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SOME ENZYMATIC PROPERTIES OF CHOLESTEROL OXIDASE PRODUCED BY *BREVIBACTERIUM* SP

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ABSTRACT

In this study we determined some properties of the cholesterol oxidase from a *Brevibacterium* strain isolated from buffalo's milk and identified the cholesterol degradation products by the bacterial cell. A small fraction of the enzyme synthesized by cells cultured in liquid medium for 7days was released into the medium whereas a larger fraction remained bound to the cell membrane. The extraction of this fraction was efficiently accomplished in 1 mM phosphate buffer, pH 7.0, containing 0.7% Triton X-100. The enzyme stability under freezing and at 45°C was improved by addition of 20% glycerol. The optimum temperature and pH for the enzyme activity were 53°C and 7.5, respectively. The only steroidal product from cholesterol oxidation by the microbial cell and by the crude extract of the membrane-bound enzyme was 4-colesten-3-one. Chromatographic analysis showed that minor no steroidal compounds as well as 4-colesten-3-one found in the reaction media arose during fermentation process and were extracted together with the enzyme in the buffer solution. Cholesterol oxidation by the membrane-bound enzyme was a first order reaction type.

Key words: Cholesterol oxidase, *Brevibacterium* sp, cholesterol, 3β-hydroxysteroid oxidase

INTRODUCTION

Cholesterol decomposition ability is widespread among microorganisms that have been explored as free and immobilized cells (15, 29) or as enzyme source (12) in steroid biotransformations. Cholesterol may be completely oxidized by microbial cells to carbon dioxide and water by the action of a complex enzyme system in which cholesterol oxidase is the first enzyme involved. Cell-free enzymes and microbial cells have been investigated for reduction of cholesterol level in foods (2, 3, 14, 10, 26, 28), for precursors production in manufacturing pharmaceutical steroids from cheap sterols (15) and for clinical assay of serum cholesterol (7, 25). Microbial cholesterol oxidases have received much attention in recent years, mainly due to its large use in medical practice for determination of free and bound cholesterol.

Cholesterol oxidase (3β-hydroxysteroid oxidase,

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EC 1.1.3.6) is a bifunctional enzyme catalyzing the oxidation of $\triangle^5$-3β-hydroxysteroids to the corresponding $\triangle^5$-3-ketosteroid and also the isomerization of this compound to the $\triangle^4$-3-ketosteroid, with reduction of oxygen to hydrogen peroxide (21, 26, 25). Although the enzyme exhibits a broad range of steroid specificities, the presence of a 3β-hydroxyl group, as in cholesterol (5-cholesten-3β-ol), 5α-cholestan-3β-ol, pregn-5-en-3β-ol-20-one, androst-5-en-3β-ol-17-one and 5α-androstan-3β-ol-7-one, is essential for activity.

In the sequence of the cholesterol oxidation by microbial cells, 4-cholesten-3-one ($\triangle^4$-3-ketosteroid) maybe oxidized with accumulation of the steroids 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) (4), which are important precursors of chemically synthesized hormones, or may be transformed to no steroid intermediates (1).

Cholesterol oxidases can be intrinsic membrane-bound enzymes located on the outside of the cell, as produced by *Nocardia rhodochrous* (8, 7, 27), *Nocardia erythropolis* (19), and *Mycobacterium* species (22), or can be isolated from broth filtrate as in cultivation of *Streptomyces violascens* (23), *Brevibacterium sterolicum* (24), *Streptoverticilium cholesterolicum* (15), *Rhodococcus equi* nº 23 (26), *Mycobacterium* ATCC 19652 (9) and *R. erythropolis* (22).

The aim of this work was to identify the cholesterol degradation products by *Brevibacterium* sp. cell isolated from buffalo's milk and to define some properties of the cholesterol oxidase produced by the microorganism.

MATERIALS AND METHODS

Strain identification - bacterial strain used throughout this work was isolated from buffalo's milk in a liquid medium containing cholesterol and mineral salts as described in Menezes *et al*. (16). Taxonomic studies were carried out by the classic methodology according to Collins (11) and Balows *et al*. (6), complemented by chemiotaxonomic tests. The phenotypic characterization using the API-50 CH system was conducted in accordance to the analytical profile of bioMerieux *API index* (50CH, 50CHB, 50CHL) and the identification of diaminopimelic acid (LL-/meso-DAP) was done by thin-layer chromatography with ninhydrin development.

Microorganism maintenance and enzyme production - stock culture of isolated strain, identified as *Brevibacterium* sp., was maintained on nutrient agar slants and stock culture of *Rhodococcus equi* ATCC 25729 (ATCC 6939), used as type microorganism, was maintained in glucose-yeast extract medium (GY). Organisms were subcultured at monthly intervals. Before flasks inoculation, cultures were transferred from maintenance medium to agar slants consisting of 1.0% peptone (Difco, Detroit, MI, USA), 1.0% meat extract (Difco, Detroit, MI, USA), 0.5% NaCl and 2.0% agar (Difco, Detroit, MI, USA), and incubated for 24 hours at 30°C. A loopful of the 24 hours bacterial cultures was inoculated in 500 ml Erlenmeyer flasks containing 225 ml of a medium composed of: 0.1% NH_4NO_3, 0.025% K_2HPO_4, 0.025% $MgS0_4.7H_2O$, 0.0001% $FeS0_4.7H_2O$, 0.5% yeast extract (Difco, Detroit, MI, USA) and 0.1% cholesterol (Sigma Chemical Co., USA), pH 7.0 (medium I) (5). After sterilization, the medium was homogenized by sonication at room temperature to disperse cholesterol. Cultivation was carried out on a rotary shaker at 37°C for 7 days and at agitation speed of 150 rpm.

Enzyme isolation - the supernatant from centrifuged broth at 10,000 rpm for 10 minutes at 5°C was used as extracellular cholesterol oxidase solution. Harvested cells were washed twice with ethyl acetate and frozen at -18°C. Membrane-bound enzyme was extracted from frozen cells in 1 mM phosphate buffer, pH 7.0, containing 0.7% Triton X-100 (v/v), for 18 hours at 5°C under stirring. The extract obtained by centrifugation of the cell suspension was used as membrane-bound enzyme solution.

Enzyme assay - the activity of the extracellular and membrane-bound enzymes was determined according to the method described in Inouye *et al*. (13). To 0.4 ml of 125 mM Tris-HCl buffer, pH 7.5, was added 0.1 ml of enzyme solution, and the mixture was incubated in water bath at 37°C. After 3 minutes, 25 µl of 12 mM of cholesterol in isopropanol solution were added to the mixture. After 30 minutes, 2.5 ml of absolute ethanol were added to the reaction medium and the amount of 4-cholesten-3-one was determined by measurement of the absorbance at 240 nm. Reaction blanks were prepared by replacing 25 µl of the cholesterol solution for 25 µl of isopropanol. One unit of cholesterol oxidase activity (U) was defined as that which brings about the formation of 1 µmol of 4-colesten-3-one in 30 minutes at 37°C.

The quantity of 4-colesten-3-one was obtained from a standard curve prepared with solutions containing 10 to 100 µg of 4-colesten-3-one dissolved in isopropanol ($\varepsilon=2.32x10^4$ M^{-1}. cm^{-1}).

Identification of the cholesterol oxidation products by thin-layer chromatography - in order to know the cholesterol degradation products accumulated in the culture medium due to the action of bacterial cells, 10 ml of medium I in 50 ml Erlenmeyer flasks were inoculated with a loopful of 24 hours cultures of *Brevibacterium* sp. or *Rhodococcus equi* ATCC 25729 and incubated for 7 days at 30°C and at agitation speed 150 rpm. After incubation time, cells were harvested by centrifugation and 10 ml of ethyl acetate were added to the supernatant. The organic phase was recovered in a funnel and evaporated at 60°C in a rotary evaporator until a white residue was formed. The dry residue was dissolved in 1ml of methylene chloride and analyzed by thin-layer chromatography. Blank of culture medium refers to no inoculated flask incubated and treated as the other ones.

Cholesterol degradation products resulted from the action of membrane-bound enzyme were also identified by thin-layer chromatography. The reaction medium and procedure used for cholesterol oxidation in this experiment were as those used to determine the enzyme activity but ten fold volumes of buffer, enzyme and substrate were actually employed and reaction were stopped by addition of 2 ml of methylene chloride at time intervals of 30, 60 and 90 minutes. The methylene chloride fractions were recovered and analyzed by thin layer chromatography. Reaction blank, where no cholesterol was added to the reaction mixture, was prepared for each time interval and treated in the same way.

Thin-layer chromatographies were carried out on pre-coated silica gel 60 F_{254} plate (0.25 mm thick, 20 cm x 20 cm) (Merck) at room temperature. Plates were activated at 110°C for 1 hour and spotted with 25 µl of samples. Benzene:ethyl acetate 9:1 (v:v) was used as solvent system and the development was carried out by spraying H_2SO_4: methanol 5:95 (v:v) solution followed by heating at 90°C until visualization of the spots. Twenty microlitres of 5.0 mg/ml solutions of cholesterol (5-cholesten-3-β-ol), cholestenone (4-colesten-3-one), 1,4-androstadiene-3,17-dione (ADD) and 4-androsten-3,17-dione (AD), from Sigma Chemical Co., USA, were spotted as standards.

Identification of the of cholesterol oxidation products by gas chromatography - a mixture of 20 ml of the membrane-bound enzyme solution (3.86 U/ml) and 80 ml of 12 mM Tris-HCl buffer, pH 7.5, was heated at 37°C. After reaching the reaction temperature, 5.0 ml of 12 mM of cholesterol in isopropanol solution were added to the mixture. Samples of 8.5 ml of the reaction medium were collected at 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes, and also at 24, 48 and 72 hours. The enzyme reaction was stopped by adding 20 ml of ethyl acetate to each sample. After saturation of the aqueous phase with NaCl, the organic phase was separated in a funnel and dried over anhydrous sodium sulfate. Ethyl acetate phase was evaporated to dryness at 60°C in a rotary evaporator. The dry residue was dissolved in ethyl acetate:methanol 9:1 (v:v). A blank, where no cholesterol was added to the reaction mixture, was prepared for each time interval and treated in the same way. Extracted compounds were quantified by gas chromatography performed with a Pye-Unicam 4600 gas chromatograph equipped with a flame ionization detector. Samples of 1 µl were injected in a poly-dimethylsiloxane column (25 m x 0.53 mm). Operating parameters were as follows: column temperature was kept at 100°C for 2 minutes and then raised, at a rate of 25°C/min, up to 250°C followed by a heating rate of 1°C/min up to 280°C, at which temperature was kept for 3 minutes; injector temperature 280°C; detector temperature 300°C; inlet pressure 14 psi and hydrogen as carrier gas. Cholesterol, cholestenone, 4-androstene-3,17-dione, 1,4-androstadiene-3,17-dione, 25-hydroxycholesterol, 5α,6α-epoxycholesterol, 3β,5α,6β-tri-hydroxycholestan, from Sigma Chemical Co. (USA), and 5α-cholestane were used as standards.

Protein concentration - protein concentration in enzyme solution, used for electrophoresis, was estimated according to the LOWRY *et al.* method (22), after extraction of Triton X-100 with Amberlite XAD-2 according to Cheethan (8). Bovine serum albumin (Merck) was used as standard protein.

Electrophoresis - a sample of the membrane-bound enzyme solution in 1 mM phosphate buffer, pH 7.0, containing 0.7% Triton X-100 was concentrated to 2 mg/ml of protein by polyethileneglycol (PEG) 20,000, dialyzed for 18 hours against the same buffer free of detergent, and analyzed in polyacrylamide gel. Electrophoresis was performed in gradient 8-25% gel (Phast gel gradient

8-25) with native buffer strip (Pharmacia LKB-Biotechnology, Uppsala-Sweden) in PhastSystem equipment (Pharmacia LKB-Biotechnology, Uppsala-Sweden). Proteins were silver stained according to File nº 210 from equipment users manual.

Enzyme properties - the effect of temperature on the crude membrane-bound enzyme activity was estimated by the enzyme assay method, lightly modified, in the temperature range from 35°C to 65°C. The enzyme was added to the reaction medium after the mixture of buffer and substrate solutions had reached the reaction temperature. The same procedure was used to evaluate the effect of pH on the enzyme activity at 37°C, using 100 mM phosphate buffer for pH range from 5.0 to 7.5 and 50 mM Tris-HCl buffer for pH between 8.0 and 8.8. Thermal stability of the enzyme at 45ºC was investigated in the presence of glycerol or not according to the standard method, only that before the enzyme activity measurement it was maintained at 45ºC for different time intervals.

RESULTS AND DISCUSSION

The isolated microorganism was identified as a strain of *Brevibacterium* sp., with characteristics shown in Table 1.

When cultured in liquid medium the isolated strain produced both membrane-bound and extracellular cholesterol oxidase as observed for three *Rhodococcus* strains studied by Aihara *et al.* (1). The membrane-bound enzyme from frozen cells was easily extracted in 1 mM phosphate buffer solution containing 0.7% Triton X-100. According to Cheetham *et al*. (8) the enzyme can be extracted in these conditions because it is composed by a water-soluble enzymically active part and a predominant hydrophobic anchor that allows the incorporation of the enzyme into the cell membrane and detergent micelles. During extraction probably occurs the breakage of hydrophobic bonds between the enzyme and membrane lipids of the cell membrane. According to the authors, frozen cells would lead to higher yields of enzyme extraction due to the weakening of lipid-protein bonding caused by taking the membrane lipids below their thermotropic transition temperature.

We studied the effect of the ratio wet cell mass: buffer volume on the effectiveness of the enzyme solubilization in two extraction steps. In these experiments the same buffer volume was used in the first and second extraction and the ratios tried out were 15, 20, 30 and 60 mg/ml. The amount of the enzyme extracted in the first buffer volume varied between 68.5% and 77.7% of the total extracted in both steps. The highest enzyme concentration, 1.93 U/ml, was observed in the first extraction solution from 60 mg/ml ratio, where the enzyme concentration was three times that in the supernatant of the culture broth.

The activity of the enzyme from *Brevibacterium* sp. was dependent of Triton X-100 concentration so that it lost completely activity at 0% detergent concentration. Similarly to the enzyme from *Nocardia rhodochrous* (8), the activity in buffer solution with 0.7% Triton X-100 (0.35 U/ml) was lower than in solution containing 0.15% detergent (0.51 U/ml). Studies on the enzyme from *Nocardia rhodochrous* activity showed that the amount of the enzyme extracted increased with detergent concentrations up to 10% (v/v) and that more enzyme was solubilized using detergent concentrations higher than 0.5% (v/v). However, as the enzyme activity was dependent of detergent concentration used, the additional activity was masked by detergent inhibition (8). This information should be considered when comparing the amount of the enzyme produced by the isolated *Brevibacterium* strain with others from literature, since it may show a similar behavior.

The eletrophoretic analysis of the crude membrane-bound enzyme showed 10 protein components (Fig. 1), showing that the enzyme was not the unique protein extracted in the detergent solution. This profile was similar to the cholesterol oxidase of *Actinomyces lavendulae* extract in Tween 80, that showed 7 components (19).

The membrane-bound enzyme in 1 mM phosphate buffer, pH 7.0, containing 0.7% Triton X-100, gradually lost its activity when stored at -18ºC. Addition of 20% glycerol to the enzyme solution stabilized the enzyme activity up to 15 days of storage and diminished the loss of activity at 30 days (Fig. 2), so that, at this time the loss of activity in the presence of the compound was around 24%, whereas in the absence was around 80%.

The temperature profile of the cholesterol oxidase from *Brevibacterium* sp. pointed out optimum temperature for enzyme activity at around 53°C (Fig. 3). This value is higher than the optimum temperature for the purified enzyme from *Rhodoccocus equi* and from *Corynebacterium cholesterolicum,* with

Table 1 - Bacteriological characteristics of the isolated strain.

Morphological:
Cells: isolated rods
Colonies: small, smoth and yellowish colored on nutrient agar
Motility: negative
Gram-reaction: positive
Spore: absent

Physiological:
Oxygen requirement: strict aerobe
Temperature relations: little growth at 5°C, good growth at 30°C
NaCl broth: growth at 4.5%, growth at 8% after 7 days, growth at 12% after 12 days
Nitrate reduction: negative
Catalase: positive
Oxidase: negative
Urease: negative
Prodution of H_2S: negative
Indol: negative
TDA: negative
Arginin dehydrolase, lisine decarboxilase, ornitine decarboxilase: negative
Casein, starch: not hydrolysed
Cell wall type: LL-/meso DAP
Gelatin hydrolysis: negative

Acid production from carbohydrates:

Control: negative	Inositol: negative	Melezitose: negative	Adonitol: negative
Glycerol: negative	Manitol: negative	D-Raffinose: negative	Dulcitol: negative
Erythritol: negative	Sorbitol: negative	Starch: negative	Galactose: negative
D-Arabinose: negative	α-m-D-Manoside: negative	Glycogen: negative	D-Glucose: positive
L-Arabinose: negative	α-m-D-Glycoside: negative	Xylitol: negative	D-Fructose: negative
Ribose: negative	N-Acetyl-glycosamine: negative	β-Gentiobiose: negative	D-Manose: negative
D-Xylose: negative	Amidaline: negative	D-Turanose: positive	L-Sorbose: negative
L-Xylose: negative	Arbutine: negative	D-Tugatose: negative	Ramnose: negative
Gluconate: negative	Esculin: negative	D-Fucose: negative	Salicin: negative
2-Keto-gluconate: negative	Melibiose: negative	L-Fucose: negative	Celobiose: negative
5-Keto-gluconate: positive	Sucrose: negative	D-Arabitol: negative	Maltose: positive
Inulin: negative	Trehalose: negative	L-Arabitol: negative	Lactose: negative
β-Methyl-xyloside: negative			

maximum activity at approximately 47°C (26) and 40°C (20), respectively. It is closer to that for the purified enzyme from *Streptomyces violascens,* with optimum temperature at around 50°C, with a plateau between 40°C and 60°C (23). 20% glycerol stabilized the enzyme at 45°C, so that after 30 minutes at this temperature the enzyme lost around 1% of the activity against a loss of 20% when the compound was not added (Fig. 4).

The enzyme was pH dependent as shown in Fig. 5, with maximum activity at around 7.5. Enzyme activity was at least 80% of the maximum value at pH range between 6.0 and 8.7. The optimum pH for cholesterol oxidase from *Brevibacterium* sp. activity was similar to that found in literature for the same enzyme from other microorganisms. Usually the optimum pH for the enzyme activity is between 7.0 and 8.0, as can be seen for the enzymes from *Actnomyces lavendulae* mycelium (19), *Corynebacterium cholesterolicum* (20), *Streptoverticillium cholesterolicum* (13), *Rhodococcus equi* nº 23 (26) and from *Streptomyces violascens* (23).

Thin-layer chromatography of the products accumulated in the culture medium fermented by *Brevibacterium* sp. and *Rhodococcus equi* ATCC

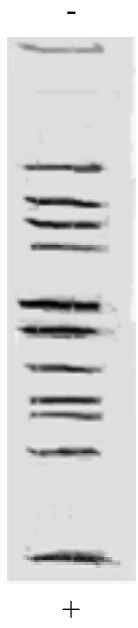

Figure 1: Electrophoresis of crude membrane-bound enzyme in gradient polyacrilamide gel 8-25%. 2 mg/ml protein in 1 mM phosphate buffer containing 0.7% Triton X-100. Silver stained proteins.

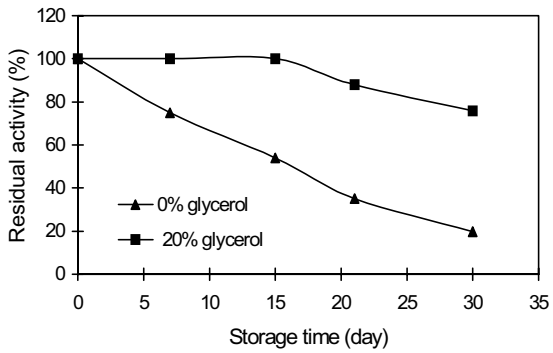

Figure 2: Stability of cholesterol oxidase at -18°C in 1 mM phosphate buffer solution containing 0.7% Triton X-100 added by 0% and 20% glycerol.

25729 cells during 7 days is shown in Fig. 6. During fermentation, cholesterol was oxidized by both strains to 4-colesten-3-one (spots 6 and 7) with very small amounts of the no oxidized compound still remaining in the culture medium. Although not visible in the photography, the samples showed a very faint spot with R_f value greater than for 4-colesten-3-one. The compound also appeared, although in lower intensity, in the control sample,

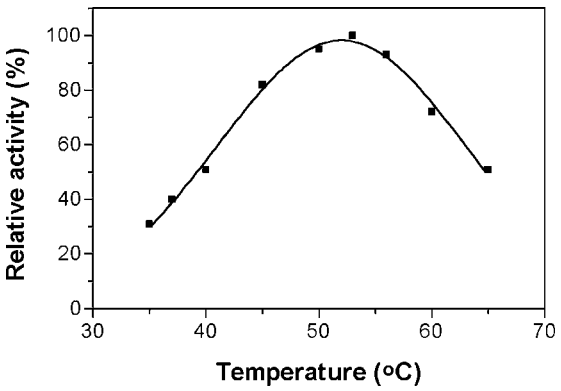

Figure 3: Effect of temperature on the cholesterol oxidase activity at pH 7.5.

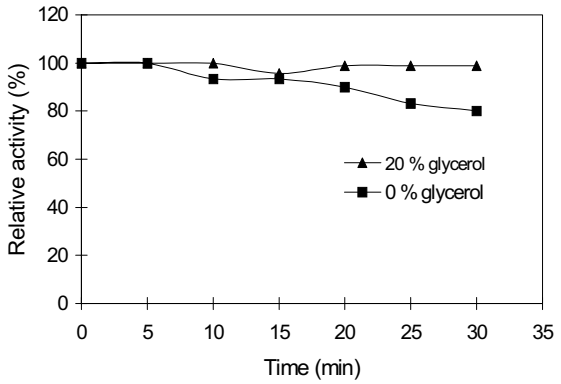

Figure 4: Thermal stability of cholesterol oxidase at 45°C in 1 mM phosphate buffer solution containing 0.7% Triton X-100 added by 0% and 20% glycerol.

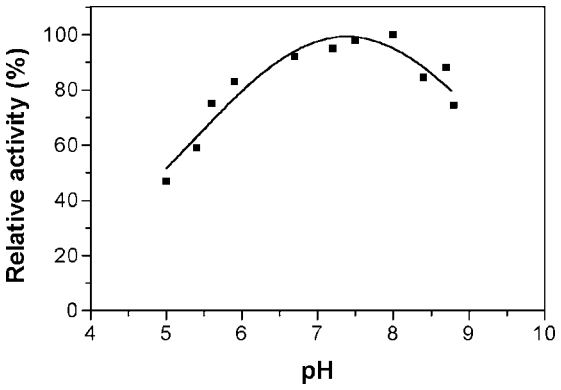

Figure 5: Effect of pH on the cholesterol oxidase activity at 37°C. 100 mM phosphate buffer was used for pH 5.0-7.5 and 50 mM Tris-HCl buffer was used for pH 8.0-8.8.

where no microorganism was inoculated, revealing that its presence was not due to the action of the microorganisms or to their enzymes on cholesterol, but probably to a metabolic compound excreted in the broth, or to a component of the medium formulation.

Cholesterol oxidation by the membrane-bound enzyme from *Brevibacterium* sp. resulted in 4-colesten-3-one as main product (Fig. 7, spots 6, 8 and 10) even after 90 minutes of reaction. Both reaction media (spots 6, 8 and 10) and controls, where no cholesterol was added, (spots 5, 7 and 9), showed very faint spots (not visible on the photography) on thin layer chromatography corresponding to a product with an R_f value higher than for 4-cholesten-3-one, the same spot detected on the chromatography of the fermented media (spots 6 and 7 in Fig. 6). As the compound was also detected in the controls of the reaction media, it was supposed to be extracted in the enzyme solution. Chromatography of the controls of the reaction media revealed the presence of 4-cholesten-3-one, probably adhered to the cell during fermentation course and extracted in the enzyme solution.

Gas chromatography analysis of the samples of reaction media and controls detected 7 to 16 peaks, most of them with retention time lower than for 5α-cholestane. Almost invariably 5 peaks, with retention times: 25.12 ± 0.06 min. (cholestenone), 11.89 ± 0.07 min., 18.78 ± 0.05 min., 26.45 ± 0.06 min. and 34.18 ± 0.07 min., were detected in the controls and in the reaction media. These four late compounds were at unchanged concentrations in all samples and were assumed to come from the membrane-bound enzyme extract. None of these compounds were identified as 25-hydroxycholesterol or 3β,5α,6β-trihydroxycholestane, both of which recognized as inhibitor of cholesterol biosynthesis and/or angiotoxic (17) or 5α,6α-epoxycholesterol, a potentially carcinogenic compound (18). 4-androsten-3,17-dione or 1,4-androstadien-3,17-dione were not detected in the samples at all. A peak at 21.91 ± 0.01 min., corresponding to cholesterol, was detected in the reaction medium at 105 minutes, showing that the conversion of the product to cholestenone was not complete until that time. The only time dependent concentration compounds in the reaction media were cholesterol and cholestenone and the rate of oxidation of that compound by the enzyme from *Brevibacterium* sp followed a first order kinetic, in which the total conversion occurred in

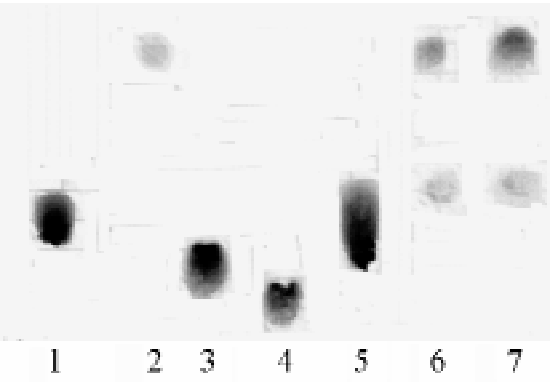

Figure 6: Thin-layer chromatography of medium I fermented by *Brevibacterium* sp. (spot 5) and *Rhodococcus equi* 25729 (spot 6) for 7 days at 37ºC at agitation speed 150 rpm. 1-cholesterol, 2-cholestenone, 3-AD, 4-ADD, 5-no inoculated medium. Running solvent: benzene : ethyl acetate 9:1 (v/v). Development system: H_2SO_4 : methanol 5:95 (v/v).

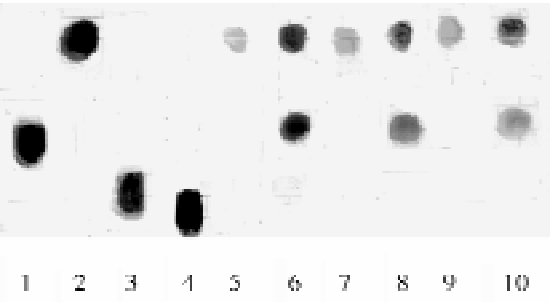

Figure 7: Thin-layer chromatography of reaction media and controls at different reaction times. 1- cholesterol, 2-cholestenone, 3-AD, 4-ADD, 5-control of reaction medium at 30 min., 6-reaction medium at 30 min., 7-control of reaction medium at 60 min., 8-reaction medium at 60 min., 9-control of reaction medium at 90 min., 10-reaction medium at 90 min. Running solvent: benzene : ethyl acetate 9:1 (v/v). Development system: H_2SO_4 : methanol 5:95 (v/v).

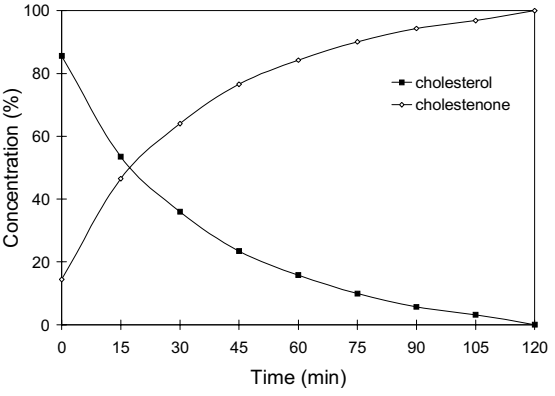

Figure 8: Cholesterol consumption and cholestenone production by membrane-bound cholesterol oxidase from *Brevibacterium* sp.

120 minutes (Fig. 8). An increase in the time reaction until 72 hours did not give rise to other compounds than cholestenone. These results show that, on the contrary of the extracellular enzyme from *Rhodococcus equi* nº 23 (2), the crude extract of the membrane-bound enzyme do not have enzymes for successive degradation of cholesterol.

ACKNOWLEDGMENTS

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RESUMO

Algumas propriedades enzimáticas da colesterol oxidase produzida por *Brevibacterium* sp.

Neste trabalho foram definidas algumas propriedades da enzima colesterol oxidase produzida por uma linhagem de *Brevibacterium* sp. isolada de leite de búfala e foram identificados os compostos resultantes da degradação do colesterol pela bactéria. Uma pequena fração da enzima sintetizada pelas células cultivadas em meio líquido por 7 dias foi liberada no meio de cultura e uma fração maior permaneceu ligada à membrana celular. A extração desta fração foi eficientemente efetuada em tampão fosfato 1mM, pH 7,0, contendo 0,7% de triton X-100. A estabilidade da enzima congelada e a 45ºC foi aumentada pela adição de 20% de glicerol. A temperatura ótima para a atividade enzimática esteve ao redor de 53ºC e o pH ótimo esteve ao redor de 7,5. O único produto da degradação do colesterol, causada pela ação da célula bacteriana e pela colesterol oxidase ligada à membrana, acumulado no meio de cultura foi o 4-colesteno-3-ona. Análise cromatográfica revelou que uma pequena quantidade de compostos não esteroídicos, além de 4-colesteno-3-ona, formados durante a fermentação, permanecem aderidos à célula e são extraídos juntamente com a enzima na solução com detergente. A oxidação do colesterol pela enzima bruta ocorreu segundo uma equação de primeira ordem.

Palavras-chave: colesterol oxidase, *Brevibacterium* sp., colesterol, 3β-hidroxiesteroide oxidase

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