III. MATERIALS AND METHODS

Plant material

The present study was carried out in fruits of eight banana cultivars, such as *Musa acuminata* cv. Palayancodan (AAB), Nendran (AAB), Poovan (AAB), Robusta (AAA), Red banana (AAA), Kadali (AA), Matti (AA) and Njalipoovan (AB). The fruits were collected from banana garden, University of Kerala, Kariavattom, Thiruvananthapuram, India. Mature green fruits were harvested from respective cultivars 9 months after plantation. The ripening period was designated as early ripened at the time of harvest, ripened, when fruits are soft and yellow in colour and late ripened when fruits developed black spots on the skin (Potter and Hotchkiss, 1998).

Methodology

3.1 Biochemical analysis of fruits

3.1.1 Total carbohydrate

Total carbohydrate was estimated using phenol sulphuric acid (Dubois *et al.*, 1956). The fruit pulp (1 g) was refluxed in 2.5 N HCl (5 ml) for 3 hours and the solution was then cooled and made up to 10 ml. Sample solution (0.2 ml) was pipetted out and made up to 1 ml with distilled water. Phenol (1 ml) and sulphuric acid (5 ml) was added to the sample and the contents were mixed thoroughly. The tubes were kept in boiling water bath for 20 minutes and cooled to room temperature. Absorbance was read in Spectrophotometer (ShimadzuUV-1700, uv-visible spectrophotometer) at 490 nm against blank. The amount of carbohydrate was calculated from the standard graph of glucose.
3.1.2 Reducing sugar

Reducing sugar in fruits was quantified using dinitro salicylic acid reagent (Miller, 1959). The fruit pulp (1 g) was treated with hot 80% ethanol (5 ml) and sugar was extracted twice. The supernatant was collected, evaporated the ethanol content, followed by adding distilled water (10 ml) to dissolve the sugars. The sample (0.2 ml) was pipetted, equalized the volume to 1 ml with distilled water and 3 ml DNS reagent was added. The content was heated in a boiling water bath for 5 minutes, cooled and the intensity of dark red colour was measured at 540 nm. Reducing sugar content was calculated from the standard graph of glucose.

3.1.3 Non-reducing sugar

Non-reducing sugar was determined by calculating the difference between total carbohydrate and corresponding reducing sugar value.

3.1.4 Amino acids

Estimation of total free amino acids was done by the method of Moore and Stein (1948). The fruit tissue (1 g) was refluxed with 80% ethanol for 10 minutes. After cooling, the extracted tissue was homogenized in a mortar and pestle for few minutes. The homogenate was filtered and centrifuged at 7500 rpm for 10 minutes and the supernatant was collected. The sample was pipetted out (0.2 ml) and made up to 1 ml with ethanol. Ninhydrin reagent (1 ml) was added, made up the volume to 3 ml with distilled water and kept in boiling water bath for 20 minutes. After cooling the intensity of purple colour was measured at 570 nm against blank. The total free amino acids in the sample were calculated using leucine as standard.
3.1.5 Total proteins

Total protein was calculated using Bovine Serum Albumin (BSA) as standard (Lowry et al., 1951). The fruit pulp (1 g) was homogenized in 0.1 M sodium phosphate buffer (pH 7.0). After centrifugation the supernatant was used as crude protein source. The sample (0.2 ml) was taken and made up to 1 ml with distilled water. Reagent C (5 ml) (50 ml 2% sodium carbonate in 0.1 N sodium hydroxide and 1 ml 0.5% copper sulphate in 1% potassium sodium tartarate) was added, mixed well and kept for 10 minutes. Folin-Ciocalteau reagent (0.5 ml) was added, mixed well and kept in dark for 30 minutes. The absorbance was measured at 660 nm against blank solution and the total protein was calculated using BSA as standard.

3.1.6 β-carotenes

Total β-carotene content was measured by the method described as Ortiz et al. (2011). The fruit tissue (5 g) was homogenized with 10 ml acetone petroleum ether (1:1) and the homogenate was centrifuged at 3000 rpm for 10 minutes at 4°C. This procedure was repeated three times, the liquid phase was collected. The colourless extracts were combined with 0.1 M NaCl (10 ml) solution and the carotenoids present in petroleum ether phase was separated from lower aqueous acetone phase. Absorbance of pooled extracts was taken at 450 nm against petroleum ether as blank. Total β-carotene content was determined using the absorption coefficient of β-carotene in petroleum ether.
3.2 Fruit firmness measurement

Naturally ripened and ethylene treated fruits of two cultivars Palayancodan and Kadali were analyzed for firmness of flesh using Penetrometer. The readings are taken three times for same fruit and values are expressed in neutons.

3.3 Extraction and assay of fruit ripening enzymes

3.3.1 Enzyme extraction

3.3.1.1 Polygalacturonase (PG)

Polygalacturonase was extracted and assayed according to the method of Sai Prasad et al. (2004). The fruit tissue (2 g) was homogenized in 0.1 M sodium acetate buffer (pH 6.0) containing 0.2% sodium dithionite and 1% PVP. After centrifugation supernatant was taken and was used as enzyme source for assay.

3.3.1.2 Pectin methyl esterase (PME)

PME was extracted and activity assayed as described by Hagermann and Austin (1986) with slight modifications. The fruit tissue (2 g) was homogenized in cold (4°C) 8.8% NaCl (w/v) and the homogenate was centrifuged at 20000 X g for 10 minutes. The supernatant was collected and pH (7.5) was adjusted with NaOH. This was used as enzyme extract for assay.

3.3.1.3 Cellulase, xylanase and invertase

Cellulase, xylanase and invertase enzymes were extracted as described by Srivastava and Dwivedi (2000). The fruit pulp (2 g) was homogenized with 15 ml of 20 mM sodium phosphate buffer (pH 7.0) containing cysteine-HCl (20 mM), EDTA (20 mM) and Triton- X 100 (0.05%). The homogenate was passed through two layers of muslin cloth to remove cell debris and the volume was made to 20 ml
with the homogenization medium. The homogenate was centrifuged at 15000 X g for 30 minutes at 4°C in a refrigerated centrifuge. The clear supernatant was used for cellulase, xylanase and invertase assay.

3.3.2 Enzyme assay

3.3.2.1 Assay of polygalacturonase (PG)
Assay was carried out by measuring reducing groups released from polygalacturonic acid. The reaction mixture containing crude enzyme (1 ml), 0.2 M sodium acetate buffer (pH 4.5) (0.2 ml), 1% pectin (0.3 ml) was incubated at room temperature for 1 hour. The reaction terminated by heating the solution at 100°C followed by the addition of 1 ml DNS reagent. The solution was boiled for 5 minutes, cooled and diluted with distilled water. Absorbance was measured at 540 nm against blank.

3.3.2.2 Assay of pectin methyl esterase (PME)
Assay mixture consisted of aqueous pectin solution (0.033%, pH 7.5), bromothymol blue (0.0016%), 0.01 M NaCl and enzyme in a total volume of 3 ml. The difference in absorbance between 0 and 3 minutes was taken immediately after adding the enzyme solution at 620 nm and this was taken as measure of PME activity. One unit of PME activity is equivalent to units of methyl ester liberated min / g / FW of tissue under the condition of enzyme assay.

3.3.2.3 Assay of cellulase
Cellulase activity was assayed as described by Ahmed and Labavitch (1980) with slight modifications. The reaction mixture consisted of 0.4 ml sodium acetate buffer (100 mM, pH 5.0), 0.3 ml carboxy methyl cellulose (1.5% w/v) and
enzyme extract (0.3 ml). The reaction mixture was incubated at 37°C for 16 hours. After 16 h, the substrate was added to the control tubes. The tubes were boiled on a water bath for 10 minutes and the colour was measured at 540 nm using spectrophotometer (Shimadzu UV-1700, uv-visible spectrophotometer). Amount of reducing sugar released was calculated from a calibration curve drawn using glucose as standard. One unit of cellulase activity was defined as the amount of enzyme liberating 1µmol of reducing sugar per minute at 37°C.

3.3.2.4 Assay of xylanase

Activity of xylanase was measured according to Singh and Singh (1993). The assay mixture consisted of 0.4 ml sodium acetate buffer (100 mM, pH 5.0), 0.3 ml xylan (0.1%) and 0.3 ml enzyme preparation. The mixture was incubated for 1 h at 37°C. The released reducing sugar was measured using DNS. One unit was defined as 1 µmol of reducing sugar released per minute at 37°C.

3.3.2.5 Assay of invertase

Invertase activity was assayed by the method of Moriguchi et al. (1991) and the assay mixture consisted of 0.4 ml acetate buffer (100 mM, pH 4.5), 0.3 ml sucrose (100 mM) and 0.3 ml enzyme extract. The reaction mixture was incubated for 1 hour at 37°C. The substrate was added to control tubes after the incubation and colour was developed using DNS. Amount of reducing sugar released was calculated from the calibration graph. One unit invertase activity was defined as micromoles of reducing sugars equivalent released per minute at 37°C.
3.4 Statistical analysis

One Way ANOVA (analysis of variance) and Duncan multiple range test were carried out at significance level of $p< 0.05$ using SPSS (version 7.5), in preliminary biochemical analysis, enzyme activity profiling and fruit firmness measurements. Three independent experiments were done in each analysis and the values were expressed as Mean ± Standard Error.

3.5 Purification of enzyme

3.5.1 Isolation of enzyme

The ripened fruit pulp (100 g) was homogenized with homogenizing medium (125 ml) consisting of 0.02 M sodium phosphate buffer (pH 7.0), containing 0.02 M EDTA, 1% Triton X-100, 0.02 M cysteine –HCl and 1 mM PMSF. Homogenate was filtered using a double layered cheese cloth and then centrifuged at 15000 X g for 30 minutes. The supernatant was collected and used as crude enzyme source for purification (Pathak and Sanwal, 1998).

3.5.2 Purification of enzyme

3.5.2.1 Purification of enzyme from Musa acuminata cv. Palayancoden

All purification steps were carried at 4°C by the method of Pathak and Sanwal (1998). Collected supernatant (crude enzyme 90 ml) was dissolved in (NH4)2SO4 with 0.9 saturation. The suspension centrifuged at 20000 X g for 45 minutes and the precipitate suspended in 0.02 M sodium phosphate buffer (pH 7.0) containing 1 mM PMSF. The suspension (55 ml) was dialysed against the same buffer and centrifuged at 15000 X g for 15 minutes. (NH4)2SO4 fraction (55 ml)
was loaded in a DEAE-cellulose column (3.2x 24 cm) pre-equilibrated with 0.02 M sodium phosphate buffer (pH 7.0). The column was washed with 3 bed volumes of the above buffer and 8 ml fractions were collected. The adsorbed proteins were eluted using a linear NaCl gradient (0.1-1 M). Each collected fractions were assayed for activity and the fractions with higher activities were pooled. Fractions eluted with 0.6 M, 0.7 M and 1 M were pooled and constituted PG1, PG2 and PG3 respectively. Pooled active fractions obtained from DEAE-cellulose chromatography were concentrated with sucrose and further purified by gel filtration over Sephadex G100 column (1.6x80 cm) pre-equilibrated with 0.02 M sodium phosphate buffer (PH 7.0). The adsorbed proteins were eluted using linear NaCl gradient (0.1-1 M) and 9ml fractions were collected. A single peak of activity was obtained for PG1 (0.4 M), PG2 (0.8 M) and PG3 (0.9 M) respectively.

3.5.2.2 Purification of enzyme from *Musa acuminata* cv. Kadali

Purification was done according to the method mentioned as above upto dialysis. After dialysis the purification was carried out using Bio-Rad Biologic LP Purification System. Dialyzed protein sample (40 ml) was loaded on DEAE-cellulose column pre-equilibrated with 0.02 M sodium phosphate buffer (pH 7.0). Fractions were eluted from the column using a linear gradient of NaCl (0.1-1 M) in 0.02 M phosphate buffer (pH 7.0) and the flow rate is 1 ml / minute. Fraction volume is 1.5 ml and 30 fractions were collected, and the 15th fraction showed a peak at 280 nm. All the fractions assayed for activity and total protein content and the fractions from 14-17 (4 fractions) showed more activity. These fractions were pooled (6 ml) and concentrated with sucrose and again loaded on Sephadex G 100
column for gel filtration. All the conditions of purification steps are same as mentioned above and the 16th fraction only showed a single peak at 280 nm and has high protein content and enzyme activity. This fraction was collected, and this represented the purified enzyme.

3.5.2.3 Enzyme assay

The enzyme assay was carried out by measuring the reducing groups released from polygalacturonic acid. One unit of the enzyme catalyzed the liberation of one mole of galacturonic acid in one second under the conditions of the enzyme assay. Specific activity was expressed as units/mg protein.

3.5.2.4 Protein quantification

Protein quantification was carried out according to the method of Lowry et al. (1951) using BSA as standard.

3.5.3 Enzyme kinetics / characterization of purified enzyme

3.5.3.1 pH - activity

Optimum pH of purified enzymes were determined by assaying the activity of enzyme in a range of pH (3.0-6.0) in 0.2 M sodium acetate buffer.

3.5.3.2 Thermal stability

Optimum temperature of purified enzymes were calculated by measuring the activity of enzyme in various temperatures (35°C - 60°C).

3.5.3.3 Effect of metal ions and EDTA

Effect of monovalent metal ions like Na⁺, K⁺, divalent ions such as Mg²⁺, Mn²⁺, Ba²⁺, Cu²⁺, Ca²⁺ and trivalent ion Fe³⁺ on the activity of purified enzymes were studied by adding various concentrations of metal ions (1-10 mM) in the
assay medium. EDTA (10 mM) also added to the assay buffer and its effect on enzyme activity was studied.

3.5.3.4 Effect of Triton X-100

Effect of non ionic detergent Triton X-100 on the purified enzyme activity was determined by measuring the activity in 0.1 to 30% of this detergent.

3.5.3.5 Km value

Km value was determined using different concentrations of polygalacturonic acid (0.25-3.0%) and Line Weaver Burk Plot was drawn by plotting 1/V on Y axis and 1/S on X axis.

3.6 Molecular analysis of purified enzymes

3.6.1 Molecular mass determination

SDS-PAGE and Native PAGE

Molecular mass of purified enzymes were determined by measuring the relative mobility of proteins in SDS-PAGE along with protein markers according to the method of Laemmli (1970). Proteins were loaded equally in each well (60 µg purified protein of Palayancodan and 30 µg purified protein of Kadali) along with protein markers such as Phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa). SDS-PAGE was carried out on 12% polyacrylamide gel and the protein bands were stained with coomassie brilliant blue R 250. Native PAGE of purified enzymes was done on 7.5% polyacrylamide gel.
3.6.2 Gene expression analysis

3.6.2.1 Treatment of the fruits

Fruits of almost same size and weight were placed in an air tight container of a ten litre capacity. The injection port was covered with a rubber stopper. Ethylene (100 µl L\(^{-1}\)) was injected through the injection port with the help of a syringe. This treatment was given for 24 hours in dark at room temperature (23° – 25°C). The container was vented after every six hours during treatment and ethylene was replaced. After 24 hours of treatment fruits were allowed to ripen for 7 days at 23°C in air. Pulp tissue was harvested at 0 hour (0 h) and every 24 hours (24 h) after ethylene treatment for 7 days, frozen in liquid nitrogen and stored at -70°C till further use. Pulp tissue was also harvested from control fruits, which were allowed to undergo post harvest ripening in air only at room temperature for 7 days without any exogenous ethylene treatment.

3.6.2.2 Total RNA isolation from banana pulp tissue

Total RNA was isolated from ethylene treated mature banana pulp harvested each day during ripening for 7 days according to Asif et al. (2000). Total RNA was also isolated from naturally ripened control fruits. Fruit tissue (2 g) was ground to a fine power in liquid nitrogen and homogenized in extraction buffer containing 0.1 M Tris-HCl (pH 8.0), 1.4 M NaCl, 0.02 M EDTA (pH 8.0), 2% CTAB and 0.1% β-mercapto ethanol. The tube was incubated at 65°C for 60 minutes with gentle vortexing after every 15 minutes. The homogenate was cooled to room temperature and extracted twice with equal volume of chloroform: isoamyl alcohol (24:1). The aqueous phase was separated each time by centrifugation at
room temperature for 15 minutes at 10000 X g in a Sorvall RC 5C centrifuge. The RNA was precipitated by adding 10 M Lithium Chloride to the final concentration of 3 M and β-mercapto ethanol to 0.1%. After overnight incubation RNA was pelleted at 12000 X g for 20 minutes at 4°C. Supernatant was discarded and RNA pellet was resuspended in 1 ml sterile water. It was subsequently purified by phenol: chloroform extractions.

Aqueous phase obtained was mixed with 1/30 volume of sodium acetate and 1/10 volume of absolute alcohol and centrifuged at 12000 X g for 10 minutes at 4°C to pellet polysaccharide. Again supernatant was taken and 1/10 volume of sodium acetate and 3 volume of absolute alcohol was mixed, kept at -70°C for 6-8 hours and centrifuged at 12000 X g for 15 minutes. Nucleic acid pellet was washed twice with 70% ethanol, dried and dissolved in 50 µl RNase free sterile water.

3.6.2.3 RFD treatment

In order to remove the contaminating DNA, the RNA preparation was treated with RNase free DNase 1 (RFD). For DNase treatment the reaction mixture containing 5 µg RNA, 5 µl RFD buffer (10X) (100 mM Tris – HCl (pH 7.5), 25 mM MgCl₂, 1 mM CaCl₂), 1 µl of RFDase 1 (10U/µl) and water was kept at 37°C for 30 minutes. After this, the reaction volume was increased to 100 µl with water and aqueous phase was extracted once with equal volume of chloroform and precipitated by adding 2.5 volume of ethanol in presence of 0.3 M sodium acetate (pH 5.2) and keeping at -70°C for 1 hour. The RNA was recovered by centrifugation, washed with chilled 70% ethanol, dried and suspended in RNase free water. Protein contamination of the RNA extract was determined by
measuring the absorption ratio at 260/280 nm and RNA integrity was assessed by electrophoresis in 1.5% agarose gel in 0.5 X TBE buffer.

3.6.2.4 Preparation of cDNA by reverse transcriptase reaction

cDNA was prepared using total RNA isolated from different days of post ethylene treated and control fruits. Total RNA (5 µg) was combined with 2 µl of 3’AP primer (10 mM) in a 0.5 ml microfuge tube. The reaction was collected by brief centrifugation and kept at 70°C for 10 minutes then chilled on ice for 1 minute. The reaction was centrifuged briefly and 8 µl of PCR buffer (5X), 2 µl of dNTP mix (10 mM), and 0.5 µl of Ribolock (RNase inhibitor) were added to the tube. The reaction was mixed and centrifuged briefly to collect the content at the bottom of the tube. The reaction was equilibrated at 42°C for 2-5 minutes and 1 µl of Super Script 11 RT (20 units/µl) was added. The tube was incubated at 42°C for 1 hour. The reaction was terminated by heating at 70°C for 15 minutes. The 5X buffer was made by 100 mM Tris – HCl (pH 8.8), 500 mM KCl and 0.8% (v/v) Nonidet- P40.

3.6.2.5 Semi quantitative RT PCR

To study the expression pattern of polygalacturonase (PG), semi quantitative RT PCR was carried out using cDNA prepared from 5 µg of DNA free RNA from 0-7 days ethylene treated and control fruits. PCR was carried out using PG4 (MAPG4 acc.no.AY603341) primers. Actin primer was used as control. PCR was initiated by a denaturation step at 94°C for 3 minutes followed by 35 cycles of amplification, each consisting of denaturation at 94°C for 10 seconds, annealing at 52°C for 20 seconds and extension at 72°C for 1 minute. Finally an extension cycle
at 72°C for 7 minutes was carried out. Following the amplification, sample (10 µl) was analyzed on 1.5% agarose gel prepared in 0.5X TBE buffer.

**Nucleotide sequences of different primers used in RT PCR**

1. **ACTIN-FOR** (sequence 5’-3’)
   
   GAGAGTTTTGATGTCCCTGCCATG

   24 mer (SQ RT PCR)

2. **ACTIN – REV** (sequence 5’-3’)
   
   CAACGTCGCAATTTCATGATGGAGT

   24 mer (SQ RT PCR)

3. **3’AP** (sequence 5’-3’)
   
   GGCCACGCGTCGACTAGTAC (T) 17

   37 mer (for first strand cDNA synthesis)

4. **PG 4 -FOR** (sequence 5’-3’)
   
   ACACTCGGGCCTGTGTGGCAAACATAA

   27 mer (SQ RT PCR)

5. **PG 4 -REV** (sequence 5’-3’)
   
   CATGCGTATGGTTTTCGAAGCTGATGGA.

   27 mer (SQ RT PCR)
3.6.3 Protein sequencing

3.6.3.1 Sequencing of purified protein fractions

Three purified protein fractions from *Musa acuminata* cv. Palayancodan and single purified protein from *Musa acuminata* cv. Kadali were sequenced at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram.

3.6.3.1.1 Trypsin digestion

The corresponding protein bands and spots in the gel (SDS-PAGE) were excised for in-gel trypsin digestion. The gel pieces were treated with 50% acetonitrile and 50 mM ammonium bicarbonate (ABC) for 30 min at room temperature for destaining. This process is repeated until the gel pieces get destained completely. The destained gel pieces were then reduced with 25 mM DTT in ABC for 20 min at 55°C followed by alkylation with 45 mM iodoacetamide in ABC for 20 min at room temperature. Overnight digestion was performed with 0.01 µg/µl trypsin (Promega, Madison WI) in 20 mM ABC at 37°C. Peptides were extracted from the gel bands with 2% tri-fluro acetic acid (TFA) and then with 70% acetonitrile, 30% water, and 0.1% TFA and finally with 100% acetonitrile. The collected solutions were pooled and vacuum dried under evaporation. The dried peptides were reconstituted with 10 µl of 0.1% formic acid and analyzed by nano LC–MSE (MS at elevated energy) using a Nano Acquity UPLC system (Waters Corporation) coupled to a Quadrupole-TOF mass spectrometer (SYNAPT-G2-HDMS, Waters Corporation).
3.6.3.1.2 Mass Spectrometry

The nano-LC separation was performed using a BEH-C18 reversed phase column (1.7 µm particle size) with an internal diameter of 75 µm and length of 150 mm (Waters Corporation). The binary solvent system had 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). All mass spectrometric analysis were performed in a positive V-mode at a resolution of about 9000 full width half maximum (FWHM). The instrument was calibrated with a MS/MS spectra of glu-fibrinopeptide B (600 fmol/µL), and the lock mass correction was done every 30s by the same peptide delivered through the reference sprayer of the NanoLockSpray source. MSE was performed by acquiring the spectra at constant low collision energy (4 eV) to generate intact peptide masses, and the collision energy was elevated (20 to 40 eV) to get product ions at an alternative 1s scan. The radio frequency voltage applied to the quadrupole mass analyser was adjusted such that ions from m/z 50 to 2000 will be efficiently transmitted.

3.6.3.1.3 Data processing

The LC–MSE data was analyzed by Protein Lynx Global Server 2.5 (PLGS; Waters Corporation) software, for protein identification. The respective proteins were identified from searching the MS/MS data against the tomato and banana data bases. The ion accounting parameters were; precursor ion tolerance 25 ppm, product ion tolerance 100 ppm, minimum number of peptide matches = 1, minimum number of product ion matches per peptide = 3, minimum number of
product ion matches per protein = 5, and the number of missed tryptic cleavage sites = 2. The false positive rate was 4%. Ion intensity threshold was set at 1000 counts.

### 3.6.3.2 Conserved domain analysis

A protein domain is a conserved part of a given protein sequence and structure that can evolve, function, and exist independently of the rest of the protein chain. Domains were usually functional unit of protein, hence are conserved during evolution. With aid of computational tools, these conserved regions of proteins (domains) was elucidated.

The Conserved Domain Architecture Retrieval Tool (CDART) of NCBI finds protein domains by using sensitive protein domain profiles across significant evolutionary distances. CDART searches across ENTREZ protein database based on domain architecture. The query was submitted in the home page. Searches can be further refined by taxonomy and by selecting domains of interest.

### 3.6.3.3 Multiple sequence alignment

Clustal W multiple alignment was done with Bioedit. Sequence similarity with beta-1,3-glucanase of *Musa paradisiaca* was done in Emboss Water at EMBL. Coloured regions show conserved regions.
3.6.3.4 Structure prediction

Three dimensional (3D) structure of the proteins were predicted by template-based homology modeling or fold-recognition using the Phyre server (Kelley and Sternberg, 2009). Secondary structure of protein was in the form of three-state prediction: alpha helix, beta strand and coil. A confidence value was represented at each position of the query of each of the three secondary structure states. These confidence values were averaged and a final, consensus prediction was calculated and displayed beneath the individual predictions. In addition to this, the programme ‘Disopred’ calculated a two-state prediction of which the regions of the query were structurally ordered (o) or disordered (d).

To determine the % of sequence identity between the query and template, the SCOP code column was used. Matches with high % sequence identity (>40%) are highlighted in red. This column also indicated the unique identifier for the template structure matched by Phyre. The identifier was in the form [d/c] [PDB code] [chain identifier] [domain number]. The initial ‘d’ or ‘c’ character indicated that the structure is a SCOP domain or a whole chain taken from the PDB respectively. The PDB code and chain identifier were self-explanatory. The domain number was an index (usually 1-9) supplied by SCOP to identify a particular domain in a multidomain, yet single chain, of a protein.