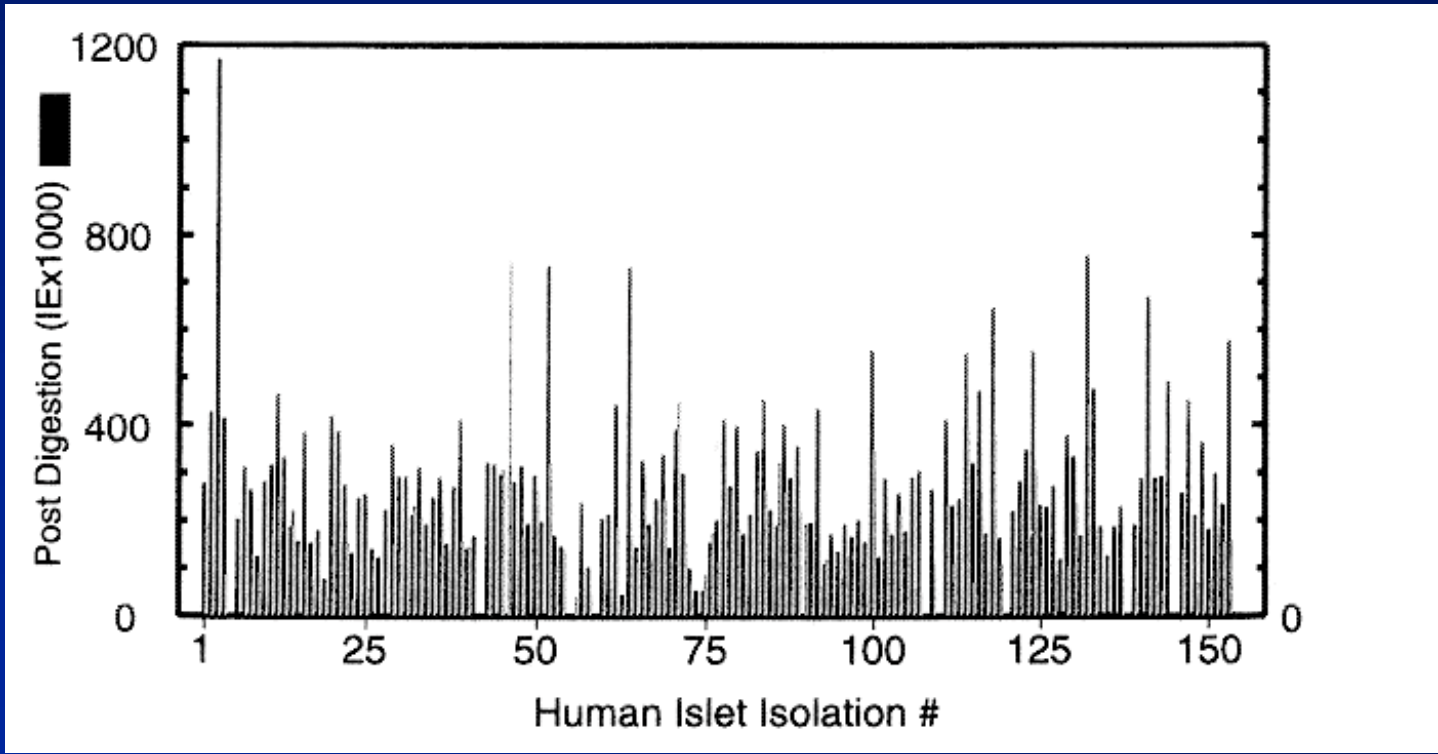
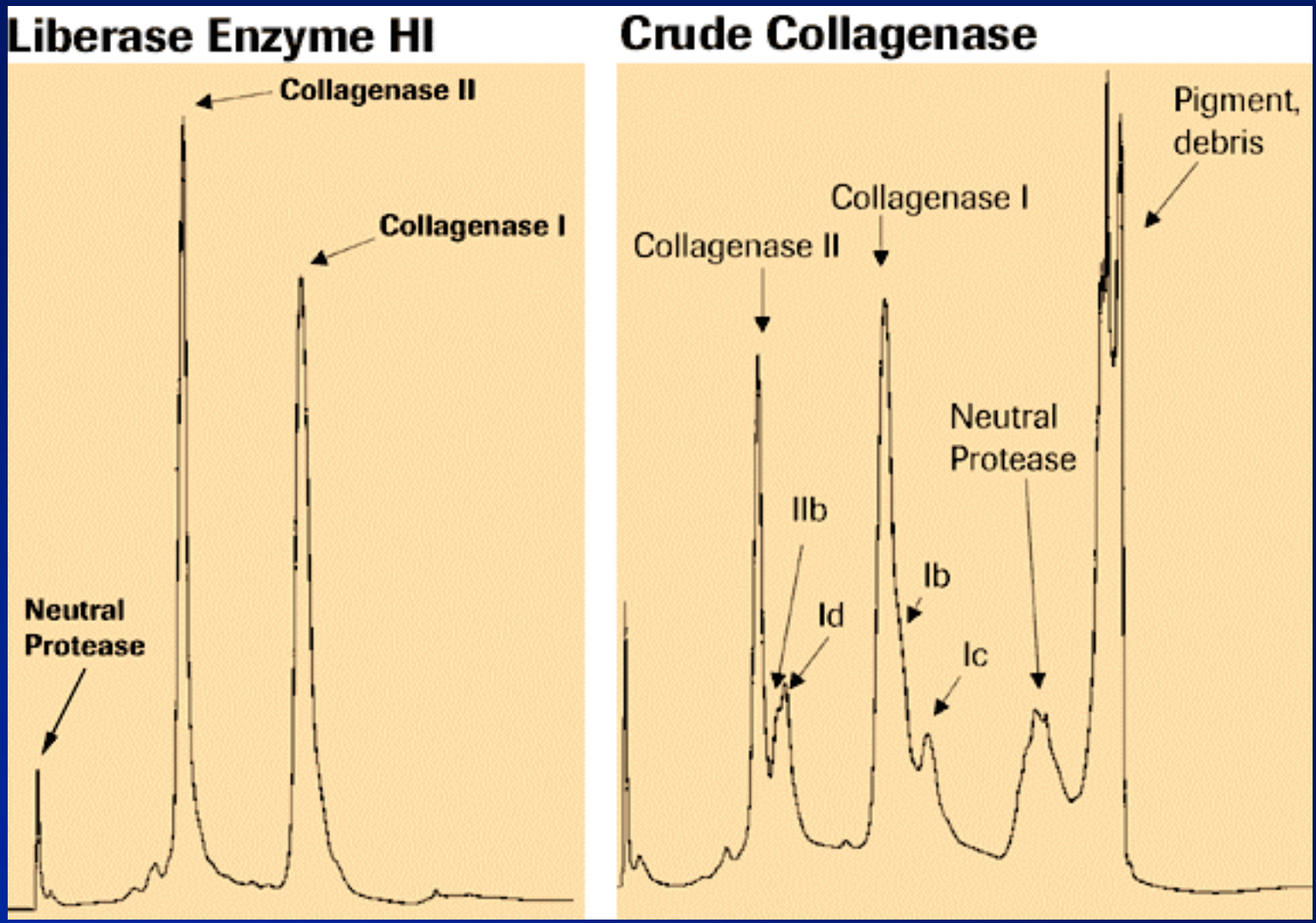
Characterization of Successful Collagenase Blend Enzymes for Human Islet Isolation

F. Bertuzzi
ISMETT, Palermo, Italy

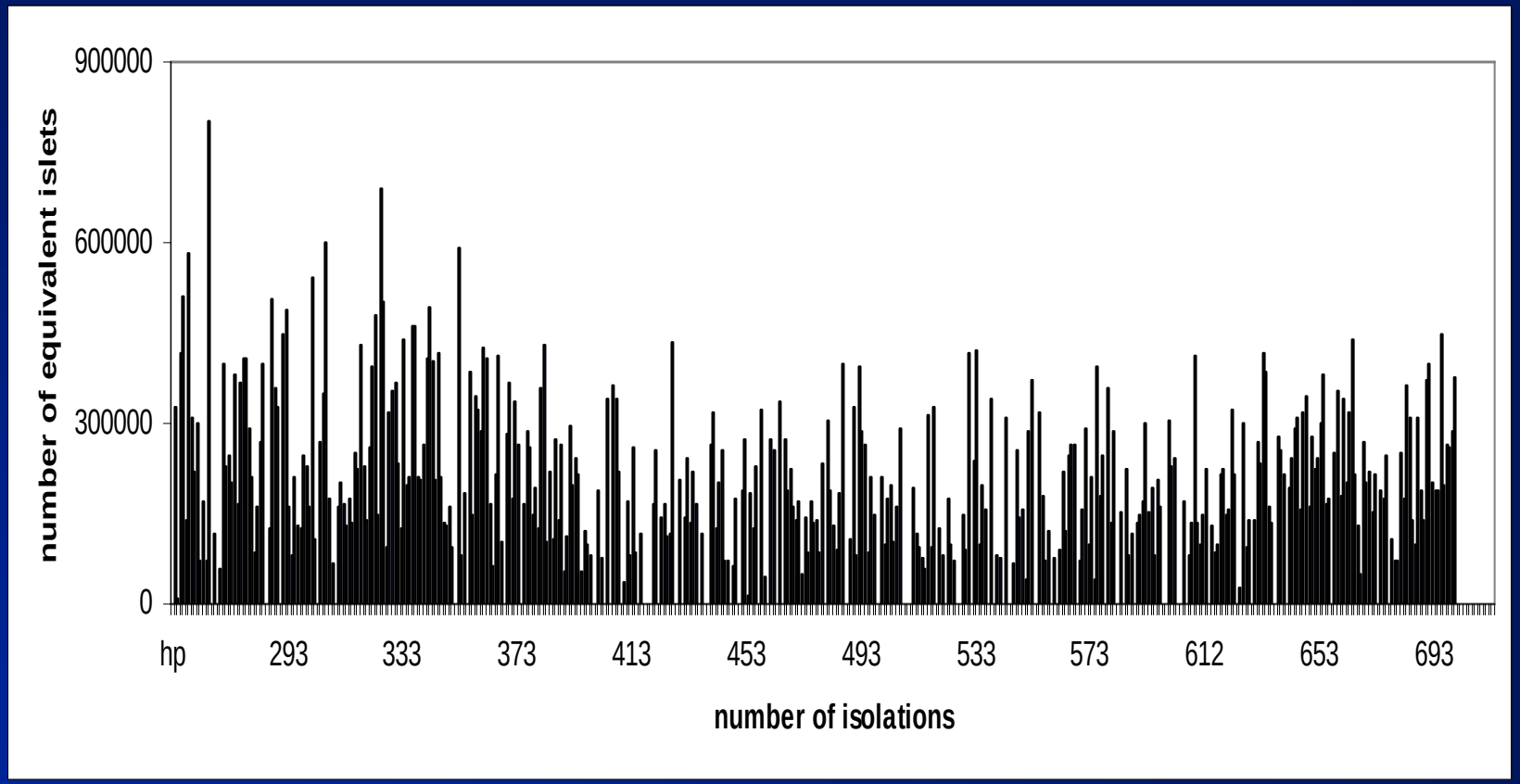
VARIABLES IN ORGAN DONORS THAT AFFECT THE RECOVERY OF HUMAN ISLETS OF LANGERHANS

Lakey, Jonathan R.T; Warnock, Garth L; Rajotte, Ray V; Suarez-Almazor, Maria E; Ao, Ziliang; Shapiro, A. M. James; Kneteman, Norman M. Transplantation 1996 vol 61:1047-1053




Liberase Enzyme HI
Collagenase II
Collagenase I
Neutral Protease
Crude Collagenase
Pigment, debris
Collagenase I
Collagenase II
Neutral Protease
IIb
Ib
Id
Ic

HSR experience 1998-2005



R. Nano · B. Clissi · R. Melzi · G. Calori · P. Maffi · B. Antonioli · S. Marzorati · L. Aldrighetti · M. Freschi · T. Grochowiecki · C. Socci · A. Secchi · V. Di Carlo · E. Bonifacio · F. Bertuzzi

Islet isolation for allotransplantation: variables associated with successful islet yield and graft function

Diabetologia (2005) 48: 906–912

Digestion				
Type of enzyme[c]	432	260 type P; 172 Liberase	0.3560	0.6366
Batches[c]	247	11	0.0280	
Collagenase activity/mg of enzyme (U/mg)	432	2.7 (2.3–4.2)	0.7356	0.9865
Collagenase activity/ solution volume (U/ml)	431	6.8 (5.4–7.3)	0.0002[d]	0.1716
Total collagenase activity (U)	431	2,160 (1,930–2,880)	0.0001[d]	0.0331
Adjusted collagenase activity/mg (U/mg)	431	4.3 (3.9–5.8)	0.0002[d]	0.0954
Collagenase activity/weight of pancreas (U/mg)	341	25 (19.2–34)	0.3257	0.5982
Neutral protease activity/mg of enzyme (U/mg)	431	138 (80–157)	0.4820	0.9074
Neutral protease activity/solution volume (U/ml)	431	244 (212–376)	0.0029[d]	0.1251
Collagenase/neutral protease activity (mU/U)	431	25.5 (20.3–28.5)	0.8846	0.8354
Clostripain/mg of enzyme (U/mg)	432	3.1 (0–8.1)	0.7241	0.9343
Trypsin activity/mg of enzyme (U/mg)	431	0.1 (0–1.1)	0.9094	0.9889
Digestion time (min)	412	25 (19–34)	0.0153	0.0379
Proteins for dilution[c]	422	338 FCS; 84 human albumin	0.1370	0.3425

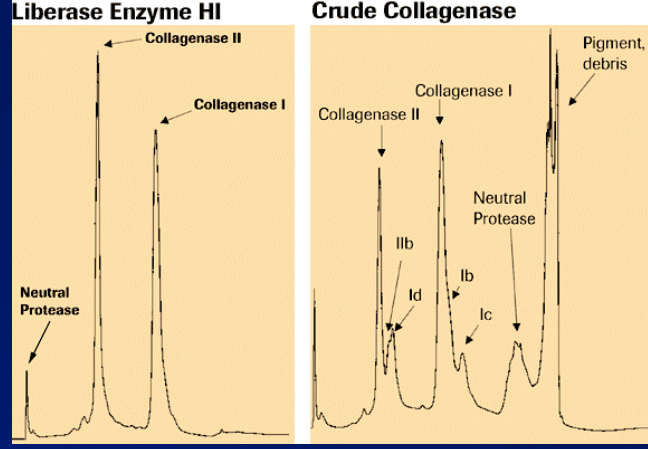
Refinement of the Automated Method for Human Islet Isolation and Presentation of a Closed System for In Vitro Islet Culture

Masafumi Goto,[1,8] Torsten M. Eich,[1] Marie Felldin,[2] Aksel Foss,[3] Ragnar Källen,[4] Kaija Salmela,[5] Annika Tibell,[6] Gunnar Tufveson,[7] Keisei Fujimori,[1] Margareta Engkvist,[1] and Olle Korsgren[1]

(*Transplantation* 2004;78: 1367–1375)

Organ procurement- and isolation-related variables			
CIT (min)	396.0±22.4	520.9±24.1	0.0002
The delivery of whole pancreas with duodenum (%)	100 (12/12) (n=12)[e]	48.3 (14/29) (n=29)[e]	0.001
Procurement team (local/distant)	(11/18)	(11/72)	0.004
Collagenase lot (efficient/nonefficient)	(16/13)	(25/58)	0.02
The use of additional (500→670 mg) collagenase (%)	41.4 (12/29)	24.1 (20/83)	0.08
The use of dye (%)	75.0 (9/12) (n=12)[e]	48.3 (14/29) (n=29)[e]	0.12
Collagenase amount per g of pancreas (mg/g)	7.31±0.35	6.81±0.26	0.22
Harvest starting time[f] (min)	24.8±1.0	26.5±0.8	0.23
Digestion rate (%)	89.2±1.4	88.4±1.1	0.64
Dissection time (min)			
Duodenum+	102.9±7.7 (n=12)	109.3±4.1 (n=14)	0.48
Duodenum−	77.6±5.9 (n=17)	79.2±2.6 (n=69)	0.80

In collagenase blend enzymes which are the key factors for isolation success?



1st key factor:

The role of collagenase class I and collagenase class II

Different Roles of Class I and Class II *Clostridium Histolyticum* Collagenase in Rat Pancreatic Islet Isolation

Gerrit H.J. Wolters, Greetje H. Vos-Scheperkeuter, Hun-Chi Lin, and Reinout van Schilfgaarde

Diabetes 44:227-233, 1995

Collagenase Class I:
- is highly active toward native collagen
- is uneffective to dissociate pancreatic tissue completely

Collagenase Class II:
- has a low affinity toward native collagen
- dissociates pancreatic tissue completely but slowly

Combining Class I and Class II:
- results in a rapid and complete dissociation of pancreatic tissue
- indicates that Class II plays the major role and Class I the supporting role

Adjustment of the Ratio Between Collagenase Class II and I Improves Islet Isolation Outcome

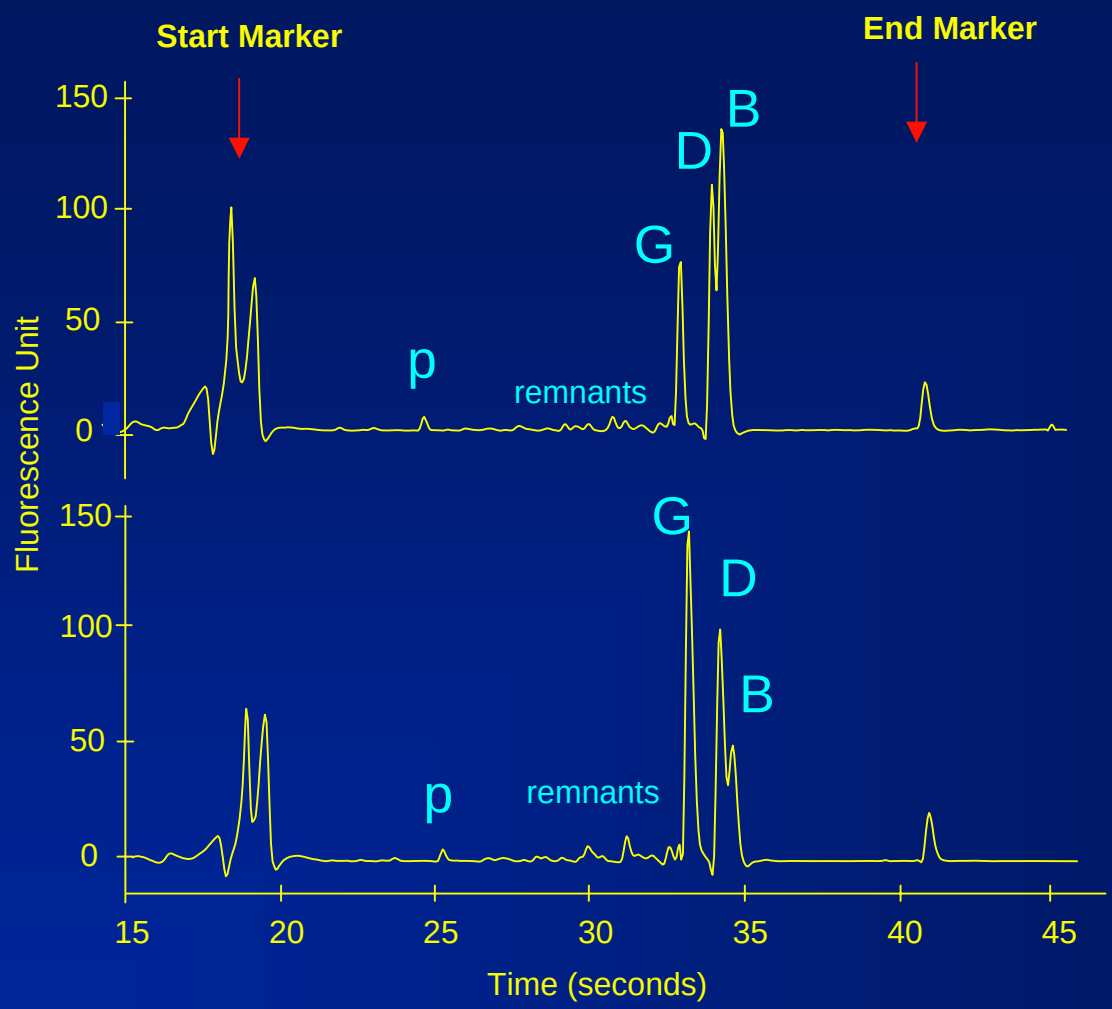
D. Brandhorst, S. Huettler, A. Alt, N. Raemsch-Guenther, M. Kurfuerst, R.G. Bretzel, and H. Brandhorst

Transplantation Proceedings, 37, 3450–3451 (2005)

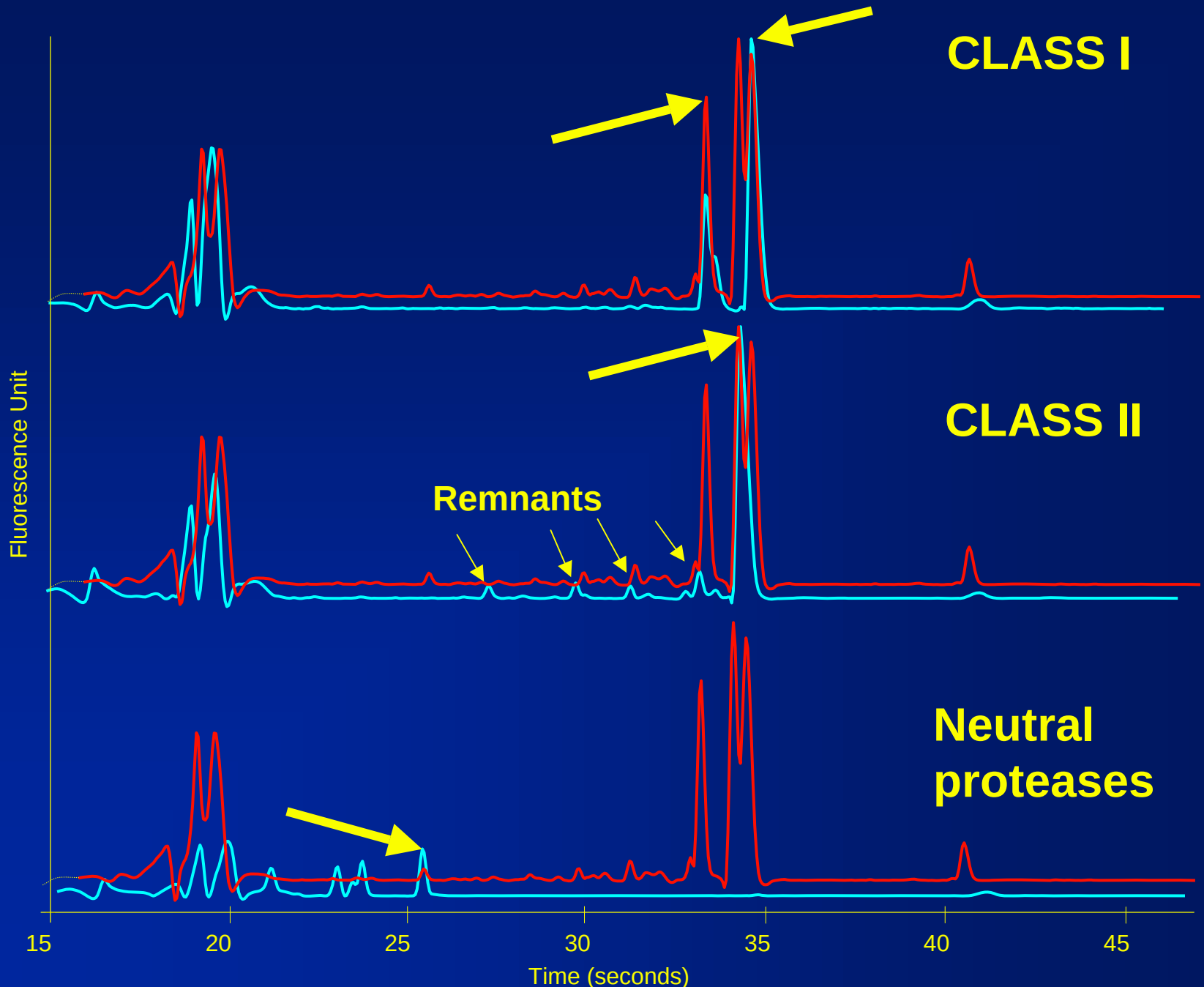
Table 1. Rat Islet Isolation Outcome Utilizing Different Ratios Between Collagenase Class II (ccII) and Class I (ccI)

ccII/I Ratio	n	Digestion (min)	Yield (IEQ/Rat)	Purity (%)	Viability (%)	Stimulation Index (20 mmol/L/2.8 mmol/L)	Transplant Survival After 32 days (n/n)
0.5	9	19 ± 0.3*	2090 ± 160*	83 ± 3	93 ± 3*	1.4 ± 0.2	7/7
1.0	7	20 ± 0.4	2730 ± 280	85 ± 6	100 ± 1	1.5 ± 0.2	6/6
1.5	7	19 ± 0.6	1960 ± 170*	84 ± 2	92 ± 2**	1.5 ± 0.2	6/6

Data are means ± SEM.
*$P < .05$.
**$P < .01$ vs a ccII/I ratio of 1.0 by Mann-Whitney test.



(Microelectrophoresis –Agylent Technology)


CLASS I
CLASS II
Remnants
Neutral proteases
Fluorescence Unit
15
20
25
30
35
40
45
Time (seconds)

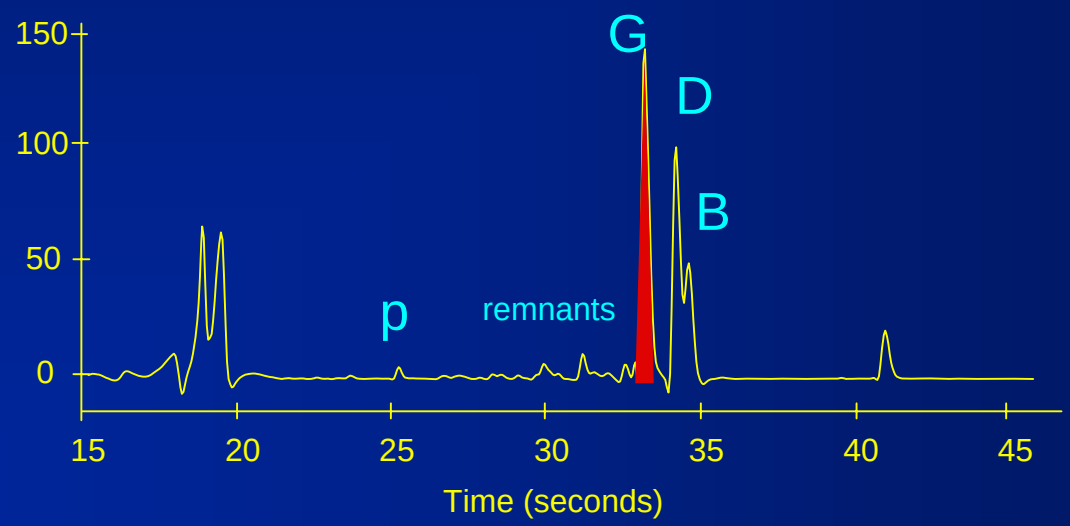
Has the relative presence of each enzyme

a role in the isolation success rate?

Index value:

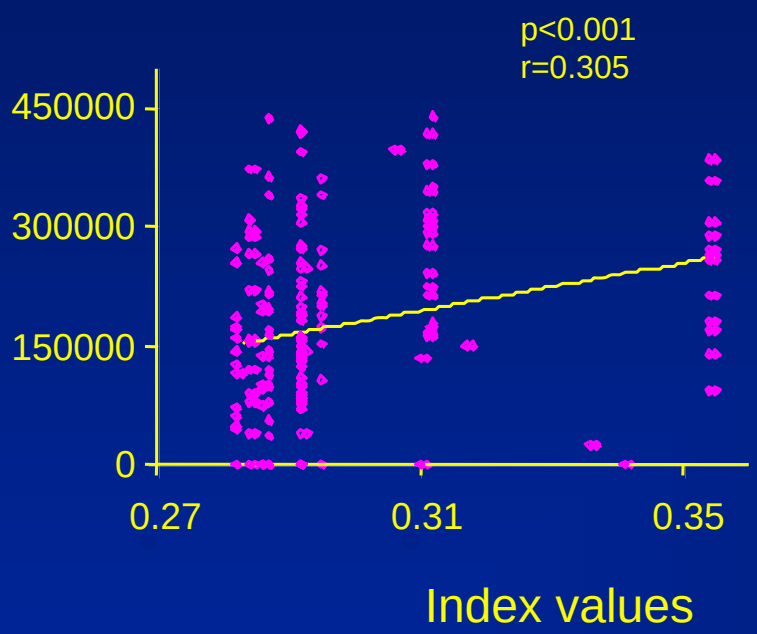
relative area under the curve of each peak

(area of each peak/sum of areas of all peaks)

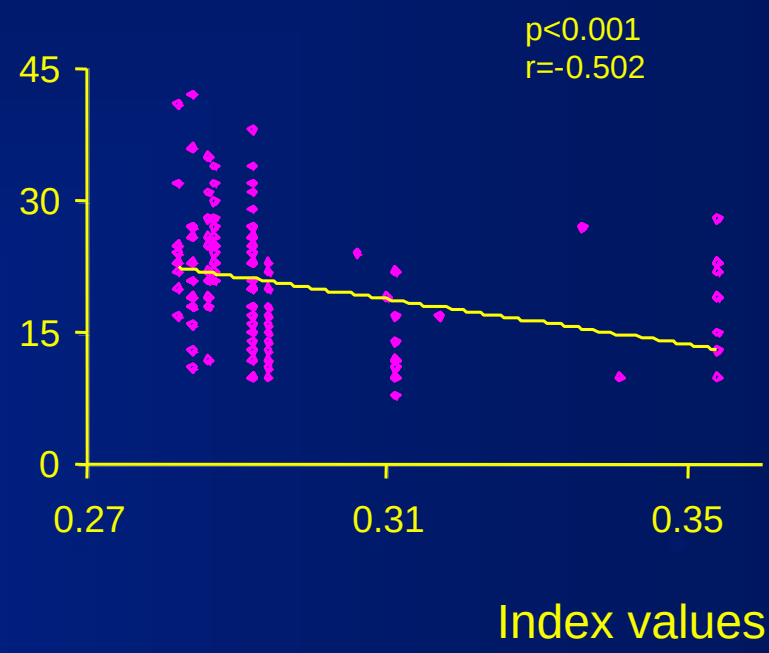


Collagenase class II

NE



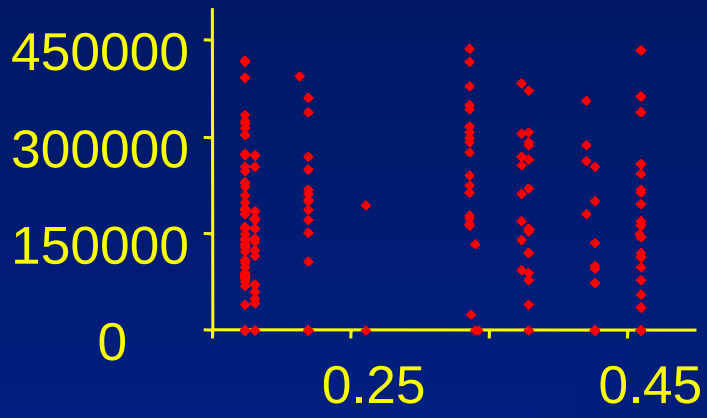
Digestion time



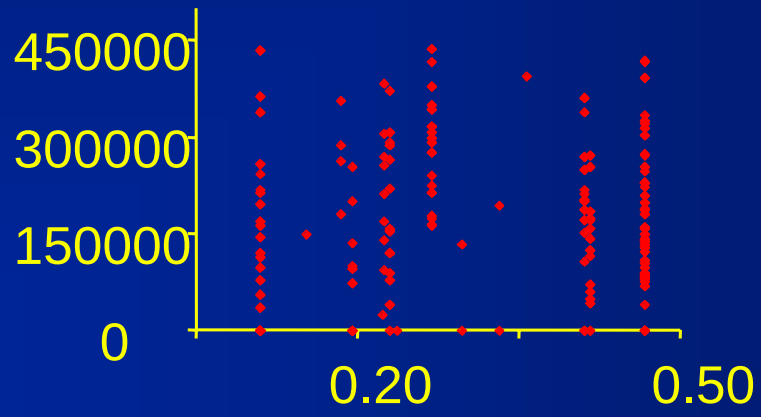
N=146 isolations of human pancreas

Collagenase class I

NE



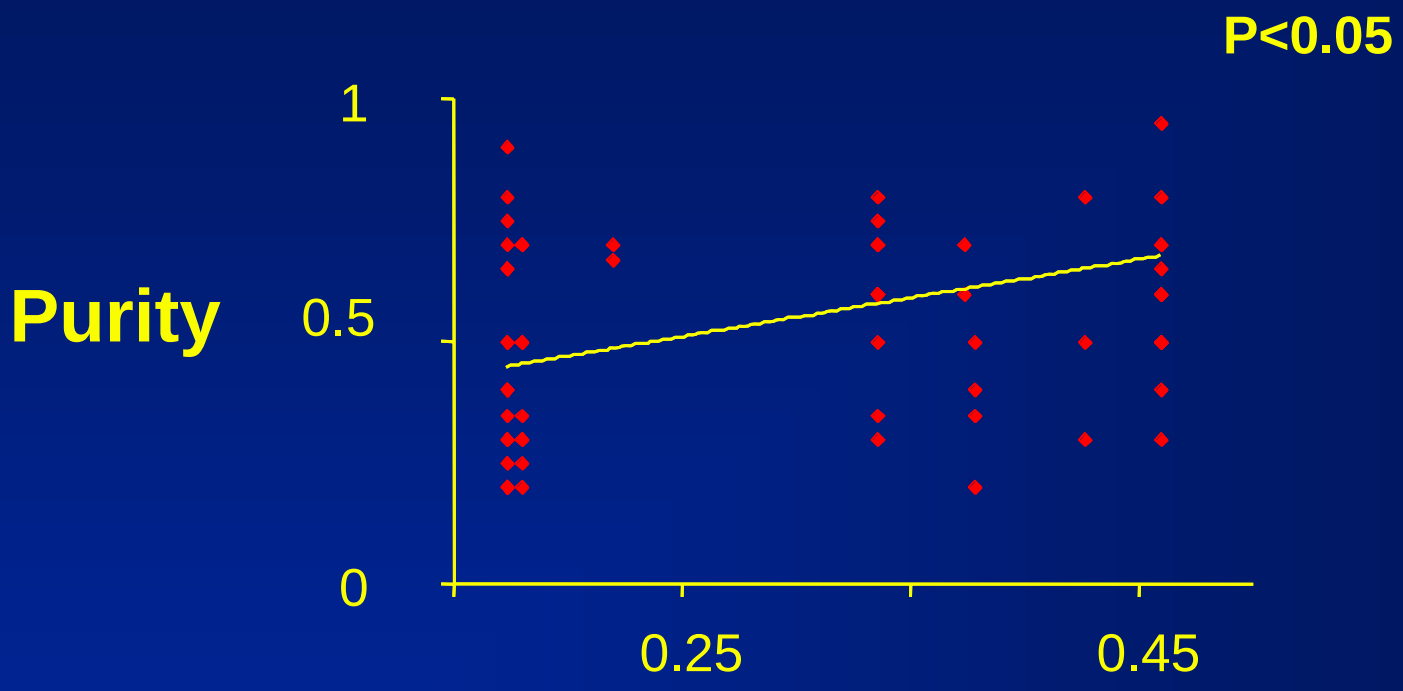
peak B



peak G

index value

Collagenase class I



Class II is the most important component of a collagenase blend

Class I peak B (the component with higher molecular weight) contributes to isolate successful islet preparations

If we consider isolations in which Liberase had

CII index values ≥50 percentile

33 out 98 were transplanted (about 1 out 3)

CII index values <50 percentile

9 out 65 were transplanted (about 1 out 7)

(χ^2 test, p=0.005).

CII and CI (B) index values ≥50 percentile

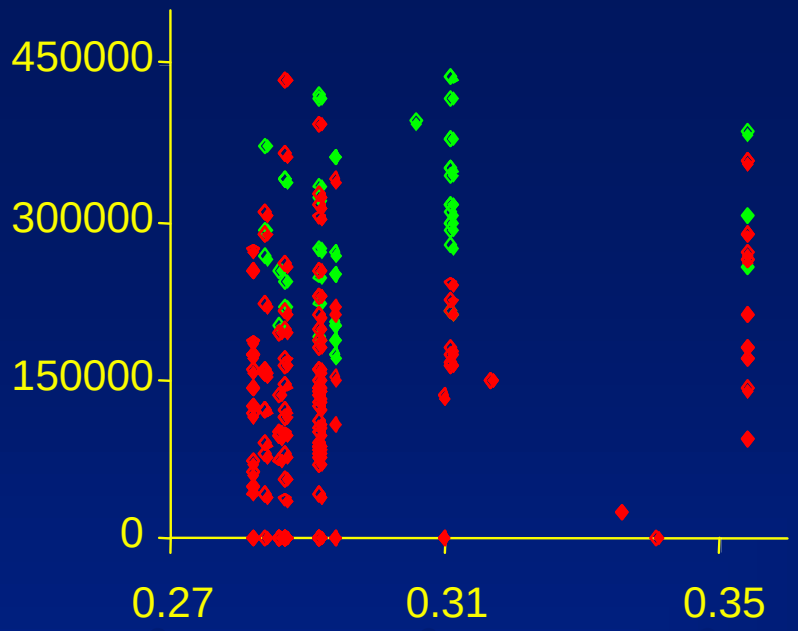
15 out 36 were transplanted (about 1 out 2)

CII and CI (B) index values <50 percentile

27 out 127 were transplanted (about 1 out 5)

(χ^2 test, p=0.013).

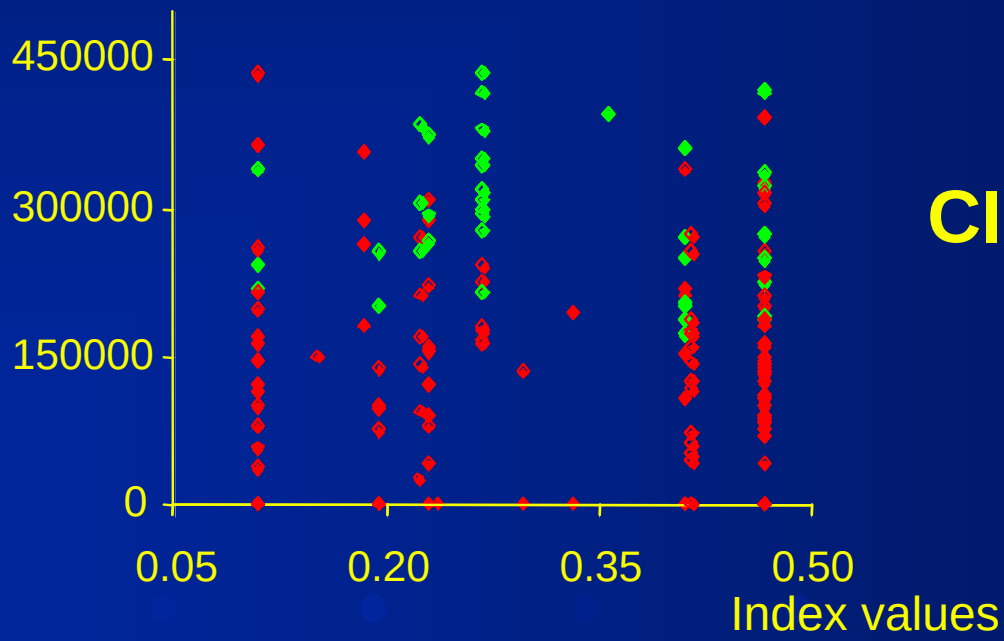
Should we really use collagenase blend enzymes with higher as possible collagenase class I (peak B) and collagenase class II content?



Tranplanted

Not transplanted

CII



CI Peak B

From our data:

Class II collagenase remains the most important component of an enzyme.

CII percentage has to be limited to 29.5-31% as well as CI peak B to 20-35%.

As consequence the remaining enzyme has to be represented by CI peak G (≅25%).

Additional consideration:

Class II is the most important component of a collagenase blend.

A proper evaluation of its role in pancreas digestion should take in account other variables of isolation procedures.

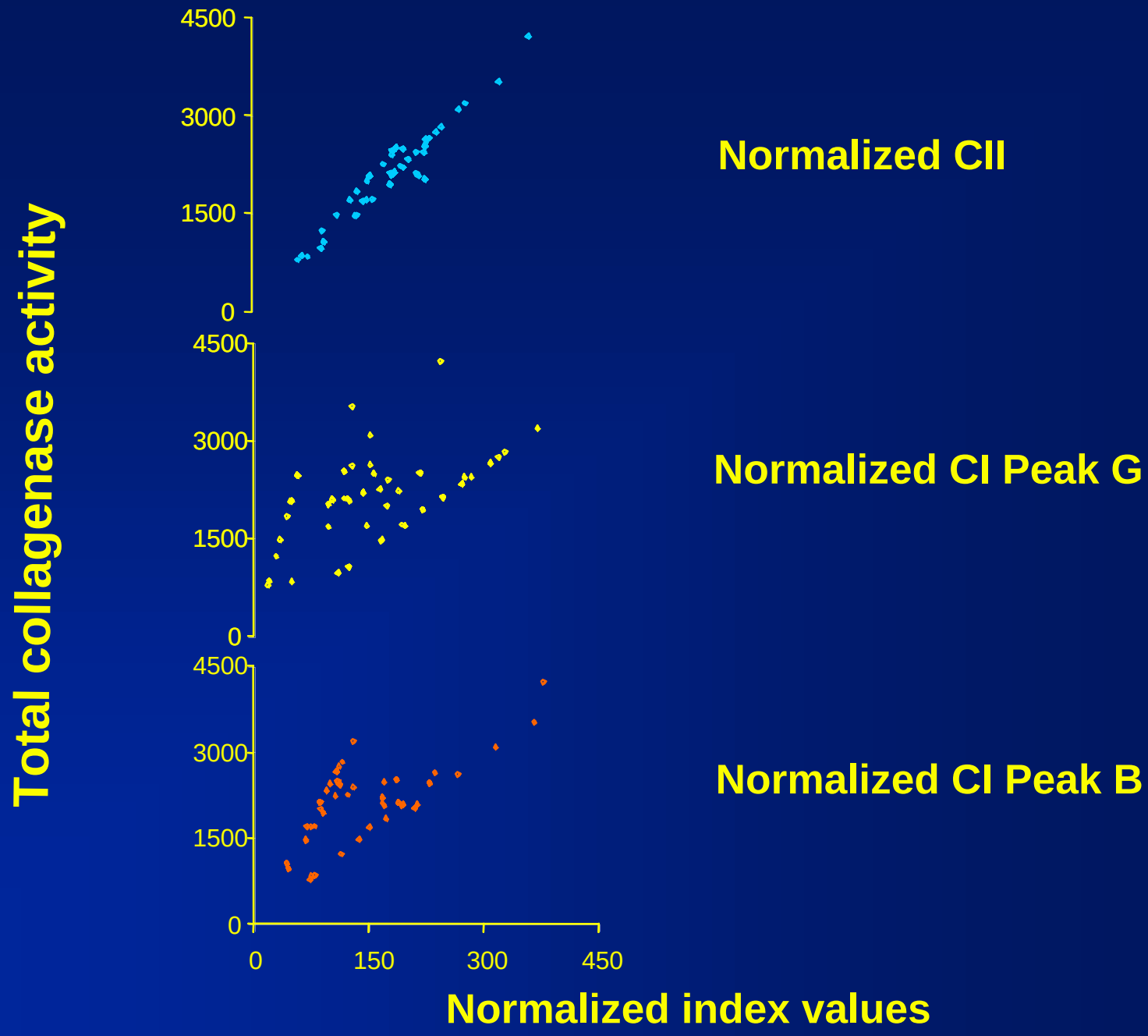
R. Nano · B. Clissi · R. Melzi · G. Calori · P. Maffi ·
B. Antonioli · S. Marzorati · L. Aldrighetti · M. Freschi ·
T. Grochowiecki · C. Socci · A. Secchi · V. Di Carlo ·
E. Bonifacio · F. Bertuzzi

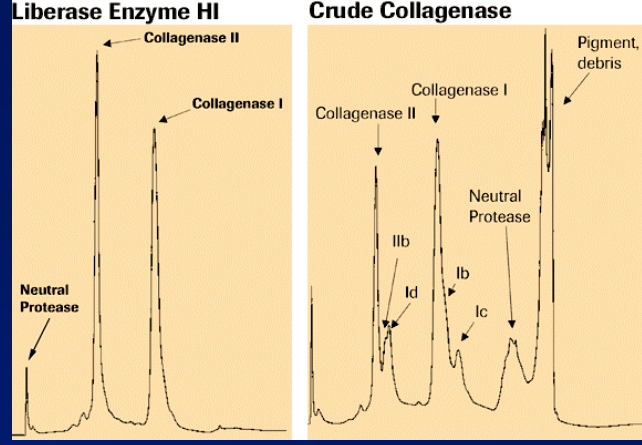
Islet isolation for allotransplantation: variables associated with successful islet yield and graft function

require standardisation. *Methods:* Since 1989 we have processed 437 pancreases using the automated method. The donor characteristics, pancreas procurement, and digestion and purification procedures including a wide enzyme characterisation of these pancreases were analysed and correlated with islet yield and transplant outcome. *Results:* By

0.0029). A statistically significant contribution to the variability of islet yield in a multivariate analysis performed on donor variables was found for donor BMI (p=0.0008). In a multivariate analysis performed on pancreas variables a contribution was found for pancreas weight (p=0.0064), and for a multivariate analysis performed on digestion variables we found a contribution for digestion time (p= 0.0048) and total collagenase activity (p=0.0001). Twenty-four pa-

Total collagenase activity=collagenase activity*mg of enzyme used for the isolation


Total collagenase activity
4500
3000
1500
0
4500
3000
1500
0
4500
3000
1500
0
0
150
300
450
Normalized index values
Normalized CII
Normalized CI Peak G
Normalized CI Peak B



2nd key factor:

the role of neutral proteases

Diabetologia (1992) 35: 735–742

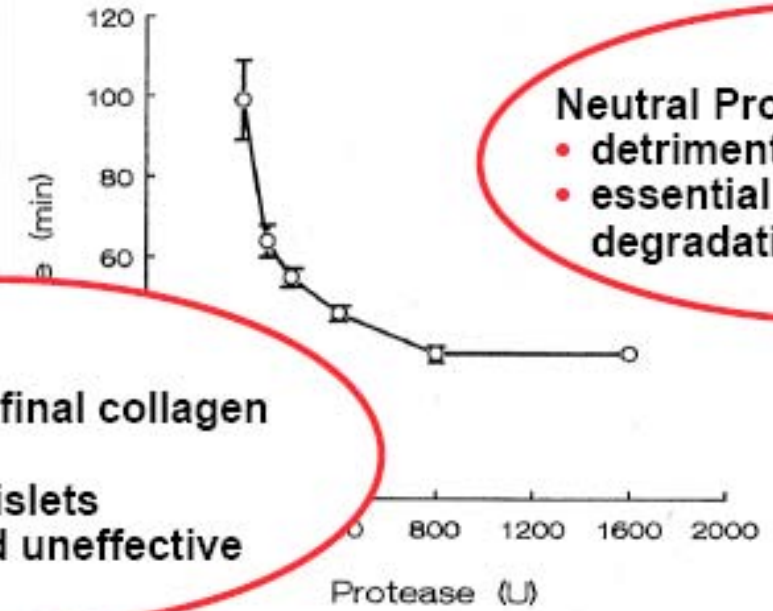
Diabetologia

© Springer-Verlag 1992

An analysis of the role of collagenase and protease in the enzymatic dissociation of the rat pancreas for islet isolation

G. H. J. Wolters, G. H. Vos-Scheperkeuter, J. H. M. van Deijnen and R. van Schilfgaarde

Department of Surgery, University of Groningen, Groningen, The Netherlands



Effect of protease concentration on pancreas dissociation time.

Neutral Protease:
- detrimental for islet integrity
- essential for initial collagen degradation

Collagenase:
- essential for final collagen degradation
- non-toxic to islets
- if singly used uneffective

Brandhorst H, Brendel MD, Eckhard M, Bretzel RG, Brandhorst D.

Influence of neutral protease activity on human islet isolation outcome.

Transplant Proc. 2005 Jan-Feb;37(1):241-2.

Brandhorst H, Alt A, Huettler S, Raemsch-Guenther N, Kurfuerst M, Bretzel RG, Brandhorst D.

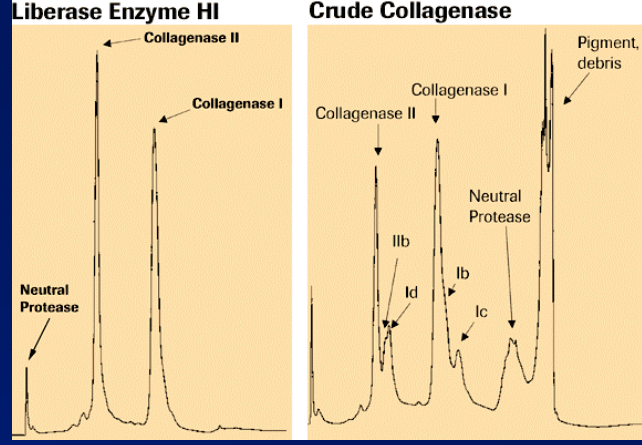
The ratio between class II and class I collagenase determines the amount of neutral protease activity required for efficient islet release from the rat pancreas.

Transplant Proc. 2005 Jan-Feb;37(1):215-6.

Bucher P, Bosco D, Mathe Z, Matthey-Doret D, Andres A, Kurfuerst M, Ramsch-Gunther N, Buhler L, Morel P, Berney T.

Optimization of neutral protease to collagenase activity ratio for islet of Langerhans isolation.

Transplant Proc. 2004 May;36(4):1145-6.



3rd key factor:

Enzyme stability

Quantitative Assessment of Collagenase Blends for Human Islet Isolation

Matthew J. Barnett,[1] Xiaojun (Wendy) Zhai,[1] Donald F. LeGatt,[2] Siew Bang Cheng,[2] A. M. James Shapiro,[3] and Jonathan R. T. Lakey[1,4]

(*Transplantation* 2005;80: 723–728)

Formulation Analysis by HPLC

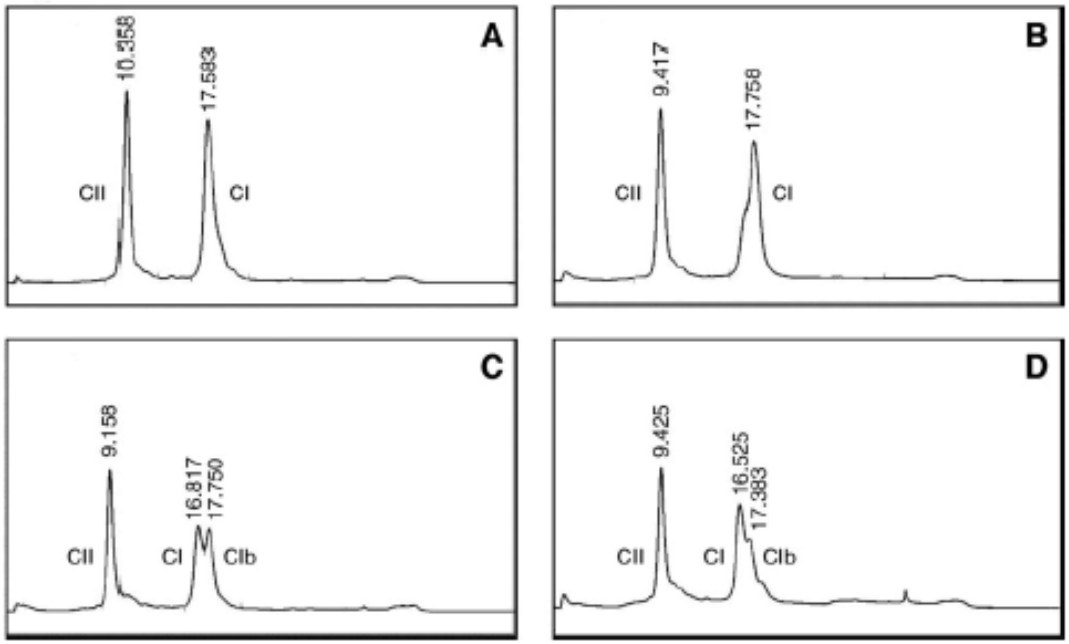


FIGURE 1. Chromatograms of Liberase HI. (A) Lot 93080820. (B) Lot 93096820 with a peak shoulder. (C–D) These lots (93131620 and 93134620 respectively) had distinct proportions of CIb.

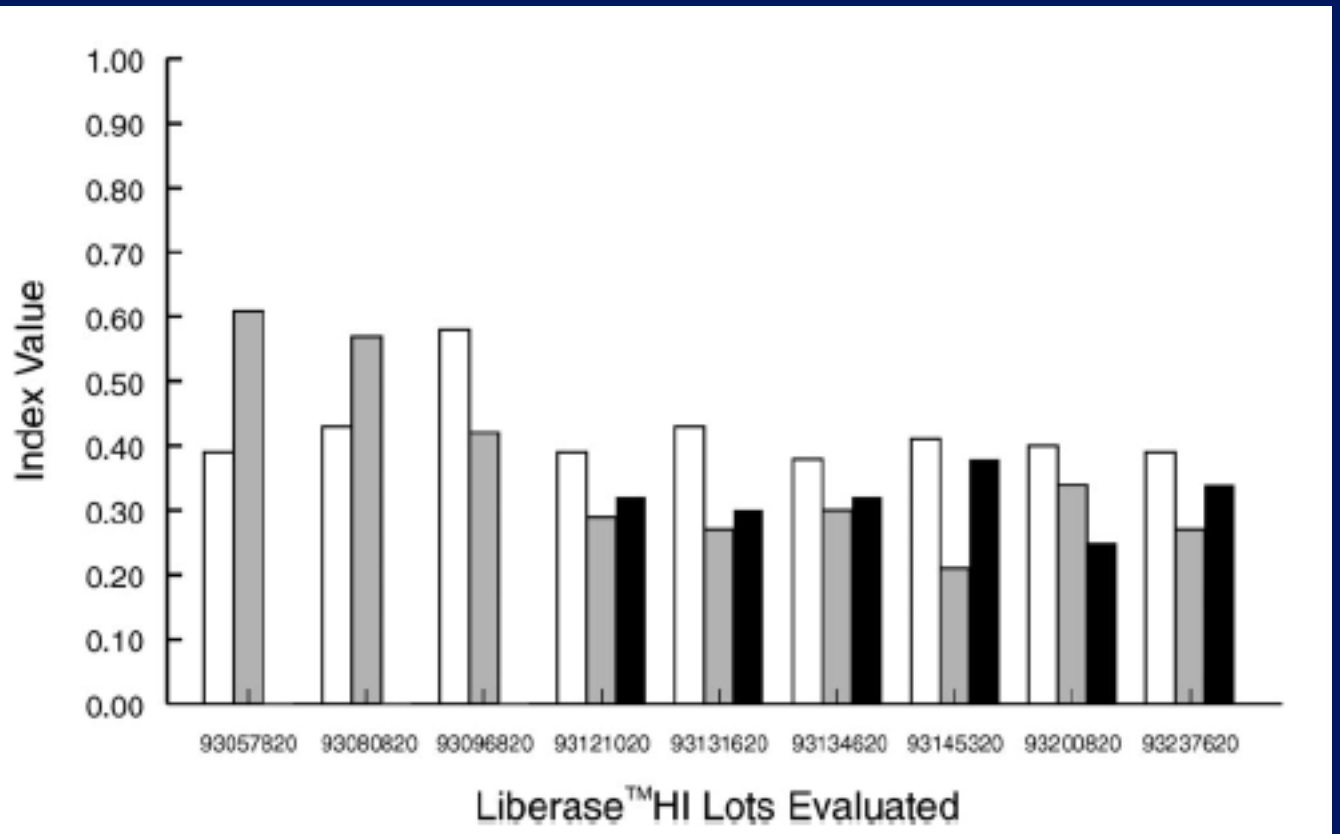
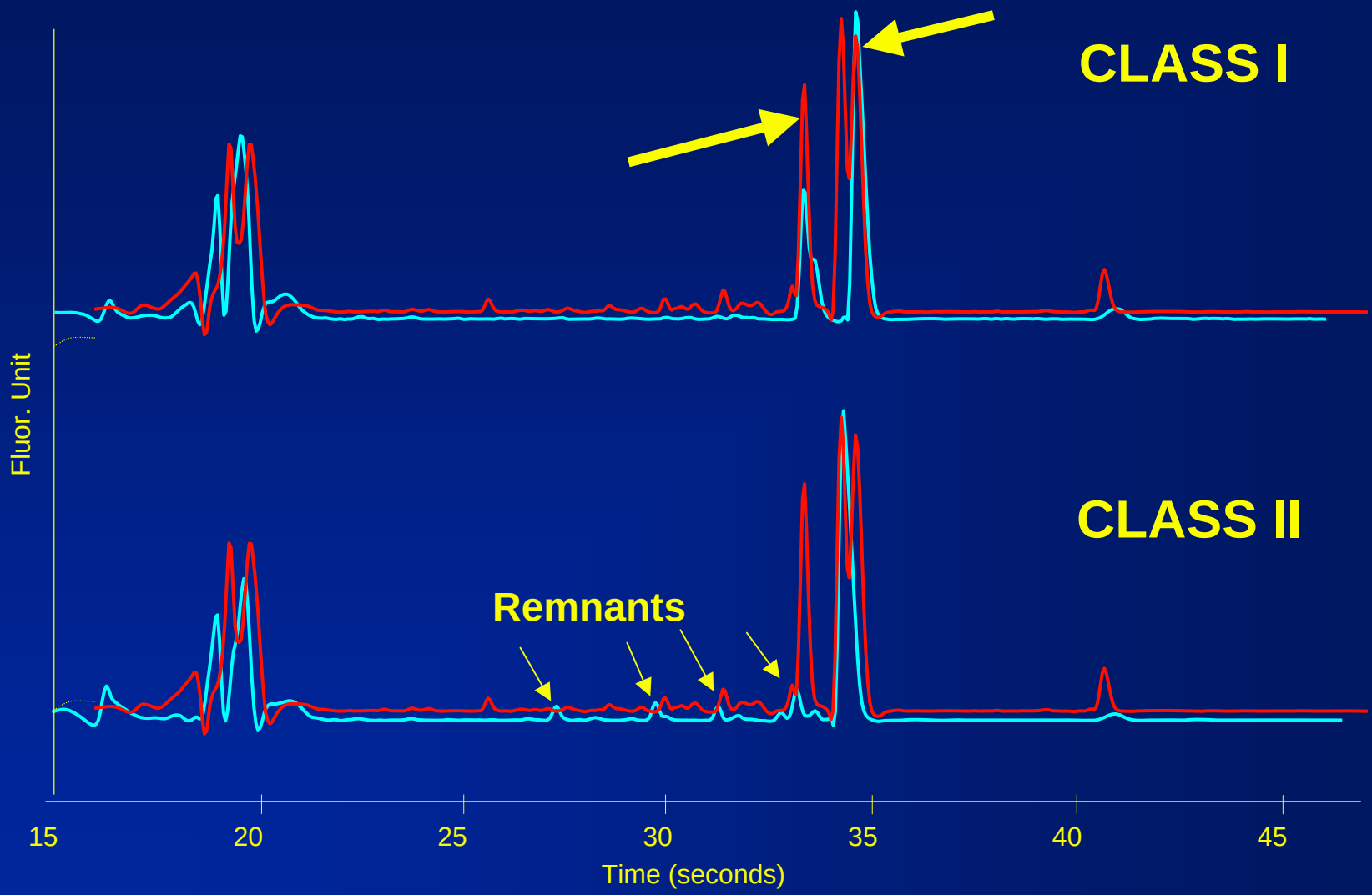
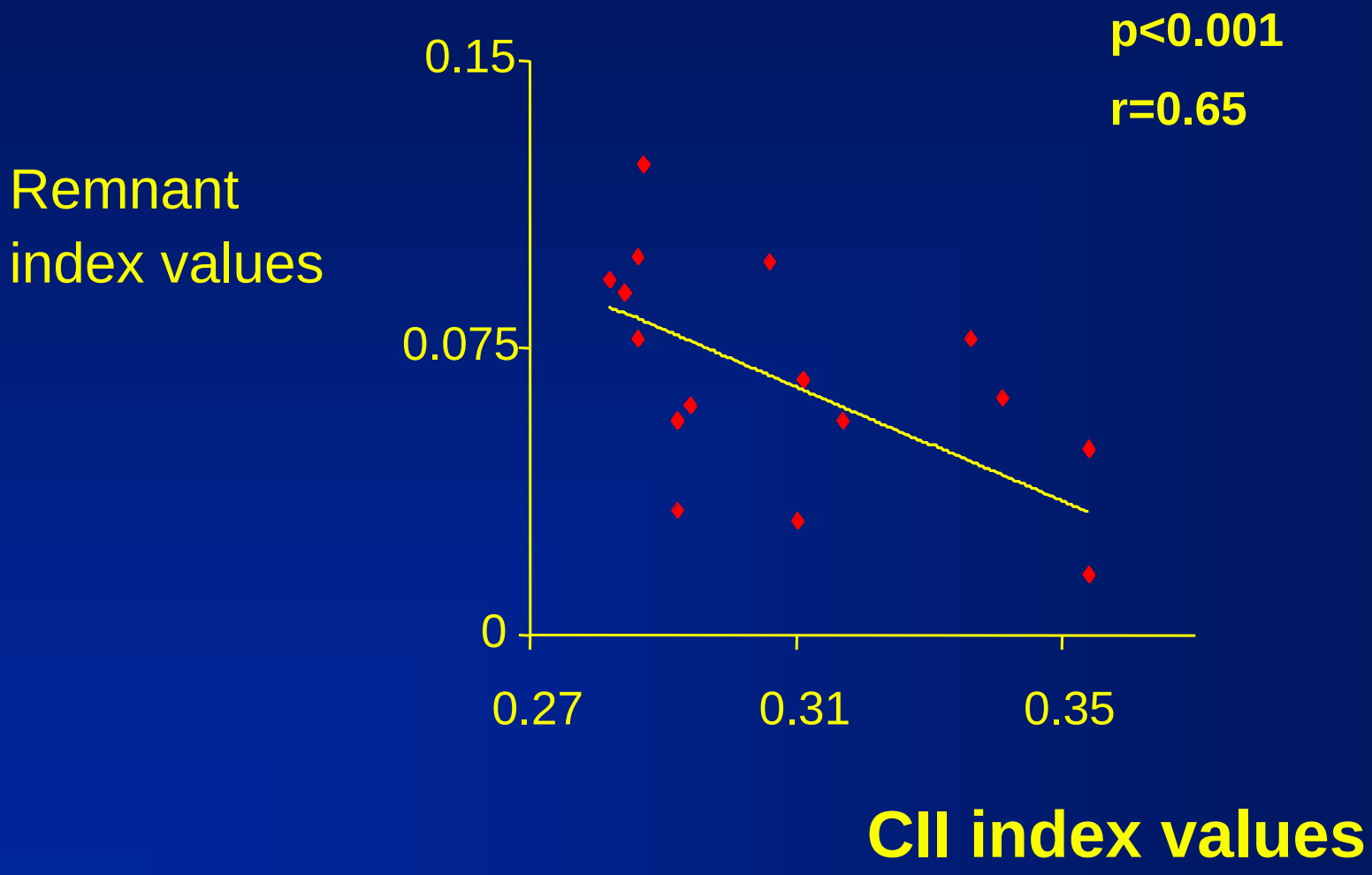


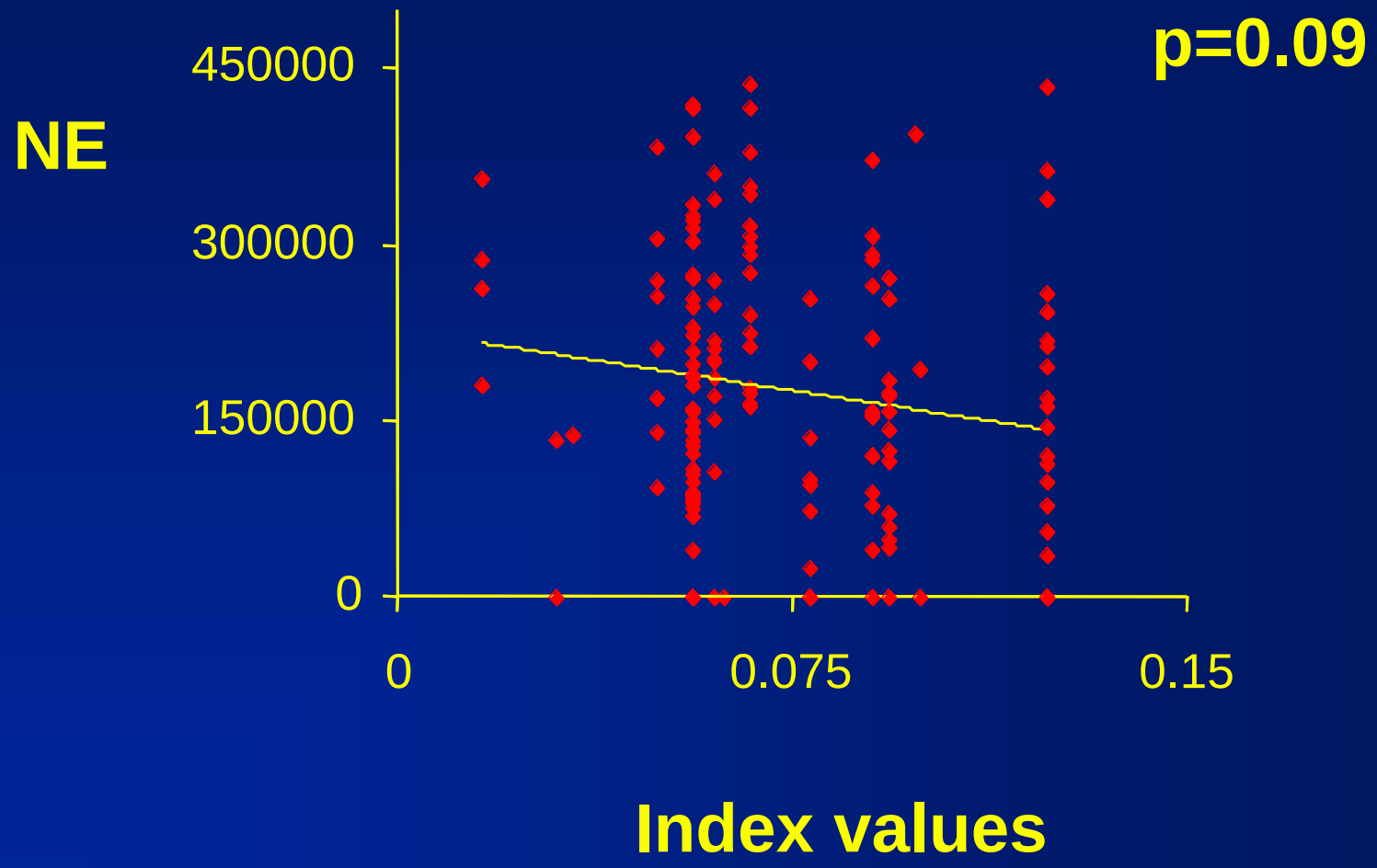
FIGURE 2. The plot of the relative index for each of CII (□), CI (▨), and CIb (■). The appearance of detectable CIb was closely related to the chronology of manufacture. The data suggests, that if CIb is a degradative product, its appearance in the blend may be related to a change in relative enzyme potency.


CLASS I
CLASS II
Remnants
Fluor. Unit
15
20
25
30
35
40
45
Time (seconds)

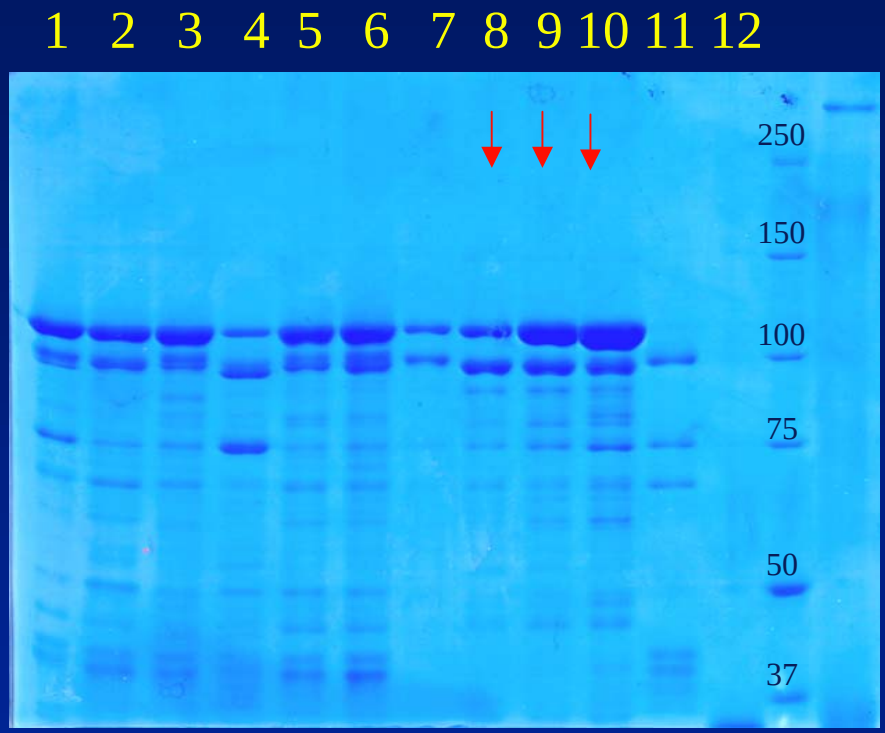
Remnants

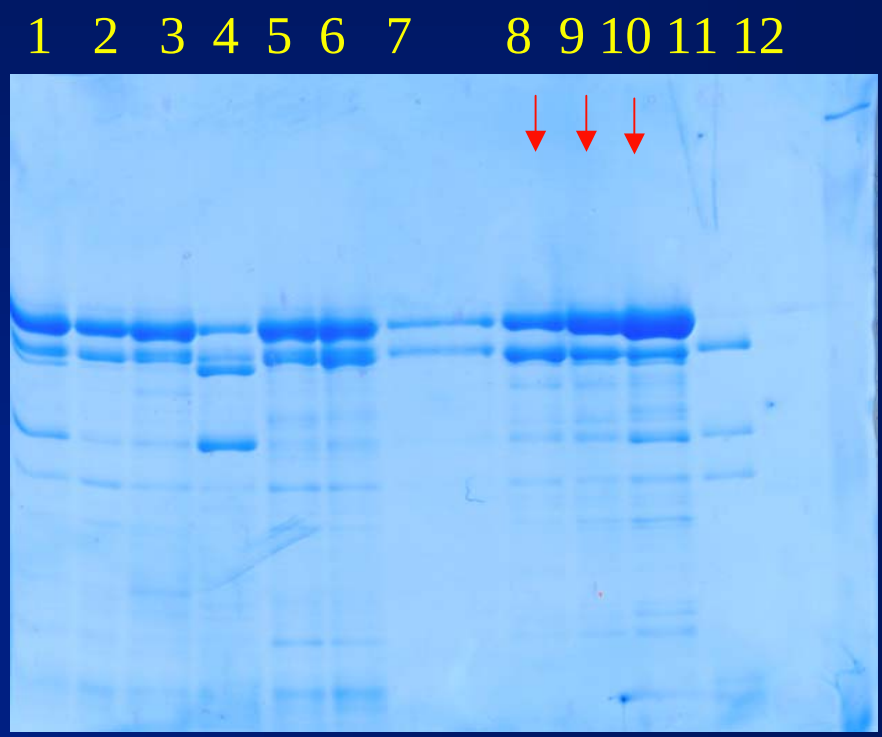

p<0.001
r=0.65
Remnant index values
0.15
0.075
0
0.27
0.31
0.35
CII index values

Remnants

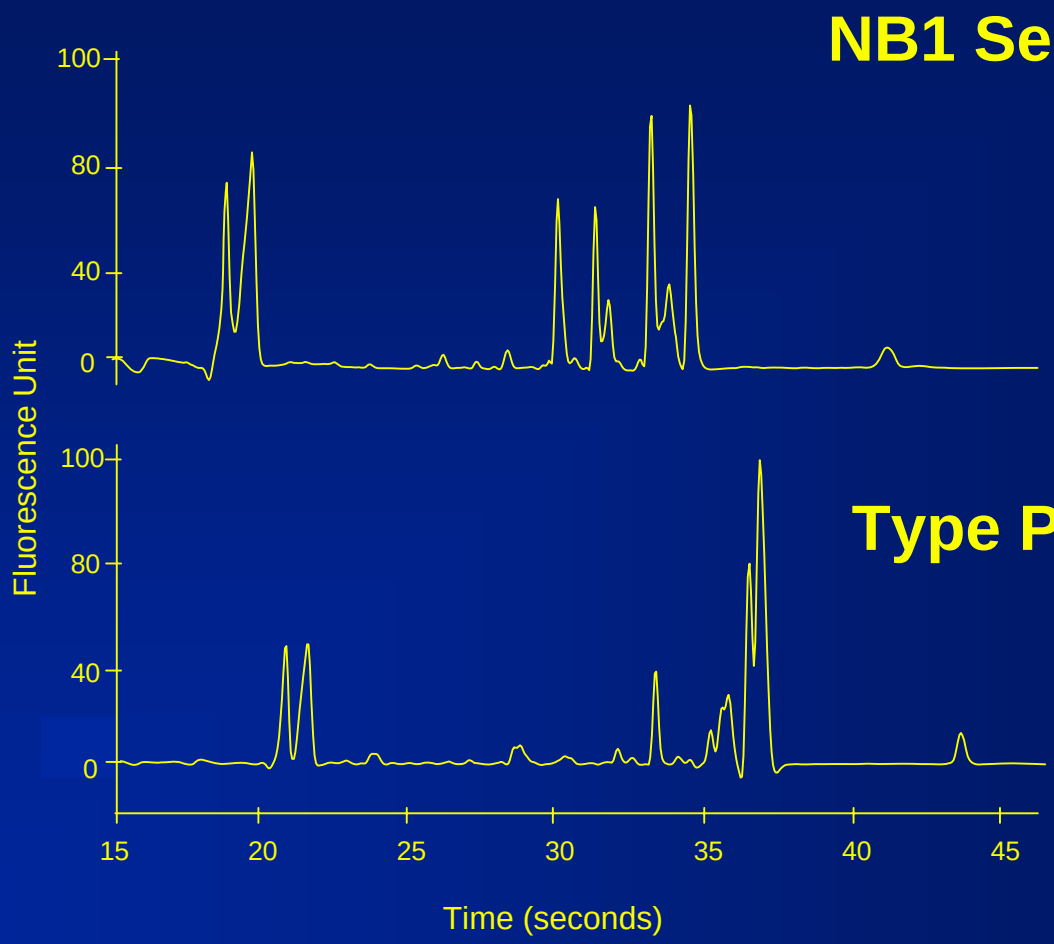


SDS-PAGE analyses



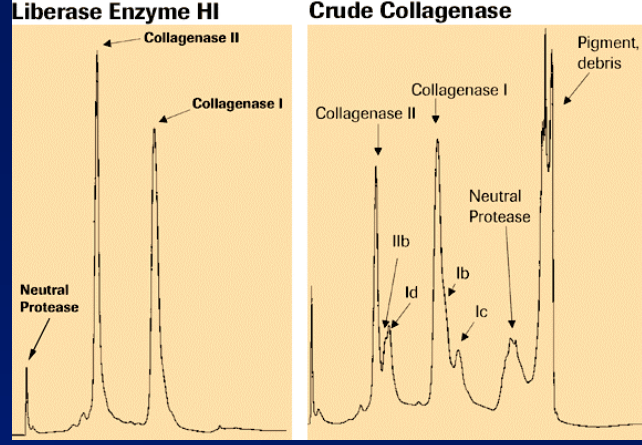


1- *92762522*
2- *852906*
3- *852905*
4- *838348*
5- *924432*
6- *994434*
7- *Coll. Serva lott. 14539*
8- *Lib. 7420*
9- *Lib. 7120*
10- *Lib. 7220*
11- *Prot. 14540*
12- *Proteasi termolisina*



Instability or unpurification?

Enzyme instability remains an unsolved problem.

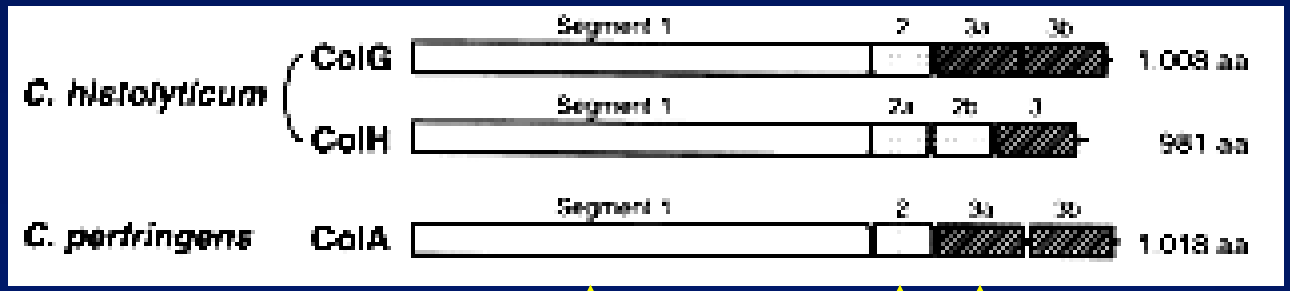


4th key factor:

The structure of collagenase class I and II

(as consequence of previous considerations)

Isoforms or degradation products?

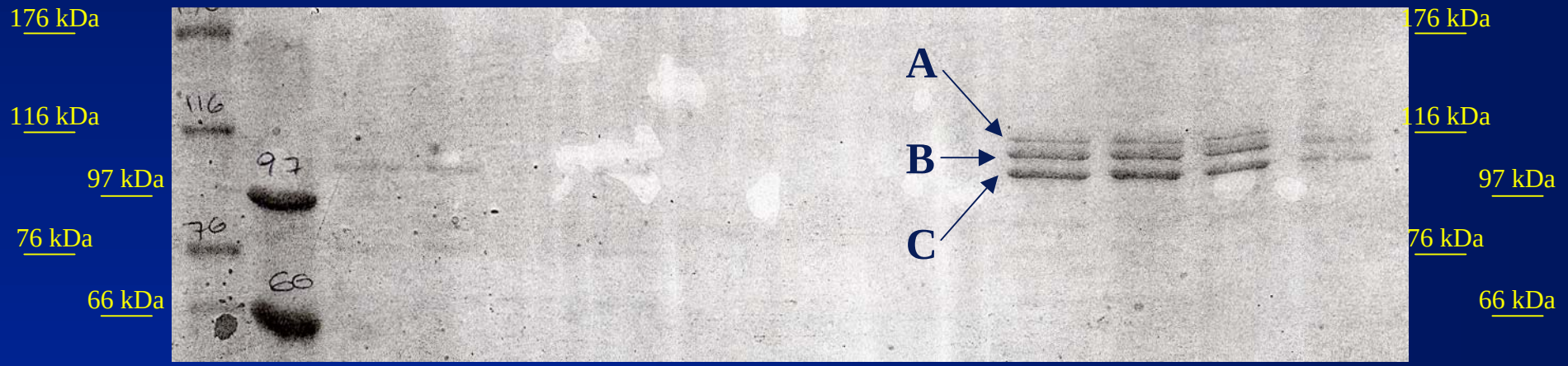


ColG → Class I collagenases → β (115 kDa) → α (68 kDa)
→ γ (79 kDa)

ColH → Class II collagenases → ζ (125 kDa) → δ (100 kDa)
→ ε (110 kDa)

Bond et al 1984

GEL SDS-PAGE AT 7.5%



LIBERASE 7420

Mascot Search Results: Band A

CAB69454. ColG. Classe I. Banda A. MW calcolato:114 kDa

1 IANTNSEKYD FEYLNGLSYT ELTNLIKNIK **WNQINGLFNY STGSQKFFGD KNRVQAIINA**
61 **LQESGR**TYTA NDMK**GIETFT EVLRAGFYLG YYNDGLSYLN DR**NFQDK**CIP AMIAIQKNPN**
121 **FKLGTAVQDE VITSLGK**LIG NASANAEVVN NCVPVLK**QFR ENLNQYAPDY VKGTAVNELI**
181 **KGIEFDFSGA AYEKDVK**TMP WYGK**IDPFIN ELK**ALGLYGN ITSATEWASD VGIYYLSK**FG**
241 **LYSTNRNDIV QSLEK**AVDMY KYGKIAFVAM ER**ITWDYDGI GSNGK**KVDHD K**FLDDAEKHY**
301 **LPKTYTFDNG TFIIR**AGDKV SEEKIKRLYW ASR**EVKSQFH RVVGNDKALE VGNADDVLTM**
361 **KIFNSPEEYK FNTNINGVST DNGGLYIEPR GTFYTYERTP QQSIFSLEEL FRHEYTHYLQ**
421 **ARYLVDGLWG QGPFYEKNRL TWFDEGTAEF FAGSTR**TSGV LPR**KSILGYL AK**DKVDHRYS
481 LKKTLNSGYD DSDWMFYNYG FAVAHYLYEK DMPTFIKMNK AILNTDVK**SY DEIIKK**LSDD
541 ANKNTEYQNH IQELVDK**YQG AGLPLVSDDY LK**DHGYKKAS EVYSEISK**AA SLTNTSVTAE**
601 **KSQYFNTFTL R**GTYTGETSK GEFKDWDEMS KKLDGTLESL AKNSWSGYK**T LTAYFTNYR**V
661 TSDNK**VQYDV VFHGVLTDNG DISNNK**APIA KVTGPSTGAV GRNIEFSGKD SKDEDGK**IVS**
721 **YDWDFGDGAT SRGKNSVHAY K**KAGTYNVTL KVTDDKGATA TESFTIEIKN EDTTTPITKE
781 MEPNDDIKEA NGPIVEGVTV KGDLNGSDDA DTFYFDVKED GDVTIELPYS GSSNFTWLVY
841 KEGDDQNHIA SGIDKNNSKV GTFKATKGRH YVFIYKHDSA SNISYSLNIK GLGNEKLKEK
901 ENNDSSDK**AT VIPNFNTTMQ GSLLGDDSR**D YYSFEVKEEG EVNIELDKKD **EFGVTWTLHP**
961 **ESNINDRITY GQVDGNKVSN K**VKLRPGK**YY LLVYKYSGSG NYELR**VNK

Class I collagenase β (115 kDa)

COLLAGENASE β ?

Sequentied peptides are in bold

Mascot Search Results : Band B

I40805. ColH. Classe II. Banda B. MW CALCOLATO: 101 kDa

1 MKRKCLSKRL MLAITMATIF TVNSTLPIYA AVDKNNATAA VQNESKRYTV SYLK**TLNYYD**
61 **LVDLLVK**TEI ENLPDLFQYS SDAK**EFYGNK TRMSFIMDEI GR**RAPQYTEI DHK**GIPTLVE**
121 **VVRAGFYLGF HNKELNEINK RSFK**ER**VIPS ILAIQKNPNF K**LGTEVQDKI VSATGLLAGN
181 ETAPPEVVNN FTPILQDCIK NIDRYALDDL KSKALFNVLA APTYDITEYL RATKEKPENT
241 PWYGKIDGFI NELKKLALYG KINDNNSWII DNGIYHIAPL GKLHSNN**KIG IETLTEVMK**V
301 YPYLSMQHLQ SADQIKR**HYD SKDAEGNKIP LDKFKK**EGKE KYCPK**TYTFD DGKVIIKAGA**
361 **R**VEEEKVK**RL YWASKEVNSQ FFR**VYGIDKP LEEGNPDDIL TMVIYNSPEE YKLNSVLYGY
421 DTNNGGMYIE PEGTFFTYER **EAQESTYTLE ELFRHEYTHY LQGR**YAVPGQ WGR**TKLYDND**
481 **RLTWYEEGGA ELFAGSTR**TS GILPR**KSIVS NIHNTTR**NNR **YKLSDTVHSK** YGASFEFYNY
541 ACMFMDYMYN KDMGILNKLN DLAK**NNDVDG YDNYIR**DLSS NYALNDKYQD HMQER**IDNYE**
601 **NLTVPFVADD YLVRHAYKNP NEIYSEISEV AK**LKDAKSEV KKSQYFSTFT LRGSYTGGAS
661 KGKLEDQKAM NKFIDDSLK**K LDTYSWSGYK** TLTAYFTNYK VDSSNR**VTYD VVFHGYLPNE**
721 **GDSK**NSLPYG KINGTYKGTE KEK**IKFSSEG SFDPDGKIVS YEWDFGDGNK** SNEENPEHSY
781 DK**VGTYTVKL K**VTDDKGESS VSTTTAEIKD LSENKLPVIY MHVPKSGALN QKVVFYGKGT
841 YDPDGSIAGY QWDFGDGSDF SSEQNPSHVY TK**KGEYTVTL R**VMDSSGQMS EKTMKIKITD
901 PVYPIGTEKE PNNSKETASG PIVPGIPVSG TIENTSDQDY FYFDVITPGE VKIDINKLGY
961 GGATWVVYDE NNNAVSYATD DGQNLSGKFK ADKPGRYYIH LYMFNGSYMP YRINIEGSVG
1021 R

Class II collagenase δ (100 kDa)

COLLAGENASE δ ?

Sequentied peptides are in bold

Mascot Search Results : Band C

CAB69454. ColG. Classe I. Banda C. MW calcolato: 106 kDa

1 IANTNSEKYD FEYLNGLSYT ELTNLIK**NIK WNQINGLFNY STGSQKFFGD KNRVQAIINA**
61 **LQESGRTYTA NDMKGIETFT EVLRAGFYLG YYNDGLSYLN DR**NFQDK**CIP AMIAIQKNPN**
121 **FKLGTAVQDE VITSLGK**LIG NASANAEVVN NCVPVLK**QFR ENLNQYAPDY VKGTAVNELI**
181 **KGIEFDFSGA AYEKDVKTMP WYGKIDPFIN ELK**ALGLYGN ITSATEWASD VGIYYLSK**FG**
241 **LYSTNRNDIV QSLEK**AVDMY KYGK**IAFVAM ERITWDYDGI GSNGK**KVDHD K**FLDDAEKHY**
301 **LPKTYTFDNG TFIIR**AGDKV SEEKIK**RLYW ASREVKSQFH RVVGNDKALE VGNADDVLTM**
361 **KIFNSPEEYK FNTNINGVST DNGGLYIEPR GTFYTYERTP QQSIFSLEEL FRHEYTHYLQ**
421 **ARYLVDGLWG QGPFYEKNRL TWFDEGTAEF FAGSTR**TSGV LPRKSILGYL AK**DKVDHR**YS
481 LKKTLNSGYD DSDWMFYNYG FAVAHYLYEK DMPTFIKMNK AILNTDVKSY DEIIKKLSDD
541 ANKNTEYQNH IQELVDKYQG AGLPLVSDDY LKDHGYKKAS EVYSEISK**AA SLTNTSVTAE**
601 **KSQYFNTFTL R**GTYTGETSK GEFKDWDEMS KKLDGTLESL AKNSWSGYK**T LTAYFTNYR**V
661 TSDNK**VQYDV VFHGVLTDNG DISNNK**APIA K**VTGPSTGAV GR**NIEFSGKD SKDEDGK**IVS**
721 **YDWDFGDGAT SR**GKNSVHAY KKAGTYNVTL KVTDDKGATA TESFTIEIKN EDTTTPITKE
781 MEPNDDIKEA NGPIVEGVTV K**GDLNGSDDA DTFYFDVK**ED GDVTIELPYS GSSNFTWLVY
841 KEGDDQNHIA SGIDKNNSKV GTFKATKGRH YVFIYKHDSA SNISYSLNIK GLGNEKLKEK
901 ENNDSSDKAT VIPNFNTTMQ GSLLGDDSR**D YYSFEVK**EEG EVNIELDKKD EFGVTWTLHP
961 ESNINDRITY GQVDGNKVSN KVKLRPGKYY LLVYKYSGSG NYELRVNK

Class I collagenase β (115 kDa) α (68 kDa) γ (79 kDa)

New isoform o degradation product?

Sequentied peptides are in bold

Further studies should be aimed at:

1) Improving enzymatic blend purification in order to separate peak B from peak G of collagenase class I.

2) Improving enzymatic blend stability in order to avoid degradation products.

3) Testing the activity of each enzymatic blend component toward the most important collagen types present in human pancreas (I, IV and VI).

4) Producing each enzymatic blend component by recombinant technique on the bases of the new data available for definitive standardization of isolation.

Thank you for your attention!