Thermophilic bacillus strain.

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The ability to hydroxylate steroids is not common amongst bacilli, but a thermophilic strain hydroxylates progesterone to form both the 6a and 6b derivatives. The hydroxylation products formed at 55°C were extracted, purified by TLC and HPLC and identified by 1HNMR. The hydroxylation was inhibited by ketoconazole and gave a reduced CO spectrum peak at 449 nm and a progesterone binding spectrum typical of cytochrome P-450. The enzyme was purified over 2000 fold in four steps including affinity chromatography with a hydroxyprogesterone ligand. The 58 KDa protein (pl = 4.65) was used to raise antibody. The N-terminal sequences of cyanogen bromide partial cleavage were used to design redundant oligonucleotide probes. Both antibody and probes are being used for screening gene libraries in an expression vector.

Cytochrome P-450 can catalyse the monooxygenation of a variety of exogenous hydrophobic substances including steroids (1). Stereoselective hydroxylations and other conversions by microorganisms are widely used during the synthesis of steroids (2). These enzymes have been identified and isolated from both eukaryotes and prokaryotes including mesophilic bacilli (3-5).

The thermophilic bacilli most of which were once allocated to Bacillus stearothermophilus are now known to be much more diverse (6). The steroid hydroxylase from this thermophile is a robust protein that would survive purification and storage. The coupling of such a thermostable protein for continuous flow hydroxylation becomes a possibility.

We have screened strains of thermophilic bacilli from culture collections and from soil samples, and find that the ability to hydroxylate steroid is uncommon (7). But a strain of thermophilic Bacillus was found to hydroxylate progesterone when incubated for 18 h at 55°C. Transformation products were analysed by TLC and HPLC as previously described (8), and structural analysis was by 1HNMR (9). The progesterone was transformed to 4 metabolites; androstenedione, testosterone, 6b- and 6a-hydroxyprogesterone. These products accounted for 25% of the extracted steroids. The 6-hydroxylase were always present in equal amounts.

Ketoconazole which is a known cytochrome P-450 inhibitor, inhibited the production of the 6-hydroxy-derivatives, but not the other activities. Further evidence for the hydroxylation in this organism being cytochrome P-450 mediated was the production of a characteristic P-450 spectra with cell-free extracts from sonically disrupted cells, with a peak at 448 nm. The extract also produced characteristic substrate binding spectra with a peak at 393 nm in the presence of progesterone.

These cell-free extracts transformed progesterone in the presence of sodium periodate and NADPH but not NADH. The periodate activity had an optimum temperature of 50°C and optimum pH of 8.0 in 50 mM Tris.Cl and 1 mM EDTA buffer.

Before fractionation of the extract, DNA was removed by streptomycin sulphate precipitation. The extract was loaded onto a DEAE-Sephacel column and the 6b progesterone hydroxylase was eluted with 0.2 M NaCl in TE buffer. This was concentrated down by ultrafiltration and loaded onto a Sephadex G-200 column. Activity was eluted from the calibrated column just after the elution point of bovine serum albumin.

The final step of purification involved fractionation on affinity column which was made by the coupling of Sepharose 6B to 11α-hydroxyprogesterone (10). The hydroxylase activity was eluted with 0.1 M NaCl in the initial batch elution. A second elution this time using a salt gradient (0.050 - 0.10 M NaCl) produced activity at about 0.08 M NaCl.

This fraction hydroxylated progesterone in the 6b position and appeared as a discrete band on silver stained SDS-PAGE and Coomassie stained IEF gels. When the protein (M, = 58 kDa, pl = 4.65) was reduced with sodium dithionite and bubbled with carbon monoxide a characteristic cytochrome P-450 spectrum was observed with a maximum at 449 nm.

The purified protein was used to raise antibody in a rabbit, but this was not specific to the P-450 in the original thermophilic Bacillus strain. There was also cross-reactivity with proteins from other thermophilic Bacillus strains that possess progesterone transformation activity (7). These proteins have molecular weights of between 50-60 kDa. The molecular weight for bacteria P-450s are usually around this region with the exception of the 119 kDa fatty acid hydroxylase found in B. megaterium (4). The possibility that these proteins are P-450 cannot be discounted due to the vast range of substrate specificity of the cytochrome P-450.

The determination of an N-terminal sequence was by the cyanogen bromide technique (11). Three major fragments resulted and when analysed gave the sequence:

Band A Leu Ala Val Ala Arg Ala Glu Leu His
Band B Tyr Phe Gln Glu Ser Ile Phe Leu Val
Band C Tyr Phe Gln Glu Ser Ile Phe Leu Val
Sequence B and C allow the design and synthesis of the following redundant oligonucleotide probe:

5‘ TAY TTY CAR GAR WSN AT

This probe is being used to test libraries that are currently being screened by immunoblotting.

The P-450 described is the first thermostable P-450 reported. It has an optimal temperature of 50°C compared to around 20°C for other bacilli (3,4). The other characteristics of this protein are not so unusual. The molecular weight for cytochromes P-450 in prokaryotes is usually 50-10 kDa and the pl values are around 4-9, both of which are close to those obtained here.

Although the sequence obtained from the purified enzyme did not match any already reported, the cloned gene should produce a more comprehensive sequence for comparisons with those already published.

References
7. Odafe Sideso unpublished work
9. Smith et al. (1992) FEMS Lett. 92, 29-34