Chapter 4: Synthesis of benzoyl-coenzyme A using recombinant biosynthetic enzymes

4.1 Introduction

Glycine N-acyltransferase uses coenzyme A esters as substrates. All investigations of the properties of GLYAT, whether it be to study substrate specificity, kinetic parameters, or the activity of mutants, depends on the use of these compounds as substrates. In the study of metabolic defects and detoxification, analysis of coenzyme A esters from clinical samples is being undertaken by our analytical laboratory. For this purpose coenzyme A esters are used as analytical standards. Our research is thus intimately dependent on a supply of these compounds. It is unfortunate that the commercially available esters of coenzyme A are very expensive, which imposes a limitation on the number of experiments that can be performed. One solution to the problem was to start to synthesise these compounds in-house from cheap starting materials. This chapter presents the synthesis of benzoyl-coenzyme A from pantetheine, using recombinant biosynthetic enzymes as the catalysts.

4.1.1 The function and synthesis of coenzyme A and its analogues

About 4% of enzymes use coenzyme A esters as substrates, including several medically important enzymes (Nazi et al., 2004). These enzymes fall into three broad categories, the acyltransferases (already discussed in this study), the Claisen enzymes (which involve deprotonation of the methyl group of acetyl coenzyme A to form a nucleophile), and enzymes that have functionality beyond the alpha carbon (such as β-oxidation enzymes). Coenzyme A dependent enzymes are involved in several fundamental biochemical reactions, including fatty acid biosynthesis and degradation, the citric acid cycle, synthesis of hormones, histone modification, and inactivation of antibiotics (Mishra & Drueckhammer, 2000).

The study of all these proteins requires coenzyme A esters as reagents or probes of enzyme function (Mishra & Drueckhammer, 2000, Nazi et al., 2004). These compounds are commonly either bought from a commercial supplier or synthesised using commercially obtained coenzyme A as the precursor. The coenzyme A itself is commonly isolated from yeast, but the large culture volumes that have to be processed and the extensive purification needed make this an inconvenient (and thus expensive) source. Given this situation, it is no surprise that several chemical and enzymatic syntheses of this complicated coenzyme have been developed.

4.1.2 Strategies for the synthesis of coenzyme A

The structure of coenzyme A, first determined in 1953, is shown in Figure 4.1 (Mishra & Drueckhammer, 2000). The structure consists of a 3'-phosphoadenosine moiety, connected by a pyrophosphate group to pantetheine. The challenges in chemical synthesis of this compound are the formation of the high energy pyrophosphate linkage and the regioselective 3'-phosphorylation. The first successful total synthesis of coenzyme A was reported in 1959. The pyrophosphate linkage was formed by coupling of an activated phosphomorpholidate derivative of 2',3'-cyclic ADP with phosphopantetheine to form 2',3'-cyclic coenzyme A (a cyclic phosphate). Acid catalysed hydrolysis then resulted in the formation of equal amounts of the 2'- and 3'-phosphates. Chromatographic purification resulted in an overall yield of 15% of the desired compound (Moffatt & Khorana, 1959).

The next advance was the use of an improved anhydride-anion exchange method in the synthesis. A mixture of 2',5'- and 3',5'-diphosphates of adenosine were treated with diphenylphosphorochloridate to form an intermediate that was then treated with pantethine 4',4'-bisphosphate to give the 2',3'-cyclic phosphate form of coenzyme A in high yields. Another advance of the method was to use the enzyme ribonuclease T2 to hydrolyse the cyclic phosphate. This resulted exclusively in the formation of the 3'-phosphate form of coenzyme A (Michelson, 1964). The enzymatic cleavage step was both expensive and inefficient, which limited the popularity of this approach.

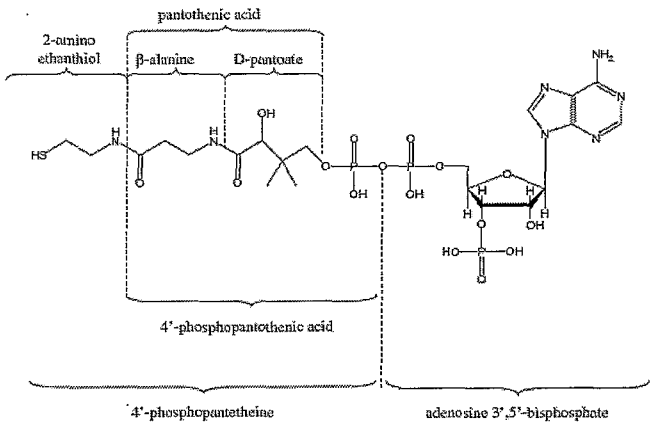


Figure 4.1 The structure of the coenzyme A molecule. The structure shows that the coenzyme A molecule is composed of three main parts, namely the adenosine moiety, a pyrophosphate linkage, and a pantetheine arm.

The first use of enzymes in the synthesis of coenzyme A was reported when crude cell extracts of *Brevibacterium ammoniagenes* (now called *Corynebacterium ammoniagenes*) were used as a catalyst for the synthesis of coenzyme A from L-cysteine, pantothenic acid and ATP (Shimizu et al., 1983). The yield was low, because of feedback inhibition on pantetheine kinase by coenzyme A itself (Figure 4.2). This problem was first bypassed by the use of mutants of the bacterium that are resistant to the toxic compound oxypantetheine, which have three fold more pantetheine kinase activity (Mishra & Drueckhammer, 2000). A more effective strategy involved the use of chemically phosphorylated pantothenic acid or pantetheine, which bypasses pantetheine kinase and its feedback inhibition (Shimizu et al., 1983).

A "one pot" chemoenzymatic synthesis of coenzyme A analogues from pantetheine derivatives and adenosine triphosphate using three recombinant biosynthetic enzymes from *Escherichia coli* as the catalysts has been reported (Nazi et al., 2004). This process is schematically represented in Figure 4.2. Stochiometric conversion of pantetheine derivatives to the respective coenzyme A analogues was achieved in under two hours. After purification of the products using column chromatography, yields of about 80% with respect to the pantetheine derivative were obtained (Nazi et al., 2004). This is the fastest, cleanest and most efficient method currently available for the preparation of coenzyme A analogues. Because purified recombinant enzymes are used for the synthesis, the product is less crude and thus easier to purify than when using a bacterial lysate as catalyst.

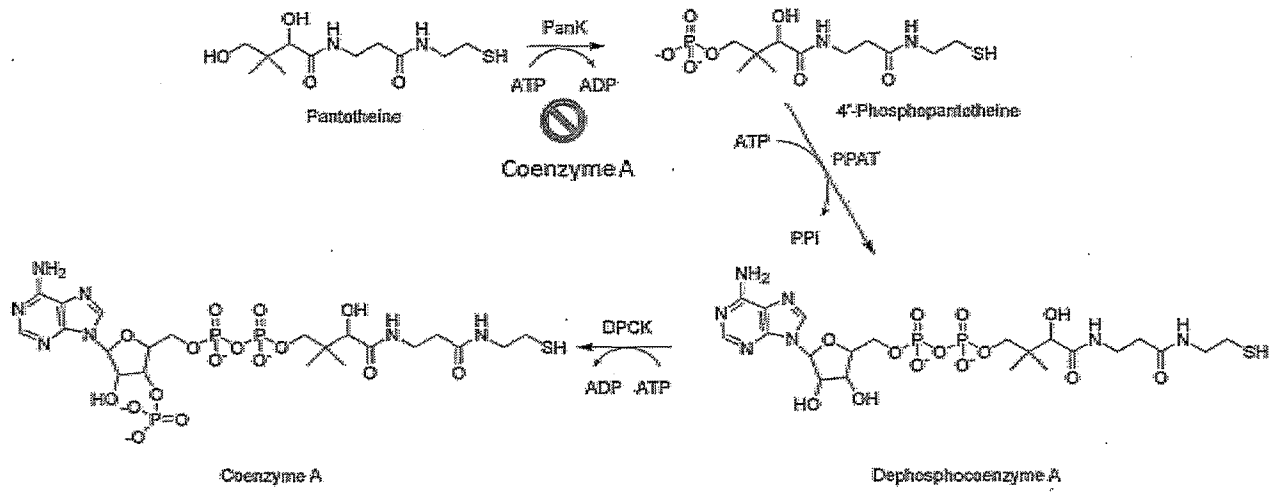


Figure 4.2 The biosynthesis of coenzyme A from pantetheine. The diagram shows the three enzymes, PanK, PPAT and DPCK, which are involved in the biosynthesis of coenzyme A from pantetheine. The symbol below PanK symbolises feedback inhibition on this enzyme by coenzyme A.

4.1.3 Objectives and experimental approach

The objective of this part of my study was to establish the system of Nazi and co-workers (2004) in our laboratory, for use in the synthesis of coenzyme A and its derivatives. The system would then be used to synthesise benzoyl-coenzyme A to facilitate our research, which is dependent on this expensive compound.

The three coenzyme A biosynthetic enzymes from *Escherichia coli*, pantetheine kinase (PanK), phosphopantetheine adenylyltransferase (PPAT) and dephospho-coenzyme A kinase (DPCK) had to be cloned and expressed in *Escherichia coli* cultures and purified for use in the enzymatic synthesis of coenzyme A derivatives.

In order to synthesise benzoyl-coenzyme A, benzoyl-pantetheine had first to be synthesised from pantethine and benzoic acid. The benzoyl-pantetheine would then be used as the starting material for the enzymatic synthesis of benzoyl-coenzyme A.

4.2 Materials and methods

4.2.1 Isolation of DNA from *Escherichia coli*

As template for the amplification of the coding sequences of the three biosynthetic enzymes, genomic DNA was isolated from *Escherichia coli* cells. To do this, the Genomic DNA purification kit from Fermentas was used. A 50 ml culture of LB medium was inoculated with a glycerol stock of *Escherichia coli* JM109 cells, and incubated at 37 °C with vigorous shaking for 12 hours. No antibiotics were included in the medium, as no plasmid to confer antibiotic resistance was present in the cells. Of the dense culture, 2 ml was placed in a centrifuge tube, and the cells were harvested by centrifugation at 10 000 g for one minute. The supernatant was discarded and the instructions of the kit followed in order to isolate the DNA. The cells were resuspended in a buffer, after which a cell lysis buffer was added to release the genomic DNA. A DNA precipitation buffer was then added to precipitate the DNA. The DNA precipitate was collected by centrifugation at 10 000 g for three minutes. The DNA was washed with 70% ethanol, dried, and resuspended in 500 µl of water.

4.2.2 PCR amplification of enzyme coding sequences

The coding sequences of the PanK, PPAT, and DPCK genes were PCR amplified from *Escherichia coli* genomic DNA using the primers listed in Table 4.1. The amplification reactions contained 1X Takara ExTaq buffer, 10 nmol of each dNTP, 25 pmol of each primer, 50 ng of template DNA, and 2 units of Takara ExTaq DNA polymerase. The final reaction volume was 50 µl. Thermal cycling was performed using an Eppendorf thermal cycler. The cycling conditions were 94 °C for 1 min, then 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute, followed by a final extension step of 10 minutes at 72 °C.

4.2.3 Cloning of enzyme coding sequences into pColdI

The PCR amplicons were cleaned up using the Machery Nagel Nucleospin II kit, as described in Section 2.2.7. The amplicons and pColdI DNA (isolated as described in Section 2.2.13), were digested with NdeI and HindIII (for PanK and DPCK) or KpnI and HindIII (for PPAT), as described in Section 2.2.6. Digested DNA was gel purified before use in ligation reactions, which were set up as described in Section 2.2.8. The ligation reactions were used to transform electrocompetent *Escherichia coli* (JM109) cells as described in Section 2.2.10. Transformants were screened for the desired recombinant plasmids, and plasmid DNA prepared for sequencing, as described in Sections 2.2.11 to 2.2.14.

Table 4.1: Oligonucleotide primers for amplification of PanK, PPAT, and DPCK

Primer	Oligonucleotide sequence (5' → 3')	Manufacturer
PanK5'	GGGAATTCCATATGACCGCCAGAAACATGCTTATGAG	Inqaba Biotech
PanK3'	CGCGGATCCAAGCTTTTATTTGCGTAGTCTGACCTCTTCTACCGC	Inqaba Biotech
PPAT5'	GTCCTCGAGGGTACCATGCAAAAACGGGCGATTTATCC	Inqaba Biotech
PPAT3'	CGCGGATCCAAGCTTCTACGCTAACTTCGCC	Inqaba Biotech
DPCK5'	GGGAATTCCATATGAGGTATATAGTTGCCTTAACGGGAG	Inqaba Biotech
DPCK3'	CGCGGATCCAAGCTTTTACGGTTTTTCCTGTGAGACAAACTGC	Inqaba Biotech

4.2.4 Expression of the coenzyme A biosynthetic enzymes in *Escherichia coli* JM109

The recombinant proteins were expressed in the *Escherichia coli* JM109 cells into which the plasmids had been transformed. Thus *Escherichia coli* JM109 cells were used both for cloning and expression of the enzymes.

Glycerol stocks were used to inoculate 150 ml cultures of LB medium containing 100 µg/ml ampicillin for plasmid selection. The cultures were grown overnight at 37 °C, with vigorous shaking. In the morning, the cells were harvested by centrifugation, and resuspended in 200 ml fresh LB medium containing 50 µg/ml ampicillin and incubated at 37 °C for one

hour. The cultures were then placed in a 15 °C incubator, with gentle shaking, for one hour. Expression of recombinant proteins was then induced by the addition of IPTG to a final concentration of 0.5 mM and incubation at 15 °C for four hours with vigorous shaking to ensure adequate aeration.

4.2.5 SDS-PAGE analysis of recombinant PanK, PPAT and DPCK expression and purification

Proteins were isolated using the BugBuster protein extraction reagent, as described in Section 2.2.16. The cells were harvested from the 200 ml culture by centrifugation, except for PPAT, where only 100 ml of culture was used. The cell pellet was then resuspended in 5 ml of BugBuster (prepared as in Section 2.2.16) and incubated at room temperature for one minute. To dilute the proteins 15 ml of ice cold Binding buffer (50 mM TrisCl, 300 mM NaCl, 20 mM imidazole, pH 7.9) was then added. The mixture was then incubated for a further five minutes on ice, before the insoluble material was separated by centrifugation at 12 000 g and 4 °C for thirty minutes.

The proteins were then purified by nickel affinity chromatography, using the His•Bind resin from Novagen (the principle was described in Section 1.6.3). Columns with a bed volume of 2.5 ml were packed and charged as described by the manufacturer. The clarified lysates were loaded onto the columns, and allowed to drain by gravity. The columns were then washed with 3 volumes of Binding buffer, followed by 4 volumes of Wash buffer. Proteins were eluted in 15 ml of Elution buffer. The PanK and DPCK enzymes were then dialysed at 4 °C against two litres of storage buffer (50mM Hepes, pH 8.0, 250mM NaCl, 2mM MgCl2), with one exchange of buffer. PPAT was not dialysed, but instead mixed immediately after elution with glycerol to a final concentration of 20% to prevent protein precipitation. Proteins were stored at 4 °C in this dilute form until needed.

When needed, a volume of each enzyme solution containing 5 mg of protein was concentrated to less than 1000 µl using Vivaspin ultra filtration membranes, as described in Section 2.2.17. This was done immediately before use in the enzymatic synthesis (please refer to Section 4.2.7). SDS-PAGE was used to monitor recombinant protein expression,

as described in Section 2.2.19. Protein quantification was carried out as described in Section 2.2.21.

4.2.6 Synthesis of S-benzoyl pantetheine

S-benzoyl pantetheine was synthesised using methods adapted from the literature. Firstly, the NHS ester of benzoic acid was synthesised (Lapidot et al., 1967). This was then used to acylate pantetheine, which was generated by reducing pantethine with DTT (Al Arif & Blecher, 1969). The procedure will be briefly outlined.

The N-hydroxysuccinimide ester of benzoic acid was synthesised as follows. Benzoic acid and N-hydroxysuccinimide (10 mmol each) were dissolved in 40 ml of ethyl acetate (freshly distilled) in a screw-top Erlenmeyer flask. An equimolar amount of dicyclohexyl-carbodiimide was dissolved in 10 ml ethyl acetate, and combined with the benzoic acid solution. The solution was thoroughly mixed by swirling the flask, and left to stand overnight at room temperature (in the dark). Dicyclohexylurea (the insoluble white crystals that form) was removed by filtration. The filtrate was dried under nitrogen to recover the white crystalline NHS-benzoic acid ester. The product was used in the next steps as is, without further purification.

The acylation of pantetheine to form S-benzoyl pantetheine was carried out as follows. Pantethine (42 mg), sodium bicarbonate (160 mg), and DTT (80 µmol) were dissolved in 3 ml of water, and left to stand for 10 minutes to allow reduction of the pantethine to pantetheine to take place. The NHS-benzoic acid ester (0.12 grams) was dissolved in 7 ml of tetrahydrofuran (THF). The THF was distilled over sodium borohydride before use, to remove peroxides that may have formed during storage. This was done by mixing 500 ml of THF with 0.2 grams of the reducing agent before transferring to the distillation apparatus.

The water and THF solutions were combined in a small glass bottle and maintained as a single phase by vigorous magnetic stirring for four hours. At this point 5 µl of the reaction mixture was removed and tested for the presence of free thiol groups. This was done using

the DTNB colour reaction for detection of sulfhydryl groups (Kolvraa & Gregersen, 1986). The 5 µl of sample is added to 495 µl of a 0.1 mM DTNB solution (in 100 mM TrisCl, pH 8.0). If the solution turned deep yellow, the reaction was left for another 30 minutes.

The reaction was transferred to a glass test tube and left to settle into two phases. The upper THF layer was then removed under a stream of nitrogen gas. The remaining water was removed by freeze-drying. To dissolve the benzoyl pantetheine, 0.5 ml of absolute ethanol was added to the tube, which was then heated to 50 °C in a water bath, with frequent shaking, for about five minutes. To this 5 ml of water was added, and the mixture was again heated to 50 °C, with frequent shaking. The mixture was left to cool, and if the solution became cloudy, more water was added in 2 ml volumes, and the process repeated. This procedure dissolves the S-benzoyl pantetheine while leaving excess NHS ester behind as a white residue on the walls of the tube. The solution is poured out, and the tube rinsed with another 5 ml of water. The water fractions were combined, and used without further purification in the enzymatic synthesis.

4.2.7 Enzymatic synthesis of benzoyl-coenzyme A

The enzymatic synthesis reaction mixtures contained 20 mM KCl, 10 mM MgCl2, 18 mM ATP, 50 mM TrisCl, pH 7.5 and 5 mM benzoyl-pantetheine in a final volume of 30 ml (Nazi et al., 2004). This reaction mixture was set up by combining the benzoyl-pantetheine solution prepared in the previous step with the other components and increasing the volume to about 25 ml. The pH was then adjusted to 7.5 before filling up the volume to 30 ml.

The reaction was initiated by addition of 5 mg of recombinant PanK and incubated at room temperature for 30 minutes. After 30 minutes 5 mg of recombinant PPAT was added, followed by another 30 minute incubation. Finally, 5 mg of recombinant DPCK was added, followed by a two hour incubation at room temperature. Gentle magnetic stirring was performed throughout the incubations. After completion, the reaction mixture was passed through a 3 ml column of His•Bind resin to remove the recombinant enzymes (Nazi et al., 2004).

4.2.8 Partial purification of coenzyme A using solid phase extraction

For purification and concentration of the benzoyl-coenzyme A solid phase extraction was used. Basically, a column packed with a resin on to which hydrophobic molecules such as C8 or C18 are immobilised is used to adsorb compounds from a solution. The column can then be washed with water to remove salts and very polar or ionic compounds. The desired compound can then be eluted from the column using methanol. If it is known at which methanol concentration the desired compound elutes, this information can be used to better purify the compound. The column can first be washed with a lower methanol concentration to elute the less hydrophobic molecules, after which the compound is eluted with the lowest possible methanol concentration. This elutes the compound of interest while leaving the more hydrophobic compounds on the column.

The reaction mixture was passed through a Zorbax XDB-C18 solid phase extraction column, which is packed with a C18 resin. The column was washed with three volumes of 18 Ω water, and the compound was then eluted with three volumes of 20% methanol. The eluate was placed under a stream of nitrogen to remove most of the methanol before being freeze-dried. The lyophilised compound was stored at -20 °C until needed.

4.2.9 HPLC-TOF analyses of the synthesis and purification of benzoyl-coenzyme A

HPLC-TOF analysis was used to monitor the synthesis and purification of benzoyl-coenzyme A and to accurately determine the concentration of benzoyl-coenzyme A in purified samples.

The analyses were performed using an Agilent 6210 Time-of-Flight LC/MS in extended dynamic range, coupled to an Agilent 1200 SL Series LC system. The LC system consisted of a binary pump, vacuum degasser, automatic liquid sampler, thermostated column compartment and MassHunter Workstation. Burdick & Jackson LC/MS grade acetonitrile and locally produced 18.1 Ω water were used for mobile phases.

Conditions for liquid chromatography will be briefly described. A Zorbax XDB-C18 column (4.6 mm x 50 mm, 1.8 µm) was used at 25 °C. Two buffers were combined in different proportions to make up the mobile phases. Buffer A contained 5 mM ammonium formate and 0.1% formic acid in water and Buffer B was composed of 0.1% formic acid in acetonitrile. A flow rate of 0.4 ml/min was used for a six minute analysis. A gradient program was used: the proportion of Buffer B used was initially 5%, kept at 5% for 1 minute, gradually increased to 100% over 15 minutes, kept at 100% for 10 minutes, and then decreased to 5% over three minutes. Sample injection volume was 1 µl.

The conditions used for time-of-flight mass spectrometry will be briefly described. Positive ionisation was used, with a nozzle voltage of 500 V. Drying gas temperature and flow rate were 320 °C and 8 L/min, respectively. A nebuliser gas pressure of 30 psi, capillary voltage of 3500 V and fragmentor voltage of 175 V were used. Reference ion masses of 121.050873 and 922.009798 were employed. A scan rate of 3 hertz was used. The MassHunter Qualitative Analysis program was used for molecular feature extraction and database searching.

4.3 Results and discussion

4.3.1 Cloning of the PanK, PPAT, and DPCK coding sequences into pColdI

The article by Nazi et al (2004) used the pET-28a vector for expression of the recombinant enzymes. Since I experienced problems cloning into this vector, the pColdI expression vector was used instead.

The agarose gel in Figure 4.3 shows the PCR amplicons for the PanK, PPAT, and DPCK coding sequences. Using NdeI and HindIII restriction enzymes for PanK and DPCK, and KpnI and HindIII restriction enzymes for PPAT, the PCR amplicons and pColdI vector were digested. After ligation and transformation, colonies were screened using restriction enzyme digestion. Plasmids extracted from positive clones were sequenced using the pCold primers (Table 2.2) to confirm that the genes were cloned without any sequence

aberrations. The sequences obtained for the three enzymes were identical to the reference sequences (GenBank accession numbers: gi 226957607 for PanK, gi 15804175 for PPAT and gi 91209166 for DPCK).

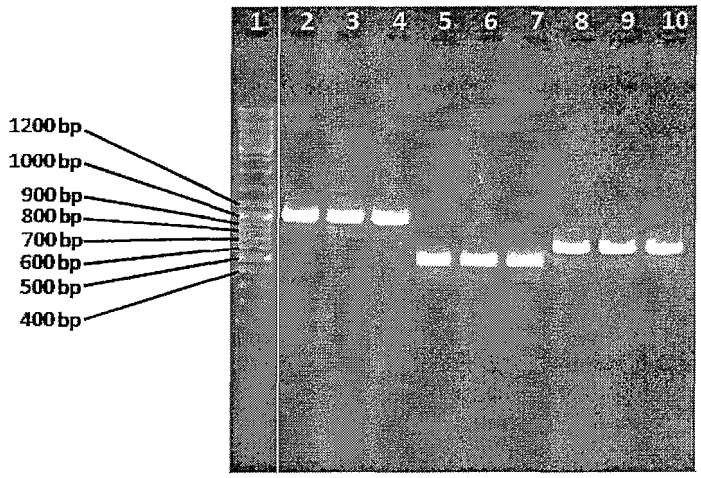


Figure 4.3 Agarose gel electrophoresis of PanK, PPAT and DPCK PCR amplicons. Lanes: 1) 5 µl of O'GeneRuler DNA marker; 2-4) PanK amplified with an annealing temperature of 50 °C, 55 °C and 60 °C respectively; 5-7) PPAT amplified with an annealing temperature of 50 °C, 55 °C and 60 °C respectively; 8-10) DPCK amplified with an annealing temperature of 50 °C, 55 °C and 60 °C respectively.

4.3.2 Expression of the recombinant PanK, PPAT, and DPCK from pColdI

The three biosynthetic enzymes were expressed as described in Section 4.2.4. Since the recombinant proteins were expressed in the host from which the coding sequences were obtained, the proteins were expressed to high levels in soluble form (results not shown).

4.3.3 Purification of recombinant enzymes using His•Bind resin

The recombinant enzymes were purified by His•Bind nickel affinity chromatography, as described in Section 4.2.5. Purification of the recombinant PanK and DPCK enzymes was straightforward, but precipitation of the PPAT enzyme was a problem at two points in the purification process.

The PPAT enzyme was difficult to purify, because it tended to precipitate very easily. In order to slow down protein precipitation, the protein concentration of the bacterial lysate

was decreased by adding 15 ml of Bind buffer to the 5 ml of BugBuster cell lysate. This slowed down precipitation, but not sufficiently. When the amount of bacteria used for purification was halved by using only 100 ml of culture instead of 200 ml, easy flow of the lysate through the column was achieved without protein precipitation.

After elution, the enzymes were dialysed against a storage buffer (Section 4.2.5). Again the PPAT enzyme precipitated during this step, despite the PPAT concentration being kept below 2 mg/ml (Nálezková et al., 2005). This effect was repeatedly found, despite precipitation of PPAT not being reported by the Nazi group (Nazi et al., 2004). The solution was to keep the enzyme in the elution buffer and adding glycerol to a final concentration of 20%. Under these conditions the enzyme was soluble for several weeks at 4 °C.

The three purified enzymes were visualised on a SDS-PAGE gel (Figure 4.4). The proteins were well purified, with negligible co-purification of other proteins. The average yields from a 200 ml culture of bacteria, after purification, were approximately 20 mg of PanK and 20 mg of DPCK. Approximately 24 mg of PPAT was usually obtained from a 100 ml culture.

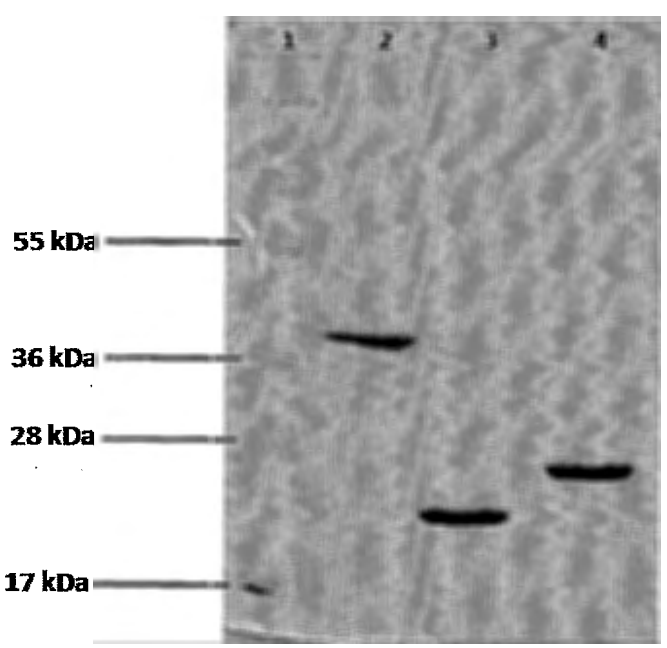
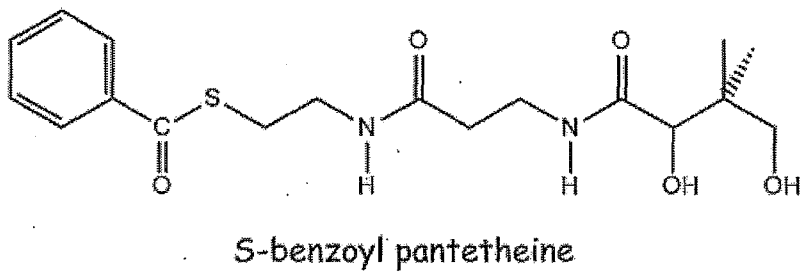


Figure 4.4 SDS-PAGE analysis of purified PanK, PPAT and DPCK. Lanes: 1) 5 µl of PageRuler protein marker; 2) His•Bind purified PanK; 3) His•Bind purified PPAT; 4) His•Bind purified DPCK.

4.3.4 Synthesis of S-benzoyl pantetheine

For the synthesis of benzoyl-coenzyme A, the pantetheine derivative benzoyl-pantetheine was used. The synthesis of this compound is briefly discussed.

The structure of S-benzoyl pantetheine is shown in Figure 4.5. It appears, from absence of literature, that this compound has not been synthesised from benzoic acid and pantetheine before. From the structure of the compound, it appears that it should be water soluble, due to its polar nature. In the development of the synthesis, it was attempted to extract the water phase with ethyl acetate, after the THF had been removed under a stream of nitrogen. It was thought that the benzoyl-pantetheine would remain in the water phase, while excess NHS ester and other organic residues would be extracted. However, after using the extracted water phase for the enzyme synthesis, it was found that only about 5% of the expected yield of benzoyl-coenzyme A was obtained. The absence of benzoyl-pantetheine and the other intermediates of the synthesis (Figure 4.2) indicated that there was no problem with the enzymatic synthesis and suggested that there was only a small amount of benzoyl-pantetheine to start with (HPLC-TOF results not shown). It turned out that the benzoyl-pantetheine preferentially enters the organic phase and that extraction with ethyl acetate removed the benzoyl-pantetheine from the water phase. Similarly, S-benzyl-pantetheine, a related compound, is soluble in water saturated butanol and can be extracted from water with chloroform (Walton et al., 1954).



S-benzoyl pantetheine

Figure 4.5 The chemical structure of S-benzoyl-pantetheine.

Subsequently, another approach was used. After removing the THF phase under a stream of nitrogen, the water was removed by freeze-drying. If 0.5 ml of absolute ethanol was added to the residue and the mixture heated to about 50 °C, some residue started to

dissolve. By adding 5 ml of water, and heating this mixture to 50 °C, more residue is dissolved. It was found that most residue benzoyl-pantetheine dissolves under these conditions. Less water soluble compounds, such as excess NHS ester of benzoic acid, are left behind on the walls of the glass tube. No standard was available for quantification of the benzoyl-pantetheine, but HPLC analysis indicated that the mixture contained high amounts of benzoyl-pantetheine, with only two unidentified contaminating peaks (results not shown).

Since enzymes, which are specific in the reactions they catalyse, are used for the subsequent synthesis of benzoyl-coenzyme A, small amounts of contaminating compounds in the benzoyl-pantetheine preparation would not interfere. For this reason the benzoyl-pantetheine synthesised did not need to be purified before use in the enzymatic synthesis, preventing losses due to inefficient purification. The synthesis of benzoyl-pantetheine was thus developed as a simple and convenient single tube protocol without any complex purification procedures.

4.3.5 Enzymatic synthesis and purification of benzoyl-coenzyme A

To synthesise benzoyl-coenzyme A, the three recombinant biosynthetic enzymes were expressed and purified, and benzoyl-pantetheine was synthesised for use as the pantetheine analogue. The enzymatic synthesis was then carried out as described in Section 4.2.7. To purify the synthesised benzoyl-coenzyme A, solid phase extraction (SPE) was used.

After the synthesis as described in Section 4.2.7 was completed, HPLC-TOF analysis showed that neither benzoyl-pantetheine nor any of the intermediates of the synthesis (Figure 4.2) were present in the reaction mixture. Furthermore, the amount of benzoyl-coenzyme A in the reaction mixture was what is theoretically expected, based on the amount of pantethine started with (Section 4.2.6). This indicated stochiometric conversion of benzoyl-pantetheine to benzoyl-coenzyme A.

HPLC analysis, as described in Section 4.2.9, was used to determine optimal methanol concentration for purification of benzoyl-coenzyme A. By eluting the HPLC column with a gradient of methanol in water, it was determined that benzoyl-coenzyme A elutes at 20% methanol (results not shown). Using this information the benzoyl-coenzyme A could be purified easily. The synthesis mixture was passed through a solid phase extraction column, as described in Section 4.2.8. The column could then be washed with water to remove salts and polar compounds such as ATP, ADP, and Tris buffer. The benzoyl-coenzyme A was then eluted with methanol at a minimal concentration of 20%. This eluted the benzoyl-coenzyme A, but left the more tightly binding contaminants on the column. These could later be removed by regenerating the column with a 100% methanol wash (Figure 4.6). The column flow through and column wash fractions contained only small amounts of benzoyl-coenzyme A (peaks are not visible on the chromatograms in Figure 4.6). The purified compound was lyophilised as described in Section 4.2.8. The benzoyl-coenzyme A powder (usually about 110 mg of powder was obtained) was dissolved in water to approximately 100 mg/ml, and aliquoted into 250 µl volumes. Since the compound was not 100% pure (Figure 4.6), the mass of the powder could not be used to determine the amount of benzoyl-coenzyme A obtained.

Rather, a sample of the benzoyl-coenzyme A was diluted to approximately 0.5 mg/ml for analysis. A commercial standard was also diluted to 0.5 mg/ml. Both the synthesised sample and the commercial standard were analysed by HPLC-TOF, as described in Section 4.2.9. Because the benzoyl-coenzyme A could be well resolved as a narrow peak containing only benzoyl-coenzyme A and no contaminating compounds (Figure 4.6), the areas and ion-intensities of these peaks could be integrated and used to compare concentrations. The concentration of benzoyl-coenzyme A in the sample could be calculated by comparing the relative ion abundance readings from the TOF analysis to that of the commercial standard (at the low concentrations analysed, the relationship between concentration and ion abundance is linear).

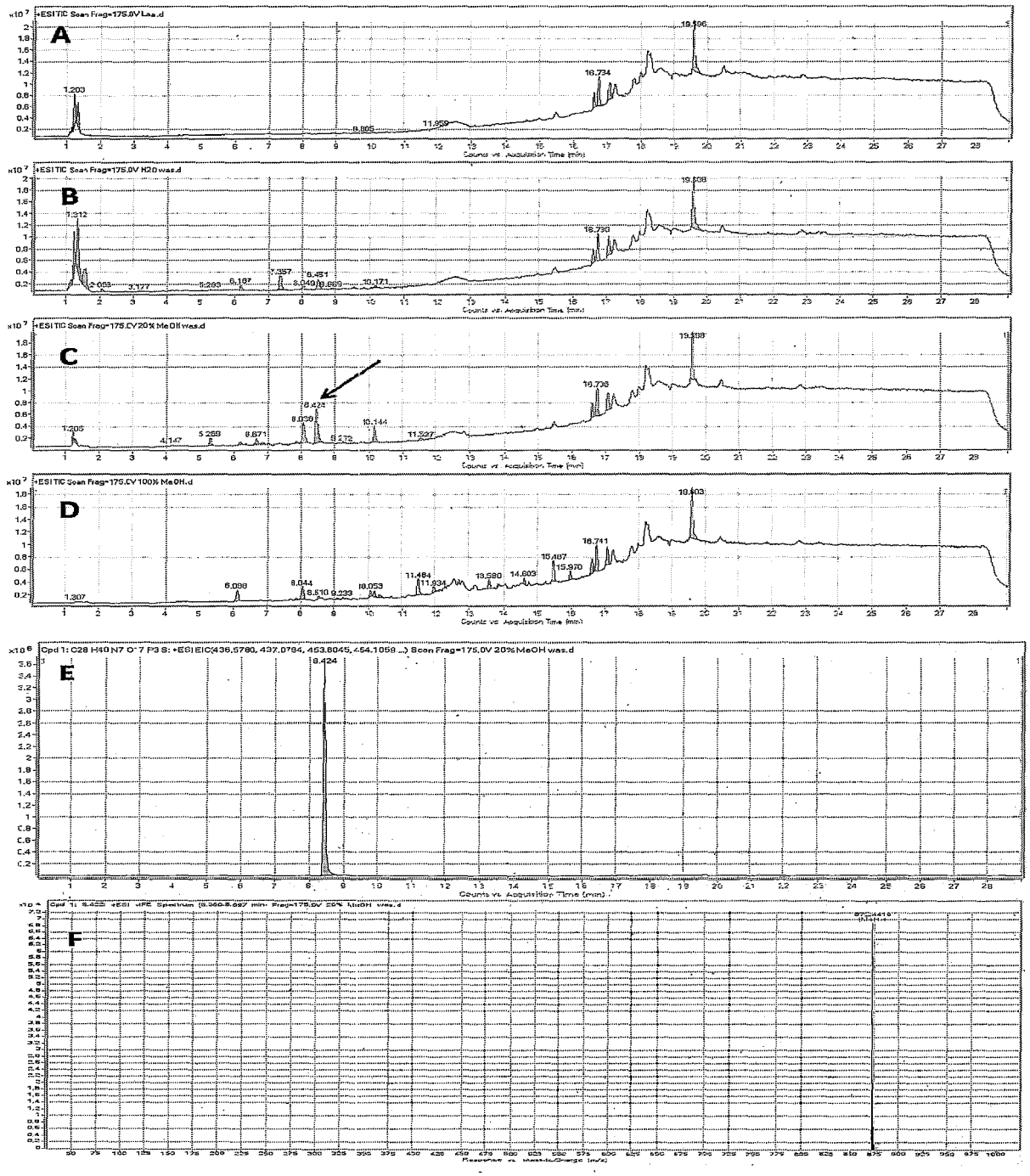


Figure 4.6 HPLC-TOF analysis of the purification of benzoyl-coenzyme A. A) chromatogram showing the flow through of the column loading step; B) chromatogram showing the water wash fraction; C) chromatogram showing the 20% methanol wash fracion; D) a chromatogram showning the 100% methanol wash fraction; E) a chromatogram showing only the benzoyl-coenzyme A peak; F) the mass spectrum of the peak in (E) showing the benzoyl-coenzyme A species with an accurate mass of 872.441.

The 250 µl aliquots of benzoyl-coenzyme A were again freeze-dried, and could be stored at -20 °C until needed. Based on the exact concentration determined using the HPLC-TOF analysis, the volume of water needed to make up the lyophilised samples to 10 mg/ml could be calculated. Approximately 85 to 100 mg of benzoyl-coenzyme A was usually obtained for a synthesis. This is a yield of approximately 70% to 85%, which is acceptable. The synthesised benzoyl-coenzyme A could be used for enzyme assays, and in this sense is indistinguishable from the commercially obtained compound.

4.4 Summary

The work presented in this chapter was done in order to establish the coenzyme A biosynthesis system of Nazi and co-workers (2004) in our laboratory. The system was successfully used to synthesise benzoyl-coenzyme A. This presents a significantly cheaper alternative to commercial coenzyme A, and makes it affordable to do large numbers of enzyme assays. The exact saving is difficult to calculate, but it appears that the synthesis is about two orders of magnitude cheaper than buying the commercial compound.

An HPLC-TOF analysis was developed for determination of benzoyl-coenzyme A from the synthesis reaction, which allowed the progress or success of a synthesis to be easily assessed. The benzoyl-coenzyme A was resolved as a pure peak (Figure 4.6). The yield of benzoyl-coenzyme A after purification varied from one synthesis to the next, but a yield of higher than 75%, based on the amount of pantetheine started with, was usually obtained.

The methods used in this part of my study were all obtained from the literature, and no part of the synthesis reported here is completely new (Al Arif & Blecher, 1969, Lapidot et al., 1967, Nazi et al., 2004). It appears, however, that this was the first synthesis of benzoyl-coenzyme A from benzoyl-pantetheine. We are currently writing an article for publication about this simple, efficient synthesis of benzoyl-coenzyme A from benzoic acid, pantetheine and ATP. Doing the synthesis via this route, instead of first synthesising coenzyme A and then acylating with benzoic acid, has advantages. Firstly, the pantethine reduction step and acylation with benzoic acid are done in one reaction mixture, which results in minimal loss of material. Further, the excess DTT used for reduction of

pantethine is inactivated in this reaction (by the excess of NHS ester of benzoic acid). It was thus not needed to purify the benzoyl-pantethine before doing the enzymatic synthesis, further avoiding loss of material. Finally, the benzoyl-coenzyme A synthesised is less susceptible to oxidation and more easily purified than un-acylated coenzyme A.

The system for synthesis of coenzyme A derivatives can be used to synthesise a variety of compounds, as shown by Nazi and co-workers, and further demonstrated by the synthesis of benzoyl-coenzyme A from benzoyl-pantetheine (Nazi et al., 2004). The system enables the simple synthesis of coenzyme A derivatives from the corresponding pantetheine analogue. Complex coenzyme A derivatives, such as bisubstrate-analogues of reaction intermediates, can thus be made by starting with the correct derivative of pantetheine. As an example, benzyl-pantethine could be synthesised, and used to synthesise benzyl-coenzyme A. This is an analogue of benzoyl-coenzyme A without the carbonyl group. The analogue has a similar structure to benzoyl-coenzyme A, but cannot be hydrolysed. This could be a powerful inhibitor of the GLYAT reaction. If such a compound is immobilised to a resin, a powerful affinity-purification column for the purification of GLYAT could be produced. This could facilitate the purification of large amounts of the enzyme from bovine liver, which could make protein crystallisation for structure determination feasible. The development of such a purification system will be attempted in the future.