Published in final edited form as:

J Liq Chromatogr Relat Technol. 2011 January ; 34(3): 182–194. doi:10.1080/10826076.2011.546151.

Partition Efficiency of High-Pitch Locular Multilayer Coil for Countercurrent Chromatographic Separation of Proteins Using Small-Scale Cross-Axis Coil Planet Centrifuge and Application to Purification of Various Collagenases with Aqueous-Aqueous Polymer Phase Systems

Kazufusa Shinomiya^{1,*}, Hiroko Kobayashi¹, Norio Inokuchi¹, Kazuya Nakagomi², and Yoichiro Ito³

¹ School of Pharmacy, Nihon University, Chiba, Japan

² Faculty of Pharmaceutical Sciences, Teikyo University, Kanagawa, Japan

³ Bioseparation Technology Laboratory, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract

Partition efficiency of the high-pitch locular multilayer coil was evaluated in countercurrent chromatographic (CCC) separation of proteins with an aqueous-aqueous polymer phase system using the small-scale cross-axis coil planet centrifuge (X-axis CPC) fabricated in our laboratory. The separation column was specially made by high-pitch (ca 5 cm) winding of 1.0 mm I.D., 2.0 mm O.D. locular tubing compressed at 2 cm intervals with a total capacity of 29.5 mL. The protein separation was performed using a set of stable proteins including cytochrome C, myoglobin, and lysozyme with the 12.5% (w/w) polyethylene glycol (PEG) 1000 and 12.5% (w/w) dibasic potassium phosphate system (pH 9.2) under 1000 rpm of column revolution. This high-pitch locular tubing yielded substantially increased stationary phase retention than the normal locular tubing for both lower and upper mobile phases. In order to demonstrate the capability of the high-pitch locular tubing, the purification of collagenase from the crude commercial sample was carried out using an aqueous-aqueous polymer phase system. Using the 16.0% (w/w) PEG 1000 – 6.3% (w/w) dibasic potassium phosphate – 6.3% (w/w) monobasic potassium phosphate system (pH 6.6), collagenase I, II, V and X derived from *Clostridium histolyticum* were separated from other proteins and colored small molecular weight compounds present in the crude commercial sample, while collagenase N-2 and S-1 from *Streptomyces parvulus subsp. citrinus* were eluted with impurities at the solvent front with the upper phase. The collagenase from *C. histolyticum* retained its enzymatic activity in the purified fractions. The overall results demonstrated that the high-pitch locular multilayer coil is effectively used for the CCC purification of bioactive compounds without loss of their enzymatic activities.

Keywords

Countercurrent chromatography; Cross-axis coil planet centrifuge; High-pitch multilayer coil; Locular tubing; Proteins; Polymer phase system; Purification; Collagenases

INTRODUCTION

The cross-axis coil planet centrifuge (X-axis CPC) has been developed mainly for performing countercurrent chromatography (CCC) for purification of macromolecules with aqueous-aqueous polymer phase systems. [1–4] This apparatus has a unique mode of planetary motion such that the column holder rotates about its horizontal axis while revolving around the vertical axis of the centrifuge at the same angular velocity. [5,6] This planetary motion provides a satisfactory retention of the stationary phase for low interfacial-tension two-phase solvent systems including aqueous-aqueous polymer phase systems which tend to produce emulsification and carryover of the stationary phase in a multilayer coil of the type-J high-speed CCC centrifuge. Our previous studies demonstrated that the floor model X-axis CPC we have built with a pair of separation columns was useful for protein separation with aqueous-aqueous polymer phase systems. [7] Recently, a new small-scale X-axis CPC has been designed and fabricated to improve the partition efficiency and promote the utility of the CCC apparatus. [8] A series of experiments revealed that this apparatus was very useful for protein separation using aqueous-aqueous polymer phase systems. This small-scale X-axis CPC has a distinctive feature such that four separation columns of similar weight are mounted symmetrically around the rotary frame to achieve stable balancing of the centrifuge under a high revolution speed. Our recent studies demonstrated that this improved apparatus was also effectively used for the purification of ribonuclease (RNase) from the extract of bullfrog egg [9] and various other types of RNases [10] using an aqueous-aqueous polymer phase system without loss of their enzymatic activities.

Partition efficiency of the small-scale X-axis CPC varies according to the type of the coiled column including multilayer coil, [8–11] eccentric coil, [7,12,13], toroidal coil [12,13], and spiral coil, [14]. Our previous studies revealed that among these coiled columns the eccentric coil is most useful for protein separation in the analytical scale and the multilayer coil for the preparative scale separations. In order to achieve sufficient solute partitioning during the separation, the two phases should repeat mixing and settling at a high frequency in the coiled column, and the newly designed locular multilayer coil assembly is found to be useful for protein separation using the small-scale X-axis CPC with the polyethylene glycol (PEG) – dibasic potassium phosphate system. [15] Present studies are focused on the effect of novel column configuration of the high-pitch locular multilayer coil on the partition efficiency as well as its application to the purification of collagenase from the crude commercial samples with an aqueous-aqueous polymer phase system.

EXPERIMENTAL

Apparatus

The small-scale X-axis CPC employed in the present study was constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design and fabrication of the apparatus were described in detail elsewhere. [8,9]

Preparation of Locular Tubing

The locular tubing used in the present study was prepared from a piece of commercial 1.0 mm I.D., 2.0 mm O.D. PTFE (polytetrafluoroethylene) tubing (Flon Kogyo, Tokyo, Japan) by compressing with a pair of hemostats at 2 cm intervals. The design and preparation of the locular tubing has been described in detail. [15]

Preparation of High-Pitch Coiled Column

Figure 1 illustrates the schematic drawing of the high-pitch locular coiled column. This separation column was prepared according to the following procedure: The locular tubing

was directly wound onto the holder hub starting on the proximal side (point S in Figure 1) to make a left-handed first coil with one and one quarter turn for forward rotation column (column I). After reaching the distal side of the flange, the tube was straightly returned to the original side (this point E in Figure 1 was quarter turned to left ($\alpha = 90$ degrees) from the starting point S). The following three coils were continuously wound by the same way to complete four coils in the first layer. The second layer was wound over the first layer and finally total eight layers were formed in each high pitch multilayer coil assembly. Neighboring column (column II) for backward rotation was wound by right-handed coils. Two pairs of left-handed (Columns I and III) and right-handed (columns II and IV) coil assemblies were alternately connected in series with flow tubes in such a way that the distal non-gear terminal of the first column assembly was connected to the proximal terminal of the second column assembly and so forth. Four coil assemblies at the total capacity of 29.5 mL were symmetrically mounted on the rotary frame for balancing the centrifuge system.

Reagents

Polyethylene glycol (PEG) 1000 (MW 1,000), cytochrome C (horse heart) (MW 12,384), myoglobin (horse skeletal muscle) (MW 17,800), and lysozyme (chicken egg) (MW 13,680) were purchased from Sigma (St. Louis, MO, USA). Dibasic and monobasic potassium phosphates were obtained from Wako (Osaka, Japan).

Commercial samples of various types of collagenase were obtained from four different companies, i.e., Wako for type I, V, and X, CALBIOCHEM (present Merck Ltd., Darmstadt, Germany) for type II, MP Biochemicals (Solon, Ohio, USA) for type IV, Nittagelatin Co. (Osaka, Japan) for type N-2 and S-1. All other chemicals were of reagent grade.

Preparation of Aqueous-Aqueous Polymer Phase Systems and Sample Solutions

A two-phase polymer phase system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate was prepared by dissolving 125 g of PEG 1000 and 125 g of dibasic potassium phosphate (anhydrous) in 750 g of distilled water. Other two-phase solvent systems used in the present studies are summarized in Table 2. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated after the two clear layers formed.

The sample solutions were prepared by dissolving each sample mixture in 1.0 mL of each phase of the two-phase solvent system used for separation.

Measurement of the Distribution Ratios of Collagenase Samples

The distribution ratio of each commercial collagenase sample was determined spectrophotometrically using a simple test tube procedure as follows: [16] Two milliliters of each phase of the equilibrated two-phase solvent system was delivered into a test tube and about 1 mg of the sample was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the two clear layers formed, a 1 mL aliquot of each phase was diluted with 2 mL of distilled water and the absorbance was measured at 280 nm using a spectrophotometer (Model UV-1600, Shimadzu). The distribution ratio (D) was obtained by dividing the absorbance value of the upper phase by that of the lower phase.

CCC Separation Procedure

Each separation was initiated by completely filling the column with the stationary phase, followed by injection of the sample solution through the flow tube leading to the head of CCC column by a syringe. Then, the mobile phase was pumped into the column using a reciprocating pump (Model LC-6A, Shimadzu, Kyoto, Japan), while the column was rotated

at 1000 rpm of revolution speed. The effluent from the column outlet was collected into test tubes using a fraction collector (Model CHF100AA, Advantec, Tokyo, Japan).

In separation of collagenase samples, the above separation procedure was modified as follows: The column was first filled with the lower stationary phase followed by sample injection. Then, the column was rotated at 800 rpm and eluted with the upper mobile phase until the polar impurities are eluted from the column. Then the mobile phase was switched to the lower phase which was eluted through the column in the opposite direction to elute the target compound in the lower phase. This mobile phase exchange not only shortening the separation time, but also facilitates SDS PAGE analysis of collected fractions, since high concentration of PEG in the upper phase would interfere with the analysis.

Analysis of CCC Fractions

Each collected protein fraction was diluted with an aliquot of distilled water and the absorbance was measured at 280 nm and 540 nm (for the myoglobin peak) with a spectrophotometer (Model UV-1600, Shimadzu).

An aliquot of the CCC fraction was analyzed by SDS-PAGE. The equipment was obtained from ATTO (Tokyo, Japan), and the analysis was carried out with 12.5% polyacrylamide gel and at 100 V constant according to Laemmli's method [17].

Measurement of collagenolytic activity was conducted with a Collageno Kit CLN-100 purchased from Cosmobio Corporation (Tokyo, Japan). The fluorescence intensity of the reacted solution was measured at Ex. 495 nm and Em. 520 nm using a spectrofluorometer (Model FP-770, JASCO Corporation, Tokyo, Japan).

Evaluation of Partition Efficiency

The efficiencies in protein separations were computed from the chromatogram and expressed in terms of theoretical plate number (N) and peak resolution (Rs) each using the conventional formula.

RESULTS AND DISCUSSION

Partition Efficiency of High Pitch Locular Multilayer Coil

In order to improve the partition efficiency of protein separation using an aqueous-aqueous polymer phase system, high-pitch locular multilayer coil was mounted on the column holder of the small-scale X-axis CPC as shown in Figure 1. Figure 2 illustrates the CCC chromatograms of a set of test samples including cytochrome C, myoglobin and lysozyme with an aqueous two-phase solvent system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate. Table 1 summarizes the analytical data calculated from these chromatograms. Using the lower phase as the mobile phase (Figure 2A), these three proteins were sufficiently separated from each other with the stationary phase retention of 53.9% at 0.2 mL/min and 48.5% at 0.4 mL/min. These values are substantially higher than 33.8% obtained from the normal locular multilayer coil used in the previous studies [15]. The resolutions (Rs) between the first and second peaks and between the second and third peaks are both 1.3 at a flow rate of 0.2 mL/min and at 1.1 at 0.4 mL/min. When the upper phase was used as the mobile phase (Figure 2B), neighboring protein peaks were separated with Rs of 1.0 at 0.2 mL/min and 0.8 at 0.4 mL/min. And the stationary phase retention was 45.8% at 0.2 mL/min and 34.9% at 0.4 mL/min which are also much higher than that obtained by a normal locular multilayer coil of 16.9% [15]. The overall results indicate that the high-pitch locular multilayer coil produces higher retention of stationary phase for either phase used as the mobile phase.

Application to Purification of Various Collagenases

Sufficient retention of stationary phase makes it possible to exchange the mobile phase during each CCC separation process. This elution procedure was applied to the purification of various types of commercial collagenase samples using the high-pitch locular multilayer coil. Table 2 summarizes the distribution ratio (K_D) of various commercial collagenase samples obtained using a simple test tube method at UV 280 nm. [16] It should be noted that high K_D values obtained from commercial crude sample represent the distribution of impurities while the collagenase activities are much more distributed into the lower phase. At higher pH of the PEG 1000 – potassium phosphate systems, most of components present in collagenase samples tend to distribute in the PEG-rich upper phase. Among those, the 16.0% (w/w) PEG 1000 – 6.3% (w/w) dibasic potassium phosphate – 6.3% (w/w) monobasic potassium phosphate system (pH 6.6) was selected as a suitable solvent system to establish suitable experimental conditions for separation, where the components present in the sample including the collagenase were more distributed in the lower phase of the present solvent system than in other solvent systems. Figure 3A illustrates the CCC separation of commercial collagenase I sample originated from *Clostridium histolyticum*. As expected, a large impurity peak was found at the solvent front by eluting the upper phase as the mobile phase, and after switching the mobile phase from the upper phase to the lower phase, two small peaks were eluted as shown in the chromatogram. Figure 3B illustrates the SDS-PAGE electrophoretogram of the CCC fractions separated from commercial collagenase I sample shown in Figure 3A. Collagenase I (M. W. 112,878) was detected at the molecular weight of near 100 kDa in the CCC fraction No. 90, 93 and 95, while the impurity protein and low molecular weight of brown colored contaminants were found at the molecular weight of around 30kDa, and under 15 kDa, respectively, in the fraction No. 26. Higher collagenase activities were measured in the small peak of lower phase mobile fractions than in the large peak eluted near the solvent front as illustrated in Figure 3A.

Figure 4 illustrates the CCC chromatograms of four types of collagenase II, IV, V and X samples originated from *Clostridium histolyticum*. The results were similar to that obtained by the collagenase I sample except that the elution patterns of the small peaks eluted with the lower phase were slightly different from each other. High collagenase activities were also found in the fractions from the small peak eluted in lower phase mobile fractions. Figure 5 illustrates the electrophoretograms of the CCC fractions obtained by the collagenase II, IV, V and X samples. The band of collagenase at the molecular weight of 100 kDa was also detected in these lower phase fractions, while the low molecular weight colored contaminants (under 15 kDa) and other proteins (around 30 kDa) were detected in the upper phase fractions.

Figure 6A illustrates the CCC chromatograms of two collagenases N-2 and S-1 (M. W. 49,356) originated from *Streptomyces parvulus subsp. Citrinus*. Different from those originated from *C. histolyticum*, these two collagenases were detected in the solvent front fractions with other impurities in the upper phase mobile while no peak was eluted with the lower mobile phase.

Figures 7 and 8 illustrate amino acid sequence of *C. histolyticum* collagenase [18], and the hydropathy profile computed from the data, respectively. Figures 9 and 10 also illustrate amino acid sequence of *S. parvulus* collagenase [19] and the hydropathy profile computed from the data, respectively. Collagenases originated from *S. parvulus* are more hydrophobic than those originated from *C. histolyticum*. The CCC elution behavior may be explained by the difference in hydrophobicity between these two enzymes.

CONCLUSION

The performance of high-pitch locular multilayer coil on protein separation with an aqueous two-phase solvent system was evaluated using the small-scale X-axis CPC. Significantly increased retention of the stationary phase was achieved with either upper or lower phase used as the mobile phase which yields higher peak resolution of proteins. When the high-pitch locular multilayer coil was applied to the purification of various commercial collagenase samples, five types of collagenase I, II, IV, V and X originated from *C. hystolyticum* were successfully purified from the impurities present in each commercial sample by initially eluting with the upper phase followed by the lower, whereas two types of collagenase N-2 and S-1 from *S. parvulus. subsp. citrinus.* were not separated from other impurities in this method. The different elution behaviors between these two groups of collagenase may be explained on the basis of difference in hydrophobicity of composed amino acids from their hydropathy profiles.

Acknowledgments

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors would like to thank Mr. Kazunori Yoshida and Mr. Kazuhiro Yanagidaira (College of Science and Technology, Nihon University, Chiba, Japan) for their technical assistance. The authors are also indebted to Mr. Susumu Kasahara (Takacho Co., Tokyo, Japan) for his helpful advice.

References

1. Mandava, NB.; Ito, Y., editors. Countercurrent Chromatography: Theory and Practice. Marcel Dekker, Inc; New York: 1988. Principles and Instrumentation of Countercurrent Chromatography.
2. Conway, WD. Theory & Applications. VCH Publishers; New York: 1990. Countercurrent chromatography: Apparatus.
3. Ito, Y.; Conway, WD., editors. High-Speed Countercurrent Chromatography. Wiley-Interscience; New York: 1996. Principle, Apparatus, and Methodology of High-Speed Countercurrent Chromatography.
4. Menet, JM.; Thiebaut, D., editors. Countercurrent Chromatography. Marcel Dekker, Inc; New York: 1999. Coil Planet Centrifuges for High-Speed Countercurrent Chromatography.
5. Ito Y. Cross-axis synchronous flow-through coil planet centrifuge free of rotary seals for preparative countercurrent chromatography. Part I. Apparatus and analysis of acceleration. Sep Sci Technol. 1987; 22:1971.
6. Ito Y. Cross-axis synchronous flow-through coil planet centrifuge free of rotary seals for preparative countercurrent chromatography. Part II. Studies on phase distribution and partition efficiency in coaxial coils. Sep Sci Technol. 1987; 22:1989.
7. Shinomiya K, Muto M, Kabasawa Y, Fales HM, Ito Y. Protein separation by improved cross-axis coil planet centrifuge with eccentric coil assemblies. J Liq Chromatogr & Rel Technol. 1996; 19:415.
8. Shinomiya K, Yanagidaira K, Ito Y. New small-scale cross-axis coil planet centrifuge. The design of the apparatus and its application to counter-current chromatographic separation of proteins with aqueous-aqueous polymer phase systems. J Chromatogr A. 2006; 1104:245. [PubMed: 16405899]
9. Shinomiya K, Kobayashi H, Inokuchi N, Kobayashi K, Oshima H, Kitanaka S, Yanagidaira K, Sasaki H, Muto M, Okano M, Ito Y. New small-scale cross-axis coil planet centrifuge. Partition efficiency and application to purification of bullfrog ribonuclease. J Chromatogr A. 2007; 1151:91. [PubMed: 17400232]
10. Shinomiya K, Kobayashi H, Motoyoshi N, Inokuchi N, Nakagomi K, Ito Y. Countercurrent chromatographic separation and purification of various ribonucleases using a small-scale cross-axis coil planet centrifuge with aqueous-aqueous polymer phase systems. J Chromatogr B. 2009; 877:955.
11. Shinomiya K, Menet JM, Fales HM, Ito Y. Studies on a new cross-axis coil planet centrifuge for performing counter-current chromatography. I. Design of the apparatus, retention of the stationary

- phase, and efficiency in the separation of proteins with polymer phase systems. *J Chromatogr A*. 1993; 644:215.
12. Shinomiya K, Kabasawa Y, Ito Y. Protein separation by cross-axis coil planet centrifuge with two different types of coiled columns. *J Liq Chromatogr & Rel Technol*. 1998; 21:111.
 13. Shinomiya K, Kabasawa Y, Ito Y. Effect of elution modes on protein separation by cross-axis coil planet centrifuge with two different types of coiled columns. *Prep Biochem & Biotechnol*. 1999; 29:139. [PubMed: 10231893]
 14. Shinomiya K, Kabasawa Y, Ito Y. Protein separation by cross-axis coil planet centrifuge with spiral column assemblies. *J Liq Chromatogr & Rel Technol*. 2002; 25:2665.
 15. Shinomiya K, Ito Y. Partition efficiency of newly designed locular multilayer coil for countercurrent chromatographic separation of proteins using small-scale cross-axis coil planet centrifuge with aqueous-aqueous polymer phase systems. *J Liq Chromatogr & Rel Technol*. 2009; 32:1096.
 16. Oka F, Oka H, Ito Y. Systematic search for suitable two-phase solvent systems for high-speed counter-current chromatography. *J Chromatogr*. 1991; 538:99. [PubMed: 2050799]
 17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680. [PubMed: 5432063]
 18. Matsushita O, Jung CM, Katayama S, Minami J, Takahashi Y, Okabe A. Gene duplication and multiplicity of collagenases in *Clostridium histolyticum*. *J Bacteriol*. 1999; 181:923. [PubMed: 9922257]
 19. Sakurai Y, Inoue H, Nishii W, Takahashi T, Iino Y, Yamamoto M, Takahashi K. Purification and characterization of a major collagenase from *Streptomyces parvulus*. *Biosci Biotechnol Biochem*. 2009; 73:21. [PubMed: 19129667]

A. Overview of high-pitch locular multilayer coil

B. Sideview of column holder (right flange)

Figure 1. Schematic drawing of the high-pitch locular multilayer coil wound around the column holder of the small-scale X-axis CPC.

Figure 2.

CCC separation of proteins obtained by high-pitch locular multilayer coiled column with the lower phase mobile (A) and the upper phase mobile (B) at two different flow rates.

Experimental conditions: apparatus: small-scale X-axis CPC with high-pitch locular multilayer coil assemblies; total column capacity: 29.5 mL; sample: (A) cytochrome C (2 mg), myoglobin (8 mg) and lysozyme (10 mg), (B) lysozyme (10 mg) and myoglobin (8 mg); solvent system: 12.5% (w/w) PEG 1000 – 12.5% (w/w) dibasic potassium phosphate; mobile phase: (A) lower phase (outward elution), (B) upper phase (inward elution); flow rate: 0.2 mL/min (left side of the figure) and 0.4 mL/min (right side of the figure); revolution: 1000 rpm (counterclockwise). SF = solvent front.

Figure 3.

CCC purification of commercial collagenase I sample (A) and SDS-PAGE analysis of the CCC fractions (B). Experimental conditions: (A) sample: commercial collagenase I sample obtained from Wako (10 mg); solvent system: 16.0% (w/w) PEG 1000 – 6.3% (w/w) dibasic potassium phosphate – 6.3% (w/w) monobasic potassium phosphate; mobile phase: upper phase (during first 140 min, inward elution) and lower phase (after 140 min, outward elution). Other experimental conditions are same as those described in Figure 2 caption. SF = solvent front, LP = starting time for elution of lower phase after switching the elution direction. (B) SDS-PAGE was carried out with 12.5% polyacrylamide gel and 100 V constant according to Laemmli's method. [16] The developed gel was stained with silver.

Figure 4. CCC purification of four different types of collagenase sample originated from *Clostridium histolyticum*. Experimental conditions are same as those described in Figure 3 caption.

Figure 5. SDS-PAGE analysis of the CCC fractions obtained from various types of collagenase sample originated from *C. histolyticum*. Experimental conditions are same as those described in Figure 3 caption.

Figure 6.

CCC purification and SDS-PAGE analysis of commercial collagenase samples, type N-2 and S-1, originated from *Streptomyces parvulus subsp. citrinus*. Experimental conditions are same as those described in Figure 3 caption.

1 70
 IANTNSEKYD FEYLNGLSYT ELTNLIKNIK WNQINGLFNY STGSQKFFGD KNRVQAIINA
 LQESGRITYTA
 71 140
 NDMKGIETFT EVLRAGFYLG YYNDGLSYLN DRNFQDKCIP AMIAIQKNPN FKLGTAVQDE
 VITSLGKLIG
 141 210
 NASANAEEVN NCVPLKQFR ENLNQYAPDY VKGTAVNELI KGIEFDFSGA AYEKDVKTMP
 WYGKIDPFIN
 211 280
 ELKALGLYGN ITSATEWASD VGIYYLSKFG LYSTNRNDIV QSLEKAVDMY KYGKIAFVAM
 ERITWDYDGI
 281 350
 GSNKKVDHD KFLDDAEKHY LPKTYTFDNG TFIIRAGDKV SEEKIKRLYW ASREVKSQFH
 RVVGNDKALE
 351 420
 VGNADDVLTM KIFNSPEEYK FNTNINGVST DNGGLYIEPR GTFYTYERTP QQSIFSLEEL
 FRHEYTHYLQ
 421 490
 ARYLVDGLWG QGPFYEKNRL TWFDEGTAEF FAGSTRTSGV LPRKSILGYL AKDKVDHRYIS
 LKKTLSGYD
 491 560
 DSDWMFYNYG FAVAHYLYEK DMPTFIKMNK AILNTDVKSY DEIIKKLSDD ANKNTEYQNH
 IQELADKYQG
 561 630
 AGIPLVSDDY LKDHGYKKAS EVYSEISKAA SLTNTSVTAE KSQYFNTFTL RGTYTGETSK
 GEFKDWDEMS
 631 700
 KKLDGTLESL AKNSWSGYKT LTAYFTNYRV TSDNKVQYDV VFHGVLTDNA DISNNKAPIA
 KVTGPSTGAV
 701 770
 GRNIEFSGKD SKDEDGKIVS YDWDFGDGAT SRGKNSVHAY KKAGTYNVTI KVTDDKGATA
 TESFTIEIKN
 771 840
 EDTTTPITKE MEPNDDIKEA NGPIVEGVTV KGDNLGSSDA DTFYFDVKED GDVTIELPYS
 GSSNFTWLVI
 841 910
 KEGDDQNHIA SGIDKNNSKV GTFKSTKGRH YVFIYKHDSA SNISYSLNIK GLGNEKLKEK
 ENNDSSDKAT
 911 980
 VIPNFNTTMQ GSLLGDDSRD YYSFEVKEEG EVNIELDKKD EFGVTWTLHP ESNINDRITY
 GQVDGNKVSNI
 981 1000
 KVKLRPGKYY LLVYKYSMSG

Figure 7.

Amino acid sequence of collagenase originated from *C. histolyticum*. The data was cited from ref. [18].

Figure 8. Hydropathy profile of amino acid sequences of collagenase originated from *C. histolyticum*.

1 21 41 60
SVLVINHTCSASLRIRAQEM TSAQLASTCRSLADQDAYFH
SIAKDNGPVAGDRNTSLEVV
61 81 101 120
VYNSSTDYQTYAGADWGINT NNGGMYLEGNPSAAGNQARF
IAYEAEWARPDFEIWNLNHE
121 141 161 180
YTHYLDGRFNMYGDFNANVS TPTIWWIEGFAEYVSYSYRN
LRYDAAITEAGRRTYALSTL
181 201 221 240
FDTTYSHDSTRVYRWGYLAV RYMLQSHRAELDTVLGYRT
GNWNTARAYLTGSIGTRYDS
241 261 281 300
DWYNWLSRCAAGDCGGSTTP GNQAPTAFAVSVSGLTANF
TDRSTDSDGTIASRSWSFGD
301 321 341 360
GTTSAANPAKTYTAAGTYT VTLKVTDDKGATSTATQSIA
VGGADATPECTSSDGRELGR
361 381 401 420
NCKRSNLSATAGNYAYLIY LPAGVQQLKVTSSGGTGNAD
LYYSNSTWATTSSYTSRSTG
421 441 456
AGNSETLTITNPPAGWNYIS LAAADFSGATVKTEY

Figure 9.

Amino acid sequence of collagenase originated from *S. parvulus*. The data was cited from ref. [19].

Figure 10.
Hydropathy profile of amino acid sequences of collagenase originated from *S. parvulus*.

Table 1

Analytical values obtained by CCC separations of proteins using small-scale cross-axis coil planet centrifuge with high-pitch locular multilayer coiled columns

A. Lower phase mobile									
Flow rate (mL/min)	Elution volume (mL) (K value)		Peak resolution (Rs)		Theoretical plate number (N)	Theoretical plate number per column capacity (N/mL)	Stationary phase retention (%)		
	Cyt C	Myo	Lys	Cyt C/Myo					
0.2	15.2	22.2	39.5	1.3	114	3.9	53.9		
0.4	16.8	23.5	39.2	1.1	93	3.1	48.5		

B. Upper phase mobile									
Flow rate (mL/min)	Elution volume (mL) (K value)		Peak resolution (Rs)		Theoretical plate number (N)	Theoretical plate number per column capacity (N/mL)	Stationary phase retention (%)		
	Lys	Myo	Lys/Myo						
0.2	24.8	40.9	1.0	139	4.7	45.8			
0.4	25.6	39.4	0.8	103	3.5	34.9			

Abbreviations: Cyt C = cytochrome C; Myo = myoglobin; Lys = lysozyme. The average K values (CU/CL) for each protein were 0.11 for Cyt C, 0.56 for Myo and 1.66 for Lys, respectively.

Abbreviations: Lys = lysozyme; Myo = myoglobin. The average K values (CL/CU) for each protein were 0.64 for Lys and 1.90 for Myo, respectively.

Table 2

Distribution ratios of various commercial collagenase samples in PEG 1000 - potassium phosphate systems at different pH

pH	9.2	7.2	9.4	7.3	7.0	6.6	5.8
PEG 1000 (g/100 g)	12.5	12.5	16.0	16.0	16.0	16.0	24.0
K ₂ HPO ₄ (g/100 g)	12.5	9.4	12.5	9.5	8.3	6.3	2.0
KH ₂ PO ₄ (g/100 g)	----	3.4	----	3.1	4.2	6.3	8.0
Sample	Distribution ratio ($D = C_U/C_L$)						
Collagenase I	3.66	8.61	11.3	7.73	5.89	2.32	2.08
Collagenase II	4.52	10.4	11.0	9.83	7.20	2.87	2.69
Collagenase IV	4.94	8.95	11.3	8.34	8.12	2.84	2.31
Collagenase V	12.1	14.8	14.4	14.1	7.38	4.96	5.22
Collagenase X	9.00	12.4	11.6	9.59	7.45	4.69	4.74
Collagenase N-2	4.62	6.26	9.15	4.85	6.24	3.54	3.74
Collagenase S-1	3.65	5.54	6.69	4.60	4.96	2.43	3.05

Distribution ratios were calculated from the absorbance of the upper phase (C_U) divided by that of the lower phase (C_L) obtained by the spectrophotometric method at UV 280nm.