Characterization of Cholylglycine Hydrolase from a Bile-Adapted Strain of *Xanthomonas maltophilia* and Its Application for Quantitative Hydrolysis of Conjugated Bile Salts†

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Purified bile salt hydrolase from bile-adapted *Xanthomonas maltophilia* displays Michaelis-Menten kinetics on cholylglycine and cholyltaurine and hydrolyzes bile salts also in crude bovine bile. The protein is a dimer and is resistant to proteinases and to heating at 55 to 60°C for up to 60 min, in agreement with calorimetric data.

Recently, we examined the metabolism of bile salts by the bile-adapted strain *Xanthomonas maltophilia* CBS 827.97, which hydrolyzes bile salt conjugates, rearranging the steroid nucleus (1, 5). Bile salts have multiple functions in digestion of lipids, solubilization, and excretion of cholesterol (2, 19, 23). Ursodeoxycholic acid is particularly interesting in this regard, solubilizing gallbladder cholesterol stones after oral administration (20). Ursodeoxycholic acid is currently prepared by alkaline hydrolysis of bile conjugates, inversion of the hydroxyl configuration at carbon 7, and resolution of the racemic mixture (reviewed in reference 1). Procedures for enzymatic synthesis would be useful for controlling stereochemistry, increasing yields, and avoiding toxic or aggressive chemicals. In this paper we describe a solution to the hydrolysis step, in which purified bile salt hydrolase (cholylglycine hydrolase [CGH], EC 3.5.1.24) from our bacterial strain is used.

Procedures for purification of CGH and of other enzymes of bile salt metabolism are covered by an Italian patent (1). For purification of CGH, the enzyme was isolated from 10 g of bacteria grown as described previously (5) and lysed by overnight stirring in 10 volumes of 20 mM sodium phosphate–1 mM EDTA–2 mM mercaptoethanol (pH 7.5) (NaP buffer) containing 0.3 mg of egg lysozyme per ml and 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation ($200,000 \times g$, 20 min), and nucleic acids were removed by precipitation with protamine sulfate (2 mg/ml). The supernatant, adjusted to pH 8.3, was chromatographed on a DEAE-Sepharose column (30 ml) and eluted with a linear 0 to 0.25 M NaCl gradient in NaP buffer (pH 8.3). The second protein peak, displaying hydrolase activity, was fractionated with ammonium sulfate in order to collect proteins insoluble between 45 and 75% saturation. After dialysis against NaP buffer, the phosphate concentration of the solution was adjusted to 200 mM at pH 7.5 by adding solid salts, and the solution was heated to 55°C with continuous swirling in a water bath at 70°C. After the solution was cooled on ice, denatured aggregated proteins were removed by high-speed centrifugation, and the supernatant, diluted with 1 volume of water, was loaded onto a phenyl-Sepharose column (2 ml) equilibrated in 100 mM phosphate buffer (pH 7.5). Hydrolase activity was eluted with NaP buffer. The CGH activity assay was performed by measuring glycine release from 5 mM cholylglycine in NaP buffer by a cadmium-ninhydrin procedure (6). For the cholyltaurine hydrolysis assay, we employed a different ninhydrin formulation (17). The protein concentration was determined as described previously (18).

The yield was 0.4 mg of CGH from 10 g of bacteria, with 20% recovery, a specific activity of 100 U/mg, and a purification factor of 168-fold (Table 1). The activity was stable for 2 weeks at 4°C and resisted prolonged heating at temperatures below 55°C. The purified hydrolase (minor contaminants were occasionally present, as shown in Fig. 1) had an M_r of 52,000 as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (16) and an M_r of 100,000 as determined by native size exclusion chromatography, suggesting that the enzyme is a dimer of identical subunits.

The enzyme was inactivated slowly by thiol (iodoacetamide and *N*-ethylmaleimide), histidyl (diethylpyrocarbonate), car-

TABLE 1. Purification of CGH from *X. maltophilia* CBS 827.97[a]

Step	Vol (ml)	Protein (mg)	Activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Extract	89	356	213	0.6	1	100
DEAE-Sepharose	35	12.9	209	16.2	27	98
Ammonium sulfate (45–70%)	7	7.7	140	18.2	30	65
Heat treatment	7	4	132.6	34	56	63
Hydrophobic chromatography	2.5	0.33	33.5	101	168	16

[a] Results of a typical purification in which the starting material was 8.9 g of *X. maltophilia*. The activity and specific activity values for the ammonium sulfate step were calculated after extensive dialysis.
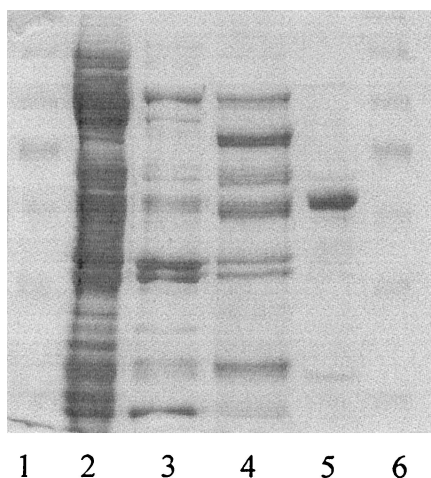
FIG. 1. SDS-PAGE of *X. maltophilia* CGH at different stages of purification. Lanes 1 and 6, M_r standards (M_rs, 172,600, 111,400, 79,600, 61,300, 49,000, 36,400, and 24,700); lane 2, extract; lane 3, DEAE-Sepharose eluate; lane 4, heated ammonium sulfate fraction; lane 5, representative fraction (protein load, 3 μg) eluted by hydrophobic interaction chromatography with NaP buffer (pH 7.5). The gel was stained with Coomassie blue R-250 and destained with aqueous isopropanol-acetic acid.

boxyl (ethyl-dimethyl-aminopropyl-carbodiimide), and arginyl (hydroxyphenylglyoxal) reagents and rapidly (15 min) by 1 mM *o*-phthalaldehyde, indicating the importance of closely spaced thiol and lysine residues for the catalytic activity. Heavy metals were inhibitory, and there was complete inactivation by 10 mM copper(II) salts.

The purified protein was resistant to narrow-specificity proteinases (trypsin, chymotrypsin, and V8 proteinase) but was slowly digested by the broad-specificity proteases elastase and pronase, with a half-life of 1.25 h at a hydrolase/protease ratio of 30:1 (wt/wt). No proteolytic intermediate that was detect-

able by SDS-PAGE accumulated, indicating the tight structure of the protein (13), and there was rapid digestion into small peptides after the initial cleavage. Proteolysis increased under mildly denaturing conditions (0.1% SDS). Automated Edman degradation (21) identified the N-terminal sequence AEGN, which was different from the N-terminal CTXY sequence (where X is Gly or Ala and Y is Ile, Leu, or Val) of other bacterial bile salt hydrolases (3, 4, 7, 22).

The enzyme hydrolyzed cholylglycine with Michaelis-Menten kinetics (K_m, 1.1 ± 0.15 mM), and there was competitive inhibition by cholyltaurine (K_i, 2.5 mM), as if both conjugated acids were hydrolyzed at a single site. The rate of inactivation by 5 mM iodoacetate was the same with both substrates. The products taurine and cholic acid inhibited uncompetitively the hydrolysis of cholylglycine, with K_i values of 2.5 and 4.7 mM. A set of parallel straight lines in double-reciprocal plots indicated that there was an inhibitory mechanism involving formation of a rate-limiting acyl intermediate or abortive ternary complexes with cholylglycine and taurine (data not shown). The pH optimum for hydrolysis was 7.9 to 8.5 with two apparent pK values (pK 7.2 and 9), which was consistent with catalytic roles for cysteine and lysine residues.

The enzyme was remarkably thermostable, with total preservation of activity during incubation for 75 min at 55°C (Fig. 2); however, it was rapidly inactivated at slightly higher temperatures, with half-lives of 9 and 5 min at 60 and 65°C, respectively. Differential scanning calorimetry revealed a transition at 64.5°C, with exothermic heat exchange and massive aggregation at temperatures above 80°C (data not shown). The thermal unfolding was clearly irreversible and complex, and deconvolution did not fit a two-state model, as shown by the large difference between the calorimetric (82.5 kJ/mol) and Van't Hoff (2.02 kJ/mol) enthalpies.

Numerous enteric bacteria express hydrolases for conjugated bile salts as protection against the toxicity of bile acids (9, 10, 15), and there are differences in cellular distribution (4, 14)
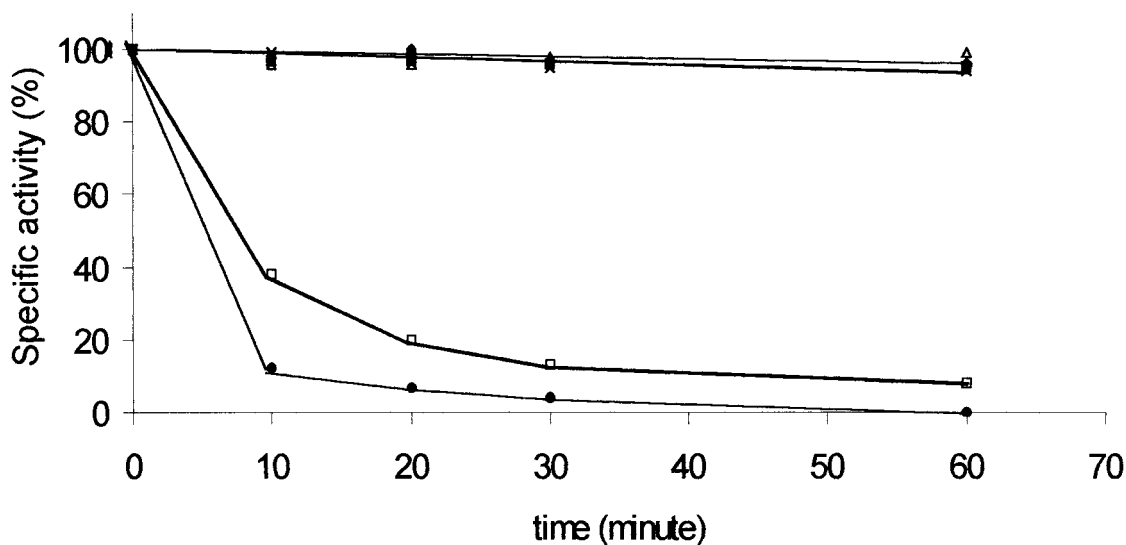

FIG. 2. Thermal stability of CGH activity. The purified enzyme was incubated at 25°C (◆), 40°C (■), 50°C (△), 55°C (×), 60°C (□), and 65°C (●), and the activity was determined in the presence of 5 mM cholylglycine at 25°C at different times.

and molecular structure (trimeric or tetrameric proteins with M_rs of 150,000 to 250,000) (3, 7, 8, 22). These hydrolases display high affinity for conjugated bile salts, have a slightly acid pH optimum, moderate thermostability, and peptide chains (314 to 329 amino acids) shorter than the peptide chain of our hydrolase (52 kDa), and are homologous to penicillin V acylase (22). The N-terminal sequence of our enzyme is different from those reported previously (3, 22), indicating that there has been appreciable divergence in these enzymes and a loss of the N-terminal cysteine, which is thought to be crucial for catalysis.

Some properties of *Xanthomonas* CGH (broad pH optimum, high hydrophobicity, appreciable thermal stability, and favorable substrate affinity) seem to be useful for carrying out biotransformations at high temperatures for prolonged times, with advantages in terms of the product yield. We examined this possibility by analyzing hydrolysis of conjugated salts in crude bovine bile (containing 35, 33, 10, and 7% taurocholic, glycocholic, taurochenodeoxycholic, and cholic acids, respectively, and smaller amounts of glycodeoxycholic, glycochenodeoxycholic, and taurodeoxycholic acids). At a concentration of 6 mM (close to the reported critical micellar concentration) (11, 12), conjugated bile salts were hydrolyzed completely by *X. maltophilia* CGH (0.04 U/ml) within 40, 22, and 12 min at 25, 37, and 50°C, respectively, without interference by other bile components (cholesterol, phospholipids) or reaction products (taurine, glycine, cholic acid).

All these features make *X. maltophilia* CGH attractive from theoretical and applicative points of view, and we are cloning this enzyme to obtain large amounts of it for complete physicochemical characterization.

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