ACTIVITY ON BILE ACIDS OF A *CLOSTRIDIUM BIFERMENTANS* CELL-FREE EXTRACT

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1. Introduction

The 7α-dehydroxylation of primary bile acids is one of the main transformations performed by intestinal microorganisms. In fact a high proportion of the bile acids are 7α-dehydroxylated during their passage through the human gut [1]. Microbial 7α-dehydroxylases of bile acids have been scarcely studied. Only Aries and Hill [2] obtained a cell-free enzyme preparation able to dehydroxylate both cholic or chenodeoxycholic acid from eight strains of *Bacillus fragilis, Clostridium welchii, Clostridium sporogenes, Escherichia coli* and *Streptococcus faecalis.*

This paper reports the results of the investigations performed on the soluble fraction of a cell-free enzyme preparation of *Clo. bifermentans,* previously isolated by us [3] from human faeces, able to 7α-dehydroxylate cholic acid. Through electrophoresis a partial purification was obtained and 7α-dehydroxylase activity on cholic acid was separated from the dehydrogenase one.

2. Materials and methods

Clostridium bifermentans SD 10 was cultivated in Todd Hewitt Broth Oxoid (T.H.) at pH 7.2. The cultures were incubated at 37°C in a Vuotomatic Bicasa TN 50 under N_2 and CO_2 (9:1) atmosphere. For cell-free extract preparation, *Clo. bifermentans* was grown in 6 litres of T.H. medium containing 0.01% of sodium deoxycholate. The cells, harvested at forespore stage after 13 h of incubation, were washed in phosphate buffer 0.02 M (pH 7.0) by centrifuging three times. Approximately 30 g of washed cells (wet wt) were then crushed in an Aminco French Pressure Cell (American Instr. Co. Inc. Washington) at 0–4°C. The disrupted cells, treated with (mg) 0.5% L-cysteine HCl (pH 7.0), were incubated with 100 μg deoxyribonuclease (Mann Research Lab. N.Y.) for 15 min at 0°C. The crude extract was then clarified by centrifuging at 23 000 × *g* for 30 min. The supernatant solution was again centrifuged twice more at 38 000 × *g* for 30 min and then purified at 100 000 × *g* for 30 min, in a Spinco L50 Ultracentrifuge, in order to separate the soluble fraction. The total protein content of this soluble fraction was determined by the Warburg and Christian method [4]. For preventing the enzymatic preparation from O_2 inactivation, all steps were performed by flushing with N_2, O_2-free. The 7α-dehydroxylase activity was kept under these conditions for about eight days at -3°C.

The reaction mixture for the enzymatic assay contained: 1 ml of the soluble fraction (30 mg protein/ml), 1.5 ml of phosphate buffer 0.02 M (pH 7.0) and 0.5 ml of a 0.3% solution of bile acid sodium salt in order to obtain a final substrate concentration of 0.05%. The reaction mixture was incubated under N_2 at 37°C and then extracted with methylene chloride/ethyl acetate (1:1, v/v) and subjected to thin-layer chromatography on silica-gel plates DC Merck, 0.25 mm, dried at room temperature. The developing solvent system was benzene/ethyl acetate/acetic acid/H_2O (46:40:10:4, v/v/v/v). Spots were revealed by spraying with sulphuric acid/H_2O (1:1, v/v). The chromatographic method of Eneroth [5] was also employed. In order to separate 3α, 12α-dihydroxy-5β-chol-6-en-24-oic acid from the deoxycholic acid, silica-gel plates treated with $AgNO_3$ were employed and developed with acetic acid/ethyl acetate/

cyclohexan (9:69:21, v/v/v). For a quantitative evaluation of the spots a densitometer Vitatron TLD 100 was used.

Bile acid products were separated as methyl esters on a chromatographic column as previously described [6] or as free acids on a preparative thin-layer chromatogram (silica-gel H Merck, 0.5 mm). An infracord spectrophotometer (Perkin-Elmer Ltd.) was used to obtain infrared spectra (in Nujol). Mass spectra were recorded on a LKB model 9000 spectrometer.

The soluble fraction was subjected to a preparative electrophoresis according to the method proposed by Davis [7] and modified by Marchesini (personal communication). The gel was prepared with 8% polyacrylamide. The working buffer was Tris–glycine 0.1 M (pH 8.6). The glass cylinder (diameter 2 cm, height 8 cm) containing the polyacrylamide gel was filled with the running buffer. The samples (50–100 mg total protein after saturating with N_2) were layered on the top of the gels. For the anodic run, electrophoresis was performed for 90 min at 4°C and the power 220 V × 4 mA was found to be optimum for the separation of enzymatic activities. The portion of the gel incorporating the yellow-brown bands were cut off (5 mm thickness) from the rest of the gel with a razor blade and then cut into pieces, under N_2, in a small beaker containing 0.5 ml of buffer phosphate 0.01 M (pH 7.0). After allowing the gel portions to stand for 30 min at room temperature, they were centrifuged and the liquid supernatant used for enzymatic assays.

3α, 12α-Dihydroxy-7-oxo-5β-cholan-24-oic acid was prepared as described by Fieser and Rajiagopalan [8], 7α, 12α-dihydroxy-3oxo-5β-cholan-24-oic acid respectively by Sihn [9], 3α, 7β, 12α-trihydroxy-5β-cholan-24-oic acid by Samuelsson [10], 3α, 12α-dihydroxy-5β-chol-6-en-24-oic acid by Kagan and Jacques [11].

3α, 7α, 12α-Trihydroxy-5β-cholan-24-oic acid was supplied by N.B.C. Cleveland, Ohio, USA, 3α, 7α-dihydroxy-5β-cholan-24-oic acid by Applied Science Laboratories Inc., State College, USA, 3α, 12α-dihydroxy-5β-cholan-24-oic acid and 3,7,12-trioxo-5β-cholan-24-oic acid Ikapharm, Ramat-Gan, Israel. Taurocholate and 3α, 12α-dihydroxy-3, 12-dioxo-5β-cholan-24-oic acid was supplied by Serva Feinbiochemica, Heidelberg, FRG and glycocholate by Calbiochem, Lucerne, Switzerland.

3. Results

The chromatographic analysis of the reaction mixtures in the presence of sodium deoxycholate already revealed the formation of deoxycholic acid after 15 min of incubation; after 4 h the maximum rate of transformation was observed. At this time the thin-layer chromatographic analysis revealed a spot with R_F = 0.63 corresponding to deoxycholic acid; cholic acid (R_F = 0.30) and deoxy-7-oxo-cholic acid (R_F = 0.45) were also detected. Traces of other metabolic products, probably corresponding to 7α, 12α-dihydroxy-3-oxo-5β-cholan-24-oic acid (R_F = 0.51), 3,12-dioxo-5β-cholan-24-oic acid (R_F = 0.73) and 3,7,12-trioxo-5β-cholan-24-oic acid (R_F = 0.70) were also revealable.

7α-Dehydroxylase activity was found in *Clo. bifermentans* cells grown in the presence of sodium deoxycholate, between pH 6.0 and pH 8.5. This activity occurred in the enzymatic reaction mixtures between pH 5.5 and pH 9 with an optimum between pH 7.0 and pH 8.5 and at a temperature range from 3–50°C. The 7α-dehydroxylase activity was inhibited by incubating the cell-free preparation with sodium deoxycholate at a concentration higher than 0.4%. It should be noted that the deoxycholic acid appeared also when the reaction mixtures were incubated in the air.

7-Epicholic acid was still not transformed by the enzymatic extract even after 24 h of incubation, while deoxy-7-oxo-cholic acid was reduced into cholic acid.

By incubating the same soluble fraction with 0.01% and 0.05% chenodeoxycholic acid, respectively, only a few traces of litocholic acid were formed, corresponding to about 5% of the amount of deoxycholic acid obtained from cholic acid. The same result was shown in the experiments performed with an enzymatic extract obtained from cells grown in the presence of chenodeoxycholic acid (0.01% or 0.05%).

When the enzymatic extract was incubated in the presence of 3α, 12α-dihydroxy-5β-chol-6-en-24-oic acid, a formation of deoxycholic acid was revealed by $AgNO_3$ thin-layer chromatography.

Taurocholate and glycocholate were rapidly deconjugated by the 38 000 × *g* supernatant cell-free preparation and the free cholic acid was immediately 7α-dehydroxylated.

A preparative electrophoresis of the soluble enzy-

matic preparation was performed, which allowed the separation of three bands (A, B, C) corresponding to a run of 8 mm, 16 mm, and 20 mm. These fractions were dissolved in phosphate buffer and separately incubated in the presence of sodium deoxycholate at 0.05% under N_2. The fraction which remained at the start was tested under the same conditions. The 7α-dehydroxylase activity was detected only in the fraction which remained at the start. Fractions A and B proved to be able to dehydrogenate cholic acid, while no activity was present in fraction C.

4. Discussion

The soluble fraction of a cell-free enzyme preparation, obtained from *Clo. bifermentans* grown in the presence of cholic acid, was demonstrated to be able to transform cholic into deoxycholic acid. This 7α-dehydroxylase activity does not occur when the hydroxyl group in C_7 is β-oriented. In fact 7-epicholic acid was not dehydroxylated. A very low 7α-dehydroxylase activity on chenodeoxycholic acid was shown, even if the enzyme preparation had been obtained from cells grown in the presence of chenodeoxycholic acid, differently from that reported by Aries and Hill [2] for the enzyme activity of their microbial strains, which transformed cholic and chenodeoxycholic acid to a similar extent. In the cell-free extract it was given evidence of the presence of enzymes responsible for the oxydative–reductive equilibrium occurring between cholic acid and its 7-oxo-derivative [3]. The transformation of 3α, 12α-dihydroxy-5β-chol-6-en-24-oic acid could be assumed as a proof that this compound may be an intermediate in the 7α-dehydroxylation of cholic acid into deoxycholic acid according to that demonstrated in vivo by Samuelsson [12, 13].

It was possible to separate 7α-dehydroxylase and 7α-dehydrogenase activities by purifying partially the soluble enzymatic extract.

Research is in progress in order to further purify the enzyme fraction active in 7α-dehydroxylating cholic acid.

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