Chapter 4

RESULTS AND DISCUSSION OF PRODUCTION OF RECOMBINANT MITE TROPOMYOSIN IN *Escherichia coli* AND *Pichia pastoris*

4.1 Amplification of Der p 10 cDNA

As described in **section 2.6.1,** a standard 25 µl PCR reagents were prepared together with 50-100 ng of DNA template, mixed with 50 pmol of upstream primer (P1: 5' GG GAT CCA TGG AGG CCA TCA AAA AG 3') which contained a *Bam*HI site, and 50 pmol of downstream primer (P2: 5' GCT CTA GAT TAA TAA CCA GTA AGT TC 3') containing an *Xba*I site (both sites underlined). The cDNA of Der p 10 was first extracted and amplified from pUC19-Der p 10, by PCR and the product was as shown in **Fig. 4.1**.

4.2 Restriction enzyme digestion

The PCR product containing Der p 10 DNA was digested with *Bam*HI to linearise the plasmid and another reaction was prepared for the vector (plasmid pGEX-4T-1); digested with *Sma*I followed by *Bam*HI.

Fig. 4.2 shows the linearized vector pGEX-4T-1 after complete digestion with *Sma*I followed by *Bam*HI. The size of the plasmid was determined to be 4.95kb (Lane 3). *Bam*HI-digested Der p 10 DNA showed a size of ~870 bp. (Lane 2).

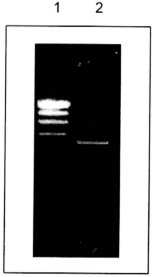


Fig. 4.1: Ethidium bromide-stained agarose gel 1% (w/v) of the eluted fragment of Der p 10 cDNA; extracted from pUC19-Der p 10.
Lane 1: DNA standard marker: 100 bp DNA ladder
2: Der p 10 cDNA after PCR (size : ~870 bp)

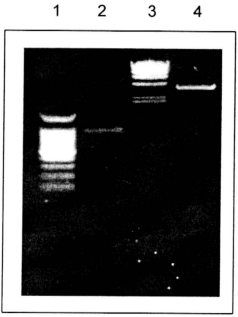


Fig. 4.2: Ethidium bromide-stained agarose gel 1% (w/v) of the insert (Der p 10) and vector pGEX-4T-1 after complete digestion with *Sma*I followed by *Bam*HI.

Lane 1 : DNA standard marker: 100 bp DNA ladder
2 : Der p 10 cDNA after PCR (~870 bp)
3 : λHindIII DNA standard marker
4 : Linearised pGEX-4T-1 vector plasmid (~4.95kb)

4.3 Restriction enzyme analysis of the pGEX-Der p 10 construct

Restriction enzymes analysis of a construct after a ligation and transformation into the host system is crucial in determining the correct insertion of the DNA into the vector plasmid. The analysis should also be able to show that the insertion of Der p 10 DNA had occurred at the sites and most importantly, at the appropriate orientation within the vector plasmid, pGEX-4T-1. **Fig. 4.3** is the combined effort of examining the purified product of the construct after a successful transformation, together with the products of the restriction enzyme analysis carried out.

Lane 2 of the gel contained the plasmid DNA of pGEX-Der p 10 extracted from the purification of the lysate of bacterial culture grown overnight (37°C) in 6 ml of LB+Amp. Thus, this plasmid was the combination of the insert (853 bp) and the vector (4940 bp) resulting in a DNA fusion product of 5793 bp i.e. ~ 5.8 kb.

The band with the size of ~700bp in lane 3; containing the digested product of the construct by *Eco*RI and *Bam*HI shows that the digestion had succeeded in cleaving the plasmid (construct) at the appropriate sites, thereby justifying that the fusion between the insert and vector had taken place. This analysis was also able to confirm the correct orientation of the insert.

4.4 Expression and characterisation of recombinant Der p 10 in *E. coli*

The cDNA cloned into the expression vector pGEX-4T-1 (Pharmacia) was transformed into expressing cells *E. coli* (DH5α). Transformant carrying the pGEX-Der p 10 construct was verified from the analysis of the expression of the minipreps. SDS-PAGE analysis of the 8 clones (**Fig. 4.4**) above showed that 80-90% of the eluted protein consisted of GST-Der p 10. The proteins in lane 2 compared to the rest of the lanes (3-10)

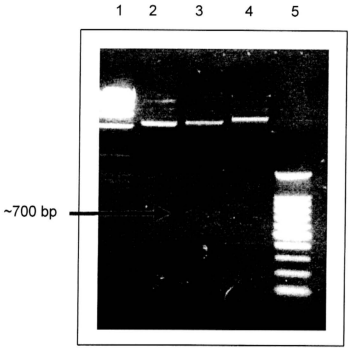


Fig. 4.3 : Ethidium bromide-stained agarose gel 1% (w/v) of the insert (Der p 10) and vector pGEX-4T-1 after digestion with *Sma*I followed by *Bam*HI.

Lane 1 : λHindIII DNA standard marker
2 : pGEX-Der p 10 construct
3 : Construct after double digested with *Eco*RI and *Bam*HI
4 : Construct after digestion with only *Bam*HI
5 : DNA standard marker: 100 bp DNA ladder

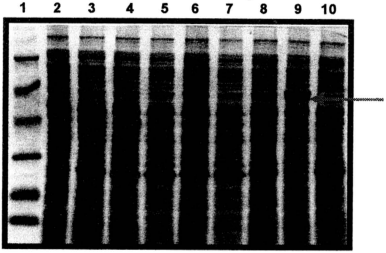


Fig. 4.4: SDS-PAGE profile of the minipreps of 8 transformants.

Lane 1: Molecular weight marker
2: Uninduced culture
3 to 10: Pre-purification lysate of 8 of the 13 cultures.

[N.B. Lane No. 9 contained lysate of clone No. 7 containing pGEX-Dp10]

in the analysis also showed that significant expression was achieved after induction by IPTG. Clone No. 7 in lane 9 showed the successful expression of the GST-Der p 10 fusion protein with the molecular weight of 58 kDa, being the fusion of Der p 10 (32 kDa) to GST (26 kDa). With only two out of 13 clones found to be the transformant carrying the putative pGEX-Der p 10 construct, the expression is considered to be low that is of about 15% efficiency only.

For this study, by using the above procedure, a yield of 0.1 mg/ml of fusion protein (GST-Der p 10) was obtained; as determined with the Bradford protein assay. SDS-separation demonstrated that the allergens were successfully purified (**Fig. 4.5**). Thrombin enables the site-specific cleavage of fusion protein containing an accessible thrombin recogniton sequence. The optimal cleavage conditions must be determined for different fusion proteins. For this purpose, 20 μg of GST-Der p 10 was incubated with 10 units of thrombin (thrombin : fusion protein ratio of 1:10) or 20 ng of thrombin at 4°C for 18 hours, on a shaker; leading to the release of Der p 10 from the fusion protein. In this study thrombin was able to initiate site-specific separation of GST affinity tag from the ~62 kDa GST-Der p 10; producing the 36 kD Der p 10 and the 26 kDa GST protein as shown lane 2 of in **Fig. 4.5** and **Fig. 4.6.**

4.5 Verification of GST-Der p 10

The verification of the expressed fusion protein, GST-Der p 10, was done upon recognition of the right size achieved through SDS-PAGE. The SDS-PAGE profile **(Fig. 4.7)** shows the product of induction by IPTG (in Lane 3) on the bacterial cell culture compared to that before induction (in Lane 2).

As the recombinant Der p 10 was produced in fusion with GST, the appropriate way to verify its presence is by means of immunoblotting assay using the anti-GST antibody to bind to it. An indirect method of verification is to challenge the adsorbed protein on the membrane with an antiserum that contains antibody against the epitope of tag signal GST. In this assay, the mouse anti-GST monoclonal antibody (Pierce, USA) was used at 1:1000 and subsequently, the alkaline phosphatase conjugated mouse anti-IgG antibody (Kirkegaard & Perry Lab., USA), serving as the secondary antibody was used at 1:3000 as recommended. Finally with BCIP/NBT, the GST-fusion was visualised as in **Fig. 4.8.** As shown in lane 3, the total protein of transformed bacteria contained the putative GST-Der p 10 protein with the right size of ~62 kDa, constituting the fusion of Der p 10 (36 kDa) and the GST portion (26 kDa). Upon centrifugation, it was found that the supernatant (lane 4) contained a small portion of the protein while the pellet (lane 5) retained a large amount of the fusion protein thus indicating that the expressed protein had accumulated in inclusion bodies in the bacteria cells and therefore appeared in the pellet. Upon sonication of the pelleted cells and subsequent purification, the collected protein was visualised through immunoblotting, showing the presence of purified GST-Der p 10.

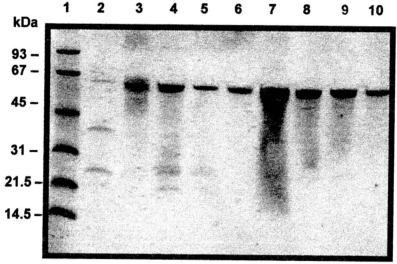


Fig. 4.5: SDS-PAGE analysis of eluted GST-Der p 10 and thrombin-cleaved protein as visualized by Coomassie staining. Eluted fusion protein had an apparent molecular weight of ~62 kDa. This SDS-PAGE analysis also indicated that the fusion protein represented 80-90% of the eluted protein.

Lane 1: Molecular weight marker
2: Thrombin-cleaved GST-Der p 10 protein
3-10: Uncleaved lysate samples

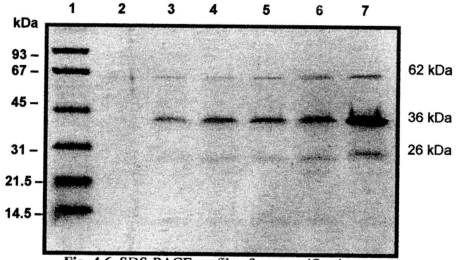


Fig. 4.6: SDS-PAGE profile of post-purification supernatant of culture proteins extracted from the lysed *E. coli* cells, after thrombin cleavage.

Lane 1: Molecular weight marker
3-7: Flowthrough samples after thrombin cleavage

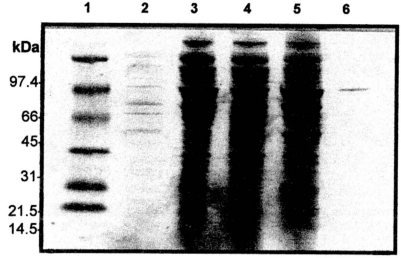


Fig. 4.7: SDS-PAGE profile of the collected proteins extracted from the *E. coli* cells.
Lane 1 : Marker
2 : Uninduced culture
3 : Pre-purification supernatant of culture
4 : Post-purification supernatant of culture
5 : Pellet after sonication
6 : Sample of eluate

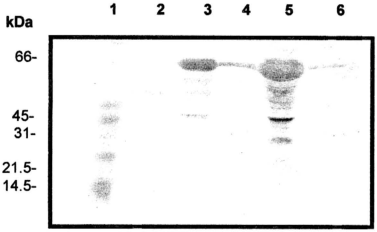


Fig. 4.8: Immunoblot of fusion proteins from bacterial culture on PVDF membrane (10μg/well) as captured by alkaline phosphatase conjugated anti-GST antiserum as visualised by BCIP/NBT
Lane 1: Molecular weight marker
2: Bacterial total protein as control
3: Transformed bacterial total protein
4: Supernatant of lysed culture
5: Pellet of lysed culture
6: Purified GST-Der p 10

As part of verifying the right expression of rDer p 10, the construct pGEX-Der p 10 was sequenced prior to the transformation into bacterial cells for expression. Sequencing of the construct by the ABI systems was carried out by another lab (Operon, USA) and results (**Appendix 3**) were then further analysed for homology through the method of alignment using the NCBI websites. The nucleic acid sequence was then examined by BLASTn and converted into amino acid sequence, and further analysed by BLASTp alignment study to check for sequence similarity to be used for subsequent homology studies. The calculated molecular mass based on the amino acid residues was ~34-36 kDa; the appropriate size for most tropomyosin proteins. Therefore this cloned and expressed protein in this study is verified as the recombinant protein representing the allergen from the group 10 of the house dust mite, *D. pteronyssinus*, i.e. the rDer p 10.

4.6 Purification and quantification of GST-Der p 10

Purification of the GST-Der p 10 was facilitated by using a column containing glutathione Sepharose 4B beads and the elution buffer used was at pH 8.0. High efficiency of elution was observed, as recombinant protein was totally eluted in the second and third vial of the eluates collected (**Fig. 4.9**).

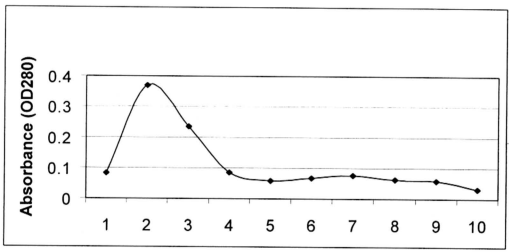


Fig. 4.9 : The elution profile of the GST-Der p 10 eluates (#1-10) after purification through the affinity chromatography method.

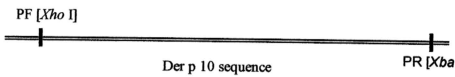
These purified protein in vials 2 and 3 were pooled and quantified by using Bradford reagents as described by Bradford (1976) using bovine serum albumin (BSA) as a standard. With the absorbance at 280 nm, subsequent calculation showed that the maximum protein collected was 1.0 mg/ml (vial #2) and 0.54 mg/ml (vial #3).

4.7 Cloning of Der p 10 in *P. pastoris*

The main cloning methods in this part of the study have been carried out according to protocols described in the manual of the EasySelect *Pichia* expression kit. The Der p 10 cDNA (size of 870bp) was obtained from Dr Thomas W.R. (Australia) and amplified by PCR using a forward primer (5'-TCC GAG AAT TCA TGG AGG CCA TCA A-3') and a reverse primer (5'- GAA TTC TAG ACG TGC TTC AGC TTC TTT-3') with an *Eco*R I and an *Xba* I restriction sites respectively, to facilitate the cloning. The PCR-amplified

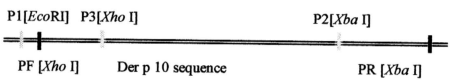
fragment was then digested by appropriate restriction enzymes before being cloned into pPICZB to form the pPICZ.Der p 10 construct. Two attempts of cloning Der p 10 in *P. pastoris* were carried out.

1st attempt:



In the first attempt whereby primers PF and PR were used; only one out of seven clones was found to have the right construct, verified through PCR. However, this clone was considered as 'tentative' due to the low efficiency (14%) of this cloning procedure. Nevertheless, the construct pPICZαA.Dp10 was later transformed into yeast with the other constructs in subsequent steps.

2nd attempt:



In the second attempt, a set of 3 new primers were designed; P1[*Eco*RI], P2[*Xba* I] and P3[*Xho* I] with their respective restriction sites in the parenthesis; thereby creating several fragments on the Der p 10 sequence. Primer PR was used again; resulting in 4 fragments of truncated Dp10 (hereafter will be referred to as the 'Z' fragments). Therefore with the Dp 10 plasmid, and 4 primers; 4 different fragments (**Fig. 4.10**) were created and amplified by PCR which were;

Fragment	Restriction sites
Z1	P1 – P2
Z2	P3 – R
Z3	P3 – P2
Z4	P1- R

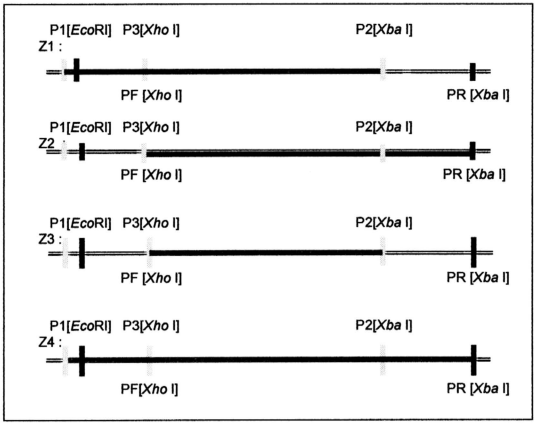


Fig. 4.10: The Der p 10 DNA fragments in a diagrammatic depiction; derivatives from the sequence of the Der p 10 DNA. Primers were designed at the inherent cloning sites based on both the Der p 10 sequence, serving as inserts.

Ligation was subsequently performed using these DNA as inserts and the pPICZ B (which has a *Xba* 1 site preceding the *myc* epitope tag) as vector. The ligated DNA was then transformed into Top 10F' *E. coli* competent cells and was plated out, and incubated at 37°C overnight. Bacterial colonies could be observed on the following day and a few clones were selected and analysed with 15 µl screening mixture.

Clones from Z1 and Z4 plates showed presence of construct when screened but all Z2 and Z3 clones were negative and therefore considered not having the gene of interest. Subsequently, four clones from the Z1 plate and only two clones from Z4 plate were chosen for further cloning steps. The PCR products from the previous screening were pooled up, and designated new references as shown in **Table 4.1.**

Table 4.1: PCR products and their designation from previous screening

Plate	Clones chosen	Pooled and given new designation	Remarks
Z1	Z1-1	Zd	All plasmids were pooled into Zd ['d' for the truncated Dp 10 sequence]. The actual construct designation is: pPICZ.dp10
	Z1-2		
	Z1-3		
	Z1-4		
Z4	Z4 -1	ZD	ZD signifies the full length DNA of Dp 10 i.e. pPICZ.Dp10
	Z4 –2		

So with Zd, ZD and ZαD (brought in from the 1st attempt), transformation into yeast competent cell host strain (X-33), by electroporation as described in **section 2.7.2** and the pulsed cells were then plated out on YPG (yeast + peptone + glycerol) + Zeocin™ agar plates. The initial use of YPD was substituted with YPG because the yeast cells no longer needed the dextrose (glucose) and instead the absence of dextrose was able to enhance expression, as energy would not be used up for the digestion. Incubation was carried out at RT, and yeast colonies appeared only after 2 or 3 days, unlike *E. coli* cells. Only a single colony appeared on ZD plate; 8 colonies appeared on Zd plate while ZαD showed a confluent plate. These colonies were picked; the single colony from ZD, 8 colonies from Zd and another 8 colonies from the ZαD plate. PCR screening using primers P1 (*Eco*RI) and P2 (*Xba* I) found the ZD clone to be negative, while from the Zd plate, only 2 clones were found to be positive. From the ZαD plate, three clones were positive.

4.8 Analysis of transformants

These plasmids were then screened by PCR to verify the integration of Der p 10 gene into yeast genomic DNA. **Fig. 4.11** shows the electrophoresis profile of the investigation. Positive colonies were selected and expressed in nutrient-rich buffered YP culture medium with 0.5% (v/v) methanol to induce expression.

4.9 Characterisation of *Pichia*- expressed rDer p 10

4.9.1 Kinetics of Der p 10 production

Overnight cultures of were diluted 1:10 and grown for 1 hour at 37°C. Expression of the recombinant protein was induced by 0.5% (v/v) of MeOH and 1 m of the culture was sampled at the time intervals as indicated at the top of the gel diagram. Samples taken at these varying intervals were pelleted and resuspended in 300 µl of SDS sample buffer, boiled for 5 minutes and then loaded 15 µl/well in a 12.5% SDS-PAGE gel, followed by Western blotting and probed with anti-Cmyc antibody. A time-frame profile was obtained as shown here in **Fig. 4.12**. Expression of recombinant Der p 10 in *P. pastoris* yeast was found to occur significantly 24 hours after induction, increasing to an optimum level at 96 hours. The concentration of the His-tag affinity purified recombinant Der p 10, harvested after 96 hours was 0.54 mg/ml, determined by using the Bio-Rad Bradford assay with bovine serum albumin as standard.

4.9.2 Analysis of expressed protein

Investigation on the expression of the *P. pastoris* cells, whether they carried the putative clones of Der p 10 was carried out by looking at the Western blot profile of proteins derived from the cells. Yeast samples containing plasmid pPICZB insert only; Der p 10 inserts; and proteins from the supernatant and pellet of selected yeast cells were run of the 12% SDS-PAGE electrophoresis and then transferred onto the PVDF membrane. The profile of proteins on the membrane was then visualised by anti-Cmyc antiserum, stained

with NBT/BCIP as shown in **Fig. 4.13**. This direct method of detection of the protein of interest served better and showed higher specificity as the putative protein was easily recognised with the help of the secondary binding to the anti-Cmyc antiserum. As seen from the profile, all three clones with the Der p 10 insert showed the presence of Cmyc, the fusion partner intended for this expression. Both supernatant and pellet showed the presence of the protein, but because this system could not direct protein secretion from the yeast cells (the absence of the α factor) most of the proteins were still trapped in the cells (therefore in the pellet) and needed thorough break-up of the cells for protein harvesting.

Finally the fusion protein was purified on metal-chelating resin i.e. Probond™ (Invitrogen). Elution occurred efficiently at pH 4.0 yielding the recombinant. Samples were collected consecutively in Eppendorf vials and later analysed through anti-Cmyc challenge, visualised by NBT/BCIP for IgG binding on the PVDF membrane (**Fig. 4.14**).

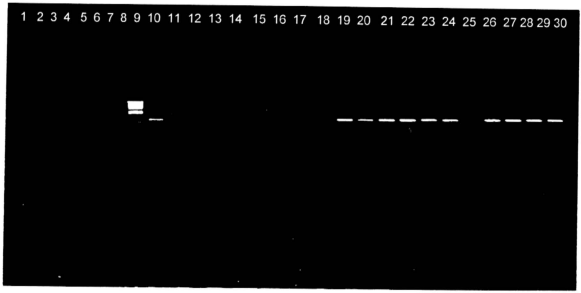


Fig. 4.11: Ethidium bromide-stained 1% (w/v) agarose gel of DNA bands from the PCR screening of colonies to examine and thus detect the correct construct in the transformed yeast cells. Detection was based on the correct size of the construct.

Lane 1-8: clones from Zd plate
9: 100 bp MW marker
10-16: clones from ZαD plate
17-30: clones from ZαD initial plate

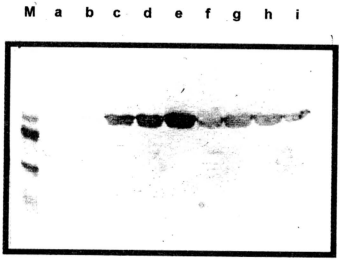


Fig. 4.12: Time-course analysis of Der p 10 expression expressed by *P. pastoris* yeast cells using pPICZB vector.
[M- molecular weight marker, a-0 hr, b-24hr,c-36hr, d-48hr, e-96hr, f- 108hr, g-120hr, h-132hr, i-144hr]

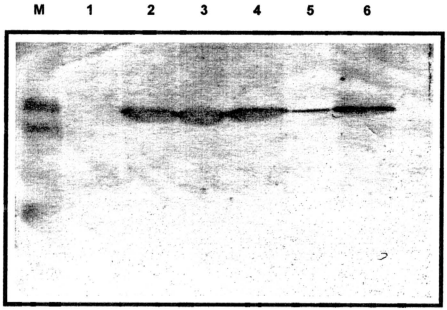


Fig. 4.13: Western blot profile of the Der p 10 expression by *P. pastoris* as visualised by anti-Cmyc antiserum, stained with NBT/BCIP.
Lane M : Molecular weight marker
1 : Yeast with plasmid pPICZB insert only
2, 3 & 4 : Yeast clones found to have Der p 10 insert
5 : Yeast supernatant having soluble protein in significant amount
6 : Yeast pellet still having substantial amount of protein.

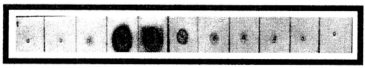


Fig. 4.14: The dot blot profile of the lysate elution after denaturation at pH 4.0

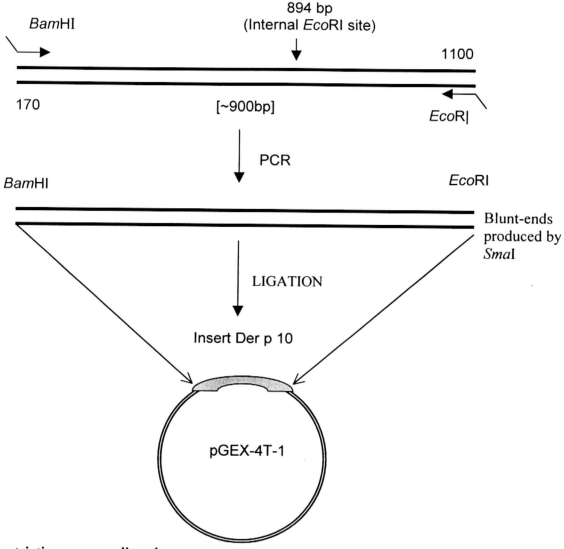
4.10 Discussion

More than 30 components produced by the mite are potential allergens. To study an allergenic component, it is best to study the allergen in isolation, without the presence of other allergens that may affect its performance adversely or even compound its actual quality. However, extracting separate native allergens requires tedious biochemical method of eluting crude extracts or through immunomagnetic means, involving monoclonal antibodies and magnetic field. Thus the option of cloning and expressing the tropomyosin gene was deemed more convenient and suitable.

In this study, the 870 bp cDNA fragment encoding Der p 10 was successfully subcloned into the multiple cloning site of the GST gene fusion vector pGEX-4T-1. With cDNA of mite tropomyosin Der p 10 as the template, amplified fragments were later cloned into the *Bam*H1 and *Eco*R1 sites of the pGEX4T-1 bacterial expression plasmid followed by transformation involving BL21(DE23) *E. coli* competent cells. Expression of this GST-Der p 10 fusion protein was controlled by the plasmid-borne *lacI*^{q} repressor and the inducible *tac* promoter. Therefore, the addition of IPTG to the culture medium not exceeding 2 hr resulted in the induction of protein expression within the *E. coli* cells. The schematic diagram of the whole cloning strategy explaining the ligation and restriction enzyme analysis is summarised and shown in **Fig. 4.15**.

E. coli cells (BL21) was a suitable host for cloning the recombinant plasmid pGEX-Der p 10 despite the low efficiency (14%) of this expression system. In the downstream processing of recombinant Der p 10, the combination of chromatographic procedures was employed for purification. The fact that site-specific separation of GST affinity tag from GST-Der p 10 protein by thrombin was able to produce two fragments of proteins, each with the correct size; also showed that the expressed fusion protein contained the thrombin-accessible recognition sequence. This result, together with the correct sizes of the bands

Ligation of Der p 10 cDNA (insert) into the pGEX-4T-1 vector:



Restriction enzyme digestion:

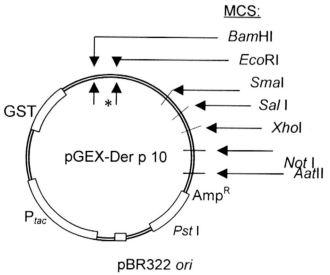


Fig. 4.15: Restriction enzyme digestion analysis following the ligation process. Double digestion was carried out with *Eco*RI and *Bam*HI (37°C; 2 hr) showing bands as in Lane 3 in **Fig. 4.4**. The faint band of ~700 bp is the fragment marked * in the construct, pGEX-Der p 10.

visualised in the SDS-PAGE profile indirectly proved that the recombinant protein produced was the putative GST-Der p 10. The verification by anti-GST antibody by Western blotting also proved that the expressed protein carried a GST peptide of the right size, when summed up with the Der p 10 protein. Sequence analysis (**Appendix 2 and 3**) revealed that this construct encoded a protein with a high degree of homology to tropomyosin. The putative clone 7, (as visualised on SDS-PAGE in **Fig. 4.4**), contained DNA sequence deduced as the 284 residues long, and showed 98%, 97% and 82% identity to tropomyosin from *D. pteronyssinus, D. farinae* and shrimp (*Metapenaeus ensis*), respectively.

E. coli expression affords a number of advantages although the protein being studied is a eukaryotic protein. Expression of Der p 10 DNA sequence in *E. coli* cells results in significant quantity of homogeneous protein with its composition dictated purely by the cloned DNA sequence. Expression of tropomyosin (as a contractile protein) in *E. coli* is not as likely to affect the viability of the bacteria as might be expected for a eukaryotic cell, which normally expresses endogenous contractile proteins too. Therefore, this might actually compensate for potential drawback of bacterial expression which the lack of post-translational modifications in *E. coli* (McNally *et al.*, 1991).

Heterologous expression systems are frequently employed for the production of recombinant allergens because of the convenience of manipulation these systems offer, and their ability to achieve levels of productions higher than the native source of protein (i.e. the original tissue or primary cell culture). However, sometimes this drive for high expression levels leads to lower quality or authenticity of the protein sequence or structure. Therefore, yeast is increasingly being used for the identification of genes as a biological readout for screening small-molecule pharmaceuticals. *P. pastoris* was chosen because of its various advantages, such as its ability to produce recombinant proteins that undergo folding closest to their native forms. As a methylotrophic yeast, *P. pastoris* is capable of

metabolizing methanol as its sole carbon source with alcohol oxidase (AOX) enzymes. With the *AOX* promoter, expression of the gene of interest could be carried out to high levels with tight regulation, before its induction by methanol. The expression vector used, pPICZ B (3.3 kb) expresses the heterologous protein in the intracellular region of the yeast cells which can then be purified from endogenous proteins by Ni^{2+}-binding affinity chromatography to bind the His-tag, fused at the carboxyl terminal of the gene construct. Der p 10 was expressed through *Pichia* expression system to compare the resulting recombinant tropomyosin expressed by a eukaryotic host with that of the *E. coli* (prokaryotic) system. This underlines the difference of recombinant protein production with and without the posttranslational modifications as offered by the respective host.

In *Pichia* expression system, the identity of the insert was verified using two independent approaches: PCR amplification of mite cDNA primed by a degenerate oligonucleotide deduced from a known segment of the amino acid sequence of Der p 10 and analysis of the specificity of an antiserum raised by immunizing a mouse with fusion protein of one of the clones containing the Der p 10 insert. Similar to the *E. coli* expression system, which had the GST tag to facilitate both purification and detection, the *Pichia* system utilises two tags on the C-terminus; the 6xHis and myc tags, each for purification and detection purposes. Unlike the *E. coli* system, this *Pichia* system proved to be rather cumbersome, not to mention the greater challenge of cloning and transforming the Der p gene into the yeast genome.

A comparison of the two expression systems (**Table 4.2**) shows a tendency to favour the bacterial system for a few reasons. Firstly, there was a relative ease in the cloning procedure compared to the *Pichia* method that eventually saw the dependence on the gene amplification role of *E. coli*. The longer expression time in the yeast system was not favourable as substantial accumulation of excretory substances from the yeast, may be detrimental to the protein of interest.

Table 4.2: A comparison of the bacteria and yeast expression systems

***E. coli* expression system**	***Pichia* expression system**
The fusion partner, GST contributed a large number of residues of size 26 kDa to the whole recombinant, making it a 62 kDa protein.	Protein was expressed with two short epitope tags; 6xHis and Cmyc. Size of protein expressed did not differ much from the native Der p 10.
IPTG was required for expression and induction must not exceed 2 hours. Therefore, expression was not time-consuming.	A longer time was needed as the maximum expression occurred only after 96 hours of culture.
Cloning procedure was relatively straightforward.	Cloning procedure was rather cumbersome; requiring the amplification phase in *E. coli* prior to being cloned into *Pichia.*

The expression system of choice has to reflect the nature and demands of the particular protein to be produced, such as specific post-translational modifications, production yield and purification requirements. Although *E. coli* offers advantages for achieving high-level regulated expression; proteases and toxins remain as by-products to avoid. Perhaps the manipulation of food-grade bacteria such as *Lactococcus lactis* and *Corynebacterium glutamicum* (which lack extracellular protease or toxins) should be attempted for the production of safe and high quality recombinant allergens for use in diagnostics and immunotherapy (Billman-Jacobe, 1996)