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Optimization of Expression and Purification of Acetoacetyl-CoA Thiolase from Sunflower

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Optimization of Expression and Purification of Acetoacetyl-CoA Thiolase from Sunflower

by

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List of abbreviations

AACT: Acetoacetyl-CoA Thiolase

CS: Citrate synthase

IPTG: Isopropyl-beta-D-thiogalactopyranoside

MDH: Malate dehydrogenase

NTA: Nitro-triacetic acid

OACT: Oxoacyl-CoA Thiolase

PCR: Polymerase Chain Reaction

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAE: Tris-Acetate-EDTA

TCA: Tricarboxylic acid cycle

Abstract

The β-oxidation system in sunflower (*Helianthus annuus L.*) cotyledons is distinguished by the existence of two different thiolase isoforms, Thiolase I (acetoacetyl-CoA thiolase, EC 2.3.1.9 AACT) and Thiolase II (3-oxoacyl-CoA thiolase, EC 2.3.1.16 OACT). Glyoxysomal AACT is the last enzyme in the β-oxidation of fatty acids in plant glyoxysomes. Glyoxysomal AACT has been successfully cloned, expressed and purified from sunflower cotyledons (Dyer et al., 2006). In this paper we investigate the optimal conditions for both expression and purification. These include the choice of vector, the growth conditions and the purification parameters for the maximum possible yield of AACT protein.

Introduction

Thiolase enzymes catalyze the catabolism of fatty acids in prokaryotes and eukaryotes via β-oxidation. β-oxidation pathway consists of a recurring cycle of four enzyme-catalyzed reactions; the first involves the oxidation of the C_{α}– C_{β} bond to form a double bond resulting in the formation of a trans-enoyl-CoA. This reaction is catalyzed by Acyl-CoA dehydrogenase, which consists of a family of three soluble matrix enzymes. The second reaction involved the hydration of the double bond. Water is added across the double bond to make a hydroacyl-CoA. The reaction is catalyzed by Enoyl-CoA hydratase, which converts the trans-enoyl-CoA to l-B- Hydroxyacyl-CoA. The third reaction is the second oxidation step in this pathway and is catalyzed by l-Hydroxyacyl-CoA dehydrogenase. It involves the oxidation of the hydroxyl group at the beta position and forms a beta-ketoacyl-CoA derivative. The fourth and final reaction is the thiolase-catalyzed reaction. The reaction cleaves the beta-ketoacyl-CoA formed in the previous step to produce acetyl-CoA and a fatty acyl-CoA that has been shortened by two carbons. 3-oxoacyl-CoA thiolase is a homodimeric enzyme, which catalyzes the thiolytic cleavage of (C_n) 3-oxoacyl-CoA and (C_{n-2}) 3-oxoacyl-CoA in the presence of CoA. Acetoacetyl-CoA thiolase catalyze the cleavage of acetoacetyl-CoA into two molecules of acetyl-CoA and the synthesis (reverse reaction) of acetoacetyl-CoA. In yeast, the cooperation of acetoacetyl-CoA and 3-oxoacyl-CoA enzymes brings about the complete degradation of fatty acids to acetyl-CoA (Kurihara et al., 1988). The formation of acetoacetyl-CoA is the first step in cholesterol and ketone body synthesis (Antonekov et al., 2000). For even numbered, saturated and straight chain fatty acids, the newly formed acyl-CoA is

completely degraded to acetyl-CoA by repeatedly going through the reactions of β-oxidation (Schiedel et al., 2004).

In eukaryotes, there are two forms of 3-oxoacyl-CoA thiolase, one located in mitochondria and the other located in peroxisomes (Vollack et al., 1996). Peroxisomes are single membrane bound metabolic organelles that are present virtually in all eukaryotic cells (Chevillard et al., 2004). There is evidence that peroxisomes are required for a number of essential metabolic functions including the β-oxidation pathway, oxidation of phytanic acid and syntheses of bile acid and ether-phospholipids (Wanders et al., 2000). In eukaryotes, peroxisomes oxidize very long chain fatty acids while mitochondria preferentially oxidize short chain and long chain fatty acids. Thus peroxisomes appear to act as chain-shortening system while the mitochondrial system completely degrades fatty acids to acetyl-CoA.

In plants, however, the β-oxidation appears to be restricted to peroxisomes (Kindl et al., 1987) and glyoxysomes but not in mitochondria (Cooper et al., 1996). Glyoxysomes are membrane-bound organelles found in some plants. They are specialized peroxisomes that are found primarily in endosperms and cotyledons of germinating oilseeds. They contain enzymes which convert stored lipids into carbon compounds and energy for the germination and subsequent growth of the seedling (Schiedel et al., 2004). In this pathway, fatty acids are hydrolyzed to acetyl-CoA for the glyoxylate bypass (Figure 1).

Thiolase also plays other important roles in plants. Expression of the β-oxidation gene 3-ketoacyl-CoA thiolase 2 is required for the timely onset of natural leaf senescence in Arabidopsis (Castillo et al., 2008). Senescence is a complex process involving physiological, biochemical and gene expression changes that are regulated by

endogenous and exogenous factors (Lim et al., 2007; Castillo et al., 2008). Leaf senescence is regulated by a complex mechanism involving positive and negative regulators. Jasmonic acid, a positive regulator accumulates in senescence leaves (Castillo et al., 2008). Jasmonic acid is synthesized in plants throught the octadecanoid pathway, a complex sequence of enzymes reactions in different subcellular locations, which require three rounds of β-oxidation reactions in the peroxisome (Schaller, 2001., Castillo et al., 2008). It has recently been reported that 3-ketoacyl-CoA thiolase 2 is responsible for the majority of jasmonic acid biosynthesis in Arabidopsis (Castillo et al., 2008; Afitlhile et al., 2005), and also that 3-ketoacyl-CoA thiolase expression was up-regulated under senescence-promoting conditions (Charlton et al., 2005; Castillo et al., 2008).

In human, thiolase activity is encoded by one gene whereas in rodents, three enzymes encoded by three distinct genes (thiolase A, thiolase B, and SCP2/thiolase) catalyze the thiolase activity (Chevillard et al., 2004). The importance of peroxisomes in humans is well established since the discovery of inherited diseases associated with defects in peroxisome biogenesis and/ or fatty acid β-oxidation (Depreter et al., 2003). The cerebro-hepato-renal syndrome of Zellweger is caused by defective importation of proteins into peroxisomes, leading to peroxisomal metabolic dysfunction and widespread tissue pathology (Wanders et al., 1993). Peroxisomal β-oxidation disorders include X-linked adrenoleukodystrophy, acyl-CoA oxidase deficiency, D-bifunctional protein deficiency and 2-methylacyl-CoA racemase deficiency (Schram et al., 1987).

Here we report on the optimal conditions for expression and purification of glyoxysomal acetoacetyl-CoA thiolase (thiolase I) from the recombinant form expressed in *E. coli*.

Materials and methods

Primer design

The full-length nucleotide sequence of sunflower thiolase was obtained by using primers designed to flank the 3'-end and 5'-end. The sunflower sequence had been previously obtained via a one step reverse transcription-PCR reaction of sunflower specific thiolase cDNA (5'-full primer 5'-TAC GTC TAG TAC CTA TTT TA-3' and 3'-full primer: 3'-TGG GAT TAG GTT CAA CATT-5'). The thiolase I cDNA was obtained by restriction digest with EcoRI from pBAD/HisB expression vector (Invitrogen), where it had been previously cloned (Schiedel et al., 2004). (Figure 2)

Production of blunt end PCR product

Blunt end PCR products of the thiolase I cDNA were produced using Invitrogen kit and thiolase I specific primers. The forward primer contained four additional nucleotides (CACC) on the 5′ end for directional cloning into pET151 vector (Invitrogen).

Amplification of thiolase I cDNA

The PCR reactions were carried out in an Eppendoff Master Cycler Gradient instrument. 12 – 20 µl reactions were set-up. One reaction was PCR control reaction using Promega kit template and primers. The rest were conducted using Invitrogen Kit template and Thiolase I specific primers. PCR was performed using 1 Unit of Platinum P*fx* polymerase (Invitrogen), thiolase I cDNA as template (4 µl), 10X Pfx amplification buffer (2 µl) as supplied with the enzyme, 10 mmol dNTP mixture (0.6 µl), 50 mmol MgSO4

(0.4 µl), and 0.3 µmol of each primer, 14 µl deionized water in a total volume of 20 µl. A three-step PCR protocol was used consisting of 94°C for 2 minutes followed by 25 cycles of 94°C for 15 s, 40°C - 50°C for 30 s, and 68°C for 90 s. The annealing temperature had a gradient from 40°C to 50°C since prior PCR runs at 55°C gave no product. 30 minutes final extension at 68°C was carried out to make sure all PCR products were completely extended.

Agarose Gel electrophoresis

The products of the PCR reaction were checked by mixing 8 µl of each product with 2 µl of loading dye and running them on a 1% agarose gel. In addition 1 µl of a DNA marker was loaded to help figure out the concentration of the PCR products as well as their sizes. To observe the results, the gel was stained with ethidium bromide and visualized under UV light (Figure 3). All samples had a band of about 1236 base pairs. The control had a band of about 100 base pairs.

Low melting agarose gel (Qiaquick Gel Extraction kit protocol – Qiagen, July 2002)

Three samples from the PCR reaction were pooled and run on a 1% low melting agarose gel. In addition, a DNA ladder was loaded on the gel. The gel was stained with ethidium bromide and observed under UV light (Figure 4). The bands corresponding to thiolase I cDNA were cut out and placed into a pre-weighed 1.5 ml microcentrifuge tube. The cut out band weighed about 0.25 gm. The gel was dissolved in .75 ml of buffer QC by placing it in a 50°C water bath for 2 minutes. The color of the mixture was yellow so

no pH adjustment was required. The remaining steps were carried out as per the protocol. The extracted DNA was eluted with 50µl of deionized water.

TOPO cloning reaction

TOPO cloning takes advantage of an enzyme catalyzed cleavage of one strand by topoisomerase I enzyme (Invitrogen Champion pET Directional TOPO Expression Kits, 2006, Shuman 1994). The normal function of topoisomerase I in the cell is to relax dsDNA for replication and transcription. In the pET151 vector, topoisomerase is designed such that it binds to duplex DNA at specific sites. It cleaves the phosphodiester backbone after 5'-CCCTT in one strand. Energy from the reaction is conserved by the formation of a covalent bond between the 3'-phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase enzyme. This covalent bond can be broken by the attack of the 5'-hydroyl of the original strand resulting into binding of the cleaved strand and release of topoisomerase enzyme. It is this reaction that directional TOPO cloning utilizes. The cloning region is flanked by CCCTT on both forward and reverse strands. 4µl (equivalent to 1ng) of the extracted DNA was used for the cloning reaction following the Champion pET Directional TOPO Expression Kit protocol (Invitrogen, October 2006). To the 4µl, 1µl of salt and 1µl of the TOPO vector were added. The reaction was mixed gently and let sit for 5 minutes at room temperature. After 5 minutes, the reaction was placed on ice. The construct is designated pET 151-AACT

Transformation into One Shot TOP10 Competent E. coli Cells

3µl of the cloning reaction were added into one vial of One Shot TOP10 chemically competent E. coli cells and mixed gently. The reaction was incubated on ice for 5 minutes followed by 30 seconds heat shock at 42°C without shaking. The cells were incubated in ice immediately. 250 µl of SOC media was added. The tube was placed horizontally in the incubator (200 rpm) at 37°C for 1 hour. Also two Carbenicillin-Agar plates were place in an incubator at 37°C to warm them. After one hour, a 50 µl, and 200 µl aliquots were spread on the pre-warmed agar plates using aseptic technique. The plates were incubated overnight at 37°C.

Plasmid prep and Restriction enzyme digest of the pET 151-AACT construct

Four colonies from the transformed TOP10 *E. coli* were incubated at 37°C with shaking (270 rpm) in 5ml Carbenicillin-LB Media. The culture was grown overnight. The bacteria were centrifuged and the pellets resuspended in resuspension solution. Plasmid prep followed (Promega mini plasmid prep kit and protocol) and the resulting plasmid DNA was eluted with 50ul of nuclease free water. A double restriction digest with EcoRV and Nhe1 followed. Each reaction had 12.8µl of deionized water, 2.0ul of 10X buffer, 0.2ul of BSA, 0.5ul of EcoRV, 0.5ul of Nhe1 and 4µl of the eluted DNA. The entire contents were placed in a 37°C water bath for 2hrs. To visualize the results, 8ul of each of the digestion reaction was added to 2ul of loading dye and run on a 1.2% agarose gel. The gel was stained with ethidium bromide and visualized under UV light (Figure 5). Based on our calculations the expected fragments sizes were, 4081, 1717, 1042, and 156 bases. With

the exception of the 156 bases band, all other bands were visible. We conclude that of the four colonies selected, all had the insert in the correct orientation.

Transformation into BL21 Star (DE3) One Shot Cells (Champion pET Directional TOPO Expression Kit protocol)

The pET 151-AACT was then transformation into BL21 star (DE3) *E. coli* cells. 5µl of plasmid DNA was mixed gently into one vial of BL21 star (DE3) One Shot cells and incubated on ice for 30 minutes. A heat-shock at 42°C for 30 seconds followed and the cells were put back on ice immediately. 250µl of room temperature SOC medium was added. The mixture was placed in the incubator at 37°C for 30 minutes with shaking (200 rpm). Also two Carbenicillin-Agar plates were placed in an incubator at 37°C to warm them. After one hour, a 50µl, and 200µl aliquots were spread on the pre-warmed agar plates using aseptic technique. The plates were incubated overnight at 37°C. On observing the plates grown overnight, many individual bacteria colonies were visible.

Expression of thiolase I

A single colony from the overnight transformation reaction mixture was picked and grown overnight in 10 ml LB carbenicillin (50ug/ml) media. Cells from the culture grown overnight were inoculated into 200 ml of same media and grown to an O.D 600nm ~0.6. The cells were divided into two 100 ml cultures (250-ml autoclaved conical flask), one of which was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (0.75mM). The cells were grown at 37°C with shaking 270 rpm for an additional 4 hours

through which 1 ml aliquots were taken every hour for further analysis. The 1 ml aliquots were centrifuged, the supernant discarded and the pellets stored at −20°C. The remaining cells were harvested by centrifugation at 5,000-× *g* for 10 min at 4°C (Avanti J-30I Beckman centrifuge). The supernatant were discarded, and the pellets resuspended in 10ml of lysis buffer (50mM NaH_2PO_4, pH 8.0, 300mM NaCl). After 30 minutes of incubation with lysozyme at 2mg/ml concentration at 4°C, the bacteria were sonicated 5 times 10s burst or until a visible clearing occurred. (W-255 sonicator). The sonicated bacteria were centrifuged at 15000-x g in a JA 25-50 rotor in an Avanti J-30I Beckman centrifuge for 30minutes.

Purification of Thiolase Protein via Metal Affinity Chromatography

The supernants were pre-incubated with stripped NTA agarose for 1 hour at 4°C to remove proteins which bind non-specifically to the agarose. The flow-through from the pre-incubated supernants was combined with 1 ml of a nickel (II)-nitrilotriacetic acid (Ni-NTA) agarose resin containing 10 mM imidazole lysis buffer. Binding occurred on a rotating wheel for 1 hour at 4°C. The entire contents were poured into a Poly-Prep Bio-Rad mini-column. The flow through was collected and saved. The column was washed with 5 column volumes of wash buffer (lysis buffer containing 10mM imidazole) Proteins were eluted with a step gradient of imidazole in 4 ml increments. Fractions were saved for further analysis.

SDS-PAGE analysis

100µl of each fraction was combined with 20 µl of 6x SDS-loading buffer (300 mM Tris-HCL, pH 6.8, 400mM DTT, 20% SDS, 3 % bromophenol blue, and 50% glycerol) and heated at 95°C for 5 minutes. The fractions were resolved on a 0.75 mm 4% stacking 10% resolving polyacrylamide gel. Proteins were visualized with Simply Blue Stain (Invitrogen) (Figure 6 A) and sizes estimated from Mark 12 Unstained Standard (Invitrogen) and also by Western blotting. (Figure 6 B)

Western Blotting Analysis

After running the SDS-PAGE, the proteins were transferred to a PVDF membrane. The membrane was rinsed with 1X PBS buffer three times. 10ml of a 5% non-fat milk solution containing 0.02% sodium azide was added as a blocking solution and placed on a shaker for 10 minutes. 2µl (1:5000 dilution) of mouse V5 primary antibody (Invitrogen) was added and left on the shaker for 1hr. The Anti-V5 Antibody is a purified mouse monoclonal antibody that detects recombinant proteins containing the 14 amino acid V5 epitope. The Anti-V5 Antibody recognizes the sequence: -Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr-. The blot was washed 3 times with 10ml of 1X PBS. A 10ml solution of 5% non-fat milk containing 4µl (1:2000 dilution) of Goat anti-mouse IGg secondary antibody conjugated to alkaline phosphatase was added and let shake for another 1hr. Goat Anti-Mouse IgG (H+L), Alkaline Phosphatase Conjugate is an affinity-purified goat anti-mouse antibody. It reacts with mouse IgG (all subclasses) as well as with light chains on other mouse immunoglobulins. Antibodies may cross-react with immunoglobulins from other species. The Alkaline Phosphatase-

Conjugated Antibodies are used in the detection of proteins in Western blotting and ELISAs. The alkaline phosphatase catalyzes colorimetric reactions using standard 5-Bromo-4-chloro-3-indolyl phosphate / Nitroblue tetrazolium reagent (BCIP/NBT). A rinsing step followed. After which a color development solution (NBT and BCIP) was added to the blot and let develop for about 10 minutes. Once the bands were visible, the blot was rinsed with deionized water and let dry at room temperature. (Figures 7A, 7B)

Thiolase assay (340nm assay)

Thiolase activity was determined spectrophotometrically by monitoring the rate of NADH formation at 340nm in a coupled enzyme assay (Oeljeklaus et al, 2002). In this assay (Figure 8), the product of the thiolase reaction, acetyl-CoA, was used as substrate for the condensation with oxaloacetate. This reaction is the first reaction in the TCA cycle, which is catalyzed by citrate syntheses (CS). The reaction that precedes the CS step in the TCA cycle, the oxidation of malate to oxaloacetate by malate dehydrogenase (MDH) produces NADH. By converting one molecule of oxaloacetate and one molecule of acetyl-CoA to citrate, the equilibrium of the MDH reaction, which is highly endergonic, is shifted towards the production of oxaloacetate and NADH. The ratio between NADH formation and acetoacetyl-CoA degradation is 2:1. The reaction mixture consisted of 175 mM Tris-HCL (pH 8.5), 0.12 mM CoA, 2.0 mM dithioerythritol (DTE), 2.6 mM malate, 0.14 mM NAD, 58nkat malate dehydrogenase, 18nkat citrate synthase, 0.05% w/v bovine serum albumin, and recombinant thiolase in a total volume of 1ml. The reaction was initiated with 20µl acetoacetyl-CoA. All reagents were purchased from Sigma.

Protein Determination Assay

The calculations of specific activities and the protein concentration were determined using the Bradford dye-binding procedure with Bio-Rad protein Assay kit and BSA as the standard (Bradford et al, 1976) (Figure 9).

Results and discussion

The full sequence of AACT cDNA was obtained from the construct pBAD/HisB-AACT via restriction digestion with EcoRI enzyme. The AACT cDNA was amplified through a 25 cycle PCR reaction. The results of the PCR reaction were verified by running 10µl of each sample on a 1% TAE agarose gel and visualized under UV light following ethidium bromide staining.The PCR product resolved on an agarose gel, extracted from the gel and then ligated into the pET 151 vector. The designated pET 151-AACT construct was then transformed into chemically competent TOP10 *E. coli* to maintain the plasmid. The transformation into the competent cells was carried out by heat shock at 42°C. Bacteria were grown on 50µg/ml Carbenicillin LB-plate overnight at 37°C. A few colonies were inoculated into 50µg/ml Carbenicillin-LB media for a plasmid prep experiment. The purified DNA was subject to a restriction digest with EcoRV and NheI to verify that the insert was ligated into the plasmid in the correct orientation. The results of the restriction digest proved the insert was in the correct orientation. The cells were then transformed into BL21 competent *E. coli* cells for expression. The plasmid construct was also sequenced. (Figure 2)

Expression of AACT was conducted by growing one recombinant colony in a 10ml LB-Carbenicillin culture overnight in an incubator at 37°C with shaking (270rpm). The overnight culture was inoculated into a 100ml LB-Carbenicillin media and grown to OD ~ 0.6 at 600nm. Optimization experiments were conducted to determine the optimal IPTG concentration and growth times. IPTG at a final concentration of 0.75mM was used to induce thiolase protein expression. The culture was grown at 37°C with shaking at 270rpm for 4 hours. The cells were spun down at 10000 x g for 5 minutes, the supernant discarded and 10 ml of lysis buffer containing 2mg/ml lysozyme added. The pellet was resuspended and left in the lysis buffer for 30 minutes at 4°C. The cells were sonicated 5 times 10s bursts with a microtip set at 70% to break open the cells and shear chromosomal DNA. PMSF was added to a final concentration of 0.5mg/ml. The cells were centrifuged at 10000-x g for 10 minutes. The supernant was added into a 15ml falcon blue tube containing stripped NTA resin equilibrated with native buffer. The contents were placed in a cold room for 1 hour. The flow through from the pre-agarose treatment was transferred into a new 15ml falcon blue tube containing Ni-NTA resin that had been equilibrated with 10mM imidazole wash buffer. The flow-through was equilibrated for another one hour in the cold room. The contents were then poured into a Poly-Prep Bio-Rad mini-column and the flow through collected. A wash with 5 column volumes was carried out with 10mM imidazole wash buffer. The proteins were eluted with increasing concentrations of imidazole buffer. A protein determination assay was run with BSA as the standard to calculate the total amount of protein in each fraction. The collected fractions were analyzed by running equal amounts of total proteins on a SDS-PAGE gel and visualize using Simply Blue stain (Invitrogen). In addition, one of

the SDS-PAGE gels on which the proteins had been separated was used to carry out a Western blotting analysis. The proteins were transferred electrophoretically onto a PVDF membrane and incubated with Anti-mouse V5 primary antibody. A secondary antibody conjugated to alkaline phosphatase– Goat anti-mouse was added after primary antibody treatment. Color development was initiated by the addition of color development buffer with Nitro-BT and 5-Bromo-4-chloro-3-indolyl phosphate. The blot showed dark bands corresponding to fusion proteins containing the V5 epitope, that is, AACT, thiolase I. The collected fractions were further analyzed for the presence of an ezymatically active AACT protein. Enzyme data analysis provided proof that the eluted thiolase was enzymatically active.

Conclusions

Sunflower Glyoxysomal Acetoacetyl-CoA thiolase was successfully cloned in pET 151 vector, expressed and purified to apparent homogeneity via agarose affinity chromatography. The apparent MW of the recombinant protein was about 49kDa which is in agreement with published literature ~ 45kDa. The extra 4kDa were due to the fusion tag attached to AACT. From a 100ml culture, we obtained 0.70mg of AACT with most of the protein eluted at 100mM imidazole concentration. The eluted fractions had high enzymatic activities as well as high specific activities. The highest specific activity was obtained from a fraction eluted at 100mM imidazole concentration of about 250 units (figure 9).

Further biochemical characterizations are underway including crystallization of AACT protein.

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Figures

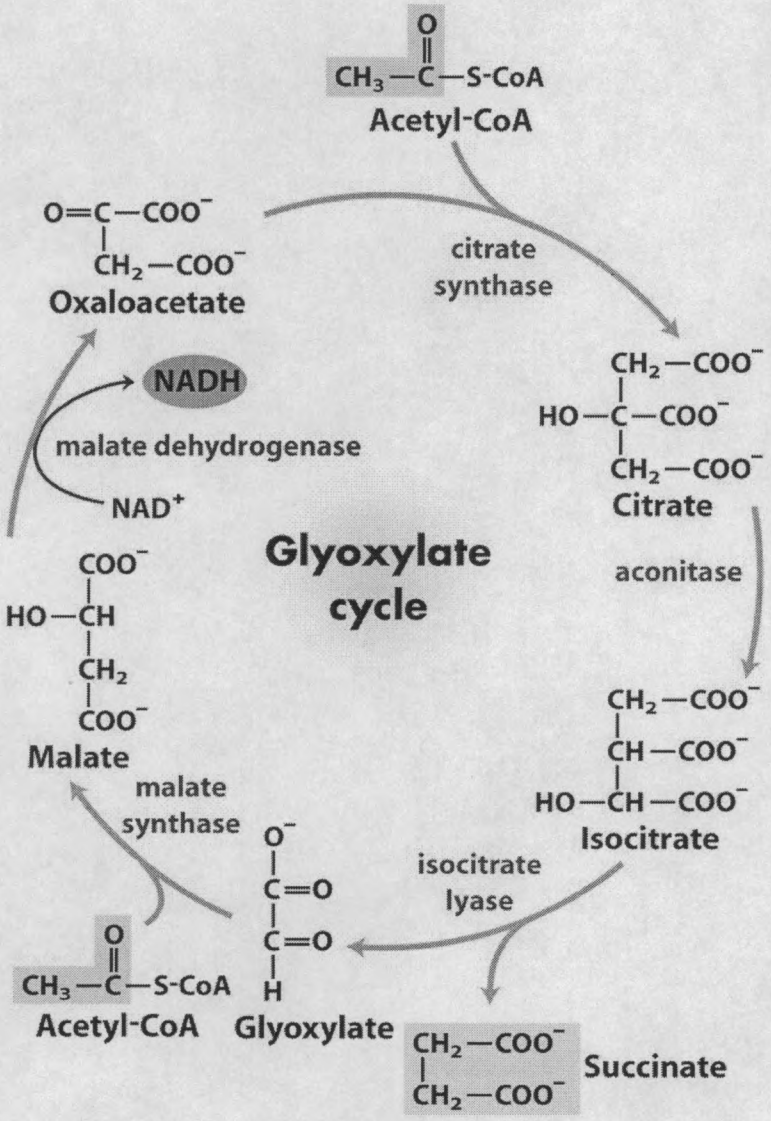

Figure 1: Glyoxylate cycle

ATGCAGATCATGGATAAAATTAACCCTCGAGATGTTTGCATTGTGGGTGTT
GCACGTACGCCTATGGGGGACTTTCTTGGTTCCCTTTCATCTTTGCCAGCA
ACCAAGCTTGGATCCATAGCTATTCAGTCTGCTCTGCAAAGAGCTAATATT
GATCCACGCCTAGTGCAAGAAGTTTTCTTTGGAAATGTCCTAAGTGCAAAC
TTAGGTCAAGCTCCTGCAAGACAGGCTGCGTTAGGTGCTGGCATACCGGAT
ACCGTCGTTTGCACAACCATCAATAAAGTTTGTTCGTCCGGGATGAAAGCA
ACCATGATTGCAGCACAGAGCATCCAAGTTGGTGCCAACGATATCGTGGTC
GCCGGTGGCATGGAAAGTATGTCTAACACACCTAAATACGTAGCGGGCTCA
AGACGTGGGTCCCGCCTAGGACATGATGCGATCATAGACGGCATGATTAAA
GATGGCTTATGGGATGTTTACAACGACTTCGGAATGGGAGTGTGTGGCGAG
TTATGCGCTGACACCTACAAGATAACAAGACAAGATCAGGATGATTATGCT
GTTAGAAGTTTCAACCGTGGAATTGCTGCGCAAAAGAACGGTGCTTTCAAA
TGGGAAATCGTTCCGGTGGAAGTTTCGGGGGGAAGAGGGAAGGTCCCTATG
GTTGTTGATAAAGATGAAGGATTGACAAAGTTTGATGCTACAAAATTGCGG
AATCTACGACCGAGTTTCAAGGTGGAAGGTGGTTCCGTTACTGCAGGCAAT
GCTTCAAGTATCAGTGACGGTGCAGCTGCGTTAGTGCTAGTGAGCGGAGAA
AAGGCGTTAAAACTTGGATTAAAAGTGATTGCTAAAATAAGAGGTTTTGCT
GATGCTGCTCAGGCCCCCGAGTTATTTACCACGGCTCCATCCCTTGCTATC
CCGAAAGCTATTTCAAATGCTGGCTTGACGGCTTCTCAAATTGATTACTAC
GAAATAAACGAAGCGTTCTCGGTTGTGGCGTTAGCAAACCAGAAGCTGCTA
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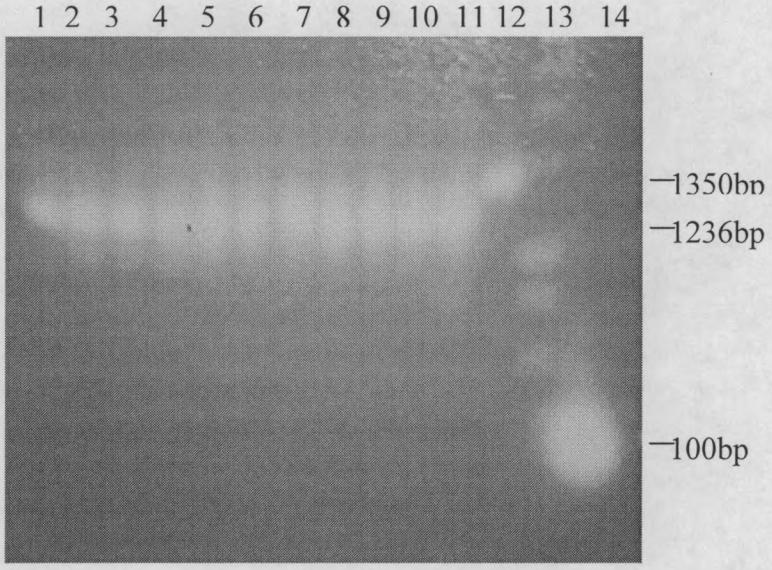
CACCCGCTAGGTTGTAGTGGAGCTCGGATTTTGGTCACATTGCTAGGGGTA
TTGAGGCAAAATAACGGAAGGTTCGGGGTTGCTGGCATTTGCAACGGGGGT
GGAGGAGCGTCTGCATTGGTTCTCGAGCTCATGCCAAGTGCAGGGACCCTA
TCCAAGTTG**TAA**

Figure 2: The full-length nucleotide sequence of sunflower thiolase I. The ATG start codon is highlighted at the 5'-end, and the TAA stop codon is highlighted at the 3'-end. The internal highlighted sequences were the ones used for the 5'-RACE and the 3'-RACE

Figure 3: Amplification of AACT cDNA

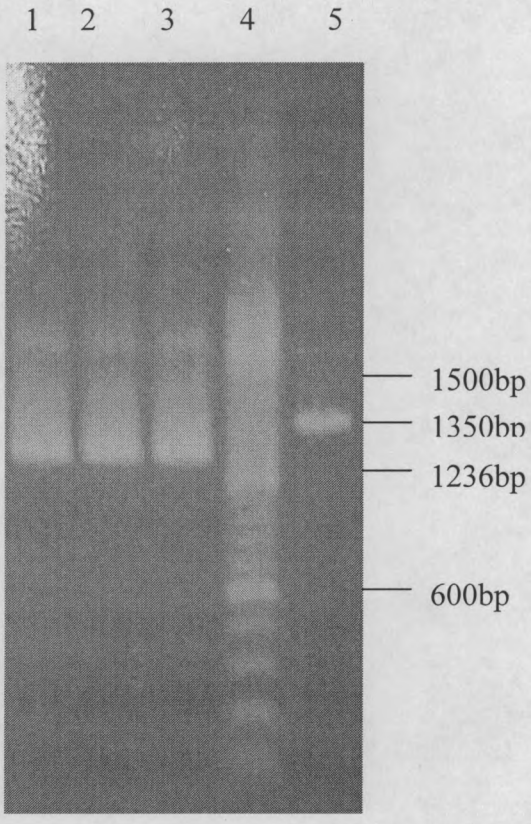
1% TAE Agarose Gel Electrophoresis of Amplified Acetoacetyl-CoA Thiolase cDNA from pBAD/HisB-AACT where it had been previously cloned.



Lanes 1 through 11 show PCR products resulting from the amplification of thiolase I (Acetoacetyl-CoA Thiolase) cDNA. Lanes 1 to 10 were run at an increasing annealing temperature gradient from 40 to 50 degrees C respectively. Lane 12 show thiolase II (3-Oxoacyl-CoA Thiolase) band of known size 1350bp. Lane 13: DNA Ladder and Lane 14: Promega control (100bp) used to verify the success of the PCR reaction

Figure 4: Extraction of AACT cDNA

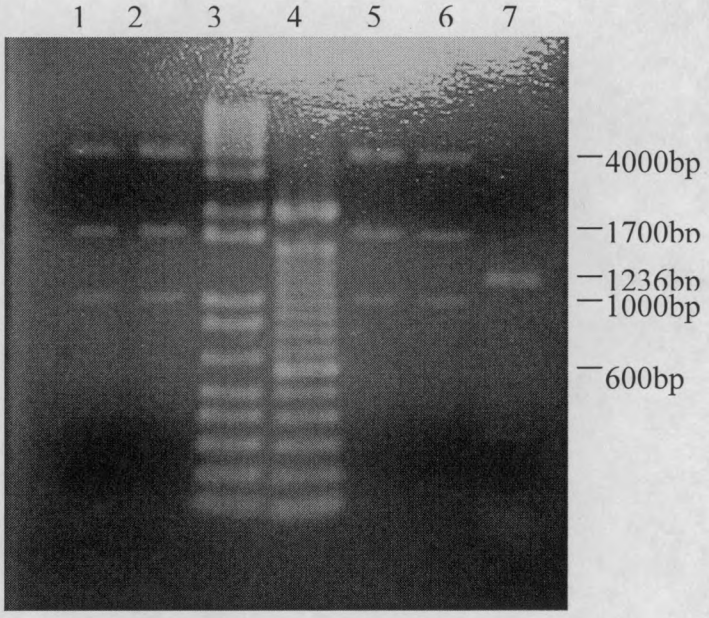
1% Low-Melting TAE Agarose Gel Electrophoresis of pooled Amplified Acetoacetyl-CoA Thiolase (AACT) cDNA from pBAD/HisB-AACT



Lanes 1, 2 and 3 show pooled PCR products. The three bands corresponding to the thiolase cDNA were excised and thiolase cDNA extracted; Lane 4 has TrackIt 100bp DNA ladder (Invitrogen), and Lane 5 show a band corresponding to 3OACT.

Figure 5: Double digest reaction with EcoRV and NheI restriction enzymes.

1.2 % TAE Agarose Gel Electrophoresis of the double digest of pET 151-AACT constructs.



Lanes: 1, 2, 5, and 6 show bands of AACT-plasmid which was digested with EcoRV & NheI. The plasmid with the construct in the correct orientation would give 4 bands upon digestion with EcoRV and NheI restriction enzymes of sizes 4081, 1717, 1042, and 156 base pairs respectively. Lanes: 3 and 4 are TrackIt 100bp and 1kb DNA Ladders respectively. Lane 7 has AACT-DNA from PCR reaction

Figure 6 (A): SDS-PAGE analysis of crude lysate and eluted fractions stained with Simply Blue Stain (Invitrogen).

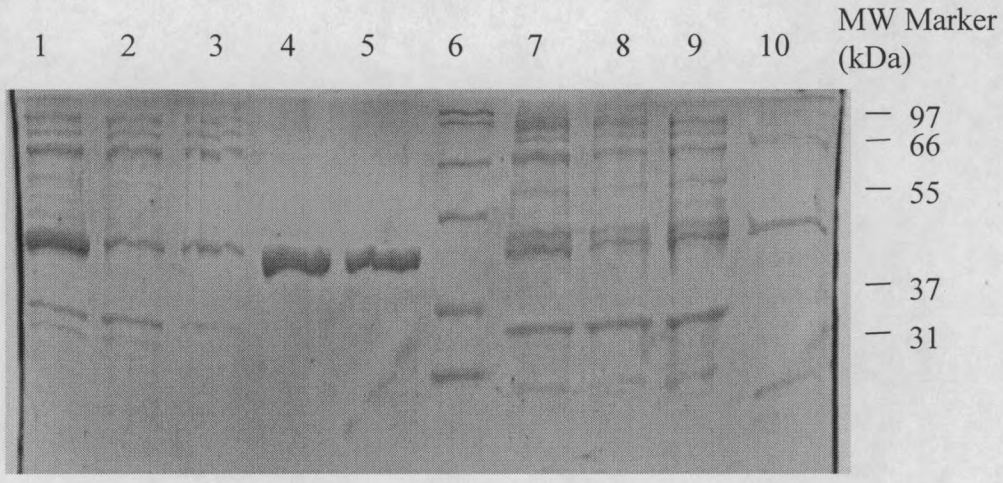
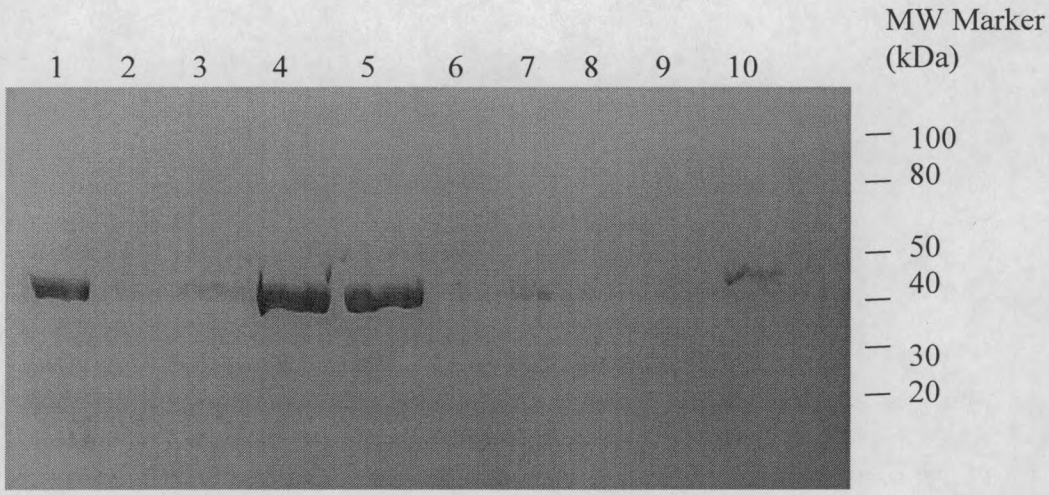


Figure 6 (B): Western Blotting. PVDF membrane of duplicate SDS-PAGE (above) showing proteins expressed that contain V5 epitope



The total amount of proteins loaded on the SDS-PAGE gel and consequently the PVDF membrane is 2.8ug. Lanes 1 and 7 contain the crude lysate from the induced (with IPTG)

and uninduced cultures. Lanes 2 and 8 have the flow-through proteins. Lanes 3 and 9 have proteins eluted at 50mM imidazole concentration. Lanes 4 and 10 have proteins eluted at 100mM and Lane: 5 eluted at 250mM only from the induced culture. Lane 6 is MW marker (Invitrogen)

Figure 7(A) and (B): Western Blot analysis of proteins from induced and uninduced cultures. All lanes contain the same amount of total protein ~5.0ug

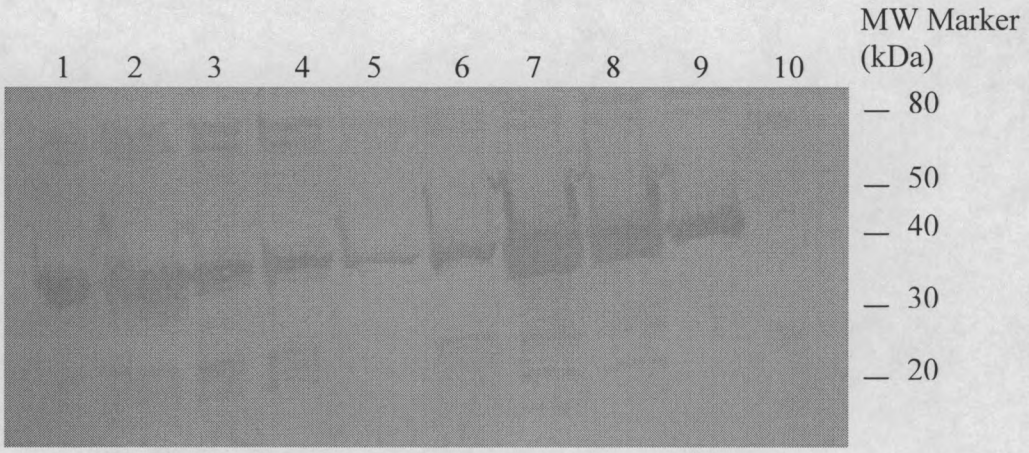


Figure 7 (A): Western Blot analysis of proteins from induced culture; Lane 1: Supernant, Lane 2: Proteins eluted from stripped agarose, Lane 3: Flow-through, Lane 4: Wash - 10mM, Lane 5: 20mM, Lane 6:50mM, Lanes 7&8: 100mM, Lane 9: 250mM, Lane10: Kaleidoscope MW marker

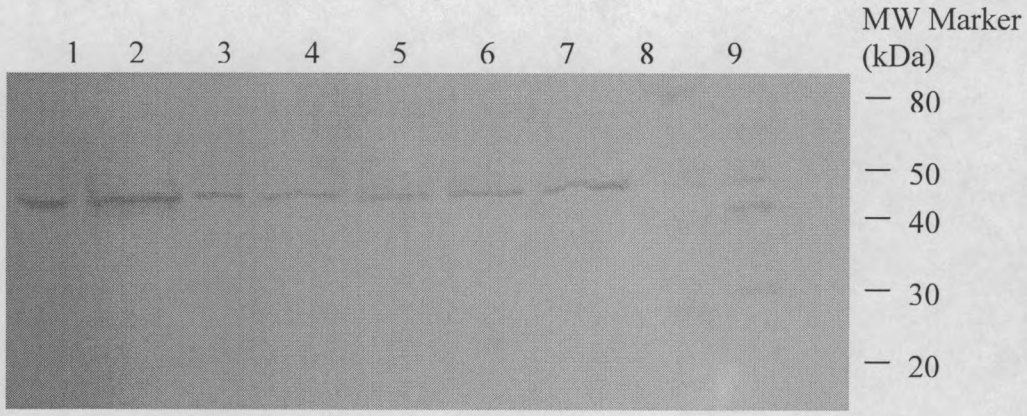
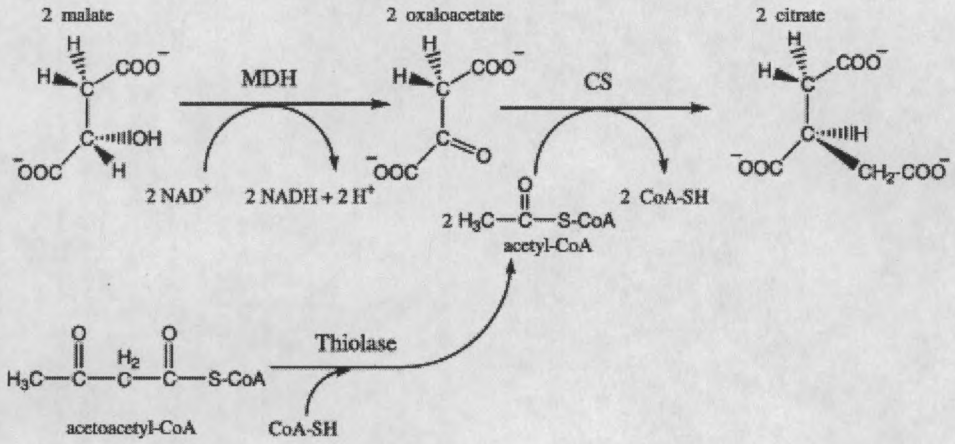


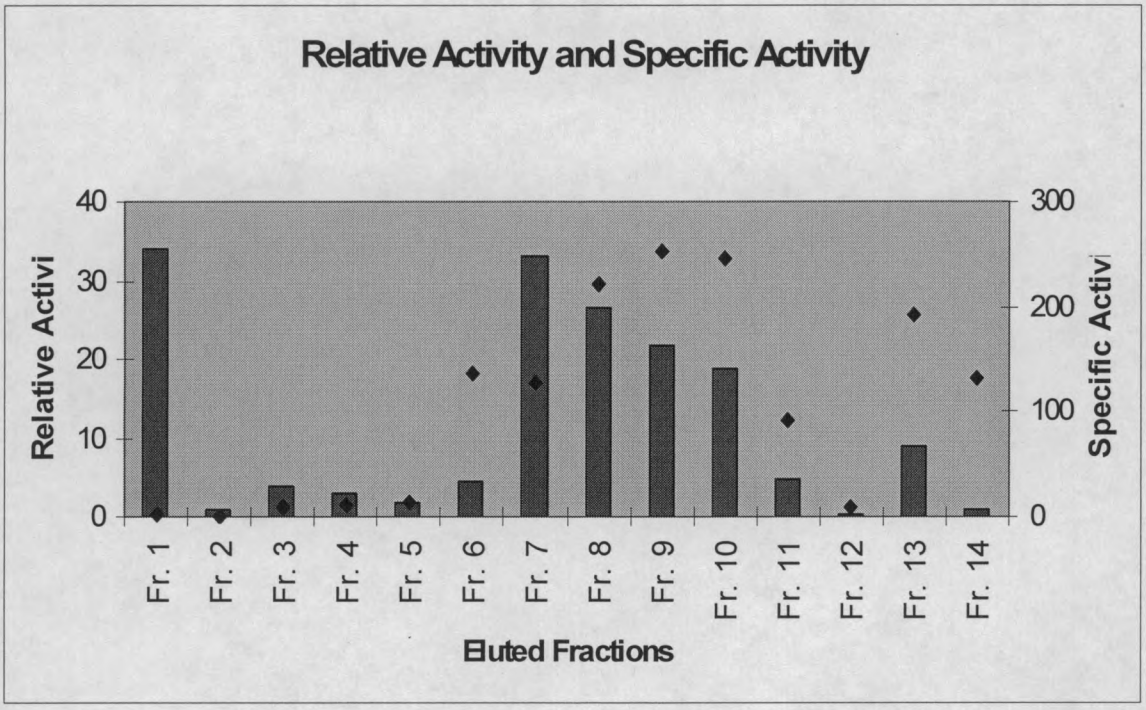
Figure 7(B): Western Blot analysis of proteins from uninduced culture; Lane 1: Supernant, Lane 2: Proteins eluted from stripped agarose, Lane 3: Flow-through, Lane 4: Wash - 10mM, Lane 5: 20mM, Lane 6: 50mM, Lanes 7: 100mM, Lane 8: Kaleidoscope ladder, Lane 9: western standards (Invitrogen)

Figure 8



Reactions involved in the assay used to determine thiolase activity. The assay uses a short sequence of the TCA cycle, namely the malate dehydrogenase (MDH) and citrate synthase (CS) reactions, to convert the product of the CoA-dependent thiolase reaction, acetyl-CoA, to citrate. Thereby, NADH is formed, which is spectrophotometrically monitored at 340 nm (Oeljeklaus et al., 2004).

Figure 9: Enzyme activity of thiolase eluted from Poly-Prep Bio-Rad mini-column. The bars represent the relative activity of the eluted proteins from the induced culture. The blue diamonds represent the relative specific activities of the eluted fractions.



Relative activity and relative specific activity from:
Fr. 1: the induced supernant
Fr. 2: the flow-through
Fr. 3: the wash at 10mM imidazole concentration
Fr. 4: eluted at 20mM
Fr. 5: eluted at 50mM
Fr. 6: eluted at 100mM – first fraction
Fr. 7: eluted at 100mM - second fraction
Fr. 8: eluted at 100mM – third fraction
Fr. 9: eluted at 100mM – forth fraction (Highest relative specific activity)
Fr. 10: eluted at 100mM – fifth fraction
Fr. 11: eluted at 100mM – sixth fraction
Fr. 12: eluted at 100mM – last fraction
Fr. 13: eluted at 250mM – first fraction
Fr. 14: eluted at 250mM – last fraction
Enzyme activities were carried out in triplicates. The standard deviations were negligible.
10µl were used from each 1ml fraction