

Synergistic Effect of Neutral Protease and Clostripain on Rat Pancreatic Islet Isolation

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Background. Islet isolation currently requires collagenase, neutral protease and other components. Thermolysin (TL) from *Bacillus thermoproteolyticus* is the gold standard neutral protease. However, we speculated that neutral protease derived from *Clostridium histolyticum* (Ch; ChNP) would be biologically superior for islet isolation. Tryptic-like activity has also been reported to be important. Therefore, we focused on clostripain (CP), since it is one of the main proteases in *Clostridium histolyticum* which possesses tryptic-like activity. We then examined the synergistic effects of highly purified ChNP and CP on rat islet isolation.

Methods. The same amount of collagenase was used in all four groups (TL, ChNP, TL+CP and ChNP+CP; n = 12/group). The efficiency was evaluated by the islet yield and function. An immunohistochemical analysis, in vitro digestion assay for each enzyme component and evaluation of the activation of endogenous exocrine proteases during islet isolation were also performed.

Results. The islet yield of the TL group was significantly higher than that of the ChNP group ($P < 0.01$). The islet yield was dose dependently increased in the ChNP+CP group, but was decreased in the TL + CP group. The islet yield in the ChNP + CP group was significantly higher than that in the TL group, but their islet function was similar. Different specificities for laminin, especially laminin-511, were observed in the TL, ChNP, and CP groups. **Conclusions.** Clostripain had a strong synergistic effect with ChNP, but not with TL. Therefore, ChNP and CP, in combination with collagenase derived from the same bacteria, may effectively increase the isolation efficiency without affecting the quality of islets.

(*Transplantation* 2015;99: 1349–1355)

Pancreatic islet transplantation can be beneficial for type 1 diabetic patients.^{1–3} However, the transplantation of islets from a single donor is currently insufficient to render a diabetic patient normoglycemic. A more efficient isolation technique is required, wherein a large number of high quality islets can be consistently isolated from 1 donor pancreas. Although several factors may be related to the results of islet isolation,^{4–7} the complexities of the pancreatic extracellular

matrix (ECM) and the tissue dissociation enzymes are crucial factors that affect islet isolation.^{4,5,8} Determining the optimal enzyme combinations is critical for obtaining proper islet release from the ECM without damaging the structural and functional integrity of the islets.

The current combination used for islet isolation comprises collagenase subtypes (collagenase G [ColG] and collagenase H [ColH]), neutral protease, and various poorly characterized components. Collagenases are the main components

Received 7 April 2014. Revision requested 13 May 2014.

Accepted 5 December 2014.

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This study was partly supported by the grant of "Coordination, Support and Training Program for Translational Research" from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors of this publication received a partial financial support by Meiji Seika Pharma Co., Ltd according to a collaborative research contract.

M.D. and H.M. contributed equally to the work. M.D. participated in the research design, the performance of the research and the writing of the paper. H.M.

participated in the performance of the research and the writing of the paper. Y.Y. participated in the performance of the research and the writing of the paper. K.M. participated in the performance of the research and the writing of the paper. K.W. participated in the writing of the paper. T.I. participated in the performance of the research. A.I. participated in the performance of the research. Y.I. participated in the writing of the paper. Y.K. participated in the performance of the research. M.E. participated in the performance of the research. K.F. participated in the writing of the paper. K.I. contributed to the analysis. N.O. participated in the writing of the paper. S.S. participated in the writing of the paper. M.G. participated in the research design, the performance of the research and the writing of the paper.

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Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

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ISSN: 0041-1337/15/9907-1349

DOI: 10.1097/TP.0000000000000662

of the islet isolation enzyme combination and are produced by *Clostridium histolyticum* (Ch). We previously reported that ColH is crucial, whereas ColG plays only a supporting role in rat islet isolation using highly purified recombinant collagenases.⁹

Thermolysin (TL) derived from *Bacillus thermoproteolyticus* is regarded as the gold standard neutral protease. This is likely due to several practical aspects, such as its low cost, stable production,¹⁰ and strong digestion efficacy for pancreatic tissues. However, the success rate of islet isolation is still less than 50%,^{4,8} even in the most advanced centers using refined procedures. Therefore, we speculated that neutral protease derived from Ch (ChNP) would be biologically superior to TL in terms of the efficiency of islet isolation because both collagenase subtypes are also produced by Ch.

Tryptic-like activity (TLA) has also been reported to be key for the isolation by Brandhorst et al.¹¹ Their findings suggest that some unknown protease(s) other than collagenases and neutral protease may affect the pancreatic dissociation because collagenases and neutral protease do not have TLA. In the present study, we focused on clostripain (CP) because it is one of the main proteases from Ch which possesses TLA.^{12,13} We speculated that CP might exhibit biological synergy for islet isolation when combined with ChNP, but not with TL because both CP and ChNP are produced by the same bacteria (Ch).

In this study, we examined the synergistic effects of highly purified recombinant CP, highly purified recombinant ChNP and TLA-free collagenase (highly purified recombinant ColG and ColH) on rat islet isolation. We also analyzed the activation of endogenous pancreatic exocrine enzymes (trypsin and chymotrypsin) during rat islet isolation in each group because trypsin has been reported to be detrimental to the islets.¹⁴ Furthermore, we investigated the target ECM of the neutral proteases (TL or ChNP) and/or CP using an immunohistochemical analysis, in vitro digestion assay, and mass spectrometry.

MATERIALS AND METHODS

Animals

Rat pancreases were obtained from 10- to 11-week-old male inbred Lewis rats (Japan SLC Inc., Shizuoka, Japan) weighing 280 to 300 g. All animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health¹⁵ and related activities at Tohoku University (approved protocol ID: 2012 NICHe-Animal-5). Maximal efforts were made to minimize suffering.

Enzyme Activity and Blending

In the present study, recombinant enzymes (ColG, ColH, ChNP, and CP) and TL (Peptide institution Inc, Osaka, Japan) were used to prepare highly pure enzyme blends. It was confirmed that no TLA was detected in any component, except CP, of the enzyme blends used in this study. The TLA was measured by the cleavage of benzoyl-L-arginine-p-nitroanilide (Bz-L-Arg-pNA) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37°C and pH 7.5. The recombinant ColG and ColH were separately produced by genetically modified *Escherichia coli* transfected with ColG or ColH genes isolated from Ch as described previously.^{16,17} The recombinant CP and ChNP

were prepared as described in the SDC, Materials and Methods (<http://links.lww.com/TP/B131>). The collagenase activity was adjusted to equal that of the crude collagenase from Ch (Sigma Chemicals, St. Louis, MO) using azocoll and 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine as substrates. The same amount of collagenase (ColG, 2.03 mg; ColH, 1.29 mg) was used in all of the experimental groups ($n = 12/\text{group}$). The amount of TL and ChNP was determined as described in the SDC, Materials and Methods (<http://links.lww.com/TP/B131>). The standard amount of CP was adjusted to equal that of the crude collagenase from Ch using Bz-L-Arg-pNA as a substrate. Five times the standard amount of CP (0.049 mg) was added to the TL+CP and ChNP+CP groups.

Rat Islet Isolation

Rat islet isolation was performed as described previously.¹⁸ The islets were cultured in Roswell Park Memorial Institute-1640 containing 5.5 mmol/L glucose and 10% fetal bovine serum at 37°C in 5% CO₂ and humidified air for 3 hours before examination.

In Vitro Evaluation of the Islet Function

Both the adenosine diphosphate (ADP)-to-adenosine triphosphate (ATP) and ATP-to-DNA ratios were measured to evaluate the energy status of the cultured islets. After picking up 60 islet equivalents (IEQs), the ApoGlow (Lonza Rockland Inc., Rockland, ME) was used for the ADP and ATP measurements as described previously.¹⁹ Using the same sample, the DNA content was measured using a DNA Quantify (Primary cell, Ishikari, Japan) as described.²⁰ A static glucose stimulation (SGS) test was performed to test the islet function in vitro. The stimulation index was defined as the ratio of the total amount of insulin secreted during high glucose stimulation (16.7 mM) to that released during low glucose stimulation (1.67 mM).²¹ The insulin:DNA ratio was measured as described previously.²⁰

Evaluation of the Activation of Endogenous Pancreatic Exocrine Enzymes During Rat Islet Isolation

Before the removal of the pancreas, 10 mL of Hanks balanced salt solutions containing enzyme combinations was injected via the bile duct. The removed pancreas was chilled on ice with 10 mL of Hanks' balanced salt solutions for 90 minutes, then the pancreas was placed into a sample pack (Eiken, Tokyo, Japan) and was digested at 37°C for 14 minutes. During the digestion, 250 μL of the digested solutions were extracted every 3.5 minutes. Then, 250 μL of the digested solution was centrifuged at 4°C, 16,100g for 10 minutes, and 200 μL of supernatant was obtained as the sample. The tryptic and chymotryptic activities were assayed as described in the SDC, Materials and Methods, <http://links.lww.com/TP/B131>.

Immunohistochemical Staining of Rat Pancreatic Tissues Incubated With the Neutral Protease (TL or ChNP) and/or CP

Rat pancreatic tissues were divided into small pieces on ice. The reaction solutions were prepared in 24-well tissue culture plates (BD Falcon, Franklin Lakes) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0), 1 mM CaCl₂ and protease inhibitor cocktail (Complete ethylenediaminetetraacetic acid-free, Roche, Basel, Switzerland). Thermolysin and ChNP were prepared at 1 mg/mL with the

reaction solution, and CP was prepared at 5 mg/mL. The tissue pieces were incubated for 1 hour in the reaction solutions with 100 μ L of each enzyme solution under 4 conditions: TL, ChNP, TL+CP, and ChNP+CP. As a control, a tissue piece was also incubated with the buffer solution. The experiments were conducted in quadruplicate ($n = 4$). The tissue pieces were then transferred to 4% paraformaldehyde solution overnight, and embedded in paraffin. After being sliced into 4- μ m sections, each section was incubated with Proteinase K (Dako, Glostrup, Denmark) for 10 minutes at room temperature, followed by immunolabeling with primary antibodies against laminin (Abcam, Cambridge, UK) for 30 minutes at room temperature. After washing the samples with phosphate-buffered saline 3 times, secondary antirabbit antibodies conjugated to horseradish peroxidase (Dako) were used, and antibody binding was localized with diaminobenzidine hydrochloride, and samples were counterstained with hematoxylin. 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 3 independent investigators. Representative photomicrographs of laminin staining and summary of scoring are shown in **Figure S2** and **SDC Table** (<http://links.lww.com/TP/B131>), respectively.

In Vitro Laminin Digestion by the Neutral Protease (TL or ChNP) and/or CP

The enzyme solutions (ChNP, TL, and CP) were prepared at 0.1 mg/mL in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0) and 1 mM CaCl_2 . Laminin digestions were performed under five enzyme conditions: TL, ChNP, CP, TL+CP, and ChNP+CP ($n=3$, respectively), using laminin-511 and laminin-411 as the substrates (BioLamina, Stockholm, Sweden). Each laminin solution (20 μ L, 0.1 mg/mL) was mixed with 2 μ L of enzyme solution and incubated at 30°C for 10 minutes. The reaction solutions (10 μ L) for each enzyme condition were mixed with 4 μ L sodium dodecyl sulfate (SDS) sample buffer containing 10 mM ethylenediaminetetraacetic acid and then applied to SDS-polyacrylamide gel electrophoresis (PAGE).

Liquid Chromatography Mass Spectrometry Analysis of Laminins

The SDS-PAGE bands of laminin-511 and laminin-411 were subjected to in-gel digestion by trypsin after alkylation with acrylamide ($n=3$, respectively). The tryptic digested samples were concentrated and desalted with GL-Tip GC and SDB (GL Sciences, Tokyo, Japan). The liquid chromatography mass spectrometry system was composed of an EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA) connected to the EASY-Spray column (25 cm length and 75 μ m inner diameter; Thermo Fisher Scientific), and a linear trap quadrupole-orbitrap Elite (Thermo Fisher Scientific). The raw data were processed with the Proteome Discoverer version 1.4.0.288 software program (Thermo Fisher Scientific) according to the standard workflow. The database search was performed using the Proteome Discoverer with Mascot search engine (Matrix Science, London, UK) against SwissProt.

Statistical Analysis

All values were expressed as the means \pm standard error of the mean and were analyzed using the Excel program

(Statcel3). The islet yield and functions were compared using a Kruskal-Wallis test. The Steel-Dwass test was used as post hoc multiple comparisons to determine the significance of differences. The activity of endogenous exocrine enzymes was compared using a 2-factor repeated-measure ANOVA. A value of P less than 0.05 was considered to indicate statistical significance.

RESULTS

The Effects of the Neutral Protease (TL or ChNP) and/or CP on the Yield, Functions and Appearance of Isolated Rat Islets

The islet yield of the ChNP group was significantly lower than that of the TL group (651.7 ± 191.4 IEQs (358.3 ± 36.7 islet number [IN]) vs 1507.8 ± 316.6 IEQs ($1,024.0 \pm 64.3$ IN), $P < 0.01$, $n = 12$, respectively) (**Figure 1A**). When CP was added to ChNP, the islet yield was the highest of the 4 groups (1967.3 ± 347.6 IEQs (1456.3 ± 59.7 IN), $n = 12$). On the other hand, the islet yield of the TL+CP group (1307.0 ± 332.9 IEQs ($1,352.1 \pm 62.3$ IN), $n = 12$) was slightly lower than that of the TL group. The islet yield was dose-dependently increased in the ChNP + CP, whereas it was found to decrease in the TL + CP group.

In the in vitro functional assays using the isolated islets, the ADP:ATP ratio of the ChNP group ($n = 8$) was significantly higher than that of the ChNP+CP group ($P < 0.05$, $n = 12$), but there were no significant differences among the other groups (**Figure 1B**). In addition, the ATP:DNA ratio of the ChNP group ($n = 8$) was significantly lower than that of the ChNP+CP group ($P < 0.01$, $n = 12$), but there were no significant differences among the other groups (**Figure 1C**). No significant differences were detected among any of the groups in terms of the insulin:DNA ratio (**Figure 1D**) or stimulation index in the SGS test (**Figure 1E**).

The proportion of small islets was apparently increased in the TL+CP group compared with the TL group. However, no obvious fragmentation was seen when CP was added to ChNP (**Figure 2**).

The Activation of Endogenous Pancreatic Exocrine Enzymes During Rat Islet Isolation

In the TL group, both the tryptic and chymotryptic activities were elevated from the initial stage of digestion. This tendency was more pronounced when CP was added to TL. In contrast, neither tryptic nor chymotryptic activity was induced in the ChNP group. Of particular interest, when CP was added to ChNP group, the chymotryptic activity markedly elevated, whereas there was only a marginal increase in the tryptic activity (**Figure 3**).

Immunohistochemical Analysis of Rat Pancreatic Tissues Incubated With the Neutral Protease (TL or ChNP) and/or CP

In the control group (**Figure 4A**), the lobular and acinar septa and the pancreatic ducts in the exocrine tissues were positively stained for laminin (score 4, 99.2%) (see **Figure S2** and **SDC Table**, **SDC**, <http://links.lww.com/TP/B131>). In the TL group (**Figure 4B**), the reaction for laminin was weak (score 1, 31.7%; score 2, 68.3%), and this tendency was further accelerated when CP was added (**Figure 4D**) (score 1,

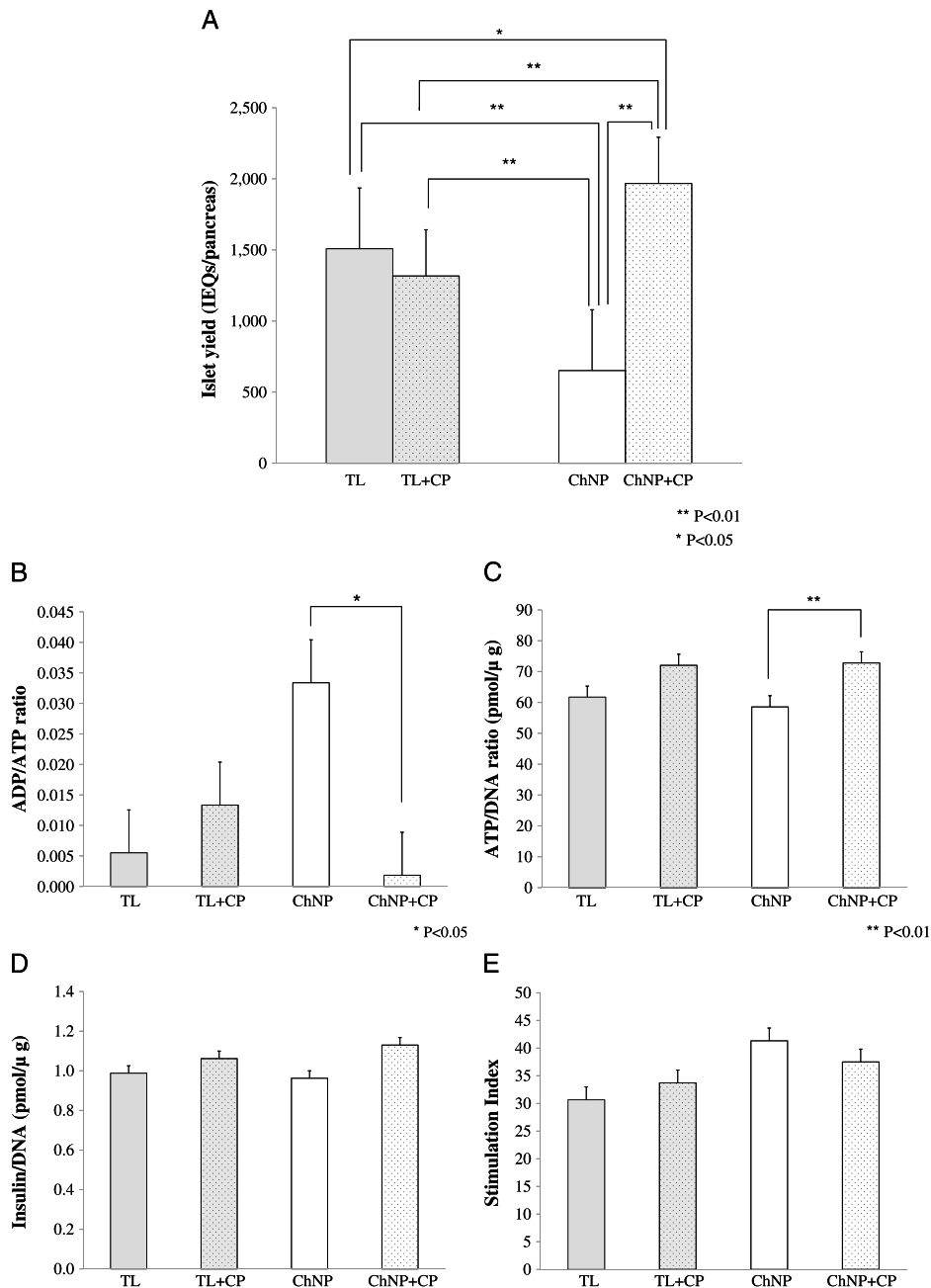


FIGURE 1. The effects of the neutral protease (TL or ChNP) and/or CP on the yield and functions of isolated rat islets. **A**, The islet yield of the ChNP group (white bar) was significantly lower than that of the TL group ($P < 0.01$). When CP was added to ChNP (white dotted bar), the islet yield was the highest among the 4 groups. On the other hand, the islet yield of the TL+CP group (gray dot bar) was slightly lower than that of the TL group. **B** and **C**, The ADP:ATP ratio (**B**) and ATP:DNA ratio (**C**) were measured to evaluate the energy status of the isolated islets. The ADP:ATP ratio of the ChNP group was significantly higher than that of the ChNP+CP group ($P < 0.05$), but there were no significant differences among the other groups. In addition, the ATP:DNA ratio of the ChNP group was significantly lower than that of the ChNP + CP group ($P < 0.01$), but there were no significant differences among the other groups. **D** and **E**, The insulin:DNA ratio (**D**) and the stimulation index in the SGS test (**E**) were measured to evaluate the functions of the islets. No significant differences were detected among the four groups in these parameters.

55.8%; score 2, 43.3%). As shown in these groups (Figure 4B and D), the tissue structure was not well maintained. In contrast, the reaction for laminin and the tissue structure was well maintained in the ChNP group (Figure 4C) (score 4, 95.0%). When CP was added to the ChNP group (Figure 4E), the laminin staining was still relatively retained (score 3, 64.2%; score 2, 32.5%), despite a substantial destruction of pancreatic tissues.

In Vitro Assay of the Laminin Digestion by the Neutral Protease (TL or ChNP) and/or CP

Laminin-511 showed 2 main bands in SDS-PAGE (Figure 5). After protease treatment, the intensity of the upper band was reduced. The digestion of the upper band was found to be in the order TL > ChNP >> CP for single enzyme digestion, and TL + CP > ChNP + CP for double enzyme digestion. On the other hand, laminin-411 showed a similar digestion pattern

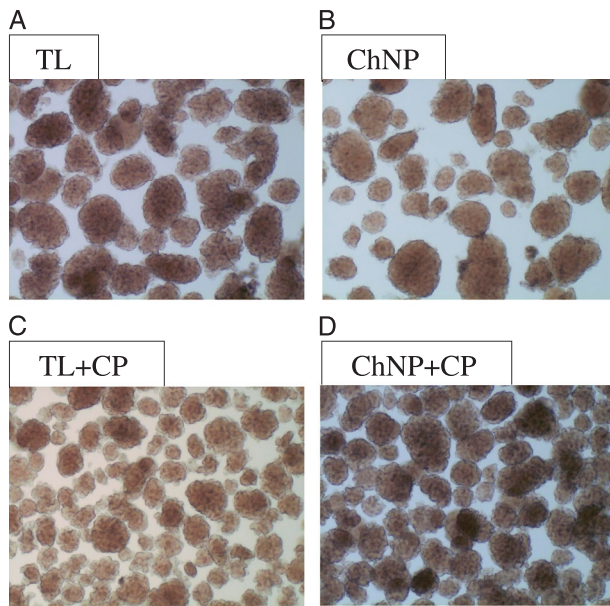


FIGURE 2. The effects of the neutral protease (TL or ChNP) and/or CP on the appearance of isolated rat islets. The proportion of small islets was increased in the TL + CP group (C) compared with the TL group (A). On the other hand, no obvious fragmentation was seen when CP was added to the ChNP group (D). Magnification, $\times 40$.

for all enzyme groups. However, enzyme treatment produced new bands that appeared under the main bands (Figure 5).

Liquid chromatography mass spectrometry analyses were conducted for the main bands of the intact protein and the new bands (asterisks in Figure 5). The upper and lower bands of laminin-511 were identified as the $\alpha 5$ and $\beta 1/\gamma 1$ chains, respectively. For laminin-411, it was verified that the single band included $\alpha 4$, $\beta 1$, and $\gamma 1$ chains. After digesting laminin-511, a new band appeared between the 2 main bands, and this band was identified as the $\alpha 5$ chain. The

band under the main band in laminin-411 was identified as the $\alpha 4$ chain.

DISCUSSION

A number of studies have investigated the roles of neutral protease during pancreatic islet isolation, but the results have been contradictory.^{8,13,22-25} One possible explanation is the enzyme combinations used in those studies. In most of the previous studies, crude enzymes were purified by anion exchange chromatography. Therefore, a risk of contamination with an unwanted collagenase subtype or unknown proteases could not be ruled out.²⁶ In the present study, we performed the first examination of the roles of TL, ChNP, and CP using highly purified enzymes. The recombinant technology used in this study provides more accurate and highly reproducible outcomes compared with the conventional anion exchange chromatography technique. We have clearly demonstrated that TL^{8,25} is more effective for dissociating rat pancreatic tissues and releasing islets from the acinar cells than ChNP. In contrast, Balamurugan et al²⁴ reported that ChNP more efficiently yielded human pancreatic islets. Although a possible involvement of species differences cannot be ruled out, this discrepancy is most likely due to the enzyme preparations used in each study. Considering that the SERVA neutral protease NB (SERVA GmbH, Heidelberg, Germany), which was used in Balamurugan's study, contains a large amount of other proteins (Figure S1, SDC, <http://links.lww.com/TP/B131>)¹⁹ and also possesses considerable TLA, the present study offers a more accurate assessment of the effects of ChNP. Balamurugan's data suggested that ChNP, combined with components that possessed TLA, but not ChNP alone, is more effective for human islet isolation than TL. In agreement with this interpretation, we have clearly shown that ChNP combined with CP (originated from the same Ch and possessing TLA) was significantly more effective for isolating rat pancreatic islets than TL,

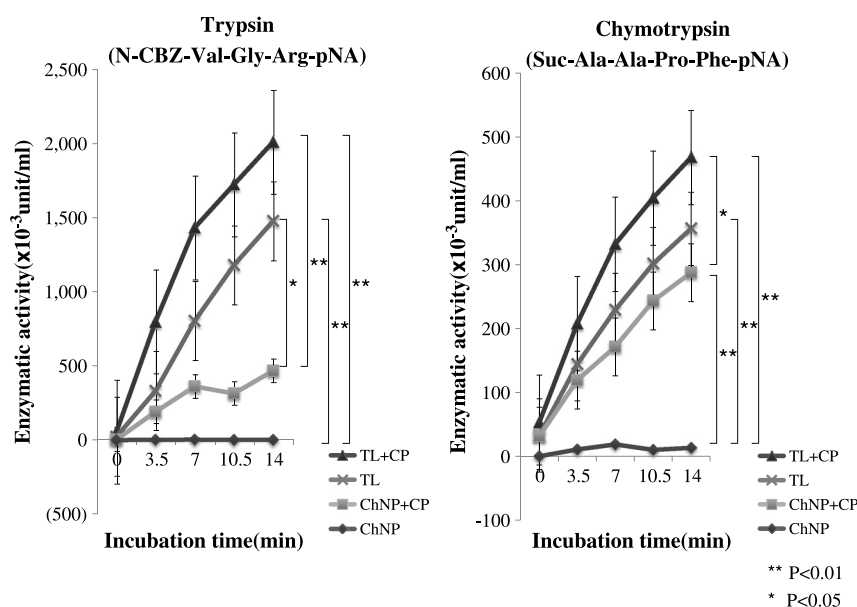


FIGURE 3. An evaluation of the activation of endogenous pancreatic exocrine enzymes during rat islet isolation. In the TL group, both the tryptic and chymotryptic activities were increased from the initial stage of digestion. This tendency was further pronounced in the TL+CP group. In contrast, neither tryptic nor chymotryptic activity was induced in the ChNP group. Of particular interest, in the ChNP+CP group, the chymotryptic activity was remarkably increased, whereas there was only a marginal increase in the tryptic activity.

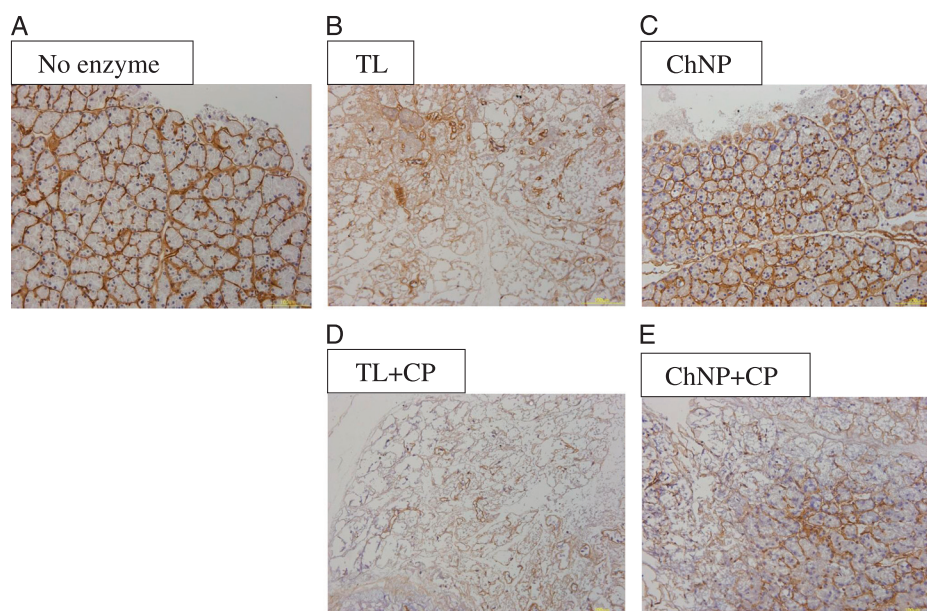


FIGURE 4. Immunohistochemical staining of rat pancreatic tissues incubated with the neutral protease (TL or ChNP) and/or CP. In the No enzyme group (A), the lobular and acinar septa, and the pancreatic ducts in the exocrine tissues, were positively stained for laminin. In the TL group (B), the reaction for laminin was weak, and this tendency was further accelerated when CP was added (D). As shown in the TL (B) and TL+CP (D) groups, the tissue structure was not well maintained. In contrast, the reaction for laminin and the tissue structure were well maintained in the ChNP group (C). When CP was added to the ChNP group (E), the laminin staining was still relatively maintained in spite of a substantial destruction of the pancreatic tissues. Magnification, $\times 200$.

which is currently the gold standard. Taken together, the present and previous data suggest that our novel findings may also be applicable in the clinical setting.

Of particular interest, the islet yield was decreased when the CP was added to TL, which originated from a different bacterium, *Bacillus thermoproteolyticus*. Furthermore, some fragmented islets were observed in the TL+CP group, suggesting that ChNP+CP could exert synergistic effects on the efficiency of rat islet isolation, possibly because the collagenase was also produced by Ch.

An analysis of the activation of endogenous pancreatic exocrine enzymes during rat islet isolation showed that both the tryptic and chymotryptic activities were increased from the initial stage of digestion in the TL group. This tendency was further pronounced when CP was added to the TL group, likely because this caused “over-digestion” in the TL + CP group. Notably, when the CP was added to the ChNP, the chymotryptic activity was remarkably elevated, whereas only a slight elevation was seen in the tryptic activity. These

results suggest that the digestion is effectively facilitated by chymotrypsin, without direct damage to the islets by trypsin, when ChNP was combined with CP. Corroborating our findings, Li et al²⁷ demonstrated an improvement of the islet yield by adding a trypsin inhibitor during rat islet isolations. Trypsin may exert indirect effects on the pancreases by activating other serine proenzymes.²⁸

In the present study, no significant differences were detected in terms of the functions or appearances of isolated rat islets between the TL and ChNP + CP groups. However, previous study has reported that the serine protease inhibitor has protective effects on rat islet viability and morphology and has a high ability to secrete insulin.^{27,29} Therefore, under more severe conditions than that of our study, such as long cold storage time and long digestion time, the beneficial effects of the ChNP + CP combination not only on islet yield, but also on preserving the cellular functions, might be observed.

Because the digestion pattern was proven to be completely different for the neutral proteases (TL or ChNP) and/or CP, we also investigated the molecular composition of the target ECM of them using an immunohistochemical analysis, in vitro digestion assay, and mass spectrometry. In the immunohistochemical analysis, when the CP was combined with the ChNP, the laminin staining was moderately maintained despite a substantial destruction of pancreatic tissues, whereas most of the laminin was digested, and the tissue structure was damaged, in the TL group. Considering that laminin expression in the pancreatic tissues was reported to be crucial for islet survival,³⁰⁻³² it may be speculated that the beneficial effects of ChNP + CP could, at least in part, be explained by its specificity for laminins.

We further focused on laminin-411 and laminin-511, and conducted in vitro digestion assays, because these were

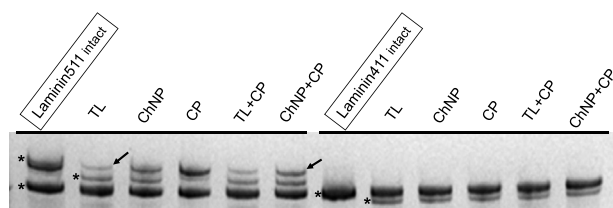


FIGURE 5. In vitro digestion of laminins by the neutral protease (TL or ChNP) and/or CP. The arrows indicate the upper band of laminin-511 showing the different intensities between the TL and the ChNP+CP groups. On the other hand, laminin-411 showed a similar digestion pattern for all enzyme groups. The bands selected for the LC-MS analyses are marked by asterisks (*) on the left side of the band. LC-MS, liquid chromatography mass spectrometry.

reported to be expressed around islet capillaries³³ and to exert crucial roles for the survival of islets.³⁴ Both the in vitro digestion assay and mass spectrometry suggested that the digestion pattern for laminin-511 (especially for the $\alpha 5$ chain), but not for laminin-411, was considerably different between the TL and ChNP + CP groups. Because the $\alpha 5$ chain of laminin is intimately related to the structure of proteoglycans, the ChNP + CP combination might be expected to better maintain the crucial proteoglycans, such as heparin sulfate,³⁵ on the islets. Further investigations of the expressed proteoglycans on the islets and the other target ECM by the neutral protease (TL or ChNP) and/or CP are topics of interest for future studies.

The present study demonstrated, for the first time, that CP has a strong synergistic effect with ChNP, but not with TL. Therefore, ChNP and CP, in combination with collagenase derived from the same bacteria, could potentially represent a promising strategy to increase the efficiency of islet isolation without affecting the quality. Further investigations using a preclinical model are required.

ACKNOWLEDGMENTS

The authors thank Kozue Imura, Takahiro Ito and Megumi Goto for their excellent technical assistance and Meiji Seika Pharma Co., Ltd. for supplying the recombinant collagenases. The authors also acknowledge the support of the Biomedical Research Core of Tohoku University, Graduate School of Medicine and TAMRIC (Tohoku Advanced Medical Research and Incubation Center), and the "Coordination, Support and Training Program for Translational Research" from the Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES

- Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*. 2000;343(4):230.
- Ryan EA, Lakey JR, Rajotte RV, et al. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes*. 2001;50(4):710.
- Shapiro AM, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med*. 2006;355(13):1318.
- Goto M, Eich TM, Feldin M, et al. Refinement of the automated method for human islet isolation and presentation of a closed system for in vitro islet culture. *Transplantation*. 2004;78(9):1367.
- Nano R, Clissi B, Melzi R, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. *Diabetologia*. 2005;48(5):906.
- Lakey JR, Warnock GL, Rajotte RV, et al. Variables in organ donors that affect the recovery of human islets of Langerhans. *Transplantation*. 1996;61(7):1047.
- Ponte GM, Pileggi A, Messinger S, et al. Toward maximizing the success rates of human islet isolation: influence of donor and isolation factors. *Cell Transplant*. 2007;16(6):595.
- Kin T, Zhai X, Murdoch TB, Salam A, Shapiro AM, Lakey JR. Enhancing the success of human islet isolation through optimization and characterization of pancreas dissociation enzyme. *Am J Transplant*. 2007;7(5):1233.
- Fujio A, Murayama K, Yamagata Y, et al. Collagenase H is crucial for isolation of rat pancreatic islets. *Cell Transplant*. 2013.
- Tajima M, Urabe I, Yutani K, Okada H. Role of calcium ions in the thermostability of thermolysin and *Bacillus subtilis* var. *amylosacchariticus* neutral protease. *Eur J Biochem*. 1976;64:243.
- Brandhorst H, Friberg A, Andersson HH, et al. The importance of tryptic-like activity in purified enzyme blends for efficient islet isolation. *Transplantation*. 2009;87(3):370.
- Tanaka H, Nariya H, Suzuki M, et al. High-level production and purification of clostripain expressed in a virulence-attenuated strain of *Clostridium perfringens*. *Protein Expr Purif*. 2011;76(1):83.
- Breite AG, Dwulet FE, McCarthy RC. Tissue dissociation enzyme neutral protease assessment. *Transplant Proc*. 2010;42(6):2052.
- Whitcomb DC, Lowe ME. Human pancreatic digestive enzymes. *Dig Dis Sci*. 2007;52(1):1.
- Bayne K. Revised Guide for the Care and Use of Laboratory Animals available. American Physiological Society. *Physiol*. 1996;39(4):199.
- Brandhorst H, Brandhorst D, Hesse F, et al. Successful human islet isolation utilizing recombinant collagenase. *Diabetes*. 2003;52(5):1143.
- Goto M, Yamagata Y, Murashima K, Yokoyama K, Fukushima T. Fusion collagenase in which affinity tag is linked and method for producing the same. vol PCT patent WO2010/058707, 2010.
- Saito Y, Goto M, Maya K, et al. The influence of brain death on tissue factor expression in the pancreatic tissues and isolated islets in rats. *Transplant Proc*. 2009;41(1):307.
- Goto M, Holgersson J, Kumagai-Braesch M, Korsgren O. The ADP/ATP ratio: a novel predictive assay for quality assessment of isolated pancreatic islets. *Am J Transplant*. 2006;6(10):2483.
- Wennberg L, Song Z, Bennet W, et al. Diabetic rats transplanted with adult porcine islets and immunosuppressed with cyclosporine A, mycophenolate mofetil, and leflunomide remain normoglycemic for up to 100 days. *Transplantation*. 2001;71(8):1024.
- Nakanishi W, Imura T, Inagaki A, et al. Ductal injection does not increase the islet yield or function after cold storage in a vascular perfusion model. *PLoS One*. 2012;7(8):e42319.
- Brandhorst H, Alt A, Huettler S, et al. The ratio between class II and class I collagenase determines the amount of neutral protease activity required for efficient islet release from the rat pancreas. *Transplant Proc*. 2005;37(1):215.
- Brandhorst H, Brendel MD, Eckhard M, Bretzel RG, Brandhorst D. Influence of neutral protease activity on human islet isolation outcome. *Transplant Proc*. 2005;37(1):241.
- Balamurugan AN, Loganathan G, Bellin MD, et al. A new enzyme mixture to increase the yield and transplant rate of autologous and allogeneic human islet products. *Transplantation*. 2012;93(7):693.
- O'Gorman D, Kin T, Pawlick R, Imes S, Senior PA, Shapiro AM. Clinical islet isolation outcomes with a highly purified neutral protease for pancreas dissociation. *Islets*. 2013;5(3):111.
- Salamone M, Seidita G, Cuttitta A, et al. A new method to value efficiency of enzyme blends for pancreatic tissue digestion. *Transplant Proc*. 2010;42(6):2043.
- Li FR, Qiu L, Yang XF, Qi H, Ren LL, Zhou HX. The protective effects of ulinastatin and adenosine triphosphate on islet function during islet isolation and after islet transplantation. *Pancreas*. 2009;38(2):227.
- White SA, Djaballah H, Hughes DP, et al. A preliminary study of the activation of endogenous pancreatic exocrine enzymes during automated porcine islet isolation. *Cell Transplant*. 1999;8(3):265.
- Tsukada M, Saito T, Ise K, et al. A model to evaluate toxic factors influencing islets during collagenase digestion: the role of serine protease inhibitor in the protection of islets. *Cell Transplant*. 2012;21(2-3):473.
- Davis NE, Beenken-Rothkopf LN, Mirsoian A, et al. Enhanced function of pancreatic islets co-encapsulated with ECM proteins and mesenchymal stromal cells in a silk hydrogel. *Biomaterials*. 2012;33(28):6691.
- Hammam E, Parnaud G, Bosco D, et al. Extracellular matrix protects pancreatic beta-cells against apoptosis: role of short- and long-term signaling pathways. *Diabetes*. 2004;53(8):2034.
- Jiang FX, Cram DS, DeAizpurua HJ, Harrison LC. Laminin-1 promotes differentiation of fetal mouse pancreatic beta-cells. *Diabetes*. 1999;48(4):722.
- Nikolova G, Jabs N, Konstantinova I, et al. The vascular basement membrane: a niche for insulin gene expression and beta cell proliferation. *Dev Cell*. 2006;10(3):397.
- Banerjee M, Virtanen I, Palgi J, Korsgren O, Otonkoski T. Proliferation and plasticity of human beta cells on physiologically occurring laminin isoforms. *Mol Cell Endocrinol*. 2012;355(1):78.
- Ziolkowski AF, Popp SK, Freeman C, Parish CR, Simeonovic CJ. Heparan sulfate and heparanase play key roles in mouse beta cell survival and autoimmune diabetes. *J Clin Invest*. 2012;122(1):132.