

Characterization, Assessment, and Autoproteolysis in Commercial Blends of Clostridial Collagenases Used for Human Islet Isolation

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Commercially available enzyme blends Liberases -HI, -PI, -CI, -RI, Blendzymes, their individual components CI, CII, Th (Roche), and Collagenases P, NB1 (Serva), etc.

are:

- **essential catalytic instruments for connective tissue disintegration and islet isolation**
- **purified from *Clostridium Histolyticum* and *Bacillus Thermoproteolyticus***
- **proteins with MM 114kDa [pI 5.36], 112kDa [pI 5.58] (Collagenases Class I, II respectively) and 34kDa [pI 5.40] (Thermolysin)**
- **encoded by known genes *ColG* for CI, *ColH* for CII, *npr* for Th**
- **Zn²⁺-metalloproteases, require Ca²⁺ for activation and binding to collagen**
- **capable of cleaving Y-Gly bond in the seq. of collagen -Pro-Y-|-Gly-Pro-, where Y-is a neutral AA**
- **optimally active at pH 7-9**
- **optimally active at a temperature of 37°C**

Liberase Enzyme Manufacture

(T. Cavanagh, 2002)

- Enrich collagenase (Dye Affinity Chromatography)
- Enrich collagenase (Cation Exchange Chromatography)
- Separate CI, CII isoforms (Anion Exchange Chromatography)
- Test specific activity of separated collagenase isoforms
- Test specific activity of thermolysin (B. thermoproteolyticus)
- Blend collagenase I, collagenase II, thermolysin to target enzyme activity ranges
- Lyophilize
- QC release testing

What is a “good lot” of Liberase HI Enzyme? (T. Cavanagh, 2002)

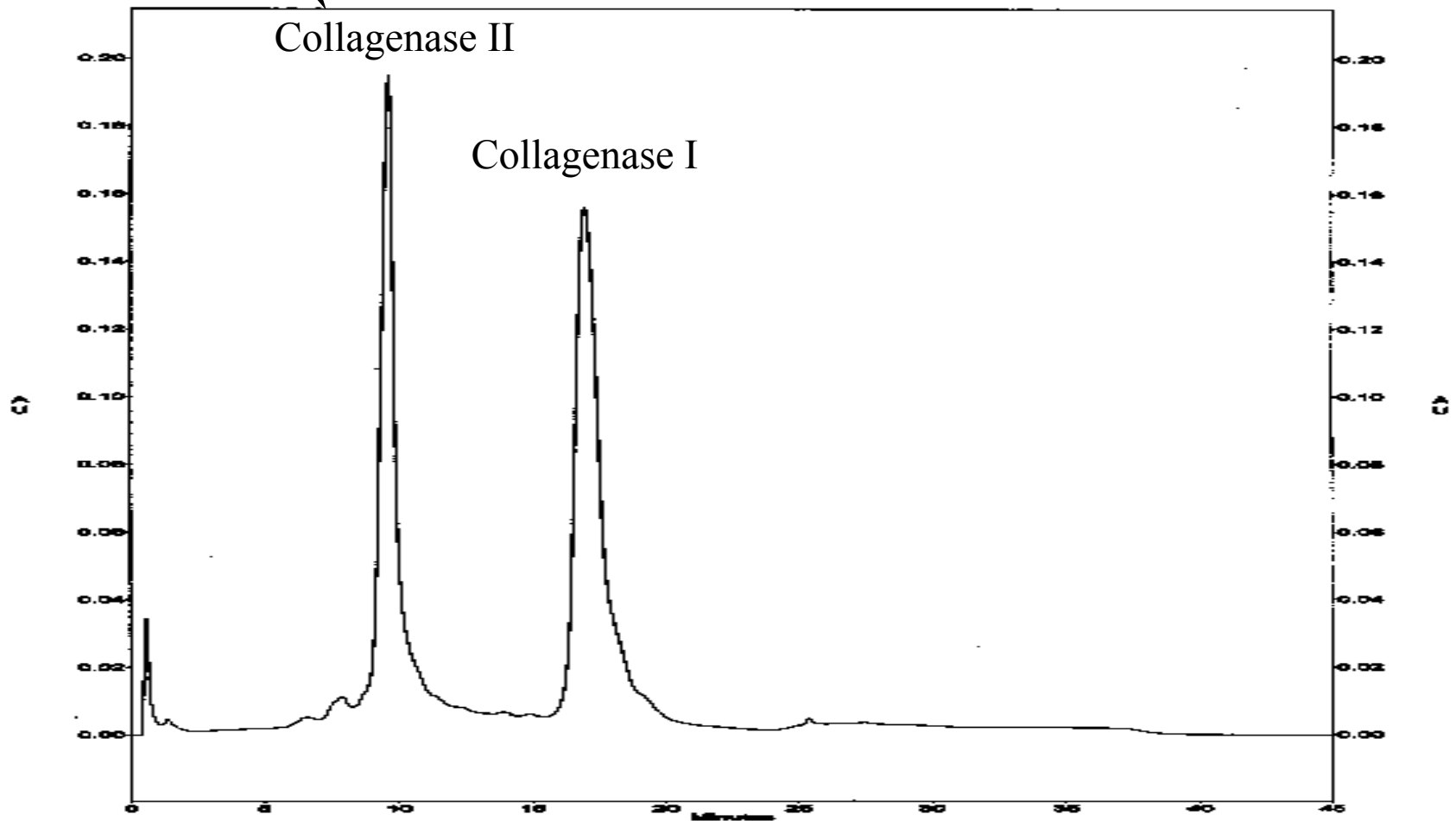
2002 Consensus

- NP 62,500 or 80,000
- Wunsch 2250 or 2800
- NP/Wunsch=28 ?
- Endotoxin ~0~
- Function tested in human
- ?

Roche manufacture / 2002

- NP 50,000 – 80,000
- Wunsch 2000 - 2650
- NP/Wunsch= 27 - 33
- Endotoxin <50 EU/mg
- In vitro enzyme activity testing

Liberase HI enzyme: mono-Q HPLC analysis (T. Cavanagh, 2002)



Sample Preparation and HPLC Conditions for Ion-Exchange Chromatography of Liberase HI on Analytical Mono Q 5/50 GL Column

(J. Lakey, 2005)

Sample: 1 vial of Liberase HI dissolved in 350 ml of 1X HBSS and 1 ml taken as a “preisolation sample”, which was diluted 5-fold with buffer A and 500 μ l (~150 μ g) injected into the column

Column: Mono Q 5/50GL, GE (Pharmacia)

Chromatographic Equipment: Beckman Gold HPLC and SC 100 Fractionator

Buffer A: 20 mM Tris-HCl, pH 7.5, 1 mM CaCl_2

Buffer B: 1 M NaCl in Buffer A

Gradient: 0%B - 2 min, 15%B – 20 min, 100%B – 10 min, 0%B - 2 min, 0%B – 10 min.

UV-Detection: 280 nm

Flow Rate: 1.5 ml/min

Peaks Intergation: 32Karat Software

Formulation Analysis by HPLC

(J. Lakey, 2005)

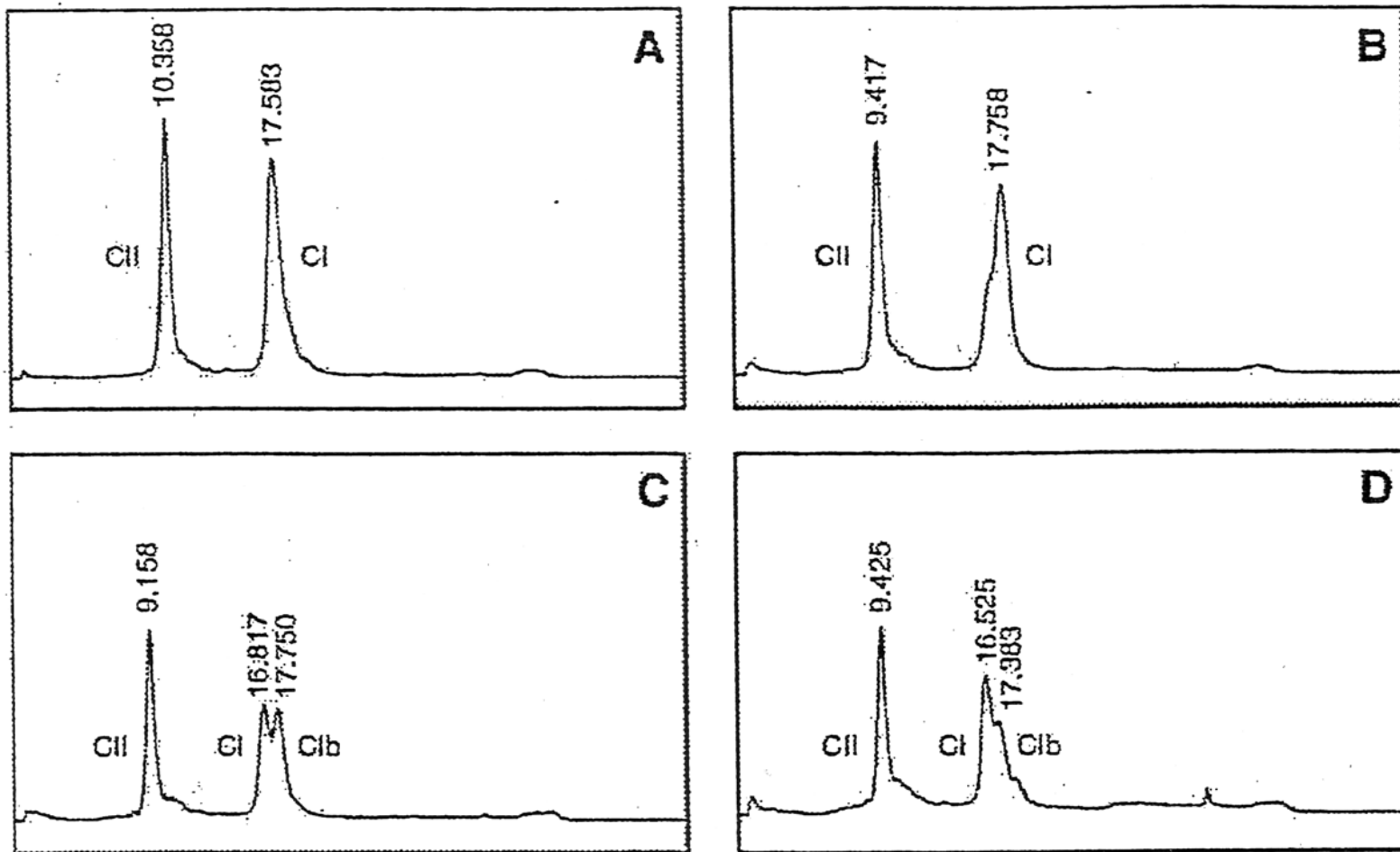
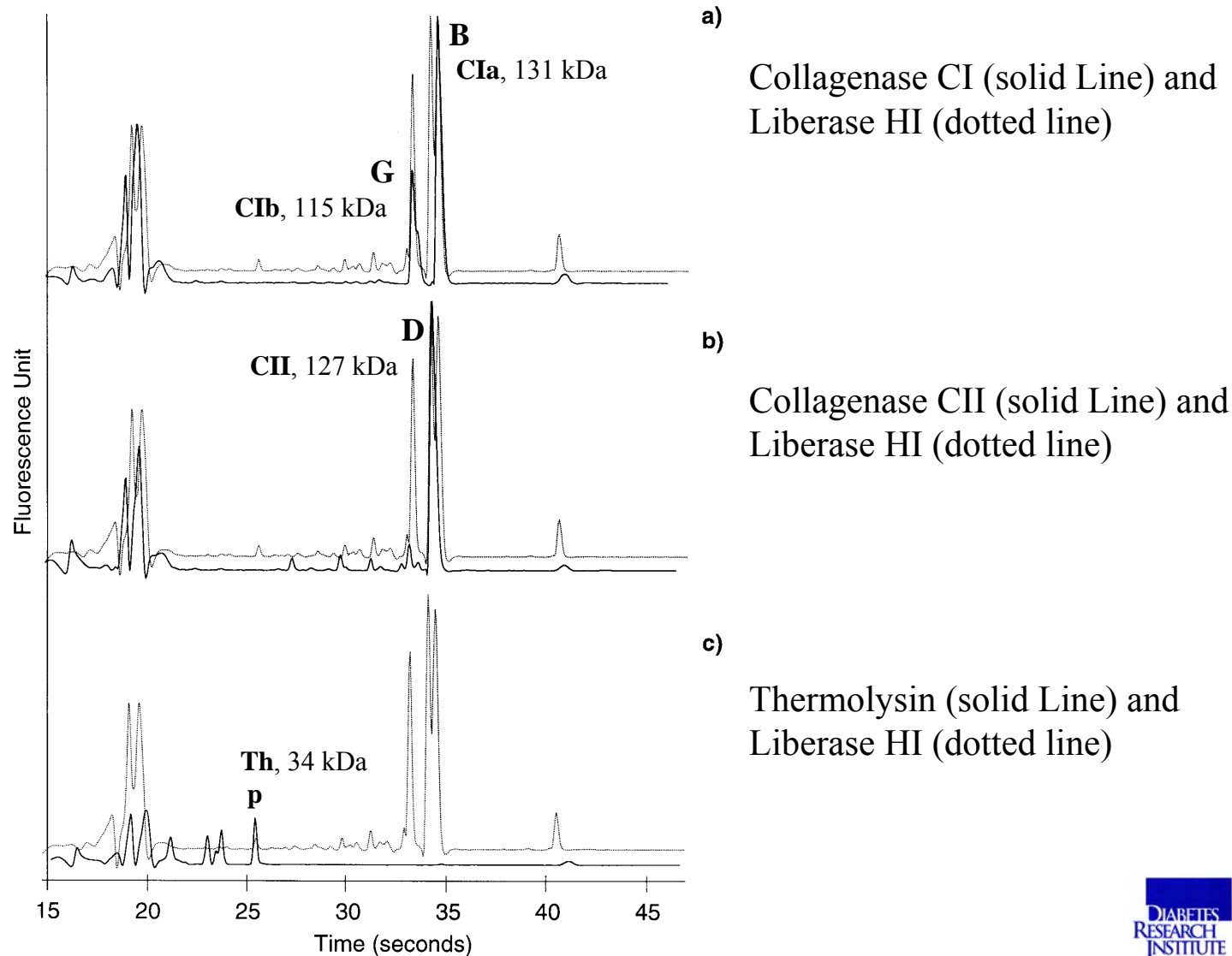


FIGURE 1. Chromatograms of Liberase HI. (A) Lot 93080820. (B) Lot 93096820 with a peak shoulder. (C-D) These lots (93131620 and 93134620 respectively) had distinct proportions of Clb.

Superposition of Protein Profiles of Collagenases CI, CII, and Thermolysin vs. Liberase HI Separated by Micro-on-Chip Electrophoresis (F. Bertuzzi, 2006)



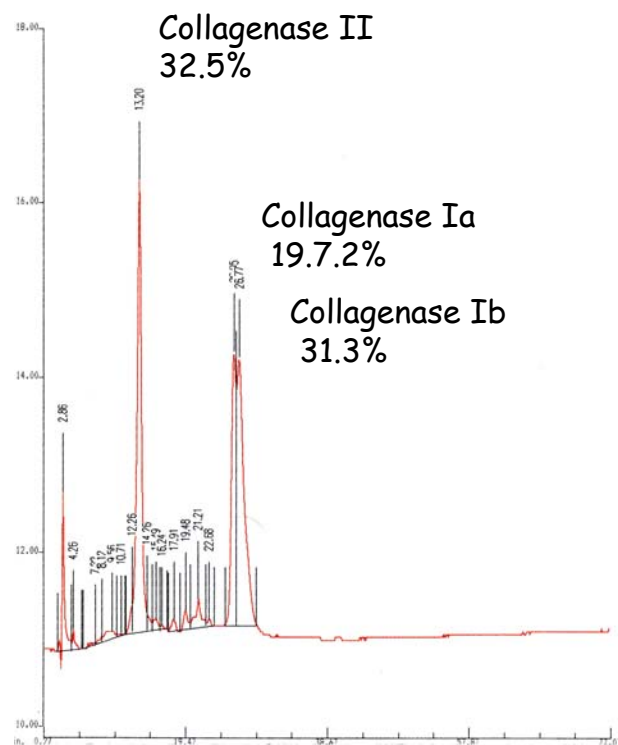
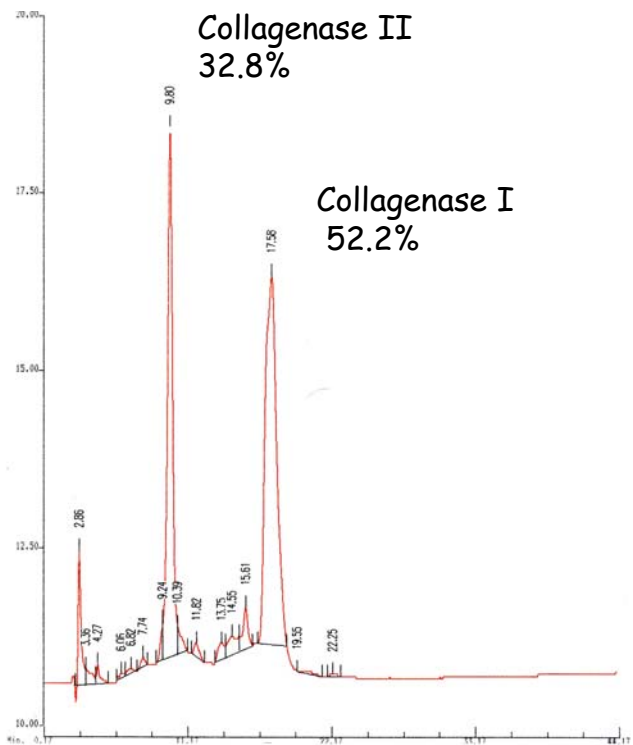
Characteristics of Different Liberaze batches and Proportion of each peak (F. Bertuzzi, 2006)

Liberase™ batches	activity/mg	neutral protease/mg	% peak p Th	% peak G CIb	% peak D CII	% peak B CIa	% remnant	% G+B CI
93047420	4.248	155.6	1.3	46.6	29.2	17.3	5.6	63.9
90287220	4.922	193.2	2.2	10.9	28.7	45.9	12.3	56.8
93017120	4.170	147.2	1.6	19.4	28.6	42.6	7.8	62.0
93114320	4.216	120.7	1.8	23.0	28.4	37.8	9.0	60.8
93126020	4.048	120.4	2.6	18.4	35.4	42.0	1.6	60.4
93145320	4.454	133.8	2.6	35.7	30.6	21.3	9.8	57.0
93152720	4.970	132.1	2.4	29.6	31.0	34.0	3.0	63.6
93028820	4.404	146.9	1.7	15.2	31.7	45.8	5.6	61.0
93045820	3.900	140.3	2.9	41.6	28.2	18.0	9.3	59.6
93142020	4.794	147.6	2.4	33.1	28.6	26.0	9.9	59.1
93167720	5.058	142.4	-	22.4	35.4	37.3	4.9	59.7
93163920	4.402	128.4	1.9	26.8	31.1	33.5	6.7	60.3
93187520	4.226	135.2	2.7	22.3	33.6	33.6	7.8	55.9
93172920	4.144	140.2	1.9	23.6	34.1	34.2	6.2	57.8
93273520	4.996	137.6	1.7	41.0	29.4	21.9	6.0	62.9

Correlation between Protein Peaks and Islet Isolation Outcome (F. Bertuzzi, 2006)

- Number of islet equivalent (IQ) correlated with CII ($p < 0.001$, $r = +0.305$).
- Digestion time (T) inversely correlated with CII ($p < 0.001$, $r = -0.502$).
- Purity of preparations (PP) inversely correlated with CIb (peak G) ($p = +0.024$, $r = -0.265$) and directly with CIa (peak B) ($p = 0.028$, $r = +0.260$).

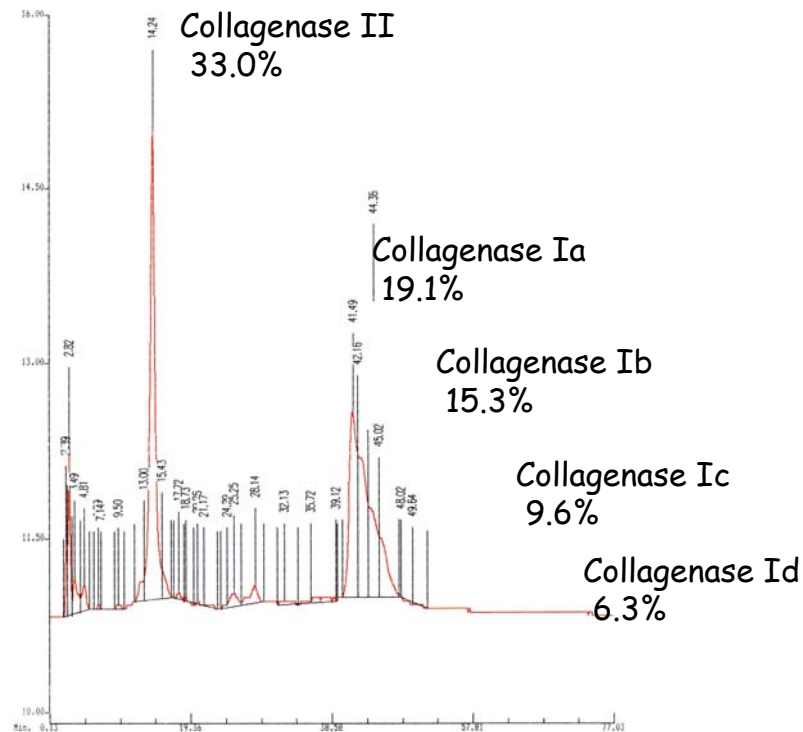
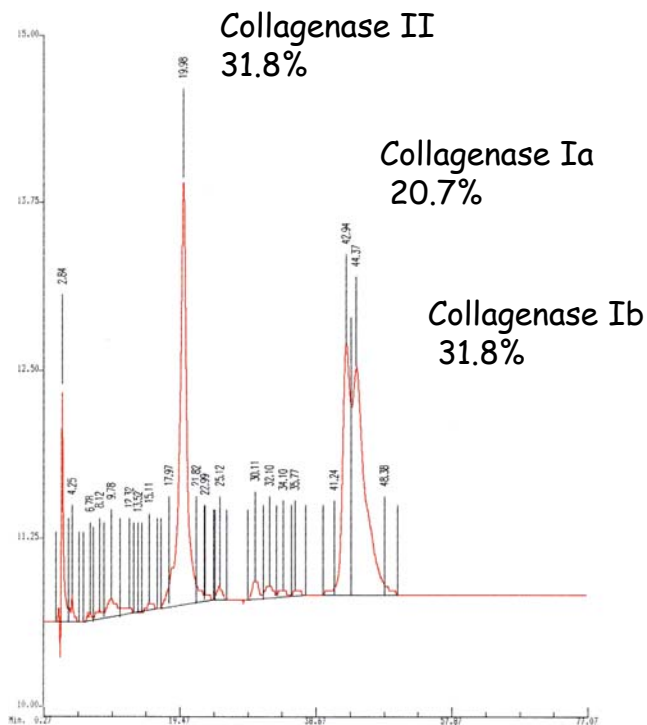
Ion-Exchange HPLC of Liberase HI on ProteinPak DEAE 5PW Column at pH 7.8 for 44 and 75 min



Sample Liberase HI (Lot #90287220 ExpNov02) 0.15ug/0.25m
Eluant A: 10 mM Tris-HCl pH 7.8, containing 1 mM CaCl2
Eluant B: 0.5 M NaCl in Buffer A
Gradient Profile 100%B-15 min>0%B-30 min>0-100% B-40 min>100%B-3>0
Flow Rate 1 ml/min
Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Detector A Model 1706 var UV/VIS, 280nm STD, 0.005 AUFS

Sample Liberase HI (Lot #90287220 ExpNov02) 0.15ug/0.25m
Eluant A: 10 mM Tris-HCl pH 7.8, containing 1 mM CaCl2
Eluant B: 0.5 M NaCl in Buffer A
Gradient Profile 100%B-15 min>0%B-30 min>0-100% B-40 min>100%B-3>0
Flow Rate 1 ml/min
Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Detector A Model 1706 var UV/VIS, 280nm STD, 0.005 AUFS

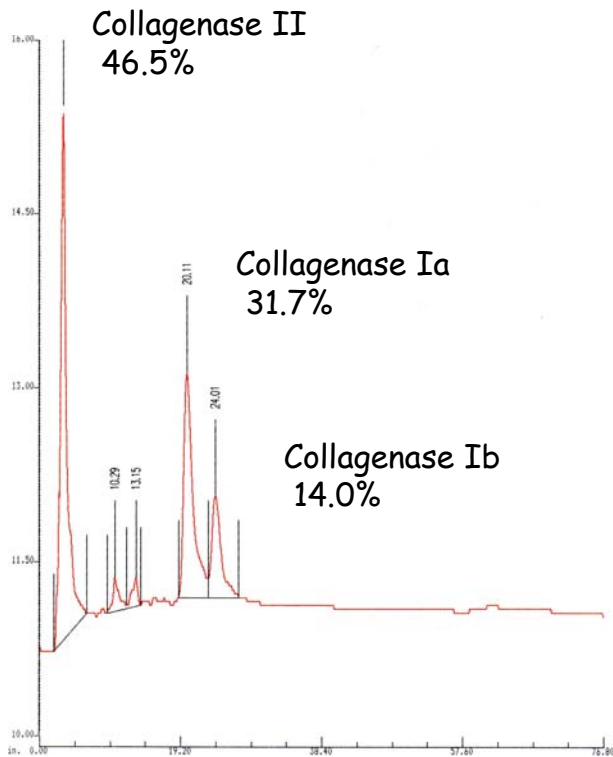
Ion-Exchange HPLC of Liberase HI on ProteinPak DEAE 5PW Column at pH 7.8 and 6.8 for 75 min



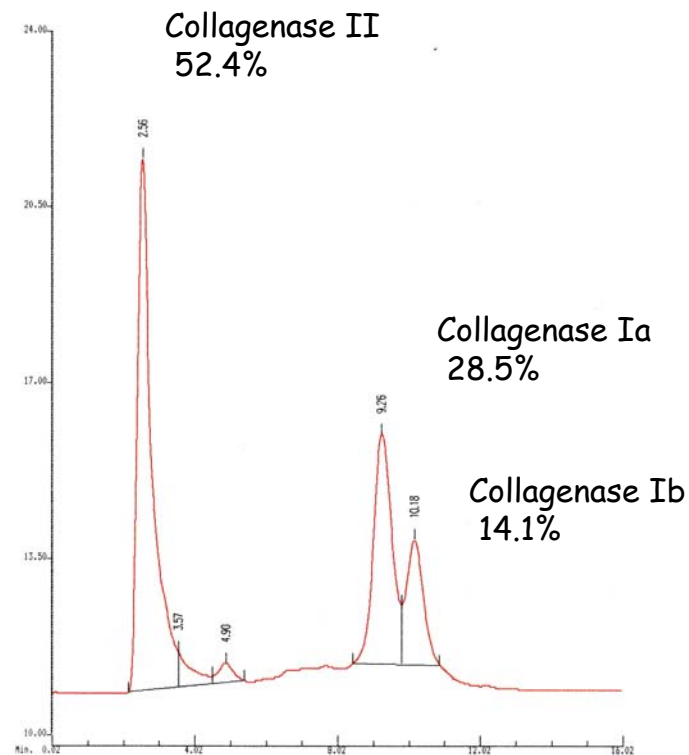
Sample Liberase HI (Lot #90287220 ExpNov02) 0.15ug/0.25m
Eluant A: 10 mM Tris-HCl pH 7.8, containing 1 mM CaCl₂
Eluant B: 0.25 M NaCl in Buffer A (Diluted 2 times from 0.5
Eluant D: The 1/2 less steep slope as mlibde0 but longer
Gradient Profile 100%B-15 min>0%B-30 min>0-100% B-75 min>100%B->0
Flow Rate 1 ml/min
Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Sample Liberase HI (Lot #90287220 ExpNov02) 0.15ug/0.25m
Eluant A: 10 mM Tris-HCl pH 6.78, containing 1 mM CaCl₂
Eluant B: 0.20 M NaCl in Buffer A
Gradient Profile 100%B-15 min>0%B-30 min>0-100% B-75 min>100%B->0
Flow Rate 1 ml/min
Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Ion-Exchange HPLC of Liberase HI on ProteinPak DEAE 5PW Column at pH 5.5 for 75 and 15 min

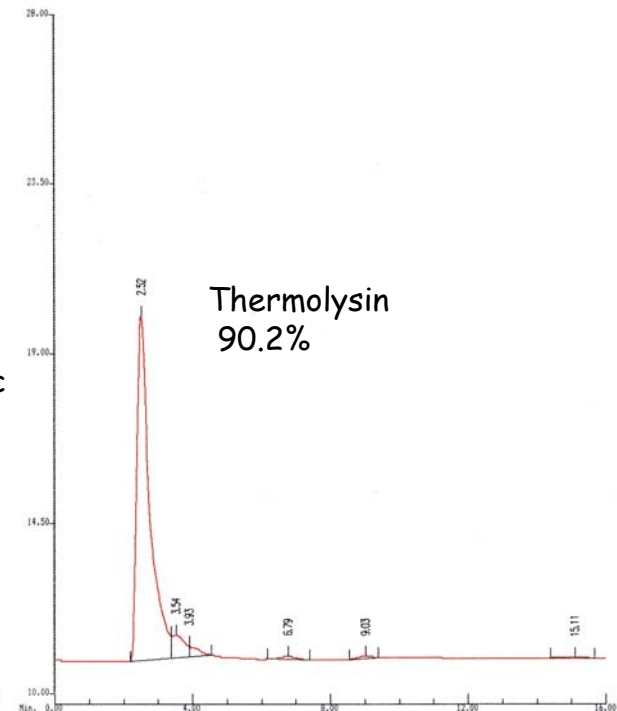
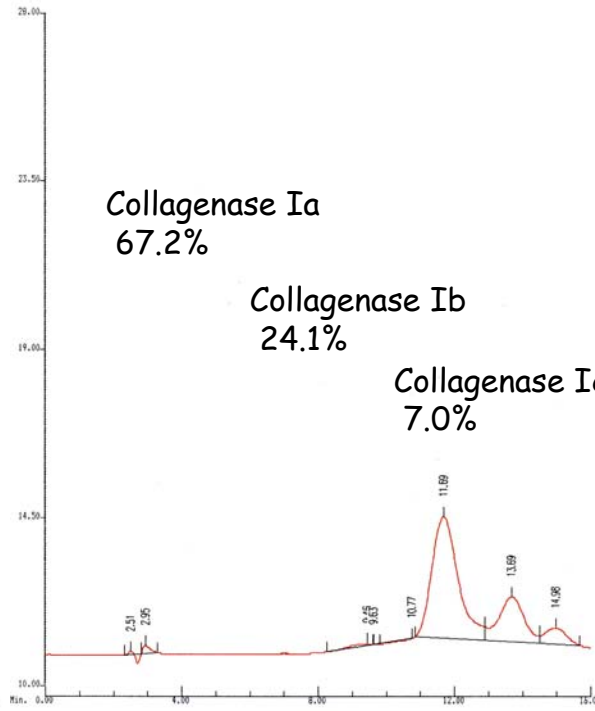
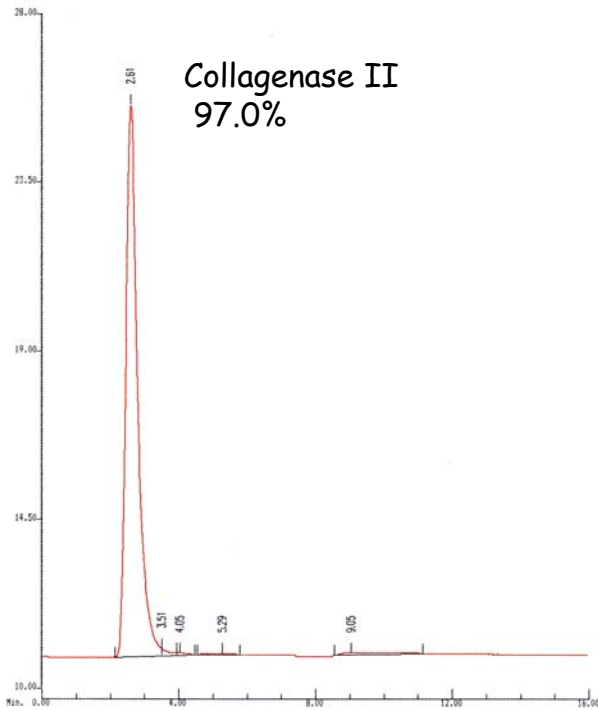


Sample Liberase HI (Lot #90287220 ExpNov02) 0.15ug/0.25m
Eluant A: 5 mM L-Histidine.HCl pH 5.5, containing 1 mM CaCl
Eluant B: 0.2 M NaCl in Buffer A
Gradient Profile 100%B-15 min>0%B-30 min>0-100% B-75 min>100%B->0
Flow Rate 1 ml/min
Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS



Sample Liberase HI (Lot #90287220 ExpNov02) 0.15ug/0.25m
Eluant A: 5 mM L-Histidine.HCl pH 5.5, containing 1 mM CaCl
Eluant B: 0.2 M NaCl in Buffer A
Gradient Profile 100%B-10min>10%B-15min>10-100% B-15min>0%B-1min
Flow Rate 1 ml/min
Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Ion-Exchange HPLC of Individual Components of Liberase HL: Collagenase Type II, Collagenase Type I, and Thermolysin on DEAE 5PW Column in L-Histidine-HCl Buffer at pH 5.5



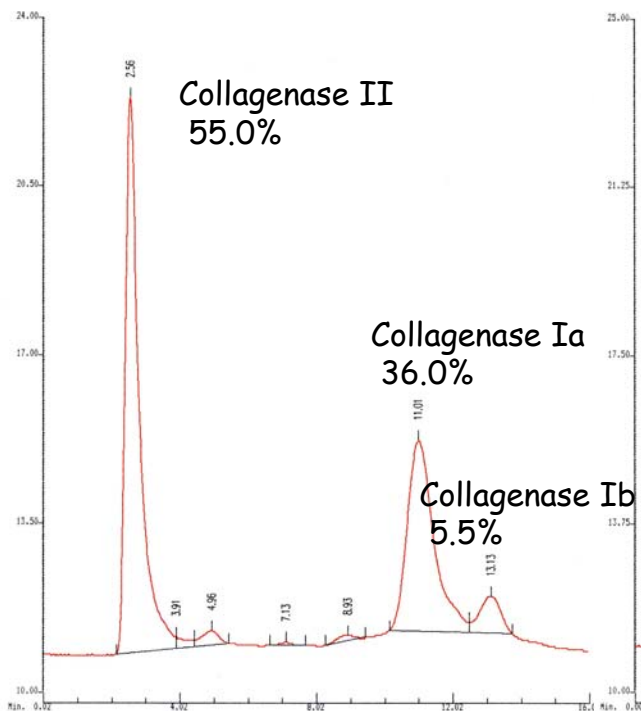
Sample Collagenase II(Lot#0000011713A ExSep07)75ug/250ul
 Eluant A: 5 mM L-Histidine.HCl pH 5.5, containing 1 mM CaCl
 Eluant B: 0.2 M NaCl in Buffer A
 Gradient Profile 100%B-10min>10%B-15min>10-50%B-15min>0%B-1min
 Flow Rate 1 ml/min
 Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
 Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Sample Collagenase I(Lot#0000012071A ExSep07)75ug/250ul
 Eluant A: 5 mM L-Histidine.HCl pH 5.5, containing 1 mM CaCl
 Eluant B: 0.2 M NaCl in Buffer A
 Gradient Profile 100%B-10min>10%B-15min>10-50%B-15min>0%B-1min
 Flow Rate 1 ml/min
 Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
 Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

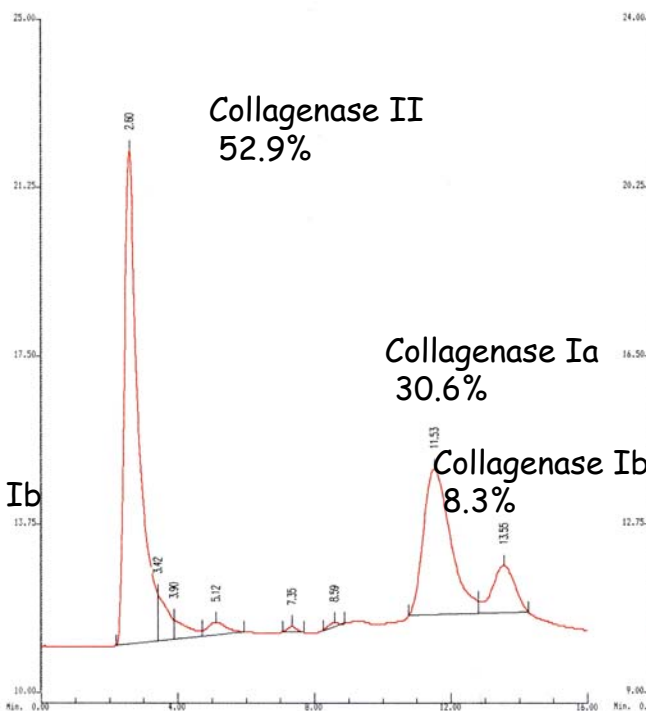
Sample Thermolysin (Lot#93467920 ExMar08)75ug/250ul
 Eluant A: 5 mM L-Histidine.HCl pH 5.5, containing 1 mM CaCl
 Eluant B: 0.2 M NaCl in Buffer A
 Gradient Profile 100%B-10min>10%B-15min>10-50%B-15min>0%B-1min
 Flow Rate 1 ml/min
 Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
 Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Time-dependent Degradative Conversion of Collagenase Ia to Ib in Freshly Prepared Liberase HI stock in 1X HBSS During up to 3 Days of Incubation at 0°C

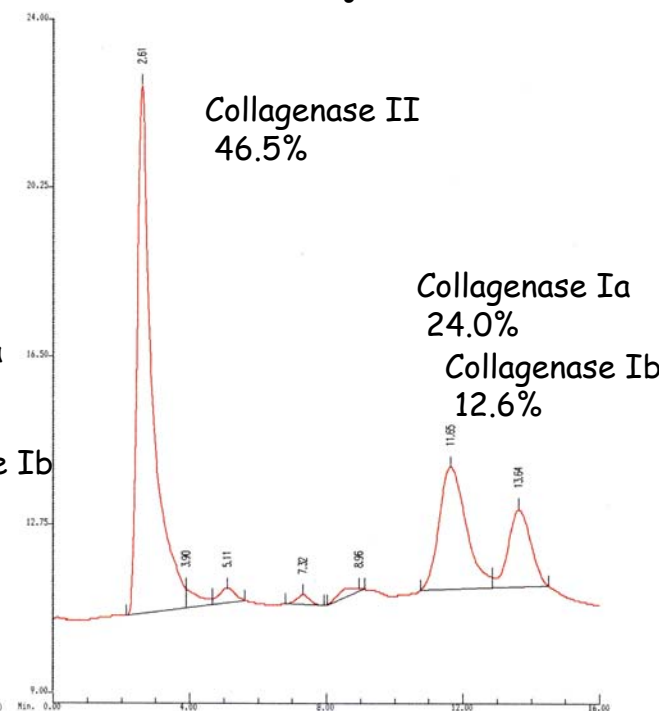
Day 1



Day 2



Day 3



Sample Liberase HI (Lot #90287220 ExpNov02) 0.15ug/0.25m
 Eluant A: 5 mM L-Histidine.HCl pH 5.5, containing 1 mM CaCl
 Eluant B: 0.2 M NaCl in Buffer A
 Gradient Profile 100XB-10min>10XB-15min>10-50XB-15min>0XB-1min
 Flow Rate 1 ml/min
 Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
 Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Sample Liberase HI (Lot #90287220 ExpNov02) 0.15ug/0.25m
 Eluant A: 5 mM L-Histidine.HCl pH 5.5, containing 1 mM CaCl
 Eluant B: 0.2 M NaCl in Buffer A
 Gradient Profile 100XB-10min>10XB-15min>10-50XB-15min>0XB-1min
 Flow Rate 1 ml/min
 Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
 Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Sample Liberase HI (Lot #90287220 ExpNov02) 0.15ug/0.25m
 Eluant A: 5 mM L-Histidine.HCl pH 5.5, containing 1 mM CaCl
 Eluant B: 0.2 M NaCl in Buffer A
 Gradient Profile 100XB-10min>10XB-15min>10-50XB-15min>0XB-1min
 Flow Rate 1 ml/min
 Column WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
 Detector A Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

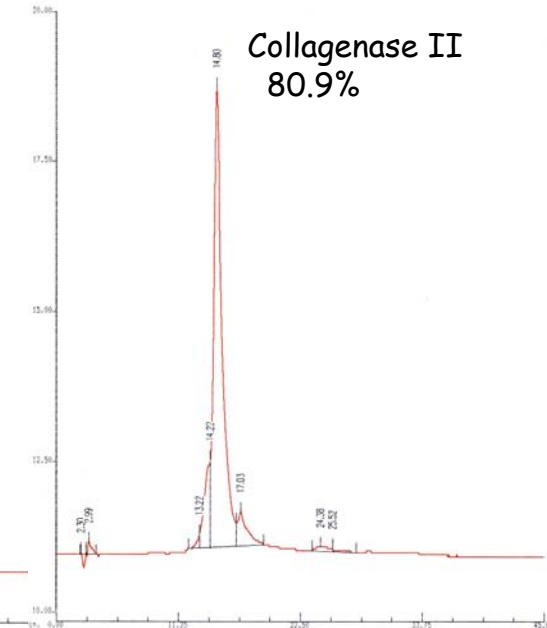
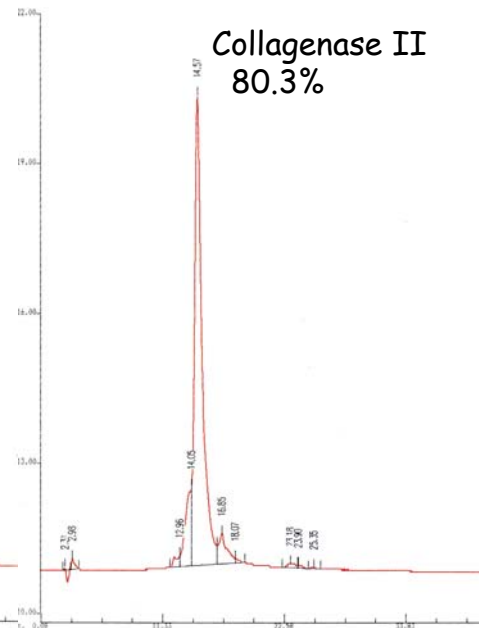
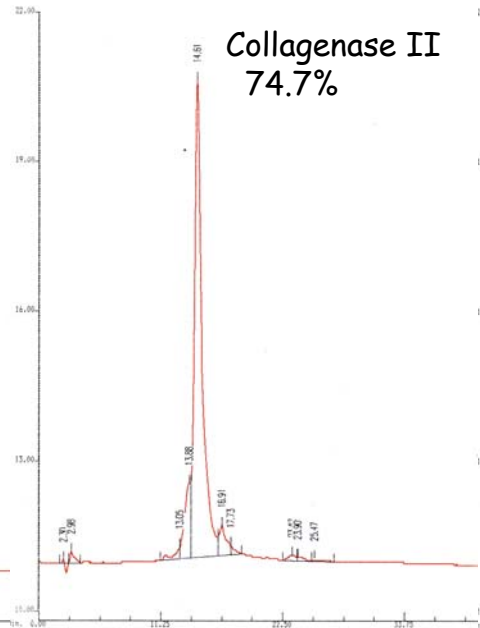
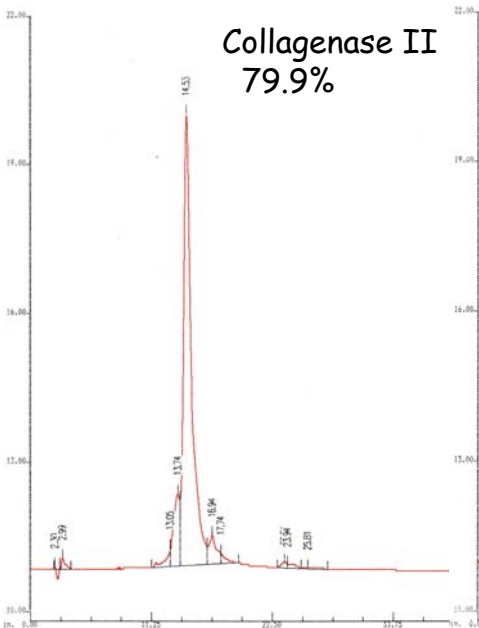
Stability of Collagenase Type II Incubated up to 1 Month in 1X HBSS at 0°C and Separated on DEAE 5PW Column in Imidazole-HCl Buffer at pH 6.3

Day 1

Day 2

Day 3

Day 30



Collagenase II (Lot#0000011713A ExSep07)150ug/250
5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
1st Day in 1X HBSS at 0°C
100XB-10'>0XB-15'>0XB-6.25XB-15'>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Collagenase II (Lot#0000011713A ExSep07)150ug/250
5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
2nd Day in 1X HBSS at 0°C
100XB-10'>0XB-15'>0XB-6.25XB-15'>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Collagenase II (Lot#0000011713A ExSep07)150ug/250
5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
3d Day in 1X HBSS at 0°C
100XB-10'>0XB-15'>0XB-6.25XB-15'>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Collagenase II (Lot#0000011713A ExSep07)150ug/250
5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
30th Day in 1X HBSS at 0°C
100XB-10'>0XB-15'>0XB-6.25XB-15'>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AU

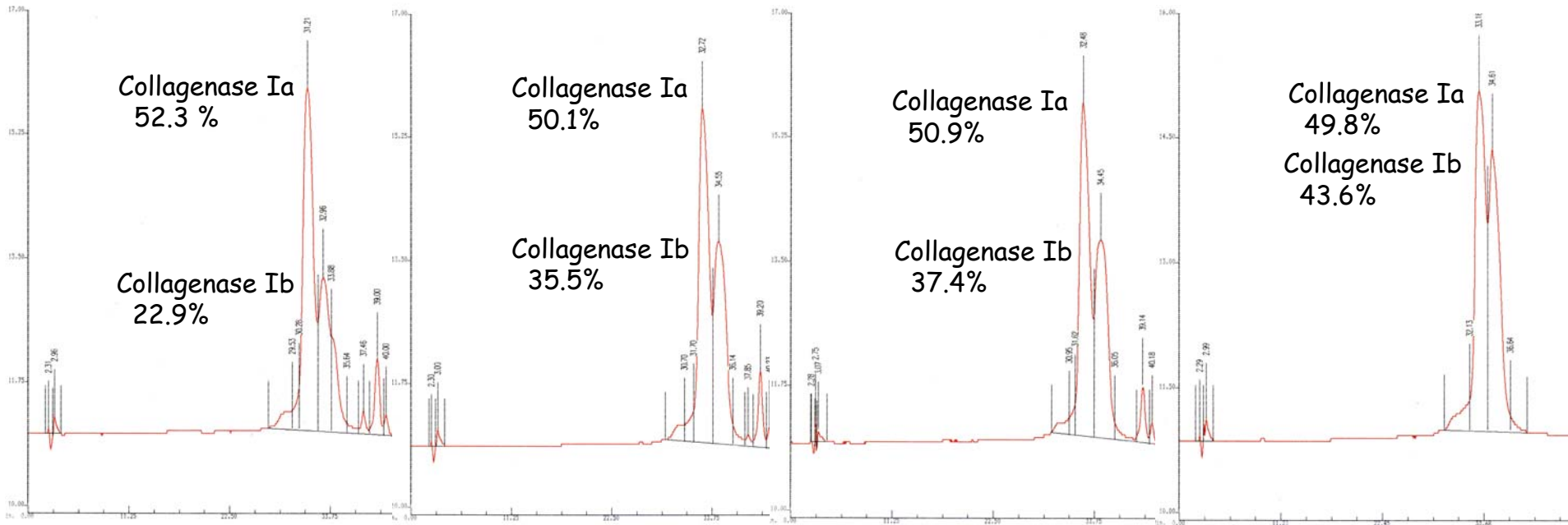
Degradation of Collagenase Type I Incubated up to 1 Month in 1X HBSS at 0°C and Separated on DEAE 5PW Column in Imidazole-HCl Buffer at PH 6.3

Day 1

Day 2

Day 3

Day 30



Collagenase I (Lot#0000012071A ExSep07)150ug/250
5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
100XB-10'>0XB-15'>0XB-6.25XB-15>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Collagenase I (Lot#0000012071A ExSep07)150ug/250
5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
2nd Day in 1X HBSS at 0°C
100XB-10'>0XB-15'>0XB-6.25XB-15>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Collagenase I (Lot#0000012071A ExSep07)150ug/250
5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
3d Day in 1X HBSS at 0°C
100XB-10'>0XB-15'>0XB-6.25XB-15>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

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5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
30th Day in 1X HBSS at 0°C
100XB-10'>0XB-15'>0XB-6.25XB-15>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Complete Degradation of Collagenase Type Ia in Liberase HI During 1 Month of Incubation in 1X HBSS at 0°C and Separated on DEAE 5PW Column in Imidazole-HCl Buffer at pH 6.3

Day 1

Day 2

Day 3

Day 30

Collagenase II
27.0%

Collagenase Ia
32.7%

Collagenase Ib
20.1%

Collagenase II
28.5%

Collagenase Ia
26.5%

Collagenase Ib
23.1%

Collagenase II
28.2%

Collagenase Ia
24.7%

Collagenase Ib
24.6%

Collagenase II
24.3%

Collagenase Ib
23.0%

Collagenase Ia
4.8%

Liberase HI (Lot#93456920 ExFeb08)150ug/250ul
5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
100XB-10'>0XB-15'>0XB-6.25XB-15>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

Collagenase HI (Lot#93456920 ExFeb08)150ug/250
5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
0.4 M NaCl in Buffer A
2nd Day in 1X HBSS at 0°C
100XB-10'>0XB-15'>0XB-6.25XB-15>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5 cm
Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

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5 mM Imidazole-HCl pH 6.3, containing 1 mM CaCl2
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100XB-10'>0XB-15'>0XB-6.25XB-15>6.25-25XB-15'>10
1 ml/min
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Model 1706 var UV/VIS, 280nm STD, 0.0025 AUFS

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0.4 M NaCl in Buffer A
30th Day in 1X HBSS at 0°C
100XB-10'>0XB-15'>0XB-6.25XB-15>6.25-25XB-15'>10
1 ml/min
WATERS PROTEIN PAK DEAE 5PW 7.5 mm x 7.5
Model 1706 var UV/VIS, 280nm STD, 0.0025

Conclusions

1. We have developed a new method for the separation of individual components in commercial blends of clostridial collagenases. The method is based on ion-exchange HPLC for the separation of target proteins at pH values close to their pIs, where enzymes are still absorbed by the column, while impurities are eluted in the void volume. Subsequent gradient elution results in separation.
2. Collagenase Type I is the most unstable component of Liberase HI. Its Ia form is rapidly autocatalytically degraded to Ib form. The ratio of these forms can be a valuable parameter for lot assessment in both blends and individual components of clostridial collagenases.
3. Collagenase Type II is homogeneous and the most stable component in the blends. Its chromatographic profile was not changed even after incubation in 1X HBSS for one month at 0°C.