Appendices

Figure A.1

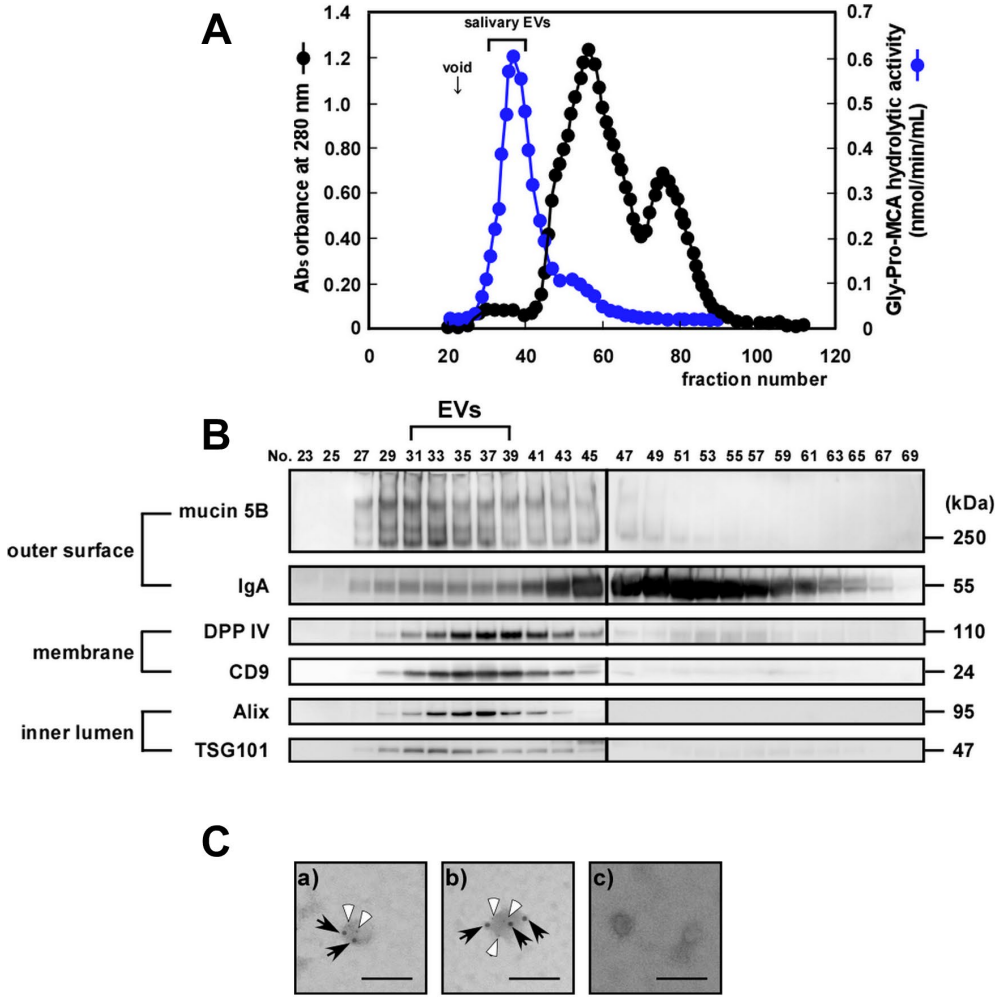
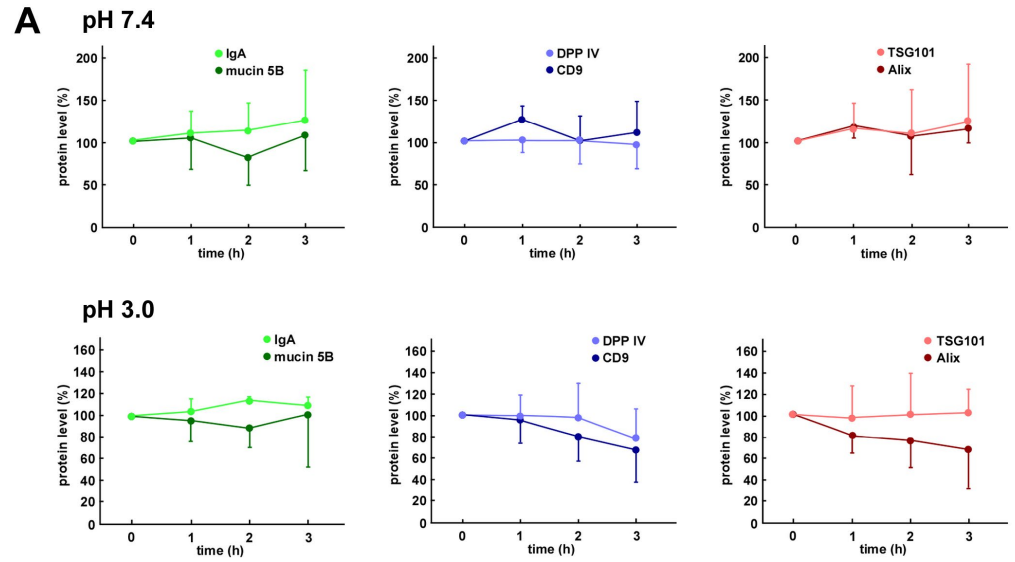
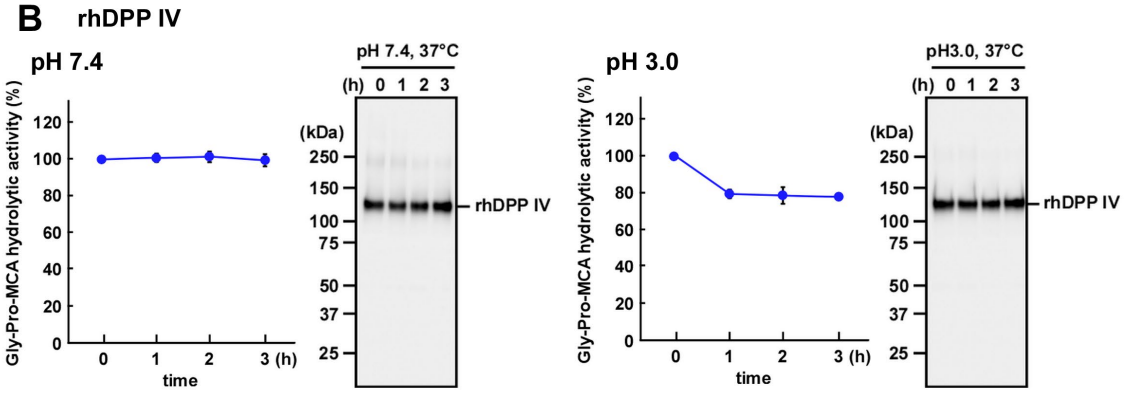
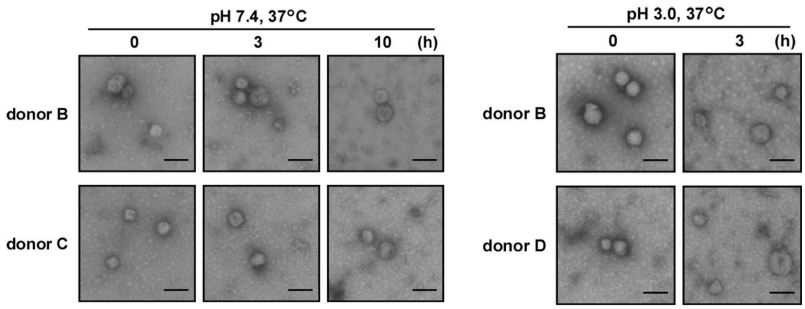


Figure A.1. Preparation of extracellular vesicles (EVs) derived from human saliva. (A) Gel filtration chromatography (Sephacryl S-500 HR) elution profiles of the EVs from fresh human saliva (donor G). (B) Western blot analysis of proteins located on the outer surface (mucin 5B and IgA), membrane (DPP IV and CD9), and inner lumen (Alix and TSG101) of the salivary EV fractions eluted from gel filtration columns (donor G). Numbers refer to the different fractions obtained via gel filtration column chromatography showed in panel A. Overall, 20 µL of each EV fraction was subjected to SDS-PAGE and analyzed using western blotting. (C) Detection of DPP IV and EV-associated proteins in the salivary EV fractions (donor A) using immunoelectron microscopy. The EV fractions were incubated with a) anti-DPP IV (black arrows) and anti-Alix (open arrowheads) antibodies, and b) anti-DPP IV (black arrows) and anti-TSG101 (open arrowheads) antibodies; c) goat and mouse immunoglobulins were used as negative controls. Scale bars, 50 nm.

Figure A.2







D

pH 7.4

time (h)	particle size (%)
0	100
3	107 ± 12

pH 3.0

time (h)	particle size (%)
0	100
3	120 ± 25

Figure A.2. Salivary EVs under acidic and neutral conditions. Salivary EV fractions were incubated at pH 7.4 and 37 °C for 1 h, or pH 3.0 and 37 °C for 3 h. (A) Western blot analysis of the proteins located on the outer surface (mucin 5B and IgA), membrane (DPP IV and CD9), and inner lumen (Alix and TSG101) of salivary EVs. The integrated density of bands in the Western blots showed in Figure 1B was measured using ImageJ and normalized to the 0-h sample as shown in the line graphs. The data represent the mean ± SD of 3 – 4 samples incubated at pH 7.4 and 4 – 8 samples incubated at pH 3.0. (B) Changes in the activity of recombinant human DPP IV (rhDPP IV). rhDPP IV was incubated at pH 7.4 or pH 3.0, 37 °C for 3 h. The remaining rhDPP IV activity was measured in triplicates and is represented as the mean ± SD. The rhDPP IV samples were then subjected to Western blot analysis using anti-DPP IV antibodies. (C) TEM images of the salivary EVs of different donors under acidic and neutral conditions, in line with Figure 1D. Salivary EV fractions (donor B and C) were incubated at pH 7.4 and 37 °C for 1 h (left). Salivary EV fractions (donor B and D) were incubated at pH 3.0 and 37 °C for 3 h (right). Scale bar, 100 nm. (D) The particle size of salivary EVs was analyzed using the DLS measurements as per in Figure 1E. Data are shown as the mean ± SD of 3 – 4 experiments. The average particle size of the EVs at 0 min was 50.6 ± 8.5 nm at pH 7.4 (3 experiments) and 51.3 ± 9.2 nm at pH 3.0 (4 experiments).

Figure A.3

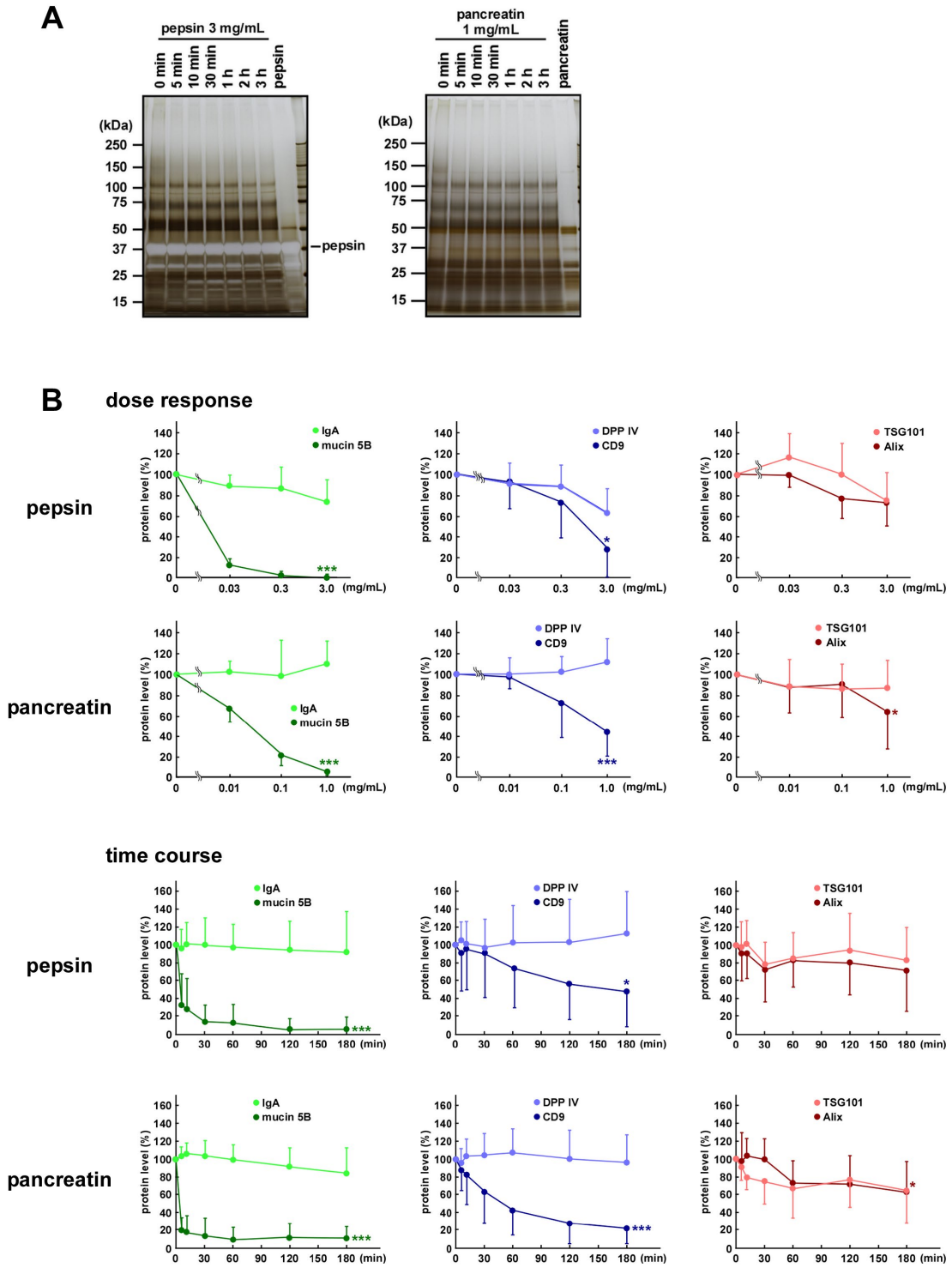
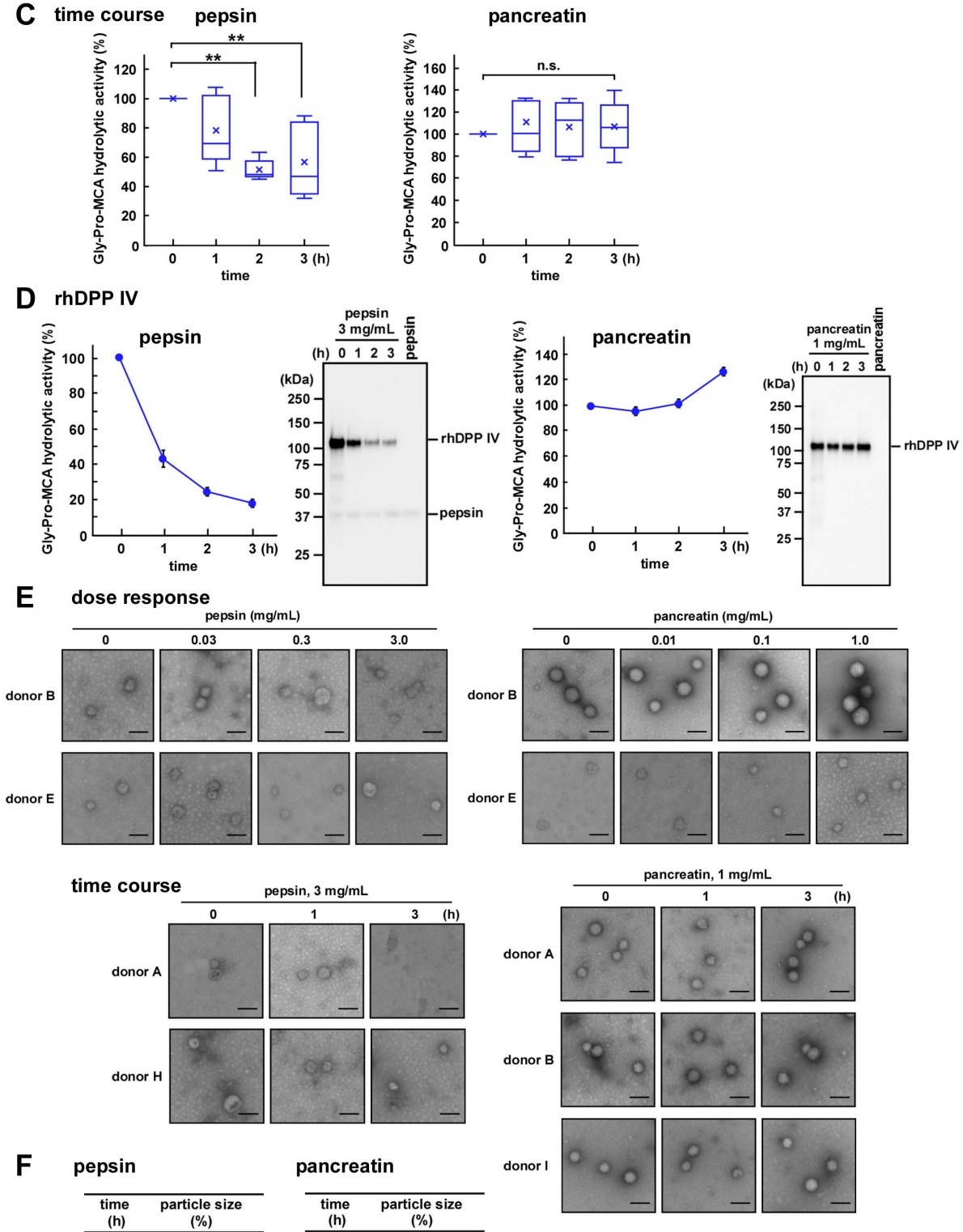

A
pepsin 3 mg/mL
0 min
5 min
10 min
30 min
1 h
2 h
3 h
pepsin
(kDa)
250
150
100
75
50
37
25
15
pepsin
pancreatin 1 mg/mL
pancreatin
B
dose response
pepsin
pancreatin
time course
IgA
mucin 5B
DPP IV
CD9
TSG101
Alix
protein level (%)
(mg/mL)
(min)

Figure A.3

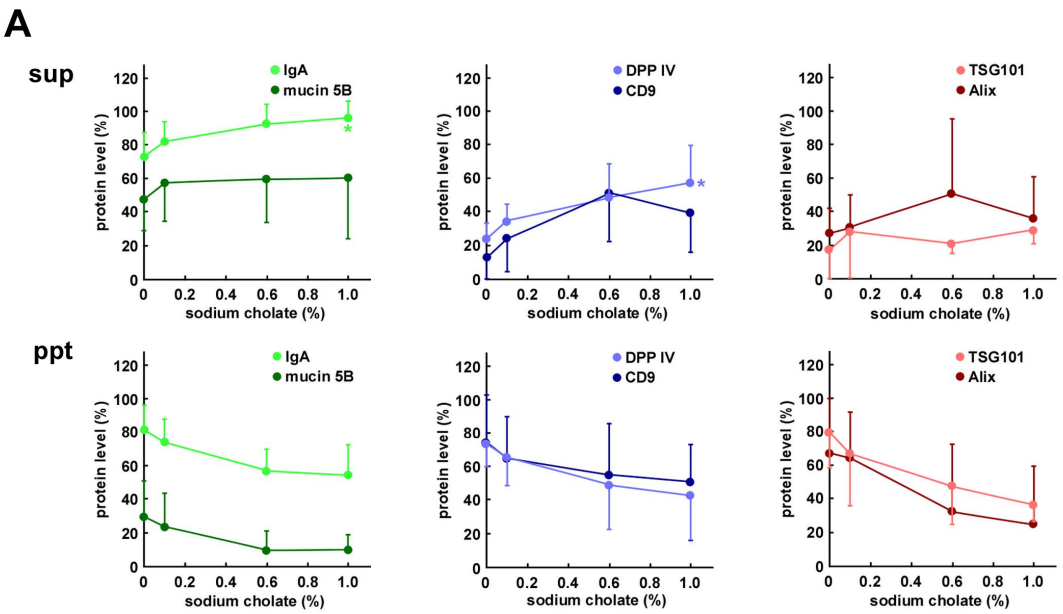


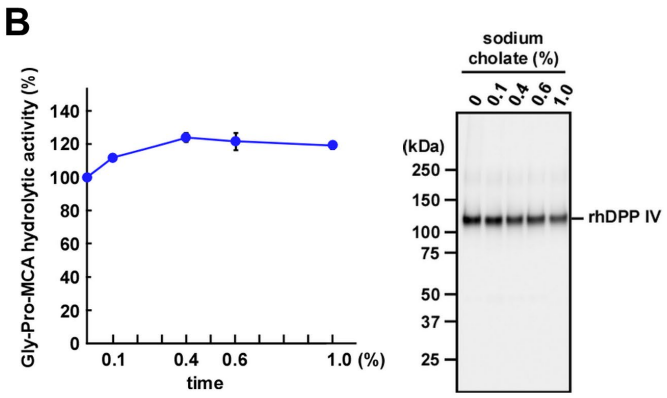
time (h)	particle size (%)
0	100
3	82.9 ± 26

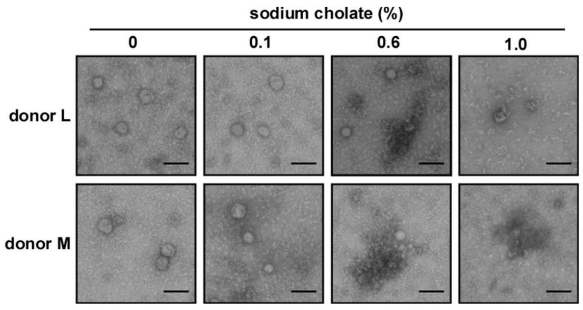
time (h)	particle size (%)
0	100
3	100 ± 30

Figure A.3. Stability of the salivary EVs in the presence of gastrointestinal enzymes. Salivary EV fractions were incubated at pH 3.0 and 37 °C in the presence of pepsin, or at pH 7.4 and 37 °C in the presence of pancreatin. (A) Pepsin was added to the EV fractions to a final concentration of 3 mg/mL and the mixtures were incubated for the indicated time periods. Similarly, pancreatin was added to the EV fractions to a final concentration of 1 mg/mL and the mixtures were incubated for the indicated time periods. Two micrograms of the EV fractions were subjected to SDS-PAGE and visualized using silver staining (donor A). (B) Western blot analysis of the proteins located on the outer surface (mucin 5B and IgA), membrane (DPP IV and CD9), and inner lumen (Alix and TSG101) of salivary EVs. For the dose-response study, the integrated density of the bands showed in Figure 2B was measured using ImageJ and normalized to that for 0 mg/mL band as shown in the line graphs. The data represent the mean ± SD of 3 – 6 samples for pepsin or 6 – 10 samples for pancreatin. For the time-course study, the data represent the mean ± SD of 5 – 9 samples for pepsin or 5 – 16 samples for pancreatin. Statistical comparisons were performed against control samples, incubated without pancreatin. *, $p < 0.05$. ***, $p < 0.001$. (C) Changes in the DPP IV activity in the salivary EV fractions. The EV fractions were incubated with pepsin or pancreatin for the indicated time periods. The DPP IV activity in fresh or treated EV fractions was measured and is represented in the form of a box plot of five (pepsin) or six (pancreatin) experiments with triplicate samples. The mean is denoted as x. **, $p < 0.01$, n.s., not significant. (D) Changes in the activity of recombinant human (rh)DPP IV. rhDPP IV was incubated with pepsin (3 mg/mL) or pancreatin (1 mg/mL). The DPP IV activity of treated rhDPP IV was measured in triplicates and represented as the mean ± SD. The samples were then subjected to Western blot using anti-DPP IV antibodies. (E) TEM images of the salivary EVs of different donors in the presence of gastrointestinal enzymes. Morphological analyses of the salivary EV fractions treated with pepsin (donor B and E) or pancreatin (donor B and E) at the indicated dose, visualized under an electron microscope in line with the results presented in Figure 2D. Scale bar, 100 nm. Morphological analyses of the salivary EV fractions treated with pepsin (donor A and H) or pancreatin (donor A, B and I) for the indicated time period, visualized under an electron microscope. Scale bar, 100 nm. (F) Particle size of the salivary EVs analyzed using the DLS measurements as per Figure 2E. Data are shown as the mean ± SD of 3 experiments. The average particle size of the EVs at 0 min was 41.0 ± 8.8 nm for pepsin treatment and 38.7 ± 11 nm for pancreatin treatment.

Figure A.4





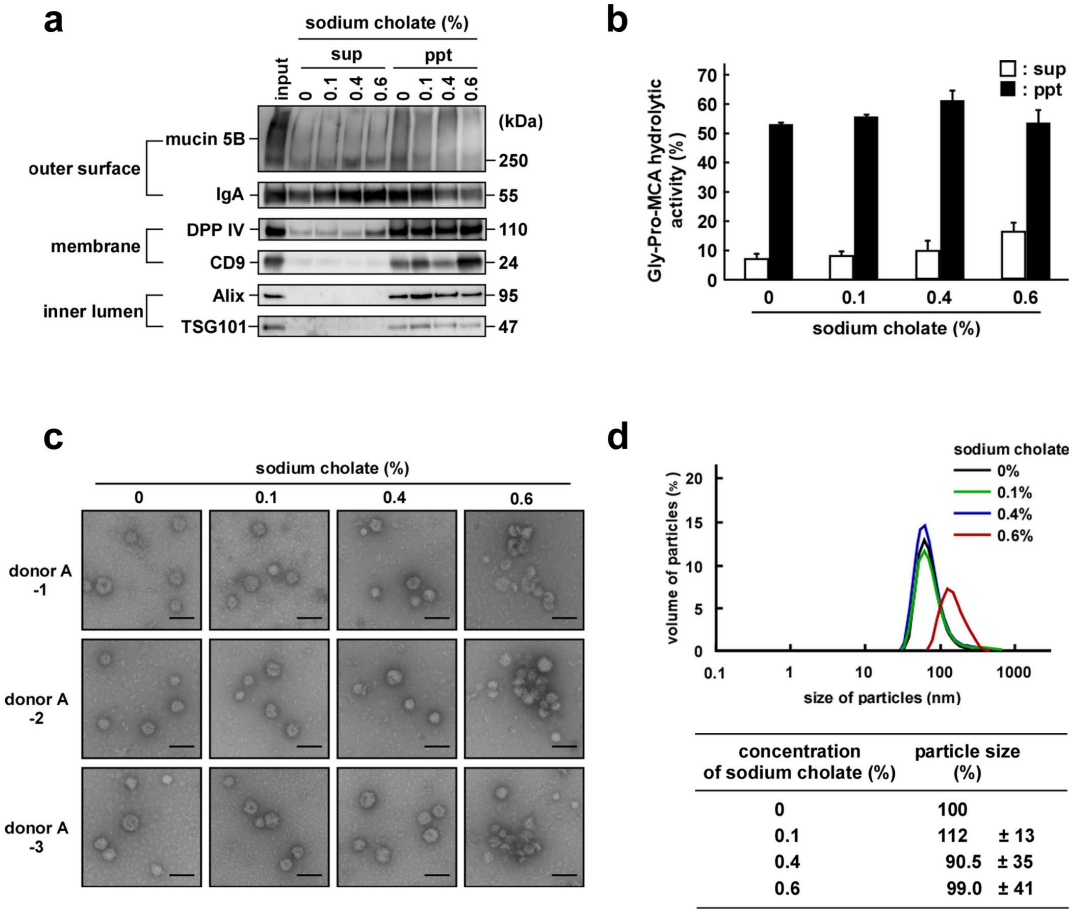


D

concentration of sodium cholate (%)	particle size (%)
0	100
0.1	83.1 ± 20*
0.6	158 ± 54*
1.0	106 ± 36

Figure A.4

E

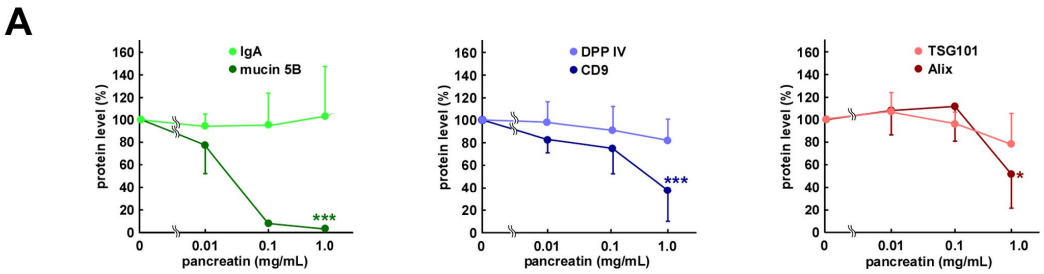


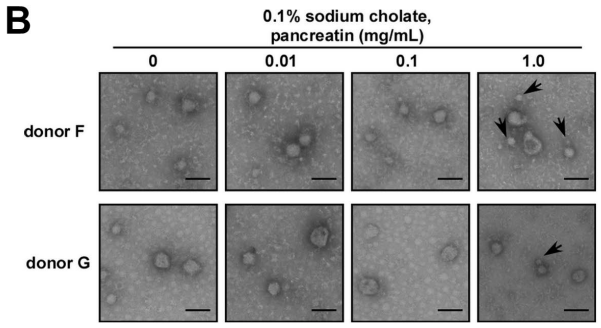
concentration of sodium cholate (%)	particle size (%)
0	100
0.1	112 ± 13
0.4	90.5 ± 35
0.6	99.0 ± 41

Figure A.4. Stability of the salivary EVs in the presence of sodium cholate. The indicated concentration of sodium cholate was added to the EV fractions and the mixtures were allowed to stand for 30 min at 25 °C. (A) Western blot analysis of the proteins located on the outer surface (mucin 5B and IgA), membrane (DPP IV and CD9), and inner lumen (Alix and TSG101) of salivary EVs. The integrated density of the bands showed in Figure 3A was measured using ImageJ and normalized to that of the bands of untreated samples, as shown in the line graphs. Data represent the mean ± SD of 3 – 5 samples. Statistical comparisons were performed against control samples, in the absence of sodium cholate. *, $p < 0.05$. (B) Changes in the enzymatic activity of rhDPP IV. rhDPP IV was incubated for 30 min at 25 °C. The remaining DPP IV activity was then measured in triplicates and is represented as the mean ± SD. The treated rhDPP IV samples were then subjected to Western blotting using anti-DPP IV antibodies. (C) TEM images of the salivary EVs of different donors in the presence of sodium cholate as per Figure 3C. Morphological analyses of the salivary EV fractions treated with the indicated concentration of sodium cholate at pH 7.4, 25 °C for 30 min (donor L and M). Scale bar, 100 nm. (D) The particle size was analyzed using the DLS measurements as per Figure 3D. Data are shown as the mean ± SD of 4–5 experiments. The average particle size of the untreated EVs after ultracentrifugation was 43.9 ± 15 nm. The

asterisk indicates a significant difference ($p<0.05$) between 0.1% and 0.6% sodium cholate. (E) Effect of sodium cholate (25 °C, pH 7.4) on the distribution of EV contents. Samples were incubated and then fractionated. (a) Western blot analysis of the proteins located on the outer surface (mucin 5B and IgA), membrane (DPP IV and CD9), and inner lumen (Alix and TSG101) of salivary EVs (donor A). (b) Changes in the DPP IV activity of salivary EVs (untreated or treated fractions). Data are represented as the mean ± SE. The experiments were performed in triplicate using a sample from donor A. (c) Morphological analyses of the salivary EV fractions visualized under an electron microscope (donor A). Scale bar, 100 nm. (d) Particle size of the salivary EVs. The particle size was analyzed using DLS measurements carried out in triplicates. A typical result is shown (donor A). The average particle size of untreated EVs after ultracentrifugation was 69.9 ± 9.6 nm.

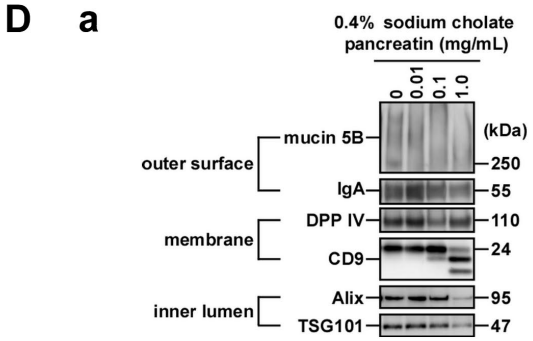
Figure A.5

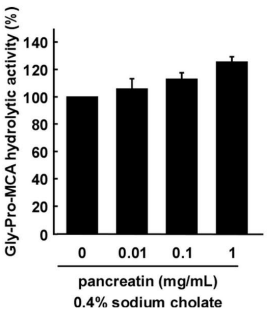


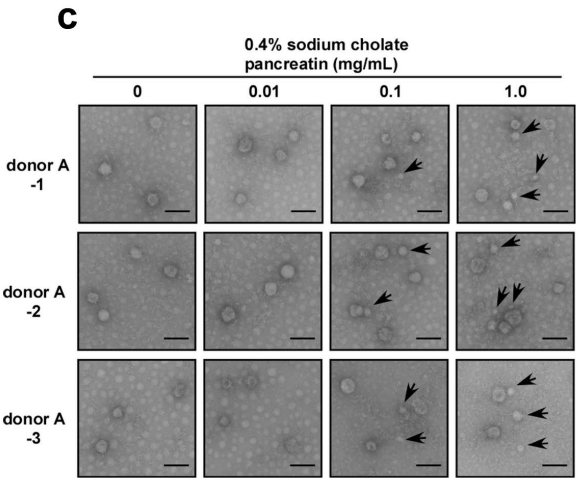


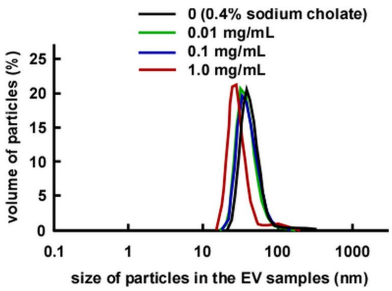
C

concentration of pancreatin (mg/mL)	particle size (%)
0 (0.1% sodium cholate)	100
0.01	92.1 ± 17
0.1	87.2 ± 16
1.0	74.2 ± 11*





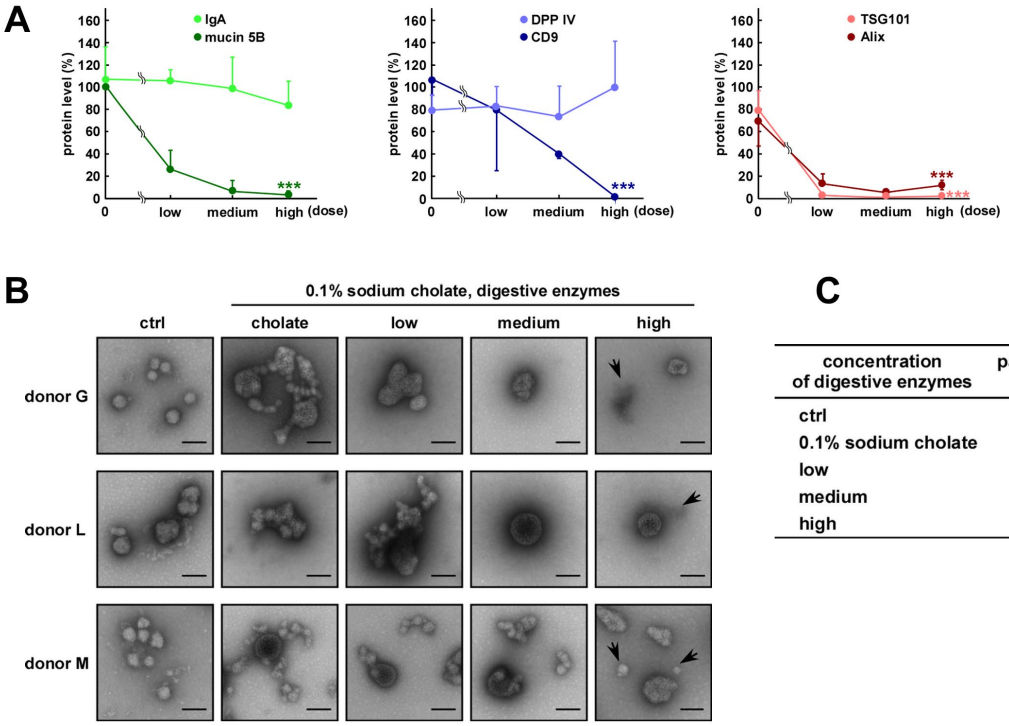




concentration of pancreatin (mg/mL)	particle size (%)
0 (0.4% sodium cholate)	100
0.01	120 ± 14
0.1	102 ± 15
1.0	91.6 ± 8.3

Figure A.5. Stability of the salivary EVs in the presence of sodium cholate and pancreatin. The indicated concentration of pancreatin was added to the EV fractions in the presence of 0.1% of sodium cholate and the mixtures were incubated at pH 7.4 and 37 °C for 1 h. (A) Western blot analysis of the proteins located on the outer surface (mucin 5B and IgA), membrane (DPP IV and CD9), and inner lumen (Alix and TSG101) of salivary EVs. The integrated density of bands showed in Figure 4A was measured using ImageJ and normalized to that of bands obtained in the context of 0.1% sodium cholate alone as shown in the line graphs. Data are shown as the mean ± SD of 5 – 7 samples. Statistical comparisons were performed against control samples, incubated in 0.1% sodium cholate in the absence of pancreatin. *, $p < 0.05$; ***, $p < 0.001$. (B) TEM images of the salivary EVs of different donors in the presence of sodium cholate and pancreatin, in line with Figure 4C. Morphological analyses of the salivary EV fractions (donor F and G) treated with the indicated concentration of pancreatin at pH 7.4 and 37 °C for 1 h in the presence of 0.1% sodium cholate. The black arrows indicate the EVs with smaller sizes. Scale bar, 100 nm. (C) The particle size was analyzed using the DLS measurements as per Figure 4D. Data are shown as the mean ± SD of 5 experiments. The average particle size of the EVs treated with 0.1% sodium cholate was 39.9 ± 8.8 nm. Asterisks indicate significant differences ($p<0.05$) between the samples incubated with or without 3 mg/mL of pancreatin in the presence of 0.1% sodium cholate. (D) Effect of 0.4% sodium cholate and pancreatin on salivary EVs. The mixtures were incubated at pH 7.4 and 37 °C for 1 h. (a) Western blot analysis of the proteins located on the outer surface (mucin 5B and IgA), membrane (DPP IV and CD9), and inner lumen (Alix and TSG101) of salivary EVs (donor A). (b) Changes in the DPP IV activity in salivary EVs (untreated and treated fractions). Data are represented as the mean ± SE. The experiments were performed in triplicate using a sample from donor A. (c) Morphological analyses of the salivary EV fractions visualized under an electron microscope (donor A). The black arrows indicate the EVs with smaller sizes. Scale bar, 100 nm. (d) Particle size of salivary EVs. The particle size was analyzed using DLS measurements carried out in triplicates. A typical result is shown (donor A). The average particle size of untreated EVs was 34.6 ± 1.9 nm.

Figure A.6



C

concentration of digestive enzymes	particle size (%)
ctrl	100
0.1% sodium cholate	111 ± 40
low	107 ± 45
medium	99.3 ± 39
high	90.5 ± 20

Figure A.6. Stability of the salivary EVs after sequential treatment. After pepsin treatment (0.03 – 3 mg/mL, pH 3.0 and 37 °C for 3 h), the pH was neutralized. Sodium cholate (0.1%) and pancreatin (0.01 – 1 mg/mL) were then added to the EV fractions and the mixtures were incubated at 37 °C for 1 h. The enzyme concentrations used were low (0.03 mg/mL pepsin and 0.01 mg/mL pancreatin), medium (0.3 mg/mL pepsin and 0.1 mg/mL pancreatin), and high (3.0 mg/mL pepsin and 1.0 mg/mL pancreatin). (A) Western blot analysis of the proteins located on the outer surface (mucin 5B and IgA), membrane (DPP IV and CD9), and inner lumen (Alix and TSG101) of salivary EVs. The integrated density of the bands showed in Figure 5A was measured using ImageJ and normalized to that of the bands obtained in the context of 0.1% sodium cholate alone as shown in the line graphs. The data represent the mean ± SD of 3 – 5 samples. Statistical comparisons were performed against control samples. ***, $p < 0.001$. (B) TEM images of the salivary EVs of different donors after sequential treatment, in line with Figure 5C. Salivary EV fractions (donor G, L, and M) were treated with pepsin at pH 3.0 and incubated at 37 °C for 3 h. Input indicates fresh and untreated EV fractions, and control (ctrl) indicates EV fractions only subjected to changes in the pH (from pH 3 to pH 7). The black arrows indicate EVs of smaller sizes. Scale bar, 100 nm. (C) Particle size was analyzed using the DLS measurements as per Figure 5D. Data are shown as the mean ± SD of 4 experiments. The average particle size of the EVs in the control group was 28.6 ± 8.4 nm.