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Cholesterol sulphate sulphohydrolase from human placenta microsomes — purification and properties of the dephosphorylated form of enzyme

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Abstract

The procedure for purification of cholesterol sulphate sulphohydrolase (ChS-ase) from human placenta microsomes was elaborated. The highy purified enzyme preparation (specific activity 2000 $nmol \times min^{-1} \times mg\ protein^{-1}$) exhibited optimal activity at pH 9.0. The K_m value was established to be $1.5 \pm 0.85 \times 10^{-5}$ M. The high molecular weight form (200 kDa) and the low molecular weight form (20 kDa) of the enzyme were separated. The interconversion of the high molecular weight variant into the low one occurs under the influence of dephosphorylation. Both forms exhibited typical Michaelis–Menten saturation kinetics. The effect of different compounds on the enzyme activity was tested.

Keywords: Cholesterol sulphate sulphohydrolase; Phosphoenzyme; Cholesterol sulphate; Microsomes; Placenta

1. Introduction

Cholesterol sulphate is widely distributed throughout nature including both invertebrate and mammalian tissues. It is present in distinct tissues, plasma, bile, stratum corneum and erythrocyte membrane [1–16]. The most abundant sources of cholesterol sulphate are liver, spleen, erythrocyte membrane and stratum corneum [4–9,15]. Daily synthesis of cholesterol sulphate ranges from 35 to 165 mg [2].

The biological functions of cholesterol are relatively well known, however, the significance of cholesterol sulphate is still not fully established.

The role of cholesterol sulphate in the protection of the erythrocyte membrane against osmotic shock [4], in the regulation of membrane hydration [17], in active transport of ions [18] and in sterol–lipid interaction processes has been recently recognized [18,19].

Cholesterol sulphate, as well as free cholesterol, serves as a substrate of the side-chain cleavage complex [20,21] and it appears to be a naturally occuring inhibitor of steroidogenesis in rat adrenal mitochondria [21,22].

Cholesterol sulphate has additionally attracted some attention when it was found that X-linked sulphohydrolase deficiency [8,13,14,23] is accompanied with increased levels of cholesterol sulphate in the erythrocyte membrane and plasma [13,14,23]. The sulphohydrolase deficiency disorder is also characterised by a decreased production of oestriol by the fetal–placental–maternal unit [23], which means that the hydrolysis of cholesterol sulphate is involved in the metabolic crossroads of the steroidogenesis.

The aim of the present work was to purify and characterize the cholesterol sulphate sulphohydrolase (ChS-ase) from human placental microsomes.

2. Materials and methods

2.1. Reagents

The reagents were purchased as follows: DEAE–cellulose, lectin from Concavalia ensiformes, α-methyl-D-glucopyranoside, dehydroepiandrosterone Coomassie

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blue R-250 and vanadium oxide(V) were from Serva (Heidelberg, Germany); Bio-gel A 1.5m (100–200 mesh) was from Bio-Rad Labs (Richmond, CA); cholesterol sulphate sodium salt, oestrone sulphate sodium salt, dehydroepiandrosterone sulphate sodium salt, pregnenolone sulphate sodium salt, cholesterol, D(−)tartaric acid, Tris and Triton X-100 were from Sigma Chemical Co. (St Louis, MO); Sepharose 6B–CNBr-activated was from Pharmacia Fine Chemicals (Uppsala, Sweden); reduced glutathione, *p*-chloro-mercuribenzoic acid sodium salt and ascorbic acid sodium salt were from Schuchardt (Munchen, Germany); sodium taurocholate and L-β-phenyloalanine were from BDH (Poole, England); pregnenolone and digitonin were from Merck (Germany); L-cysteine and oestrone were from LOBA Chemie (Wien, Austria); sodium glycocholate was from C.B. Carl Roth (Karlsruhe, Germany); oxidized dithiothreitol was from Calbiochem (Los Angeles, USA); amonium molybdate, sodium sulphite anhydrous, zinc acetate, magnesium acetate, calcium chloride were from PPH "POCh" SA (Gliwice, Poland). Centriprep YM-30 were from Amicon (Milipore, Canada).

2.2. Enzyme material

The human placenta microsomes were prepared as described previously [24]. Enzyme solubilization was effected by Triton X-100. The microsomal suspension (480 mg of protein) in 50 mM Tris–HCl buffer pH 7.6 was homogenized in a Potter glass homogenizer with an equal volume of 50 mM Tris–HCl buffer pH 7.6, containing 0.05% Triton X-100. The suspension was incubated at 37°C for 1h and centrifuged at 30 000 × *g* for 1h. The supernatant was discarded. The sediment was suspended in 50 mM Tris–HCl buffer pH 7.6, containing 0.5% Triton X-100 and was homogenized in a Potter glass homogenizer. The homogenized suspension was incubated at 37°C for 1h and centrifuged at 30 000 × *g* for 1h. The supernatant (extract) was used in further experiments. In order to remove the Triton X-100 the extract was filtered through the centriprep filter (Amicon Centriprep YM-30). The residue was dissolved in 50 mM Tris–HCl buffer pH 7.6 and used in further experiments.

2.3. DEAE–cellulose chromatography

10 mg of protein from the previous step was applied to the DEAE–cellulose column (1 × 11 cm) equlibrated with 50 mM Tris–HCl buffer pH 7.6. The enzymatically active fractions unadsorbed by DEAE–cellulose were combined, concentrated, dialysed against Tris–HCl buffer pH 7.6 containing 0.1% Triton X-100 and stored at −20°C until use.

2.4. Bio-gel A 1.5 m chromatography

Protein (0.6 mg in 0.5 ml) was applied to the column (1.8 × 46.0 cm) of Bio-gel A 1.5 m equilibrated with 50 mM Tris–HCl buffer pH 7.6, containing 0.1% Triton X-100. The same buffer was used for elution. Three millilitre fractions were collected. The enzymatically active fractions, revealing the activity at pH 9.0, were combined, concentrated and used in further experiments.

2.5. Affinity chromatography

Concanavalin A–Sepharose 6B was prepared by mixing a solution of Concanavalin A (ConA 20 mg per 30 ml of the solution containing: 10 mM $MnCl_2$, 10 mM $CaCl_2$ and 10 mM $MgCl_2$) with 1g Sepharose 6B–CNBr-activated in 1ml of 1mM HCl. The pH was adjusted to 8.0 by the addition of sodium acetate. The 1 × 10 cm column was packed with the suspension of ConA–Sepharose 6B and equlibrated with 50 mM Tris–HCl buffer pH 7.6. The enzymatic fraction (1.0 mg of protein) from Bio-gel chromatography was applied to the ConA–Sepharose column and fractionated with the use of the following solutions: 50 mM Tris–HCl buffer of pH 7.6 (fractions 1–8), 20% α-methyl-D-glucopyranoside (fractions 9–15), 50 mM Tris–HCl buffer of pH 7.6 (fractions 16–22), 50 mM Tris–HCl buffer of pH 7.6 with 0.5% Triton X-100 (fractions 23–28); 0.5 M Tris–HCl buffer of pH 7.6 with 0.5% Triton X-100 (fractions 29–38); 0.5 M Tris–HCl buffer of pH 7.6 containing 0.5% Triton X-100 and 5% α-methyl-D-glucopyranoside (fractions 39–48). Fractions of 2 ml were collected.

The protein content and enzyme activity were determined in each fraction.

2.6. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed under nondenaturing conditions by the Bollag and Edelstein procedure [25]. Gel slabs 6 × 8 cm × 0.75 mm in 25 mM Tris–glycine buffer of pH 8.8 were used. The stacking gel and separating gel contained 4 and 7% of polyacrylamide respectively. The initiation of electrophoresis was performed at 100V (50mA) for 10 min; the separation was done at 150V (50 mA) for 1h. The gel slab was stained with Coomassie blue R-250 and destained with 5% acetic acid.

2.7. Enzyme activity determinations

The assay of ChS-ase activity was performed by the procedure described by Roy [26]. The enzyme activity was determined in the presence of 0,4 mM cholesterol sulphate in 25 mM Tris–HCl buffers at pH 6.4 and pH

Table 1
Purification of cholesterol sulphate sulphohydrolase from human placenta microsomes

Purification step		Protein (mg)	Total activity nmol min^{-1}	Specific activity nmol min^{-1} mg $protein^{-1}$ (at pH 9.0)	Recovery %
Microsomal suspension				14.0	
DEAE–cellulose chromatography		4.62	119.5	25.7	100
Bio–gel A 1.5m chromatography		1.08	168.0	155.6	141
ConA–sepharose chromatography	Fraction I	0.48	121.4	289.0 (580.0)[a]	102
	II	0.25	147.4	667.0 (2000.0)[a]	124
	III	0.28	64.8	231.0 (231.0)[a]	54
Total I+II+III		1.00	333.6		280

[a] Specific activity after alkaline phosphatase digestion.

9.0 at 37°C. The effect of substrate concentration on the intial value (v) of cholesterol sulphate hydrolysis was tested within the range 0.01–0.50 mM concentration of cholesterol sulphate in 25 mM Tris–HCl buffers at pH 6.4 and pH 9.0.

The effect of alkaline phosphatase on the ChS-ase activity was tested with the use of alkaline phosphatase from human placental microsomes (prepared by a procedure which is not yet published). Subfractions I, II and III, separated by ConA–Sepharose 6B were incubated (30 min) with the alkaline phosphatase preparation (0.15 μg of protein, specific activity 2300 nmol min^{-1} mg $protein^{-1}$). The effect of this treatment is expressed as percentage of the activity in the control samples to which no alkaline phosphatase was added.

The ChS-ase activity estimations, as a function of pH were performed in 25 mM acetate buffers at pH 3.0–5.8 and Tris–HCl buffers at pH 6.0–10.0. The effects of typical sulphohydrolase inhibitors, compounds reacting with –SH groups, phosphatase inhibitors, steroids and bile acids on the purified ChS-ase activity were estimated in 25 mM Tris–HCl buffer of pH 9.0. The samples containing the substance tested were incubated for 1h at 37°C and the unhydrolysed cholesterol sulphate was determined by the procedure of Roy [26].

2.8. Protein determination

Protein concentration was measured by the method of Bradford [27]. Bovine serum albumin was used as a standard. Protein concentration in Triton X-100 extracts and after Bio-gel A 1.5m chromatography were determined with the procedure of Bensadoun and Weinstein [28].

3. Results and discussion

The procedure presented in this paper allowed us to prepare highly purified ChS-ase from human placental microsomes. The specific activity of the purified enzyme preparation was 2000 nmol min^{-1} mg $protein^{-1}$ (Table 1).

The purification was performed in four stages. The first stage consisted of the extraction of the enzymatic activity(ies) from the microsomal fraction with the use of Triton X-100 in the concentration range 0.05–0.5%. The Triton X-100 was removed from the extract by filtration through a centriprep filter (Amicon Centriprep YM-30). The second stage, which was DEAE–cellulose chromatography, allowed us to separate the enzyme fraction, which was not adsorbed by the ion exchanger, from the bulk of proteins bound by DEAE–cellulose. The unbound fraction contained 0.7% of proteins and 13.5% of ChS-ase activity of the extract, respectively.

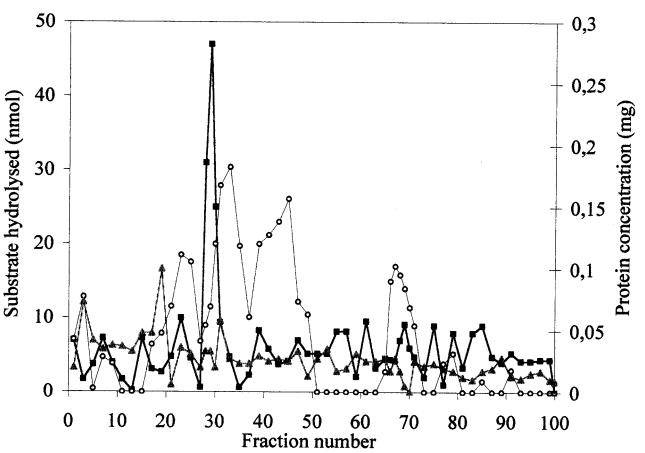

Fig. 1. Bio-gel A 1.5 m chromatography of ChS-ase preparation from human placental microsomes (the unbound by DEAE–cellulose enzyme fraction). A solution (0.5 ml) containing 0.6 mg of protein was applied to the column (1.8 × 46.0 cm) equilibrated with 50 mM Tris–HCl buffer pH 7.6 containing 0.1% Triton X-100. The column was eluted with the same buffer. Fractions (3 ml) were collected. The enzyme activities are expressed in nmoles of cholesterol sulphate hydrolysed in the presence of 0.1 ml aliquots of the respective fractions after 1h incubation at 37°C. Cholesterol sulphate released after incubation at pH 9.0 —■— and at pH 6.4 —○—; protein content —▲—.

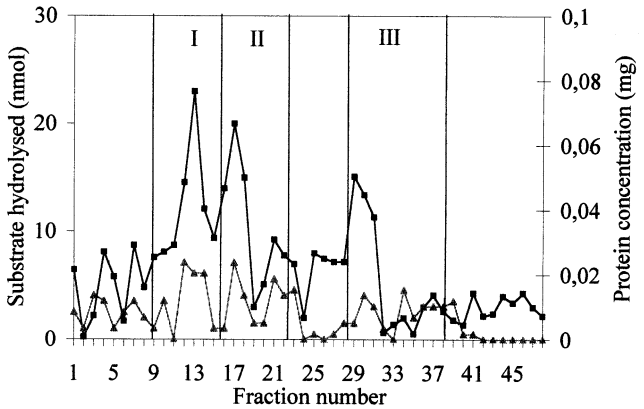

Fig. 2. ConA–Sepharose 6B chromatography of the enzyme present in the unbound by DEAE–cellulose fraction. The unbound by DEAE–cellulose enzyme fraction (1.0 mg of protein in 1.0 ml) was applied to a column of ConA–Sepharose 6B (1.3 × 10 cm) equilibrated with 50 mM Tris–HCl buffer pH 7.6. The column was eluted as described in Section 2.5. Fractions (2 ml) were collected. The ChS-ase activity is expressed in nmoles of cholesterol sulphate hydrolysed in the presence of 0.1 ml aliquots of the respective fractions after 1h incubation at 37°C. Cholesterol sulphate hydrolysed at pH 9.0 —■—; protein —▲—.

The third stage of the purification procedure consisted of the filtration on Bio-gel A 1.5 m. This procedure resulted in a partial separation (Fig. 1) of the enzyme subfraction which exhibited optimal activity at pH 9.0, from that one which revealed optimal activity around pH 6.4. The fourth stage of the purification procedure was affinity chromatography on ConA–Sepharose 6B, which gave three subfractions (I, II and III) with ChS-ase activity (Fig. 2). The first subfraction (I) was eluted with 20% α-methyl-D-glucopyranoside, the second subfraction (II) was eluted with 50 mM Tris–HCl buffer pH 7.6 and the third subfraction (III) was eluted with the solution of 0.5% Triton X-100 in 0.5 M Tris–HCl buffer pH 7.6.

As presented in Fig. 3a–c, the separated enzyme subfractions (I, II and III) responded differently to alkaline phosphatase digestion. The enzymes of subfractions I and II (Fig. 3a and b) were activated, while the enzyme of subfraction III (Fig. 3c) was unaffected by alkaline phosphatase treatment. Alkaline phosphatase also caused a substantial change of the pH activity profiles (Fig. 4a and b). The optimal pH value of the enzyme activity of subfraction I which appeared at pH 6.4 diminished, while the activity peak at pH 9.0 increased significantly (Fig. 4a). The pH activity profile of the enzyme present in subfraction II (as presented in Fig. 4b), was only modified; the slightly distinguished optimal activity peak at pH 6.4 disappeared and the prominent one at pH 9.0 increased pronouncedly. The alkaline phosphatase treatment did not substantially change the pH activity curve of the enzyme present in subfraction III (Fig. 4c).

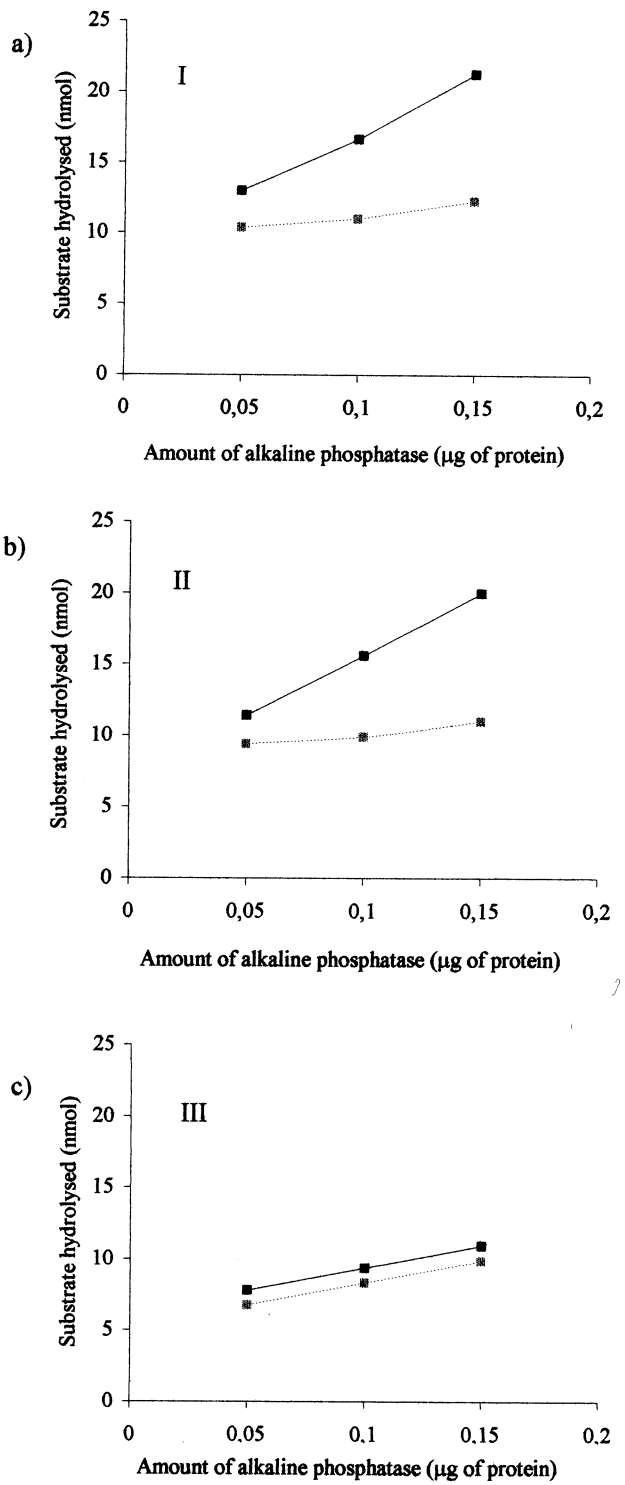

Fig. 3. The effect of different amounts of alkaline phosphatase (AP) (specific activity of 2300 nmol min^{-1} mg $protein^{-1}$) on the activity of ChS-ase separated by ConA–Sepharose 6B into subfraction I, II and III (3a, b and c). The experimental conditions are described in Section 2.7. ···■··· control (enzyme fractions I, II and III without pretreatment with AP) —■— enzyme fractions I, II and III digested with AP.

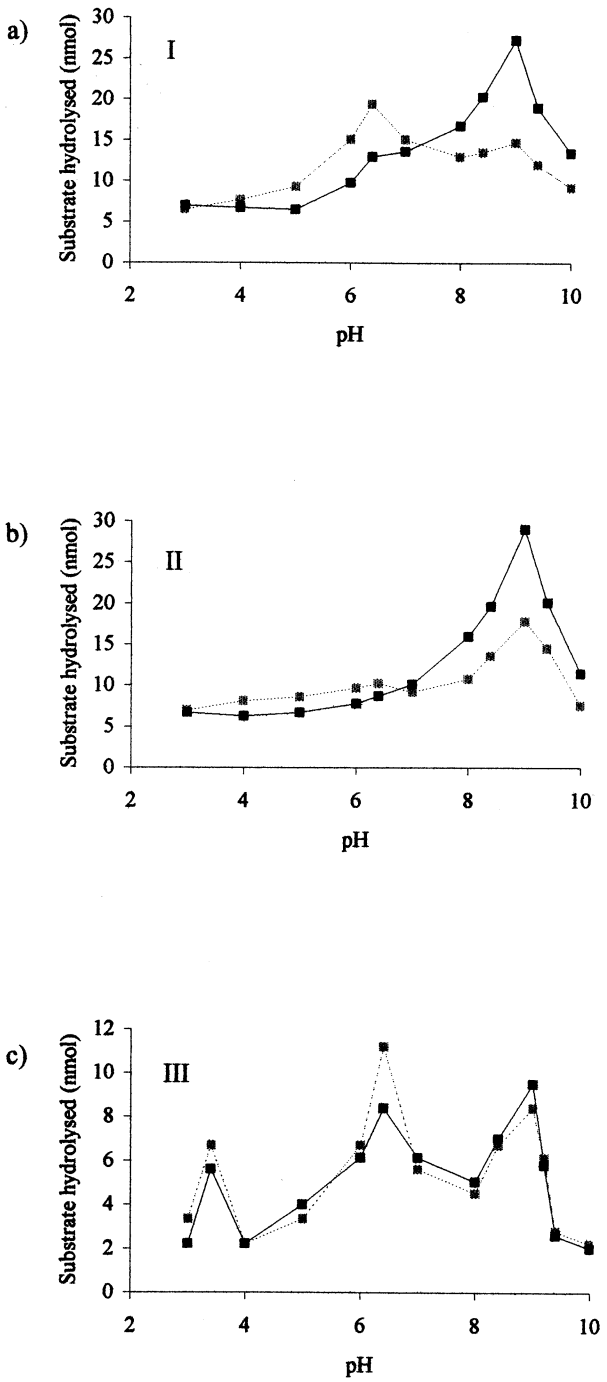

Fig. 4. The effect of pH on the activity of ChS-ase separated by ConA–Sepharose 6B into subfractions I, II and III (4a, b and c).The experimental conditions were described in Section 2.7. ···■··· control (enzyme fractions I, II and III without pretreatment with AP) — ■— enzyme fractions I, II and III digested with AP.

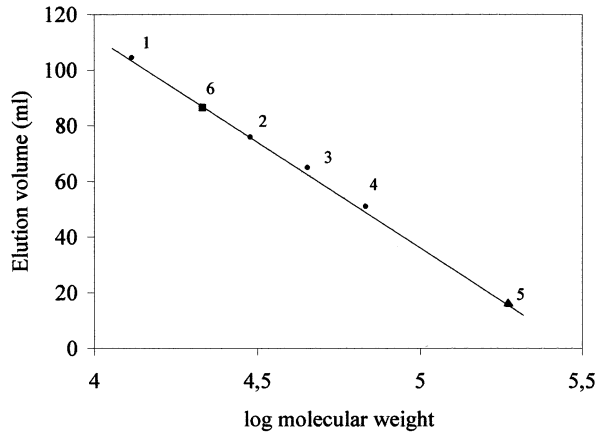

Fig. 5. Molecular weight estimation of the ChS-ase (enzyme of subfraction II after ConA–Sepharose 6B chromatography).The Biogel A 1.5 m column (1.8 × 46.0 cm) equilibrated with 50 mM Tris–HCl buffer of pH 7.6 containing 0.1% Triton X-100 was used for separation. Elution was effected with the same buffer; 1.5 ml fractions were collected. The following proteins were used as molecular weight standards: 1 — cytochrome c; 2 — carbonic anhydrase; 3 — ovoalbumin; 4 — albumin; 5 —▲— ChS-ase of fraction II; 6 —■— ChS-ase of fraction II after alkaline phosphatase digestion.

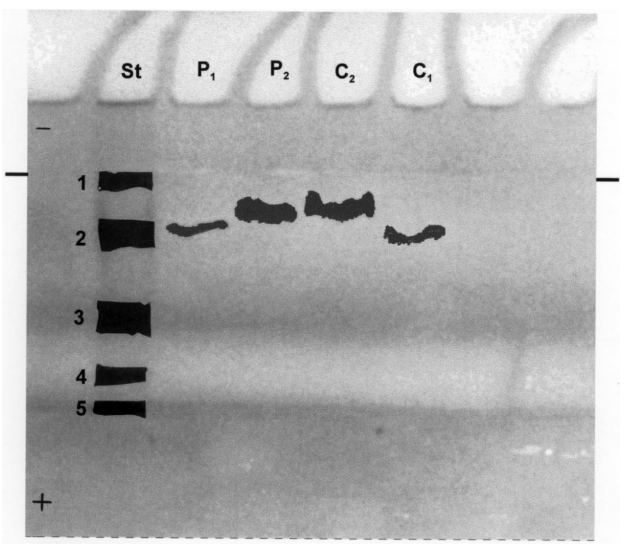

Fig. 6. Electrophoresis of purified ChS-ase preparation on acrylamide gel. The enzyme sample from ConA–Sepharose 6B (specific activity 2000 nmol min^{-1} mg $protein^{-1}$) was separated on acrylamide gel as described in Section 2.6. The following proteins were used as mobility markers: 1 — cytochrome c; 2 — myoglobin; 3 — carbonic anhydrase; 4 — ovoalbumin; 5 — albumin; C_1 and P_1 — mobilities of the purified ChS-ase (1 μg of protein), C_2 and P_2 — mobilities of the purified ChS-ase (7 μg of protein); C_1 C_2 — enzyme of fraction II-without pretreatment with AP, P_1 P_2 — enzyme of fraction II-digested with AP.

Alkaline phosphatase digestion (Fig. 5) caused the conversion of the high molecular weight form of ChS-ase (200 kDa) to the low molecular weight form (20 kDa). The interconversion process did not cause any change in the electrophoretic mobility. The acrylamide gel electrophoretic patterns of the ChS-ase digested and undigested by alkaline phosphatase were identical (Fig. 6). The K_m values established for the ChS-ase unmodified and modified by alkaline phosphatase digestion were $1.72 \pm 0.25 \times 10^{-5}$ and $1.5 \pm 0.85 \times 10^{-5}$ M, respectively. The V_{max} was doubled after phosphatase treatment (Fig. 8).

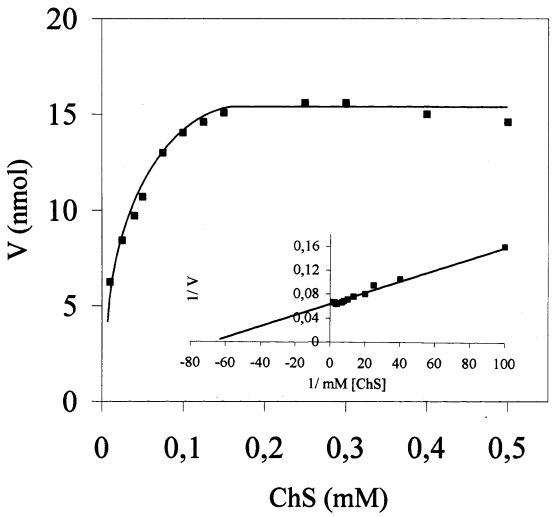

Fig. 8. The influence of cholesterol sulphate concentration on the activity of highly purified ChS-ase (specific activity 2000 nmol min^{-1} mg $protein^{-1}$). The assays were carried out in the presence of 0.01–0.5 mM concentrations of cholesterol sulphate in 25 mM Tris–HCl buffer pH 9.0. The activity is expressed as nmoles of cholesterol sulphate (ChS) hydrolysed at 37°C after 1h.

The highly purified and dephosphorylated enzyme preparation (specific activity 2000 nmol min^{-1} mg $protein^{-1}$) exhibited optimal activity at pH 9 (Fig. 7), the K_m value for cholesterol sulphate calculated from the Lineweaver–Burk plot was $1.5 \pm 0.85 \times 10^{-5}$ M (Fig. 8). The molecular weight determined by gel filtration on Bio-gel A 1.5 m was established to be 20 kDa (Fig. 5).

The effect of different compounds on the activity of the purified ChS-ase is summarised in Table 2. The typical sulphohydrolase inhibitors, such as phosphate ions and sulphite ions, effected inhibition of ChS-ase

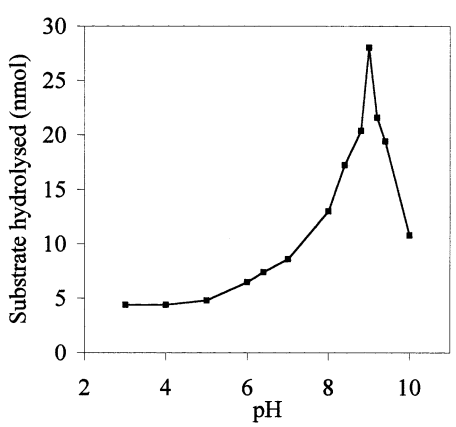

Fig. 7. The effect of pH on the activity of the highly purified ChS-ase (specific activity 2000 nmol min^{-1} mg $protein^{-1}$). The assays were carried out in the presence of 0.4 mM cholesterol sulphate in 25 mM acetate buffers of pH 3.0–5.8 and 25 mM Tris–HCl buffers of pH 6.0–10.0. The activity is expressed in nmoles of cholesterol sulphate (ChS) hydrolysed, after 1h incubation at 37°C.

Table 2
Effects of some inorganic and organic compounds on the purified cholesterol sulphate sulphohydrolase activity of human placenta microsomes[a]

Substance tested	Final concentration in the incubation mixture	Activity (% of the control)
Control	–	100
KH_2PO_4	5 mM	76 ± 15
Na_2SO_3	5 mM	36 ± 5
Na_2SO_4	5 mM	69 ± 5
Ag^+	1 mM	68 ± 10
β-mercaptoethanol	10 mM	114 ± 4
Ascorbic acid	10 mM	64 ± 7
Glutathione (red.)	10 mM	130 ± 4
Cysteine	10 mM	124 ± 3
PCMB	10 mM	150 ± 8
Dithiothreitol	10 mM	105 ± 4
Vanadium oxide (V)	50 μM	66 ± 5
Amonium molybdate	10 μM	86 ± 4
Zn acetate (II)	10 mM	63 ± 9
L-β-phenyloalanine	5 mM	99 ± 6
D(−)tartaric acid	15 mM	91 ± 5
Cholesterol	1 mM	68 ± 4
Oestrone	1 mM	61 ± 10
Dehydroepiandrosterone	1 mM	74 ± 13
Pregnenolone	1 mM	77 ± 5
Taurocholate	1 mM	127 ± 9
Glycocholate	1 mM	115 ± 4
Digitonin	1 mM	214 ± 7

[a] The incubation mixture contained: 0.4 mM cholesterol sulphate in 25 mM Tris–HCl buffer of pH 9.0 and the substance tested in concentrations specified in the Table. The incubation time was 1h at 37°C. The enzyme activity is expressed in nmoles of cholesterol sulphate hydrolysed. The data presented are average for four series of experiments (± SD).

activity. The effect of phosphate ions (at 5 mM concentration), however, was not as profound as on placental arylsulphatase A and B activities [29,30]. Sulphates at 5 mM concentration exhibited no inhibitory effect on arylsulphatases A and B [29,30], but caused 30% inhibition of ChS-ase. Most compounds reacting with –SH groups appeared to be activators of ChS-ase (mercaptoethanol, glutatione, PCMB, cystein) while ascorbic acid and Ag^+ ions caused inhibition.

Among the phosphatase inhibitors tested, vanadate, molibdate and zinc ions were effective inhibitors. Tartaric acid induced only slight inhibition. All steroids tested caused inhibition of ChS-ase activity. It is worth mentioning that taurocholate and glikocholate, which were potent activators of ChS-ase activity in microsomal suspension [31], resulted in only a slight activation of the purified enzyme preparation, which means that

bile acids resulted in a release of the membrane inhabited enzyme as a mixed phospholipid–bile acid micelles. Digitonin effected pronounced activation of the purified and membrane resided enzyme [31].

The data presented in this paper showed that ChS-ase from the microsomal fraction of human placental tissue (subfraction which was not adsorbed by DEAE–cellulose) may be separated either as the high molecular weight or the low molecular form revealing molecular mass of about 200 and 20 kDa, respectively. Alkaline phosphatase digestion caused conversion of the high molecular weight form of ChS-ase to the low molecular weight form (Fig. 5). The most purified and depolymerized (by alkaline phosphatase treatment) enzyme revealed a molecular mass of 20 kDa (Fig. 5). The enzyme recovery (assuming that the initial activity — 100% — was that unadsorbed by DEAE–cellulose fraction) was about 300%. This means that the procedure presented in this paper allowed us to remove the ChS-ase inhibitors or cause concentration of its activators. Also one could not also exclude that the observed activation was effected by depolymerization of the enzyme.

The data presented previously [32–38] showed that microsomal steroid sulphate sulphohydrolases exhibited various molecular weights. The molecular weight variants may represent distinct enzymes, heterogeneously glycosylated forms of the same enzyme, proteolyticaly modified enzyme(s), clusters of the enzyme with membrane proteins and lipids or enzyme–detergent complexes.

Steroid sulphohydrolase of rat liver microsomes, separated by Burstein [34], showed a molecular weight of 600 kDa and tended to associate to form larger aggregates. The oestrone sulphate sulphohydrolase from human placenta and rat kidney revealed a molecular mass of about 130 kDa [25,32,35] and the ChS-ase prepared by Bleau et al. [16] and by Iwamori et al. [36] from rat liver and the enzyme separated in this work from human placenta have a molecular mass of about 20–23 kDa. These data suggest the presence of specific enzymes.

From the data presented here it is evident that the differences in molecular weight of ChS-ase may also arise from polymerization and depolymerization phenomena which are triggered by phosphorylation and dephosphorylation processes.

The purified ChS-ase from human placental microsomes after the dephosphorylation of the 200 kDa form exhibited a molecular weight of about 20 kDa. The enzyme revealed the optimal activity at pH 9.0, the K_m value of the enzyme with cholesterol sulphate as substrate was established to be $1.5 \pm 0.85 \times 10^{-5}$ M.

The biochemical conditions putting in motion the interconvestion processes of phosphorylation and dephosphorylation should be investigated.

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