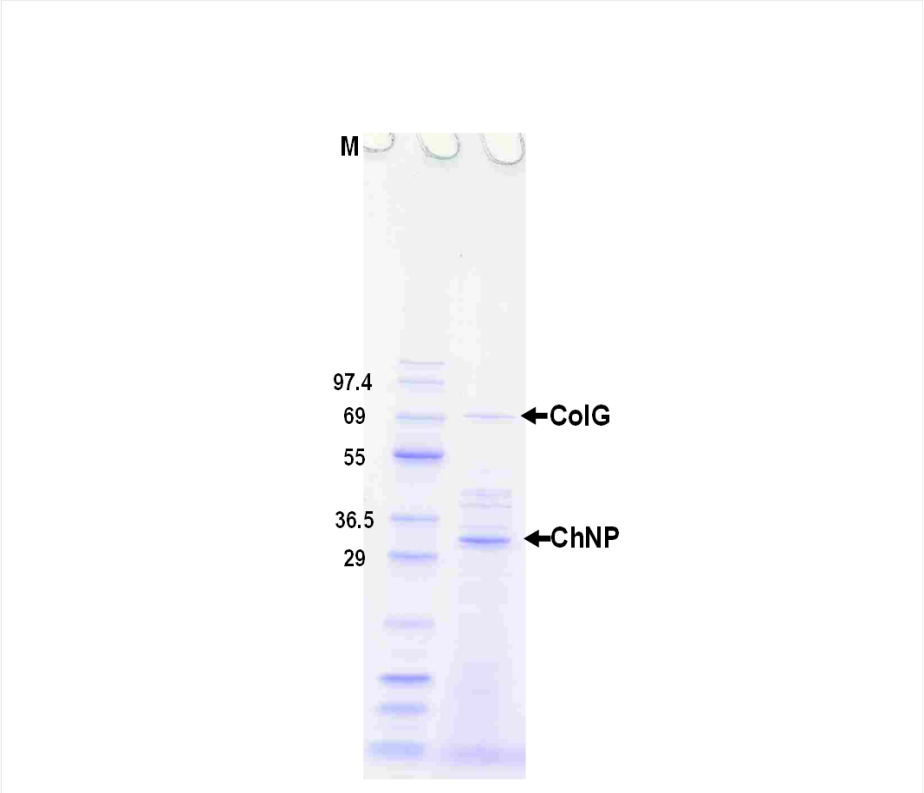

Figure S1. SDS-PAGE of SERVA Neutral protease NB

Neutral protease appeared as the main band. However, the high molecular weight impurity was identified as ColG. Also, some other impurity bands were observed.

Figure S2

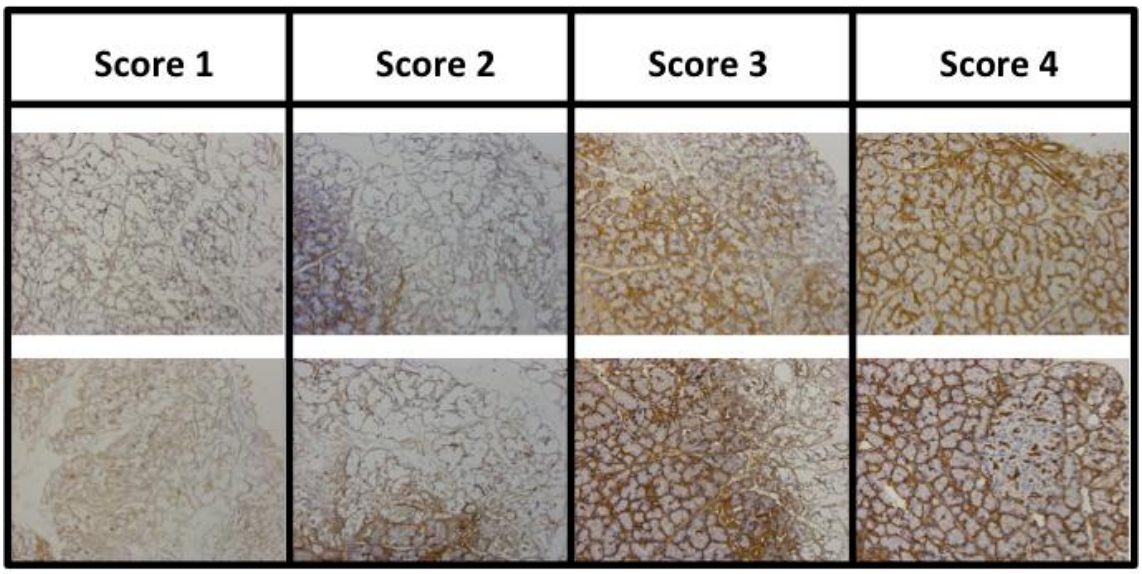

Figure S2. Representative photomicrographs of laminin immunohistochemical staining

Forty sections from the each experimental group were evaluated and scored in terms of the intensity of laminin staining and the maintenance of tissue structure using double-blind systems by independent 3 investigators. Score 4 was defined by strong laminin staining and well maintenance of the tissue structure. Low scores indicated weak staining of laminin and tissue structural collapse in a step-by-step manner.

SDC, Table

Group	Score				
	Number / %	1	2	3	4	
No Enzyme		0	0	1	119	120
		0.00	0.00	0.83	99.17	
TL		38	82	0	0	120
		31.67	68.33	0.00	0.00	
TL+CP		67	52	1	0	120
		55.83	43.33	0.83	0.00	
ChNP		0	0	6	114	120
		0.00	0.00	5.00	95.00	
ChNP+CP		3	39	77	1	120
		2.50	32.50	64.17	0.83	
		108	173	85	234	600

SDC, Table. Summary of laminin staining score in each experimental group

In the no enzyme group and the ChNP group, score 4 accounted for more than 95%. In the TL group and the TL+CP group, score 1 or 2 accounted for more than 99%. In the ChNP+CP group, score 3 and score 2 indicated 64.2% and 32.5%, respectively.