CHAPTER VI

EXPRESSION OF PROTEINASE K IN *DICTYOSTELIUM DISCOIDEUM*

6.1 Introduction: Proteinase K (PK)

(Jany *et al.*, 1986; Gunkel and Gassen, 1989)

Proteinase K is an extracellular endopeptidase which is synthesized by the mold *Tritirachium album* Limber. This fungal proteinase belongs to the class of the serine endopeptidase. The enzyme consists of a single peptide chain containing 277 amino acid residues, corresponding to 28.9 kD. It contains two disulfide bonds and a free cysteine residue. Proteinase K is used as a reagent to digest cellular proteins from genomic DNA preparations.

6.1.1 Gene sequence

(Sequence of PK has been optimized according to *D. discoideum*'s codon preference, Kastein and Dingerman, T., unpublished)

CTGCTCAAACCAATGCTCCATGGGGTTTAGCTCGTATTTCATCAACCTCACCA
GGTACTTCAACCTATTATTATGATGAATCAGCTGGTCAAGGTTCATGTGTTTAT
GTTATTGATACTGGTATTGAAGCTTCACACCCAGAATTCGAAGGTCGTGCTCA
AATGGTTAAAACCTATTATTATTCATCACGTGATGGTAATGGTCACGGAACCCA
CTGTGCTGGTACTGTTGGTTCACGTACCTATGGTGTTGCTAAAAAAACCCAATT
ATTCGGTGTTAAAGTTTTAGATGATAATGGTTCAGGTCAATATTCAACCATTATT
GCTGGTATGGATTTCGTTGCTTCAGATAAAAATAATCGTAATTGTCCAAAAGGT
GTTGTTGCTTCATTATCATTAGGTGGTGGTTATTCATCATCAGTTAATTCAGCT
GCTGCTCGTTTACAATCATCAGGTGTTATGGTTGCTGTTGCTGCTGGTAATAAT
AATGCTGATGCTCGTAATTATTCACCAGCTTCAGAACCATCAGTTTGTACCGTT
GGTGCTTCAGATCGTTATGATCGTCGTTCATCATTCTCAAATTATGGTTCAGTT
TTAGATATTTTCGGTCCAGGTACTTCAATTTTATCAACCTGGATTGGTGGTTCA
ACCCGTTCAATTTCAGGTACTTCAATGGCTACCCCACACGTTGCTGGTTTAGC
TGCTTATTTAATGACCTTAGGTAAAACCACCGCTGCTTCAGCTTGTCGTTATAT
TGCTGATACCGCTAATAAAGGTGATTTATCAAATATTCCATTCGGTACTGTTAA
TTTATTAGCTTATAATAATTATCAAGCTTAA

6.2 Construction of pA15S(1)PK

First step in expression of PK in *D. discoideum* was cloning of PK gene into pA15S(1). cDNA for PK cloned in pERPK^{Dd} (unpublished result) was a gift from Prof. Dingermann. In order to isolate PK cDNA from pERPK^{Dd}, it was PCR amplified **(section 2.3.11)**. Twentymer primers MR06 and MR07 **(section 2.1.2)** were designed for *SacI* site at both ends of PK cDNA. *SacI* site was incorporated in the amplified PK since *SacI* site present in the MCS of pA15S(1) was planned to be used for cloning of PK cDNA.

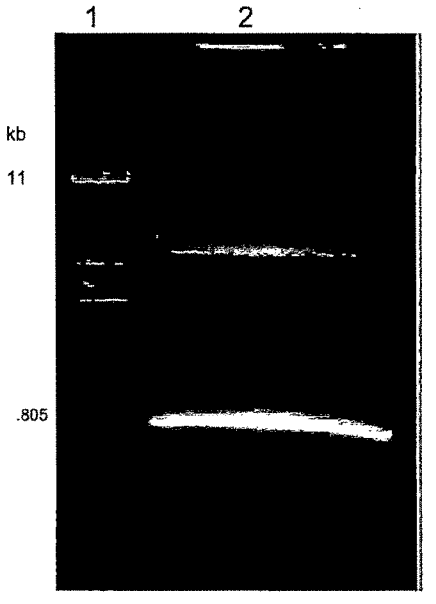
Amplification of PK was confirmed by the presence of 840 bp band on the gel **[Fig. VI(a), lane 2]**. Amplified DNA was eluted from gel, purified using Quagene DNA elution kit and ligated using poly[A] tail of the recovered band and poly[T] region of pGEM-T (Promega) vector. The new vector formed was henceforth named as pMR#9 (3.840kb). *E.coli* cells were transformed with pMR#9 by electroporation method **(section 2.3.1.2)** and blue/white selection of transformants was done on amp-Xgal-IPTG plates **(Section 2.3.12).** Many white colonies were obtained, out of which 12 were screened for the insert by digestion with *SacI*. To check for any point mutation in PK during PCR, PK along with HP1:HP2 was sequenced from pMR#9 **(section 2.3.14)** and showed no mutation (data not shown).

After confirming the sequence of PK cDNA in pMR#9, pMR#9 was digested with *SacI* to release the PK cDNA of 840 bp **[Fig. VI(d), lane 2]**. PK gene was gel eluted and purified using Quagene DNA elution kit. Purified DNA was ligated **(section 2.3.10)** to *SacI* digested and dephosphorylated pA15S(1) **(section 2.3.7) [Fig. VI(d), lane 3]**. The new vector formed was henceforth named as pA15S(1)PK (6.849 kb) **[Fig. VI(b)]**. *E.coli* cells were transformed with pA15S(1)PK by electroporation method **(section 2.3.1.2)**. Since there was no antibiotic selection for the recombinants, colonies obtained on ampicillin plates were replica plated for colony blot assay **(section 2.3.13)**. PK cDNA radiolabelled with ^{32}P by nick translation was used as a probe for detecting recombinants **[Fig. VI(c)]**.

Positive clones containing PK insert in pA15S(1)PK were further confirmed by RE digestion **(section 2.3.5) [Fig. VI(d)]**:

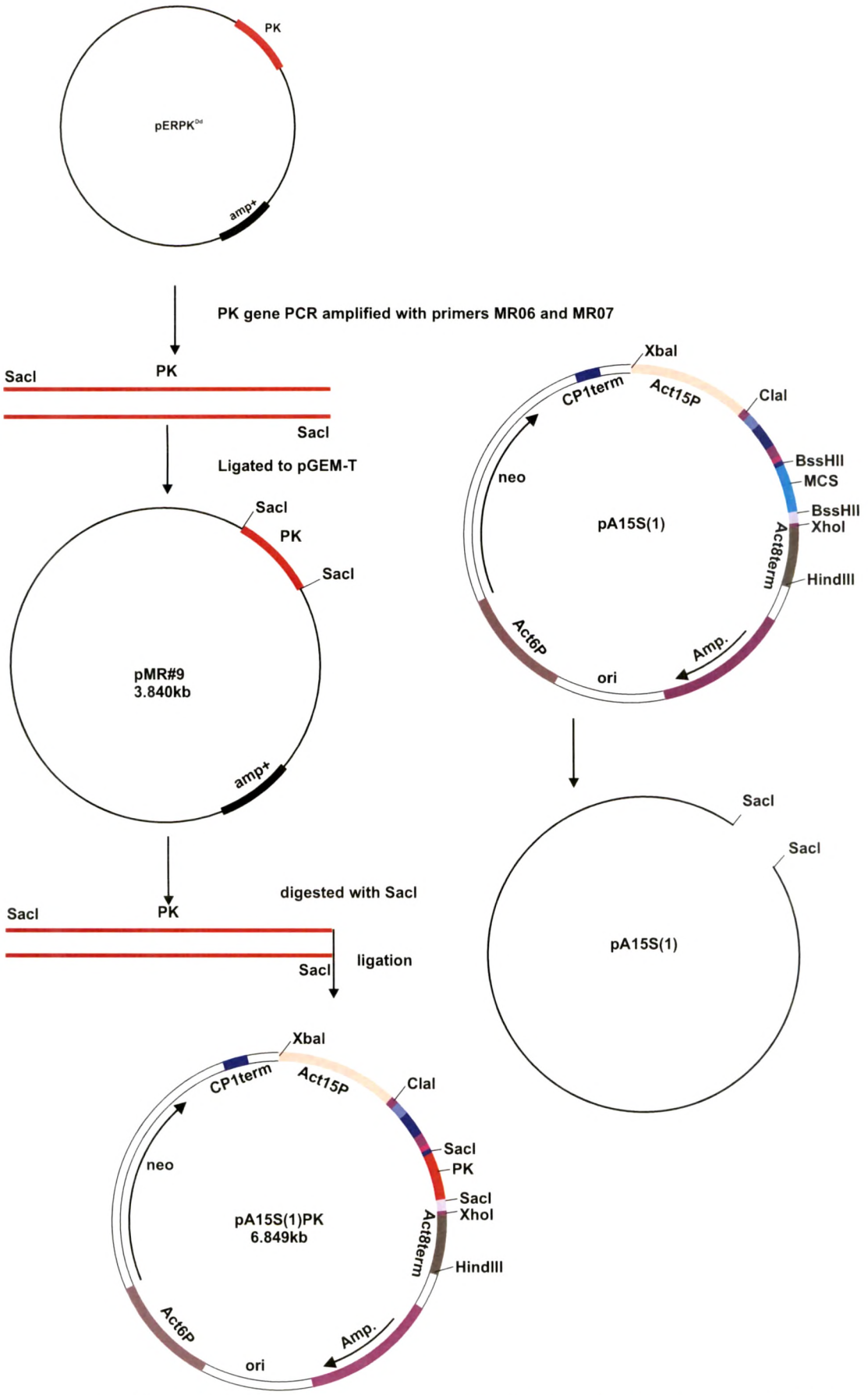
i) *SacI*: *SacI* was used for cloning of PK in pA15S(1)PK. Release of two fragments of 6 kb and 840 bp from pA15S(1)PK on digestion with *SacI* confirmed the presence of PK in pA15S(1)PK **[Fig. VI(d), lane 4]**.

ii) *XbaI-EcoRI*: *EcoRI* site is present in the PK sequence and *XbaI* site is present in the pA15S(1) backbone. Release of lower fragment of 630 bp from pA15S(1)PK on digestion with *XbaI-EcoRI* confirmed the correct orientation of insert in pA15S(1)PK **[Fig. VI(d), lane 5]**.

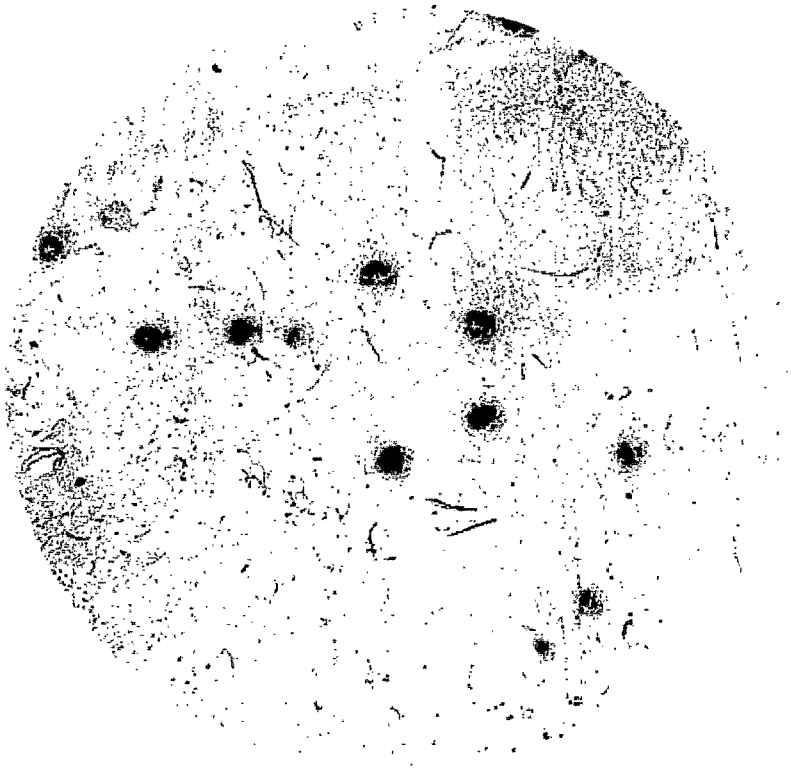


6.2 Fig. VI (a) - PCR amplified PK gene from pERPK^{Dd} on 0.8% agarose gel

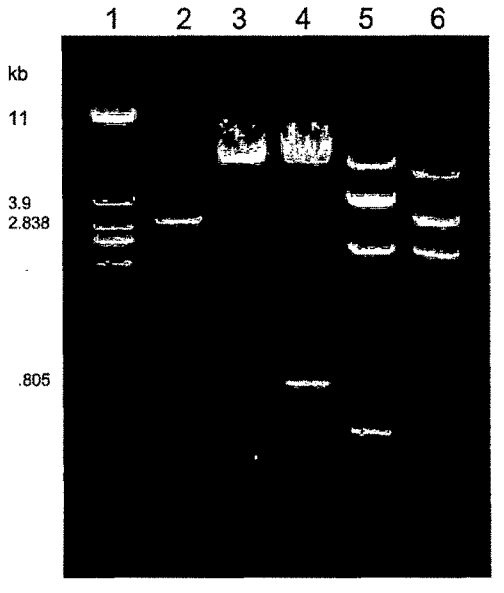
Lane 1: lambda DNA digested with *PstI*
Lane 2: PCR amplified PK from pERPK^{Dd}



6.2 Fig. VI(b) - Schematic summary of construction of pA15S(1)PK



6.2 Fig. VI(c) - Colony blot of *E.coli* cells transformed with pA15S(1)PK. PK gene radiolabelled with ^{32}P was used as a probe for detecting recombinant colonies.



6.2 Fig. VI(d) - Construction and RE digestion pattern of pA15S(1)PK on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pMR#9 digested with *SacI*
Lane 3: pA15S(1) digested with *SacI*
Lane 4: pA15S(1)PK digested with *SacI*
Lane 5: pA15S(1)PK digested with *XbaI-EcoRI*
Lane 6: pA15S(1) digested with *XbaI-EcoRI*

To further confirm the PK sequence and its reading frame in pA15S(1)PK, PK along with HP1:HP2 was sequenced **(section 2.3.14)** from pA15S(1)PK using primer MR01 **(section 2.1.2)**. The PK sequence showed no mutation and was in proper reading frame **[Fig. VI(e)]**.

6.3 Transformation of *D. discoideum* (Ax-2) cells with pA15S(1)PK

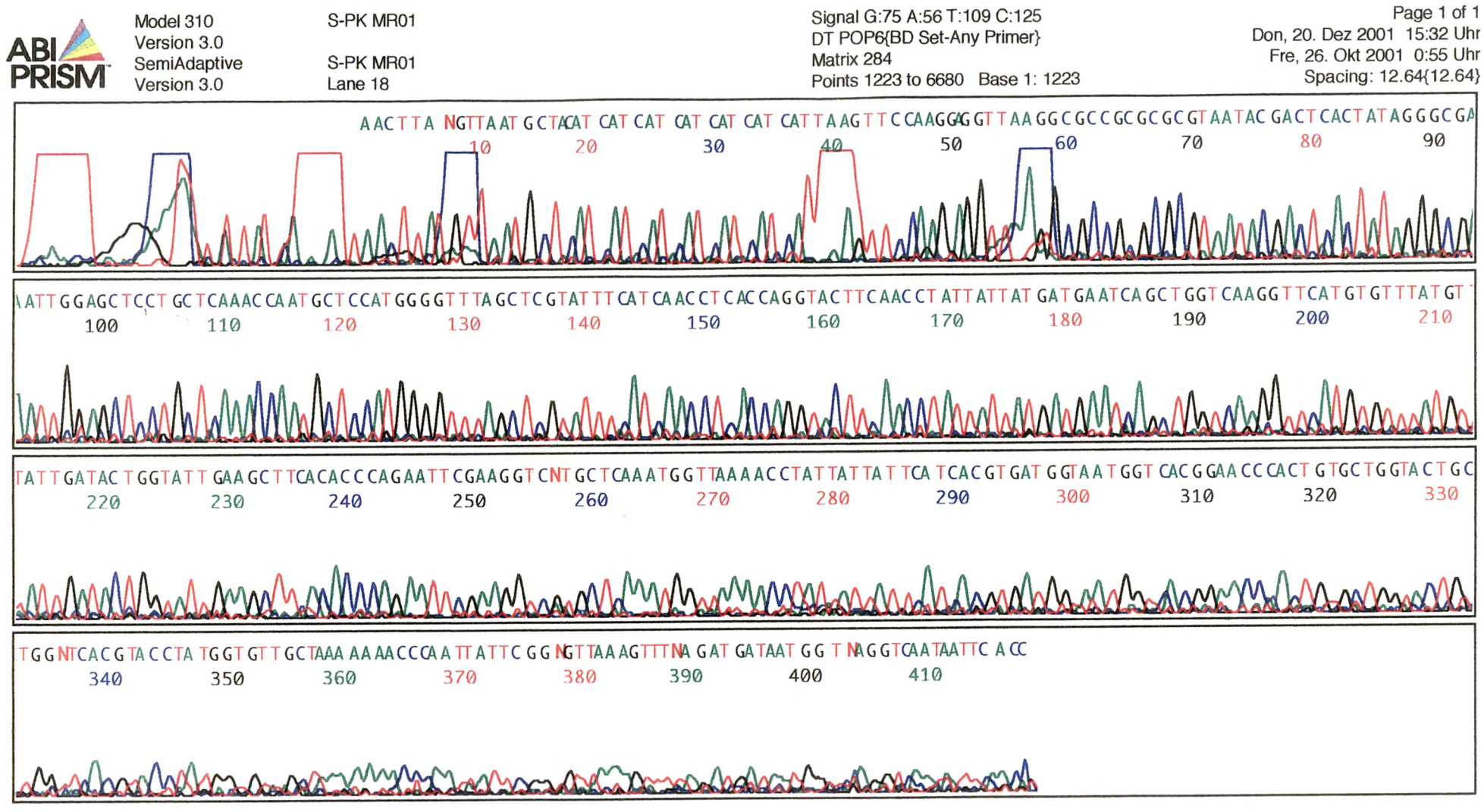
Cells of Ax-2 strain of *D. discoideum* in log phase (2 X 10^6/ml, 10 ml per plate) were transformed with pA15S(1)PK (7-8 μg) by $CaPO_4$ method **(Section 2.4)** along with proper controls. pA15GFP transformed cells were used as positive control and untransformed cells as negative control for antibiotic resistance. Transformants growing in media containing G418 at 10 μg/ml concentration were selected and subsequently maintained at 5 μg per ml. Clones were observed after 10-15 days and positive clones growing on plates containing G418 were transferred to 24 well plates for further growth and observation.

6.3.1 Morphology of cells transformed with pA15S(1)PK

Initially many small clones were observed but after some time most of them disappeared and few grew. As compared to positive control (pA15GFP), few transformants (2-10 clones/plate) were obtained with pA15S(1)PK. In 24 well plates, cells grew till 10^5/ml density and then growth was arrested. Under microscope transformants showed altered phenotype as compared to the positive control as well as untransformed cells. The cells were able to grow to a density of ~10^5 /ml and then they started getting detached from the plate, finally becoming round like beads - characteristic of dying cells **[Fig. VI(f)]**. Cell death indirectly may indicate the lethal effect of PK expression.

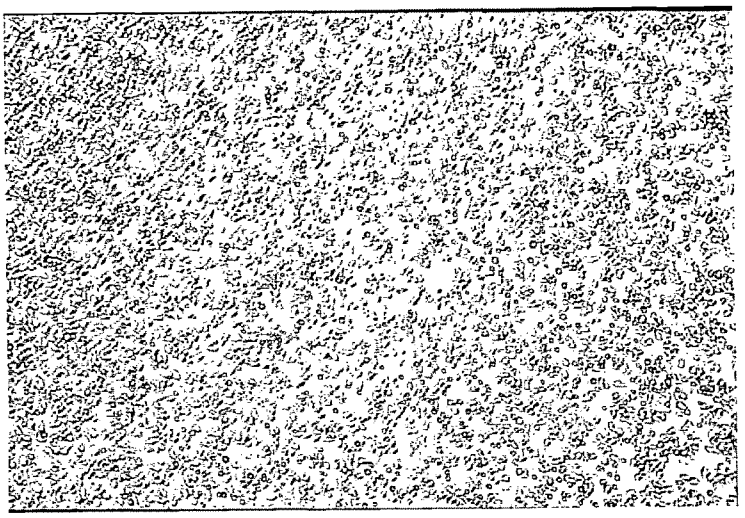
6.3.2 Drop test for viability and impaired development

To check for the altered growth pattern and morphology, drop test was done with the transformants along with control. 10^4 cells were dropped on *Klebsiella* lawn and grown at 21°C for four days **(Section 2.5.6)**. Transformants showed delayed and less clearance of *Klebsiella* lawn lacking aggregation and fruiting bodies as compared to control **[Fig.V (g)]**.



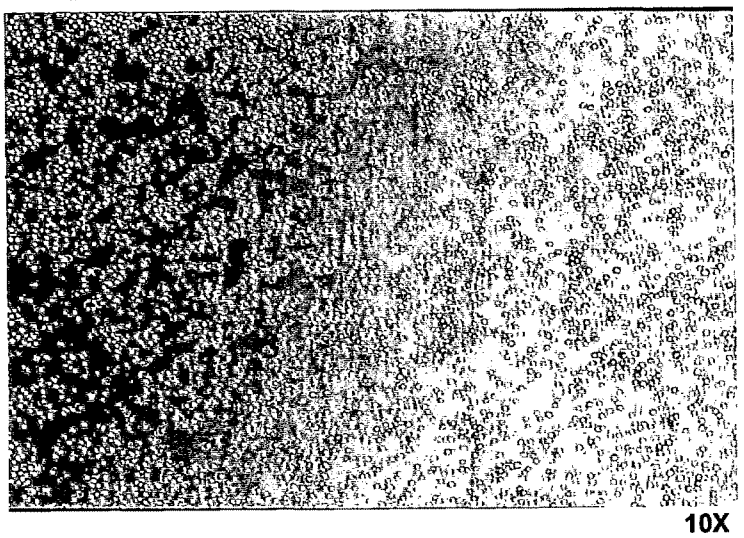
6.2 Fig. VI(e) - Sequence of pA15S(1)PK : In pA15S(1)PK, PK gene has been cloned in the *SacI* site of pA15S(1) and was found to be in frame with the secretory sequence (104 onwards).

AX2 Cells



10X

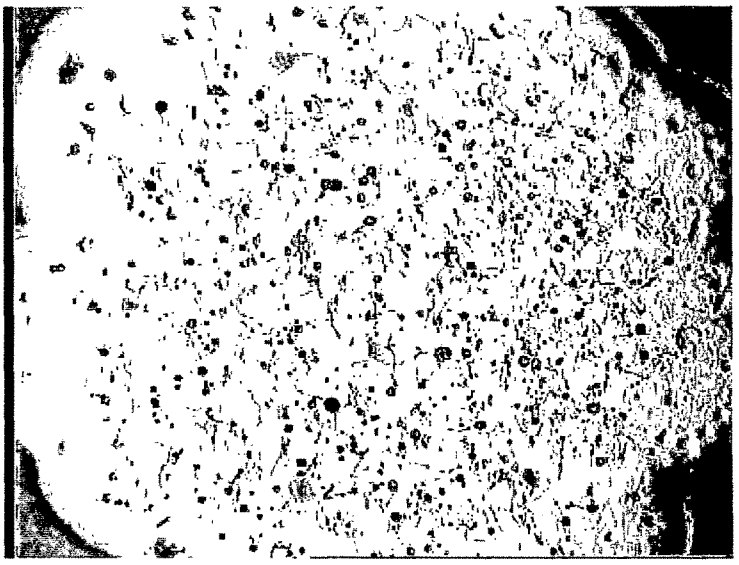
AX2 [A15S(1)PK] Cells



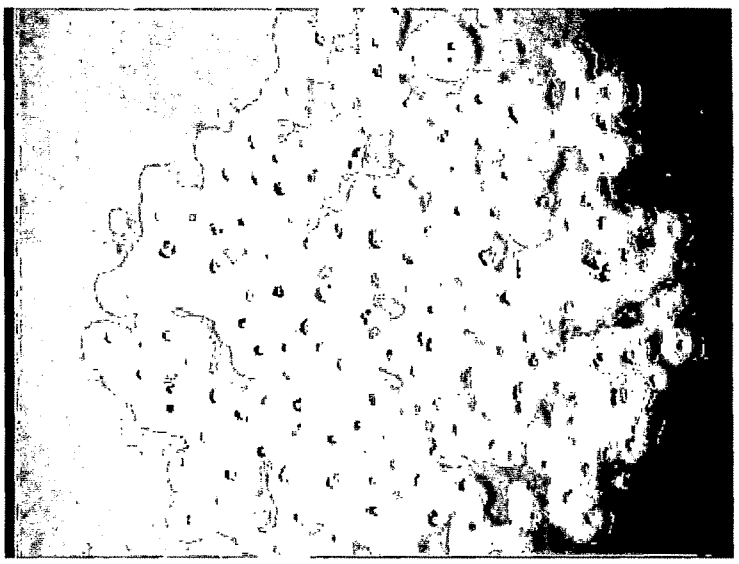
10X

6.3 Fig. VI(f) - Morphology of Ax2 cells transformed with pA15S(1)PK

AX2 Cells



AX2 [A15S(1)PK] Cells



6.3.2 Fig. VI(g) - Drop test for viability and impaired development of Ax2 cells transformed with pA15S(1)PK

6.3.3 Trypan blue staining for viability

Trypan blue is a vital dye used to distinguish living cells from dead cells. PK transformants as well as control were mixed with Trypan blue solution (0.4% stock) in 1:1 ratio and observed under the microscope. Cell death due to PK expression was reconfirmed by majority of transformants taking up the stain and hence appearing blue under the microscope **[Fig. VI(h)]**.

6.3.4 SDS-PAGE of cells transformed with pA15S(1)PK

Due to insufficient number of cells in each clone (10^5 instead of 10^6), it was not possible to do proteomics of each clone individually. Therefore several clones were pooled up for the sample preparation. 100 μg of protein/ well was loaded on 10% PAGE and the gel was run and stained with Coomassie blue **(Section 2.5.7)**. Transformants showed endogenous cellular protein degradation suggesting Proteinase K expression and its activity, but no major band at 28.9 kD corresponding to PK was observed which might be due to the difference in the size of the expressed protein undergoing post-translational modifications **[Fig. VI(i)]**.

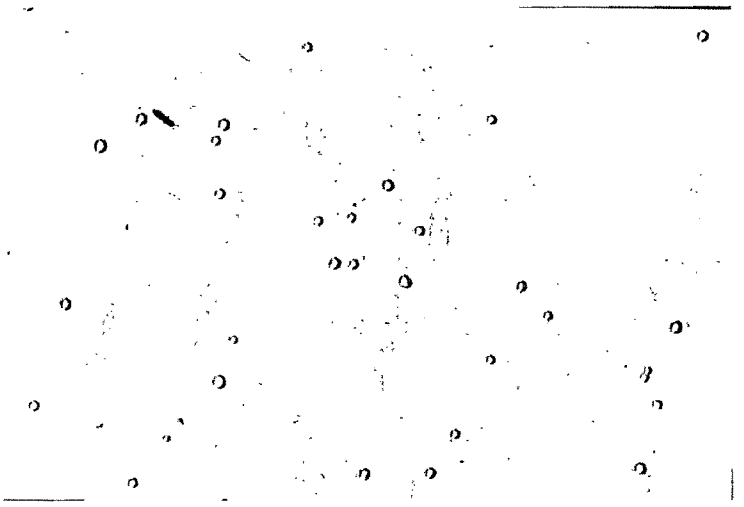
6.3.5 Detection of PK by Western blotting

Since no distinct polypeptide corresponding to 28.9 kD appeared in AX2 (A15S(1)PK) SDS-PAGE, Western blotting was done with antibodies developed against PK **(Section 2.5.8)**. Commercially available PK was used as positive control. No immunoreactive band was observed at 28.9 kD in transformed cells on western blot **[Fig. VI(j)]**. This indicates that either PK is not a major protein in transformants or it is quickly degraded.

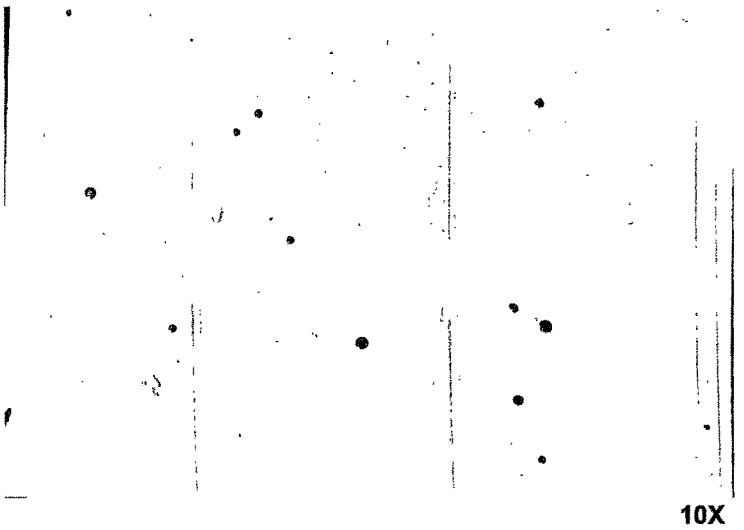
6.5 Disscussion

Proteinase K was used as an illustrative gene to emphasis the versatility of *D. discoideum* as a host for expression of recombinant proteins since it contains di-sulfide linkages (two) and is also a commercially important molecule used as a reagent to digest cellular proteins from genomic DNA preparations. While choosing PK as a test protein, toxicity i.e. endogenous protein degradation due to its expression was considered not to be a hindrance since the gene was cloned in a

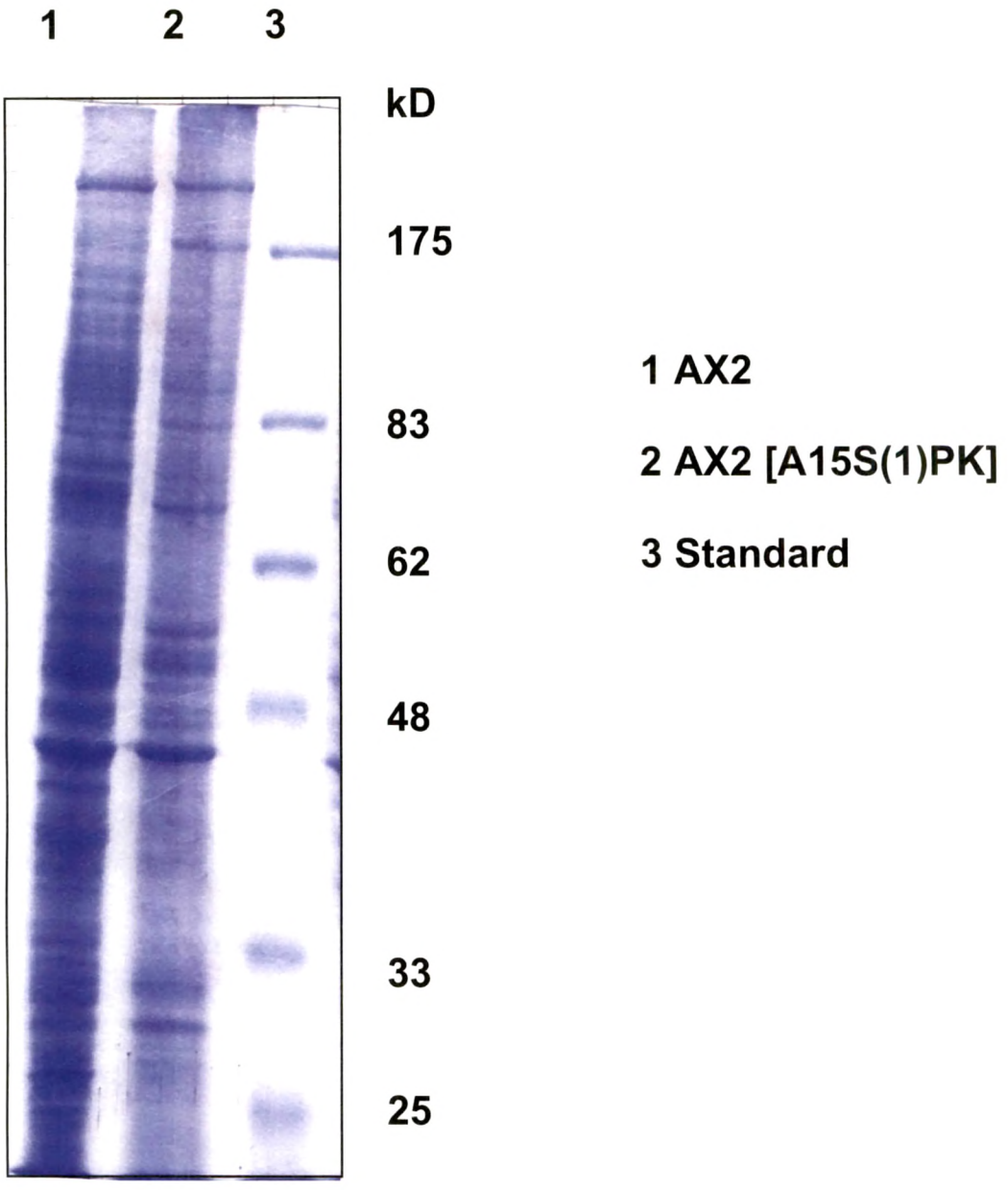
AX2 Cells



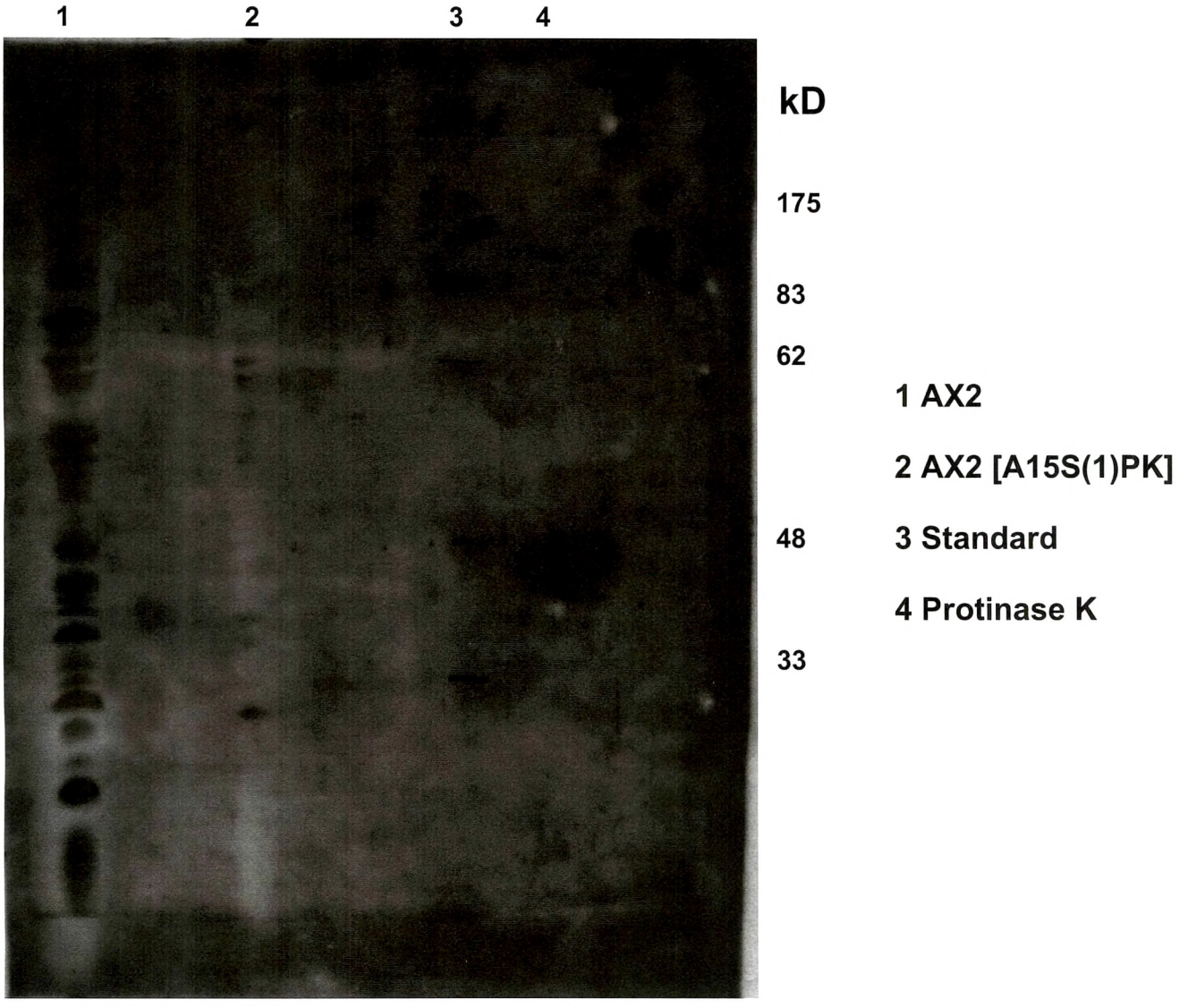
AX2 [A15S(1)PK] Cells



6.3.3 Fig. VI(h) - Trypan blue staining for viability of Ax2 cells transformed with pA15S(1)PK



6.3.4 Fig. VI(i) - 10% SDS-PAGE of Ax2 cells transformed with pA15S(1)PK



6.3.5 Fig. VI(j) - Western blotting of PK expressed in *D. discoideum*

secretory vector [pA15S(1)] having *D. discoideum* secretory signal sequence (PsA signal sequence), which directs the active protein outside the cell, into the medium.

Change in phenotype (round and smaller cells) indicates cell death of the pA15S(1)PK transformants. The phenotypic changes were because of the expression of PK and not due to any other factor involved in transformation was confirmed by taking pA15GFP transformed cells as a control which appeared healthy and grew at rates comparable to AX2. Cell death was confirmed by Trypan blue staining.

Transformants showed endogenous cellular protein degradation on SDS-PAGE (confirmed by Coomassie staining) suggesting Proteinase K expression and its activity and cellular protein degradation might be leading to cell death of the transformants. Although DNase I transformants and PK transformants showed similar morphology (round and smaller cells) and finally reduced viability, SDS-PAGE protein profile of the two were different- DNase I transformants showed few alterations as compared to untransformed cells i.e. appearance of new bands with disappearance of others whereas PK transformants showed endogenous cellular protein degradation. This indicates that cell death in each case is caused due to the activity of their respective expressed proteins having different biological activity.

Death of transformants indicated that the expressed protein was not being secreted out. Cellular retention of PK could be due to different signal used in fungal cells to control secretion. No further confirmation of PK activity in media/cell was possible due to death of transformants at an early stage of growth. No immunoreactive band was observed at 28.9 kD in transformed cells on Western blot. This indicates that either PK is not a major protein in transformants or it is quickly degraded. However, it is not clear whether the low amount of expressed protein was due to its low expression level or caused by its own proteolytic activity.

Expression of PK as indicated by death of pA15S(1)PK transformants suggests that *D. discoideum* is capable of making active recombinant protein. Activity of the expressed PK in Ax2 [pA15S(1)PK] has been further confirmed by endogenous cellular protein degradation on SDS-PAGE as compared to the

nontransformant. Cellular retention of this protein could be due to an inappropriate signal used in fungal cells to control secretion. Biologically active protein indicates that it had been processed through part of the secretory pathway.