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APPENDICES

APPENDIX A

Partial Purification of chitinase from *Burkholderia cepacia*

Crude chitinase was partial purified by two major consecutive steps, precipitation at 50-80% ammonium sulfate saturation, and DEAE-cellulose column chromatography. For the production of chitinase, *Burkholderia cepacia* was grown in LB medium at 37 °C overnight. The culture was transferred into 1% colloidal chitin minimum medium, with 1:100 dilution and cultivated for 2 days at 30 °C. The culture was centrifuged at 4 °C and the supernatant collected. Ammonium sulfate was added to the supernatant to 50% saturation. The resulting precipitate was removed by centrifugation at 4 °C. Ammonium sulfate was further added to the supernatant until 80% saturation was reached. Protein precipitated at 80% saturated ammonium sulfate was dissolved in 25 mM Tris-HCl (pH 7.6), and then dialyzed in the same buffer overnight. The enzyme solution was loaded into an equilibrated DEAE-cellulose column (2.3 x 20 cm). The column was eluted with 25 mM Tris-HCl buffer (pH 7.6) at a flow rate of 45 ml/hour. Fractions of 5 ml were collected. After the unbounded proteins were wash though the column, 0-500 mM NaCl linear gradient was applied to elute the bounded protein from the column. Chitinase activity was measured, in each fraction, by the increase of %T at 650 nm during chitinolysis.

The protein profile (Figure 27A) shows three protein peaks. The first peak came out in the flow through, fractions 13-18, while other two peaks were eluted from the column between fractions 55 to 60 and 62 to 69, respectively. Chitinase activity was eluted from the column between fraction 13 to 54. The highest chitinase activity was detected in fraction 20. Fractions 28-53 were pooled and used in further studies.

Crude chitinase from fraction 20 and pooled fractions 28-53 were analyzed by SDS-PAGE. When stained with Comassie brilliant blue (Figure 28), both fraction 20 and pooled fractions 28-53 showed a major protein band, with an molecular weight of approximately 62 kDa, and 4-5 minor bands. A chitinase activity band in fraction 20 was observed on

SDS-PAGE, with the estimated molecular weight of 47.5 kDa, after chitinase activity staining. Pooled fractions 28-53 also showed a faint chitinase activity band with similar size.

The yield of chitinase obtained at each purification step are shown in Table 7. Specific activity was increased through each step. After the final step of purification, in fraction 20 yield of 4% was obtained with 27 fold of purity.

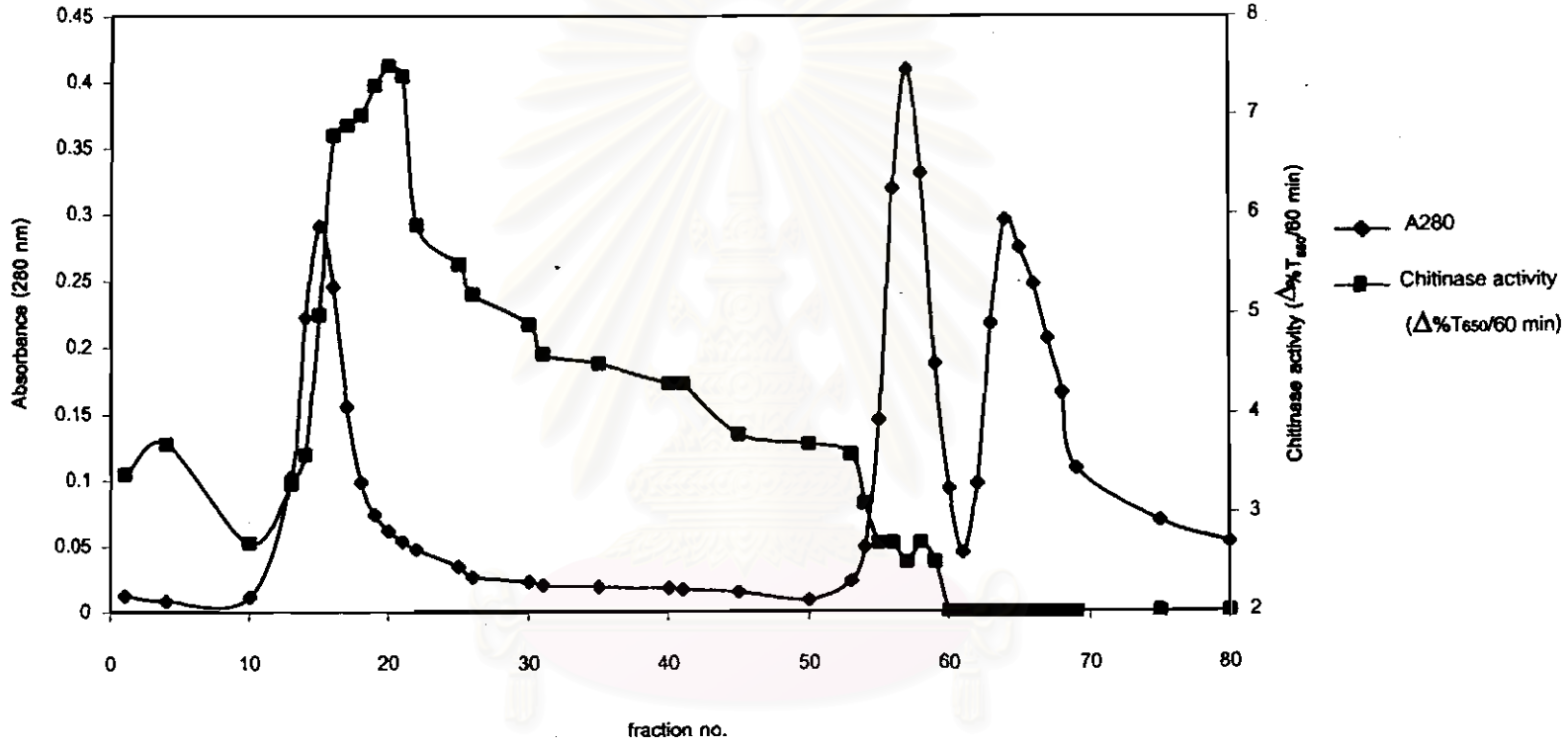


Figure 27A Profile of DEAE-cellulose column chromatography of chitinase, from *Burkholderia cepacia* at pH 7.6. Column size 2.3 x 20 cm), flow rate 45 ml/hr, and 5 ml fractions were collected.

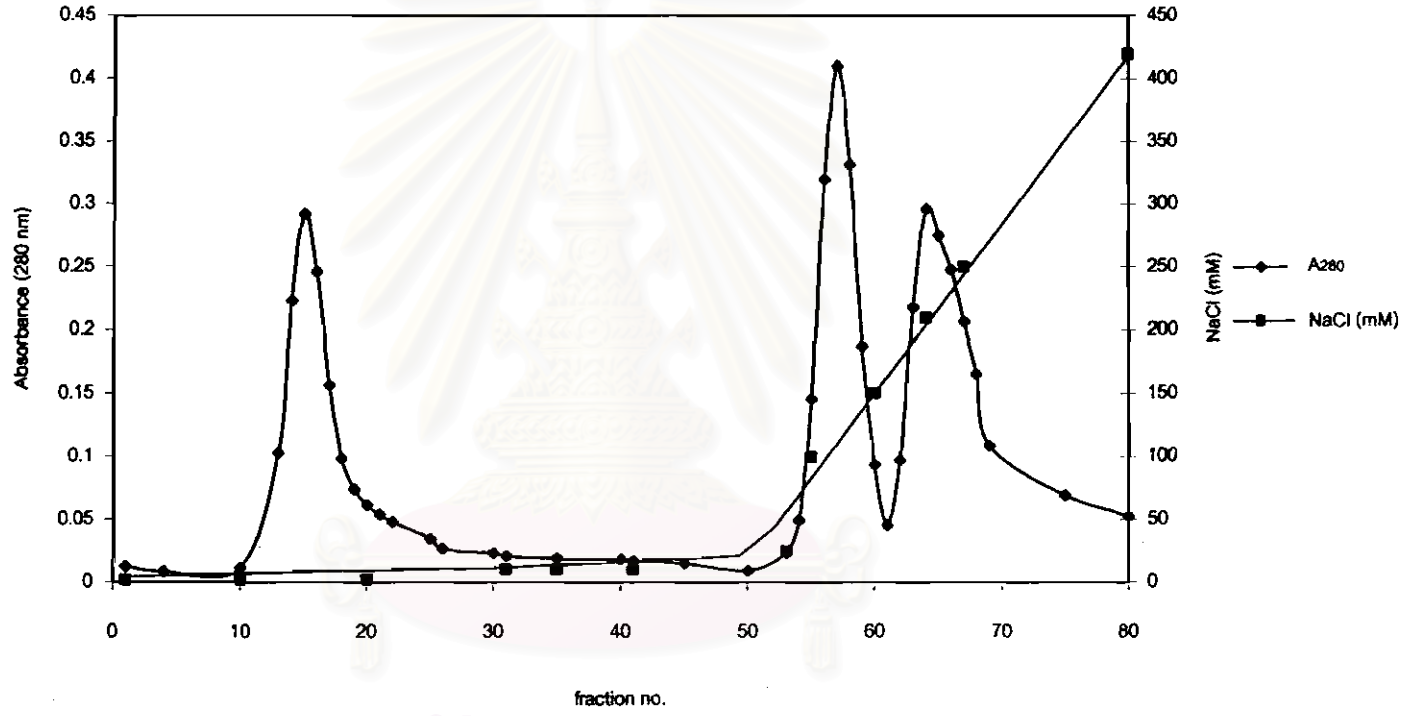


Figure 27B Profile of DEAE-cellulose column chromatography of chitinase, from *Burkholderia cepacia* at pH 7.6. Column size 2.3 x 20 cm), flow rate 45 ml/hr, and 5 ml fractions were collected.

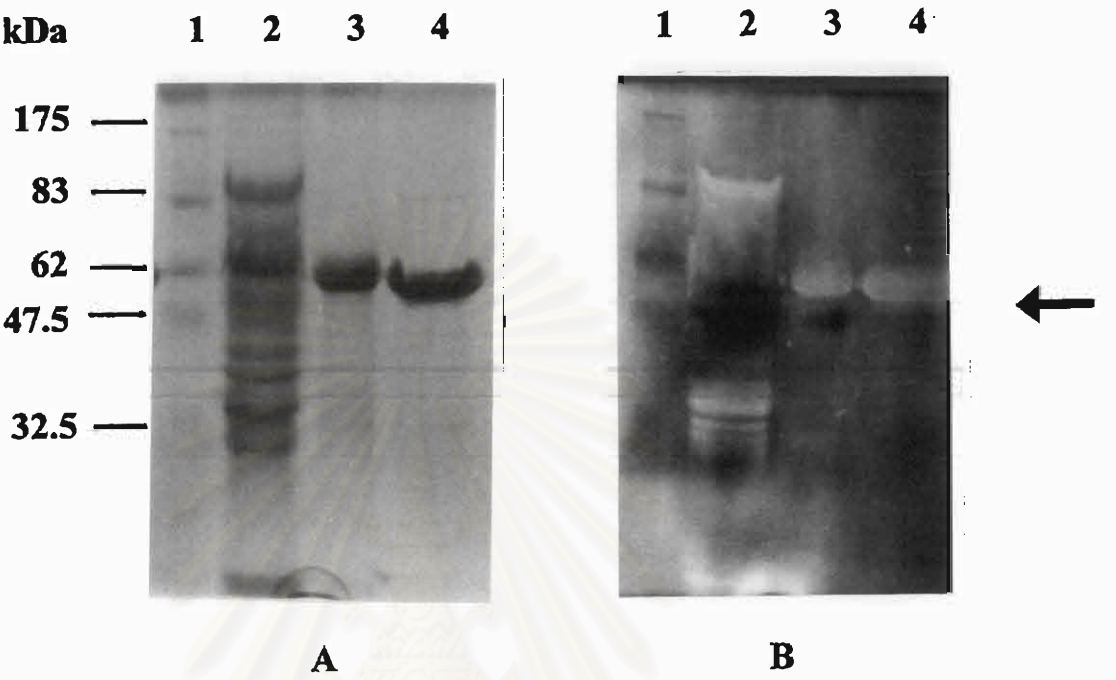


Figure 28 SDS-PAGE of Chitinase from *Burkhloderia cepacia*
The fractions from DEAE column was loaded on SDS-PAGE and stained with Coomassie brilliant blue (Protein staining) and Calcofluor white M2R (Chitinase activity staining)
Panel A (Coomassie blue staining), lane 1 shows protein marker,
lane 2 shows crude protein in *Burkholderia cepacia*'s cultured medium,
lane 3 shows fraction 20, and lane 4 shows pooled fraction 28-53.
Panel B (Chitinase activity staining), lane 1 shows protein marker. Lane 2, 3 and 4 shows chitinase in cultured medium, fraction 20 and pooled fraction 28-53, respectively.
Arrow shows chitinase activity band.

Table 7 Purification table of Chitinase from *Burkholderia cepacia*

Type	Total activity ($\Delta\%T_{650}$/min)	Specific activity ($x10^{-3}$) ($\Delta\%T_{650}$/min/μg protein)	% yield	Purification fold
Crude enzyme	151	4.4	100	1
50% ammonium sulfate precipiatation	37.5	63	24.83	14.19
80% ammonium sulfate precipiatation	92.3	13	61.13	2.93
Fraction 20	6.25	120	4.13	27.03

APPENDIX B

Preparation for polyacrylamide gel electrophoresis

1) Stock reagent

30% Acrylamide 0.8% bis stock solution

Acrylamide	30.00 g
N,N′-methylene-bis-acrylamide	0.80 g

Adjust volume to 100 ml with distilled water

Tris-SDS stock solution, pH 6.8

Tris (hydroxymethyl)-aminomethane	3.94 g
SDS	0.20 g

Adjust pH to 6.8 with 1 N HCl and adjust volume to 100 ml with distilled water

Tris-SDS stock solution, pH 8.9

Tris (hydroxymethyl)-aminomethane	11.82 g
SDS	0.20 g

Adjust pH to 8.9 with 1 N HCl and adjust volume to 100 ml with distilled water

Ammonium persulfate solution "Make up fresh each time"

Ammonium persulfate	1.00 g

Dissolve in 1 ml distilled water

Sample buffer (5x)

Tris-SDS stock, pH 6.8	5.0	ml
SDS	0.40	g
Glycerol	3.0	ml
β-mercaptoethanol	1.0	ml
1% Bromophenol blue	0.5	ml

Adjust volume to 10 ml with distilled water

Tris-glycine electrode buffer stock solution (5x)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g
SDS	1.00	g

Adjust pH to 8.3 with 1 N HCl and adjust volume to 200 ml with distilled water

Staining solution

Dissolve 1.25 g of Coomassie Blue R250 in 500 ml of 95% methanol. Stir for one hour, add 500 ml of 15% acetic acid, and filter.

Destaining solution

7% acetic acid and 5% methanol

2) **SDS-PAGE**

12.5 % Separating gel

30% Acrylamide solution	4.3	ml
Tris-SDS stock solution, pH 8.9	1.2	ml
TEMED	2.7	µl
10% Ammonium persulfate	70.0	µl
distilled water	4.4	ml
Total volume	10.0	ml

3% Stacking gel

30% Acrylamide solution	0.7	ml
Tris-SDS stock solution, pH 8.9	2.0	ml
TEMED	2.0	µl
10% Ammonium persulfate	20.0	µl
distilled water	1.3	ml
Total volume	4.0	ml

One part of sample buffer(5x) was added to four parts of sample. The mixture was heated 5 minutes in boiling water before loading to the gel.

APPENDIX C

Stock solutions for Southern hybridization

50x Denhardt's solution

Ficoll type 400	5.0	g
Polyvinylpyrrolidone	5.0	g
BSA	5.0	g

Dissolve Ficoll type 400, polyvinylpyrrolidone and BSA in 500 ml deionized water, filter and store at –20 °C

20x SSC

NaCl	175.3	g
Sodium citrate	88.2	g

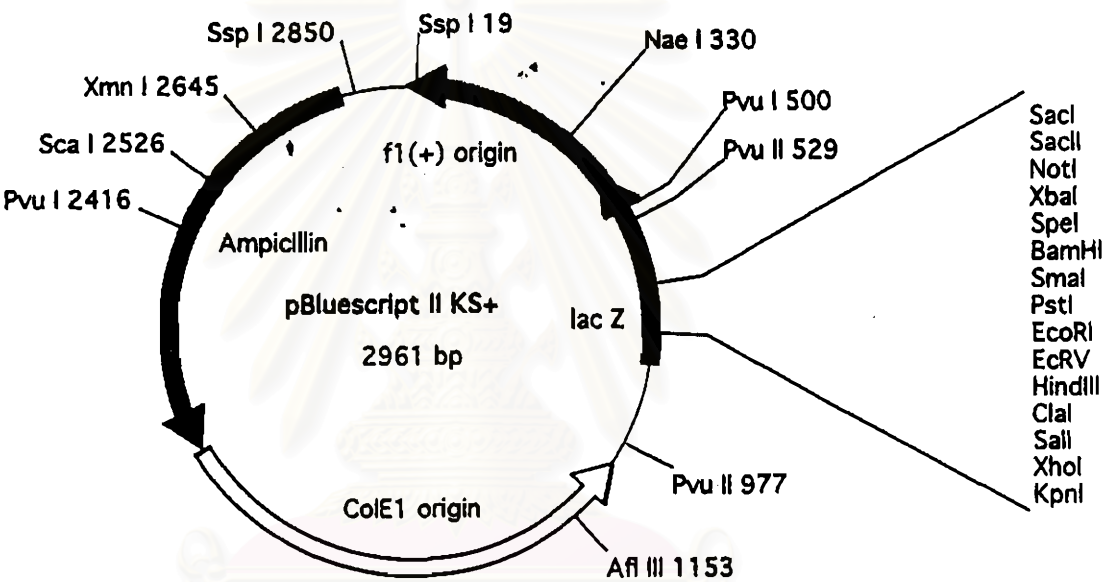
Dissolve NaCl and sodium citrate in deionized water and adjust pH to 7.0 with 10N NaOH. Adjust volume to 1 litre, sterilized by autoclaving and store at room temperature.

Prehybridization solution

5x Denhardt's solution
6x SSC
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA

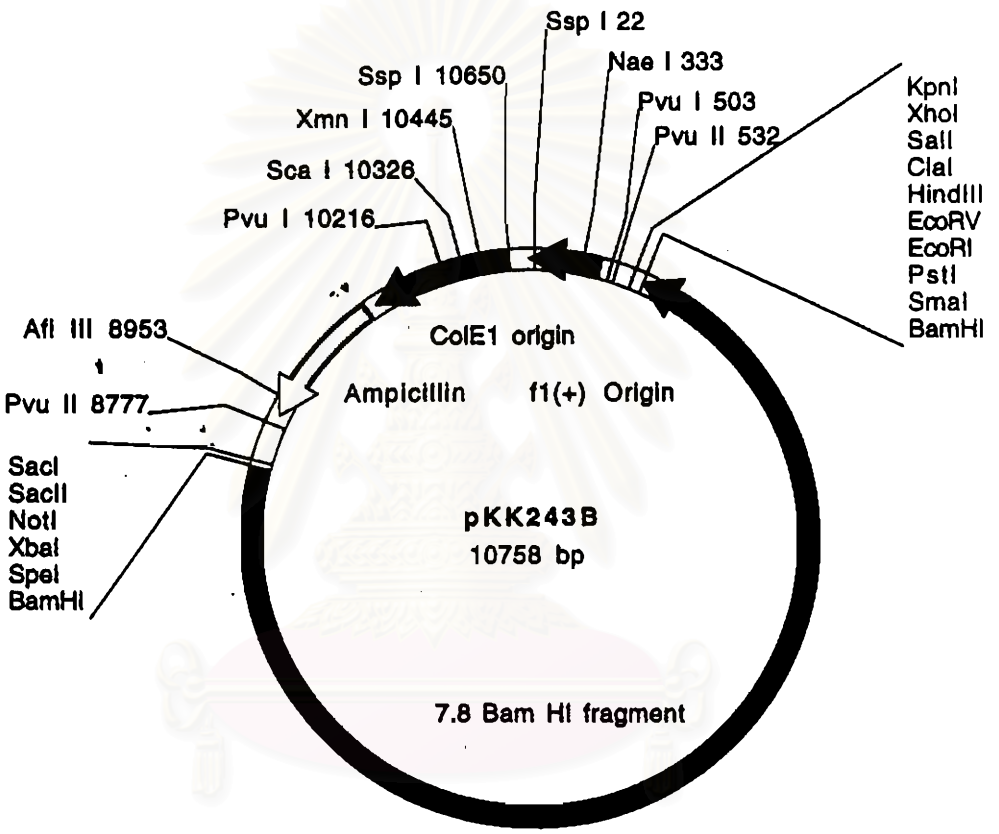
APPENDIX D

Restriction map of pBluescript II KS^{+}



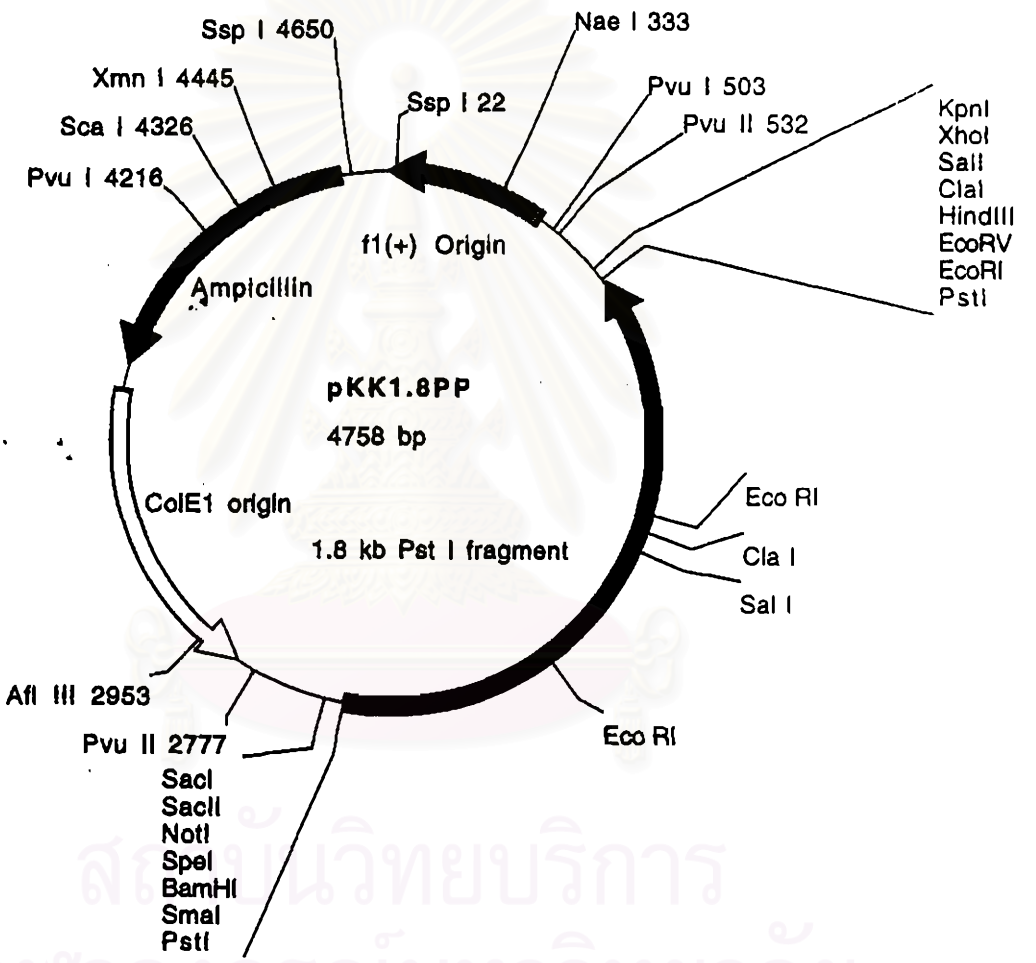
APPENDIX E

Restriction map of pKK243B



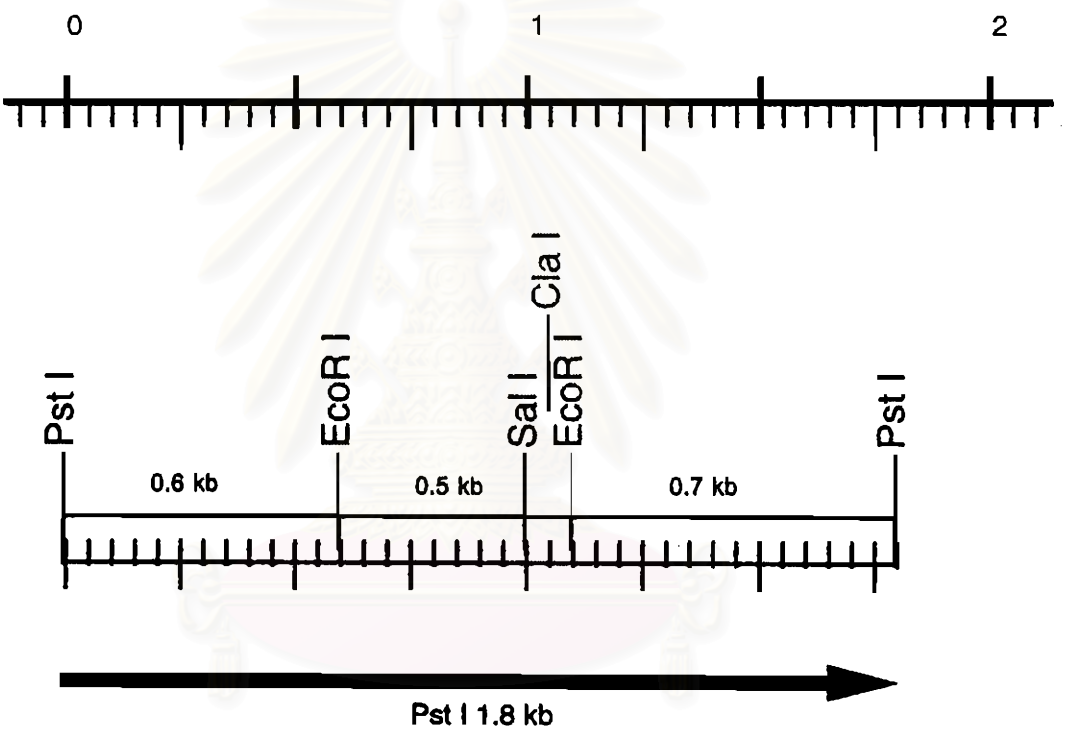
APPENDIX F

Restriction map of pKK1.8PP



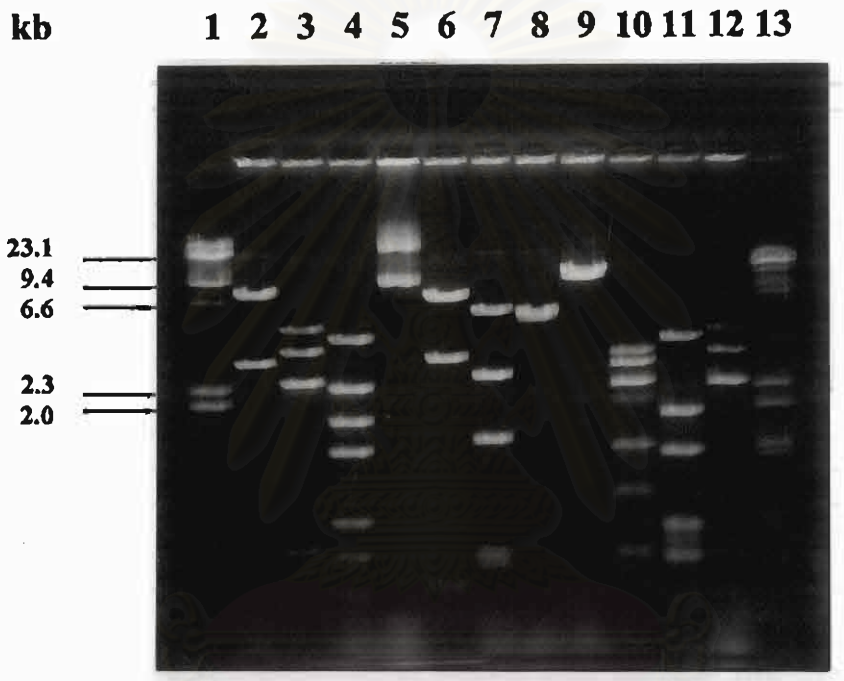
APPENDIX G

Restriction map of 1.8 kb insert fragment from pKK1.8PP



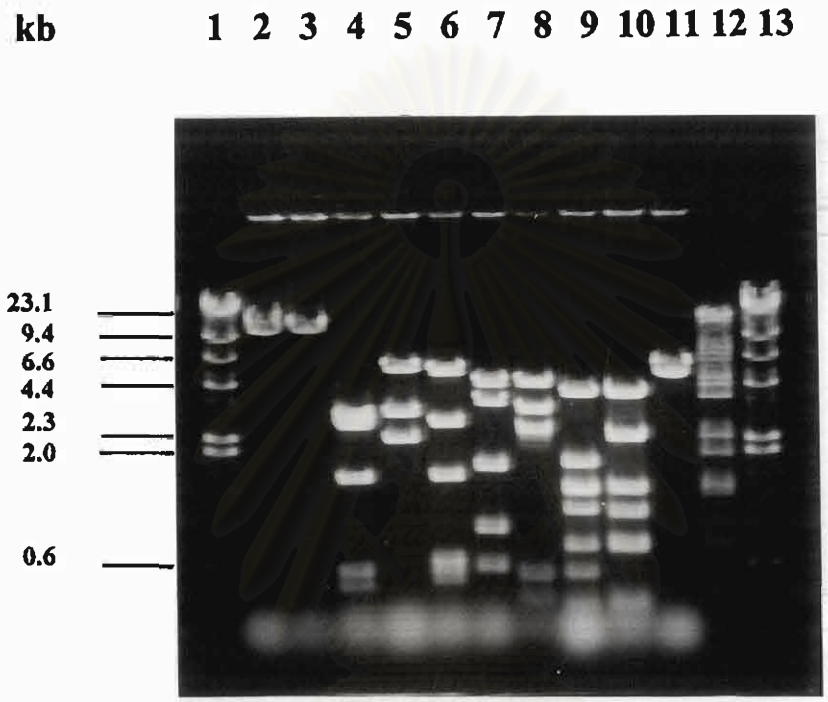
APPENDIX H

Restriction analysis of pKK243B by various restriction enzymes



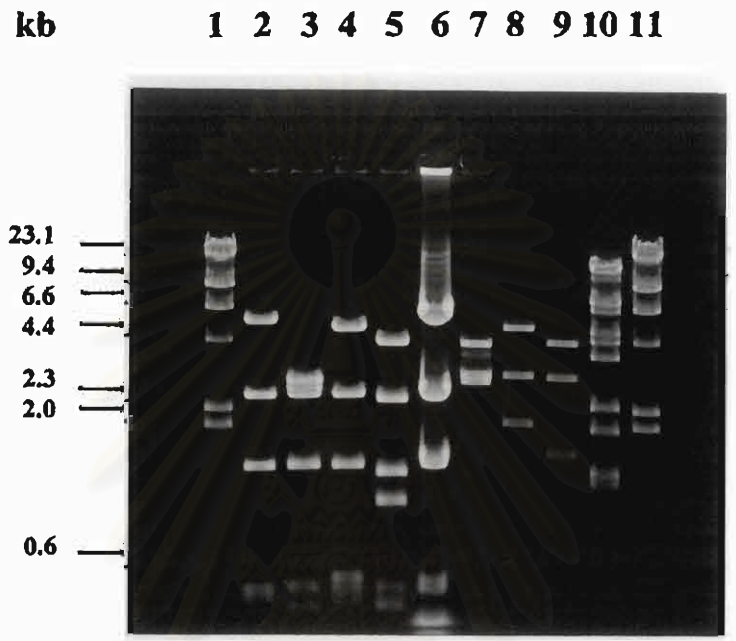
Agarose gel Electrophoresis of single and double digested pKK243B with various restriction enzymes.

Standard DNA markers, λ/*Hind* III and λ/*Bst*E II, were loaded in lane 1 and 13. Lane 2-9 are pKK243B digested with *Bam*H I, *Eco*R I, *Pst* I, *Bgl* II, *Cla* I, *Sal* I, *Hind* III and *Sma* I. Lane 10-12 shows pKK243B double digested with *Bam*H I-*Eco*R I, *Pst* I-*Eco*R I and *Eco*R I-*Bgl* II, respectively.



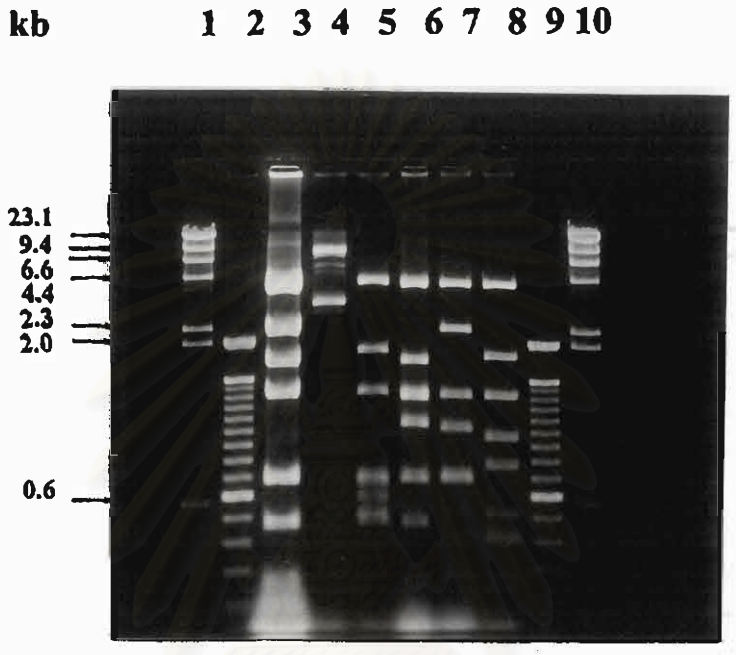
Agarose gel Electrophoresis of single and double digested pKK243B with various restriction enzymes.

Standard DNA marker, λ/*Hind* III, was loaded in lane 1 and 13. λ/*Bst*E II, standard DNA marker, was also loaded in lane 12. Lane 2 and 3 are pKK243B digested with *Kpn* I and *Spe* I. Lane 4-11 shows pKK243B double digested with *Sal* I-*Bam*H I, *Hin*d III-*Bam*H I, *Hin*d III-*Sal* I, *Hin*d III-*Eco*R I, *Eco*R I-*Cla* I, *Hin*d III-*Pst* I, *Cla* I-*Pst* I, and *Nde* I-*Hin*d III, respectively.



Agarose gel Electrophoresis of single and double digested pKK243B with various restriction enzymes.

Standard DNA marker, λ/*Hin*d III, was loaded in lane 1 and 11. λ/*Bst*E II, standard DNA marker, was also loaded in lane 10. Lane 2 is pKK243B digested with *Sal* I. Lane 3-8 shows pKK243B double digested with *Sal* I-*Bam*H I, *Sal* I-*Hin*d III, *Sal* I-*Eco*R I, *Sal* I-*Cla* I, *Cla* I-*BamH* I, and *Cla* I-*Hin*d III, respectively. Lane 9 shows pKK243B digested with *Cla* I-*Hin*d III-*Eco*R I.



Agarose gel Electrophoresis of single and double digested pKK243B with various restriction enzymes.

Standard DNA marker, λ/*Hin*d III, was loaded in lane 1 and 10. Ladder marker (100 bp) was also loaded in lane 2 and 9. Lane 3 and 4 are pKK243B digested with *Pst* I and *Bam*H I, respectively. Lane 5-8 shows pKK243B double digested with *Pst* I-*Eco*R I, *Pst* I-*Hin*d III, *Pst* I-*Cla* I, and *Pst* I-*Sal* I, respectively.

BIOGRAPHY

Miss.Kuakarun Krusong was born on April, 17th (1977) in Bangkok, Thailand. She graduated with a Bachelor of Science Degree in Biochemistry, Faculty of Science, Chulalongkorn University in 1997. She was enrolled in the M.Sc.Biochemistry Program since 1997.