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Purification and Characterization of a Cephalosporin Esterase from *Rhodosporidium toruloides*

MICHAEL POLITINO,[1]* SEAN M. TONZI,[2] WILLIAM V. BURNETT,[2] GUNA ROMANCIK,[1] AND JOHN J. USHER[1]

Departments of Enzyme Development[1] and Genetic Engineering,[2] Biotechnology Development Laboratories, Bristol-Myers Squibb Company, Syracuse, New York 13221-4755

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A novel cephalosporin esterase (EC 3.1.1.41) from *Rhodosporidium toruloides* was purified to gel electrophoretic homogeneity. The enzyme is a glycoprotein with a molecular mass of 80 kDa. Upon deglycosylation, several forms of the enzyme were observed with a molecular mass range between 60 and 66 kDa. The isoelectric point of the enzyme is approximately 5.6, with the pH optimum for activity occurring at 6.0. The optimal activity of the enzyme occurred at 25°C, with the enzyme rapidly losing activity at temperatures above 25°C. The enzyme deacetylated a variety of cephalosporin derivatives, including cephalosporin C; the K_m for this substrate is 51.8 mM, and the V_{max} is 7.9 μmol/min/mg. In addition to cephalosporins, the enzyme hydrolyzed short-chain *p*-nitrophenyl esters, with the activity decreasing with increasing ester chain length. The enzyme also has the ability to acetylate desacetyl cephalosporins in high yields under mild conditions in the presence of various acetyl donors. A comparison of the physical properties of the esterase with those of other well-characterized cephalosporin esterases indicates that the enzyme is unique in this class.

Esterases which can act on cephalosporins to form desacetyl cephalosporins have been isolated from many sources. A particularly rich source of cephalosporin esterase has been *Bacillus subtilis* (1, 12, 17); this activity has also been found in citrus peel (10), fungi (6, 9), other bacterial sources (4), and mammalian tissues (14). Few of the enzymes have been extensively characterized, with the notable exception of two distinct enzymes from *B. subtilis* (1, 17). Enzymatic deacetylation of cephalosporins is preferable to chemical deacetylation because of the instability of the cephalosporin nucleus at the extreme pH values required to chemically deacetylate these compounds. The deacetylated cephalosporins are the starting material for many of the current expanded-spectrum semisynthetic cephalosporins.

The cephalosporin C esterase activity of the pink yeast *Rhodosporidium toruloides* was first reported by Smith and Bailey at Glaxo Laboratories (16). In the presence of a donor such as isopropenyl acetate, this enzyme has the ability to specifically acetylate the C-3′ position of desacetyl cephalosporins without the need for blocking groups on other reactive groups in the molecule (19). The yields for this reaction are high, and few or no side products are produced during the course of the reaction. In this paper, we describe the purification and biochemical properties of the cephalosporin C esterase from *R. toruloides* as well as some of the synthetic properties of the enzyme.

MATERIALS AND METHODS

Microorganisms. *R. toruloides* (ATCC 10657) seed culture was initiated from the inoculation of frozen preservation cultures at 2% into 500-ml Erlenmeyer flasks containing 100 ml of the following medium: 2% glucose–1% yeast extract–1% Bacto Peptone–0.5% KH_2PO_4, pH 6.0. Seed flasks were cultured for 24 h at 28°C and 250 rpm; 2% inoculum volume was used to start production-stage fermentations. Production-stage medium was composed of 8% corn steep liquor, 1% KH_2PO_4, and 3% glucose, pH 6.2. Fermentor broth was cultured for 3 or 4 days at 16 to 21°C with high aeration. Specific activities of whole broth were typically in the range of 20 to 37 IU/ml. *B. subtilis* (ATCC 9466 and ATCC 6633) seed culture was initiated from the inoculation of culture from a Trypticase soy medium and shaken at 250 rpm and 28°C for 24 h. Seed stage was inoculated at 1% into flasks containing 100 ml of Trypticase soy medium per 500-ml flask. The cultures were shaken at 250 rpm and 28°C for 48 h.

Assays. Esterase activity was usually measured with *p*-nitrophenyl ester substrates. The enzyme was incubated at 30°C (unless described otherwise) with *p*-nitrophenyl acetate, 10.0 mM, in 100 mM potassium phosphate buffer (pH 6.5) or 10.0 mM *p*-nitrophenyl esters ranging in carbon chain length from C_2 to C_{18} in 100 mM potassium phosphate (pH 6.5)–2% acetonitrile. pH 6.5 was chosen because of the low extinction coefficient of the *p*-nitrophenylate ion at the pH optimum of the enzyme. Enzyme activity was monitored spectrophotometrically by measuring the increase in optical density at 405 nm due to the formation of the *p*-nitrophenylate ion. Alternatively, the esterase was assayed with cephalosporin derivatives as substrates. Enzyme was added to the reaction mixture containing the potassium salt of the cephalosporin (25 to 400 mM) and 100 mM potassium phosphate (pH 6.5) in a final volume of 0.5 ml. The mixture was incubated at 30°C (unless described otherwise), and the reaction was stopped by addition of 2.0 ml of 50% acetonitrile. The reaction was monitored at 254 nm by high-pressure liquid chromatography (HPLC) on a 5-μm C_{18} column (50 by 4 mm) with the mobile phase consisting of 25 mM octane sulfonic acid–0.1% phosphoric acid–12% methanol, pH 2.5. Activity values are expressed as international units: micromoles of product formed per minute at the indicated temperature and pH.

Protein was assayed by the dye-binding method of Bradford (2) with bovine serum albumin as the standard.

Enzyme purification. The esterase was released from *R. toruloides* cells by treatment of the fermentation broth with 100 mM EDTA at pH 4.0 for 8 h. Approximately 50% of the enzymatic activity could be released from the cells in this manner. The broth was centrifuged at 5,000 × *g* to remove the cells and the corn steep solids. The enzyme was diafiltered through an Amicon hollow-fiber cartridge with a molecular weight cutoff of 30,000. The pH was brought up to 7.0 by addition of 2 M ammonium hydroxide, and the enzyme solution was added to DEAE-Trisacryl (100 g of resin/50 ml of enzyme solution), which had been washed with 50 mM potassium phosphate buffer, pH 7.0. The enzyme does not bind to DEAE and was obtained in the filtrate, which was then brought to pH 4.5 with 1.0 M acetic acid. This solution was then loaded onto a carboxymethyl-Sepharose column (18 by 3 cm) and washed with 50 mM ammonium acetate, pH 4.5, until the absorbance at 280 nm was less than 0.1. The esterase was eluted with a linear gradient of 50 to 500 mM ammonium acetate, pH 6.5 (flow rate, 1.0 ml/min). Fractions of 7.0 ml were collected, and the fractions containing esterase were pooled and concentrated on a 50,000-molecular-weight-cutoff Centricon concentrator.

Deglycosylation of esterase. Removal of carbohydrate with recombinant peptide *N*-glycosidase (5) and endoglycosidase H (18) was performed as described elsewhere. Native and deglycosylated enzymes were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine carbohydrate loss.

* Corresponding author. Mailing address: Biotechnology Development Laboratories, Bristol-Myers Squibb Company, P.O. Box 4755, Syracuse, NY 13221-4755. Phone: (315) 432-4818. Fax: (315) 432-4785. E-mail: mpolitin@usccmail.uscc.bms.com.

Determination of molecular weight and pI. Molecular weight was determined by gel permeation chromatography and gel electrophoresis. Native PAGE was run according to the method of Davis (3) with a 10% running gel and 4% stacking gel. Activity was detected by incubation of the gel for 10 min in 100 mM potassium phosphate buffer, pH 6.0, containing 1.25 mM α-naphthyl acetate. Activity was visualized with 0.5% Fast B salt. SDS-PAGE gels (gradient, 8 to 25%) were run according to the method of Laemmli (13). Proteins were stained with Coomassie brilliant blue. Gel permeation chromatography was performed by HPLC on a 75- by 300-mm TosoHaas TSK-GEL GS3000SW XL column with a mobile phase of 200 mM potassium phosphate (pH 6.8)–150 mM sodium chloride. Bio-Rad gel filtration standard mixture (molecular weight, 670,000 to 1,350) was used as the marker. The flow rate was 1.0 ml/min, and the eluate was monitored at 280 nm. Fractions were collected and assayed for esterase activity. Isoelectric focusing gels were run with the Ampholine PAGplate system developed by Pharmacia in the pH range of 3 to 9. pI was also determined with the MinpHor system developed by Rainin with the broad-range ampholyte mixture, pH 3 to 9.

N terminus and peptide sequences. Esterase peptides were generated by treatment of the enzyme with lysine endopeptidase and purified by HPLC at the Cornell University Biotechnology Analytical Facility. Sequencing of the peptides and N terminus was performed by automated Edman degradation at the Cornell University Biotechnology Analytical Facility.

Cloning and sequencing of cDNA. A cDNA clone was produced by 3′ rapid amplification of cDNA ends (BRL Life Technologies, Gaithersburg, Md.). Total RNA from *R. toruloides* was isolated with Trizol reagent (BRL Life Technologies) and further purified by lithium chloride precipitation. First-strand cDNA was prepared by reverse transcription from an adapter primer. The RNA template was digested with RNase H, and the cDNA was amplified by PCR with a gene-specific primer and an adapter primer. The coding region was amplified and mutagenized by a second round of PCR with an internal gene-specific primer which included the putative translation start site and an *Nco*I restriction site at the translation start site. This produced a 1.9-kb fragment which was gel purified. The nucleotide sequence was determined by the dideoxy chain termination method (15) with the *Taq* Track femtomole DNA sequencing systems (Promega, Madison, Wis.). T3, T7, and synthesized internal primers were used to sequence the entire gene from both strands. Electrophoresis was performed on a 7% Long Ranger (AT Biochem, Malvern, Pa.) polyacrylamide gel containing 7 M urea in Tris-borate-EDTA buffer at 2,700 V.

Acetylation of desacetyl 7-amino cephalosporanic acid (desacetyl 7-ACA). Cell extract of *B. subtilis* (ATCC 9466) was prepared by sonicating the cells and removing the cell debris by centrifugation at 5,000 × *g*. The cell-free supernatant was concentrated with a 30,000-molecular-weight-cutoff Centricon concentrator to a final specific activity of 40 IU/ml. *R. toruloides* cell extract was prepared as described under "Enzyme purification," and the activity was concentrated with a 30,000-molecular-weight-cutoff Centricon concentrator to a final specific activity of 80 IU/ml. *B. subtilis* (ATCC 6633) cells were concentrated by centrifugation to a final activity of 0.63 IU/ml. The reaction conditions for acetylation were as follows: 400 mM desacetyl 7-ACA, 2.5 M acetyl donor, 22 IU of enzyme (0.23 IU for *B. subtilis* ATCC 6633), and 24°C. The pH was maintained at the pH optimum for the individual enzyme. The reaction was monitored by HPLC, and additional substrate and enzyme were added as the reaction rate plateaued. Final yields were calculated after no additional 7-ACA was formed upon further addition of enzyme and acetyl donor.

Chemicals. Cephalosporin C and derivatives thereof were obtained from Bristol-Myers Squibb. Endoglycosidases were purchased from Boehringer Mannheim (Indianapolis, Ind.). DEAE-Trisacryl was purchased from IBF Biotechnics (Villeneuve-la-Garenne, France). All other chemicals were purchased from Sigma Chemical (St. Louis, Mo.) unless otherwise indicated.

Nucleotide sequence accession number. The *R. toruloides* cephalosporin esterase-encoding gene sequence was submitted to GenBank under accession no. AF025410.

RESULTS

Purification and physical characterization of the esterase. Release of active enzyme could not be achieved by physical or enzymatic means of cell disruption. Treatment of whole cells with 100 mM EDTA at pH 4.0 for 8 h released approximately 50% of the activity from the cells without lysing them. The mechanism by which the enzyme is released is not understood, and attempts to further define it were not successful. The results of the purification are summarized in Table 1. After carboxymethyl-Sepharose chromatography, a single band at 80,000 Da was observed by SDS-PAGE. Native PAGE showed two active bands initially. However, after repeated freeze-thaw cycles of the enzyme solution, the two distinct active bands appeared as a single very broad band (data not shown). Treatment of the enzyme with endoglycosidases resulted in a 25 to 30% reduction of molecular weight (Fig. 1) with several deglycosylated forms of the enzyme present. The two endoglycosidases gave slightly different band patterns, reflecting the differences in their ability to cleave hybrid carbohydrates. Gel filtration chromatography of the enzyme indicated that the enzyme is a monomer in the native state. The isoelectric point of the protein was determined to be approximately 5.6.

TABLE 1. Purification of cephalosporin C esterase[a]

Step	Total activity (μmol/min)	Total protein (mg)	Sp act (μmol/min/ml)
Extract	8.4	13.9	0.5
DEAE-Trisacryl	6.4	5.5	1.2
Carboxymethyl-Sepharose	5.8	1.3	4.4

[a] The enzyme was incubated with *p*-nitrophenyl acetate, 10.0 mM, in 100 mM potassium phosphate buffer, pH 6.5. Enzyme activity was monitored spectrophotometrically, and protein was assayed by the Bradford method as described in Materials and Methods.

The deduced amino acid sequence of the enzyme is shown in Fig. 2. The amino-terminal sequence obtained from the purified enzyme was H_2N-Thr-Asn-Pro-Asn-Glu-Pro-Pro-Pro-Val-Val-Asp-Leu-Gly-Tyr-Ala-Ala. The N-terminal threonine is 28 amino acids from the translation start site, indicating that the peptide is cleaved to produce a mature active enzyme. This cleavage appears to be a simple posttranslational modification. The 27-amino-acid peptide removed does not contain the consensus sequence found in eukaryotic signal peptides (20). The molecular mass of 61,315 Da deduced from the sequence information is similar to that of the lowest-molecular-weight

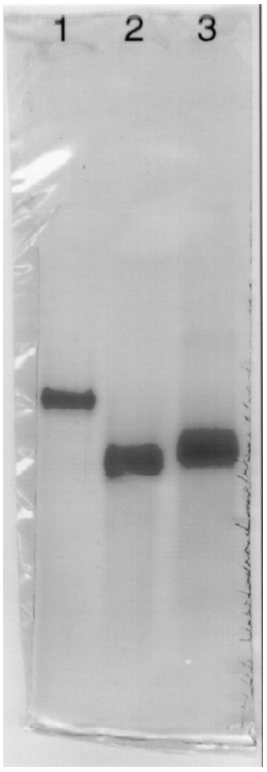

FIG. 1. SDS-PAGE of intact and deglycosylated esterase. Carbohydrates were removed from the enzyme as described in Materials and Methods. Lanes: 1, untreated esterase; 2, esterase treated with peptide-*N*-glycosidase; 3, esterase treated with endoglycosidase H.

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1    MLLNLFTLASLAATLQLAFASPTSLVRRTNPNEPPPVVDLGYARYQGYLN

51   ETAGLYWWRGIRYASAQRFQAPQTPATHKAVRNATEYGPICWPASEGTNT

101  TKGLPPPSNSSSSAPQKQASEDCLFLNVVAPAGSCEGDNLPVLVYIHGGG

151  YAFGDASTGSDFAAFTKHTGTKMVVVNLQYRLGSFGFLAGQAMKDYGVTN

201  AGLLDQQFALQWVQQHVSKFGGNPDHVTIWGESAGAGSVMNQIIANGGNT

251  VKALGLKKPLFHAAIGSSVFLPYQAKYNSPFAELLYSQLVSATNCTKAAS

301  SFACLEAVDAAALAAAGVKNSAAFPFGFWSYVPVVDGTFLTERASLLLAK

351  GKKNLNGNLFTGINNLDEGFIFTDATIQNDTISDQSQRVSQFDRLLAGLF

401  PYITSEERQAVAKQYPISDAPSKGNTFSRISAVIADSTFVCPTYWTAEAF

451  GSSAHKGLFDYAPAHHATDNSYYIGSIWNGKKSVSSVQSFDGALGGFIET

501  FNPNNNAANKTINPYWPTFDSGKQLLFNTTTRDTLSPADPRIVETSSLTD

551  FGTSQKTKCDFWHGSISVNAGL
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FIG. 2. Amino acid sequence of cephalosporin esterase. The amino acid sequence of the enzyme was deduced from the cDNA sequence. The arrow (threonine 29) represents the beginning of the mature peptide. The underlined sequences represent the sequences determined by lysine endopeptidase digestion of purified esterase. The amino acid sequence from position 231 to 235, G-X-S-X-G, is boxed. Potential glycosylation sites are highlighted (**N**).

band observed after deglycosylation. There are eight potential glycosylation sites (Asn-X-Ser or Asn-X-Thr) on the enzyme. The enzyme also contains the G-X-S-X-G motif found in many esterases, lipases, and serine proteases (17). A search for related sequences with the National Center for Biotechnology Information's BLAST facility revealed sequence similarity with several lipases from yeast and filamentous fungi. In addition, much weaker similarity was seen with mammalian carboxyl, choline, and sterol esterases. The highest-scoring segment pairs (score of 200 to 226) were observed with lipases from *Candida rugosa*, *Candida cylindracea*, *Geotrichum candidum*, and *Galactomyces geotrichum*. As expected, the peptide segment including the active site (amino acids 120 to 250) showed the highest degree of relatedness (up to 75%) between the esterase and all of the lipases listed. However, few peptide sequences outside of this putative active-site segment showed any sequence similarity. Based on the matching sequences found with BLAST, it was estimated that the greatest overall similarity was approximately 28% with the lipase from *C. rugosa*.

Biochemical characterization of the esterase. The esterase preferentially hydrolyzed short-chain esters of *p*-nitrophenol and did not hydrolyze carboxylic esters of more than four carbons (Table 2). The esterase also showed only slight selectivity among the cephalosporins tested. The group at the 7 position of the cephalosporin ring system did not greatly influence the ability of the enzyme to hydrolyze the acetyl group at the 3′ position. As shown in Table 3, with the exception of *N*-acetyl 7-ACA, the cephalosporins with smaller substituted acetyl side chains were hydrolyzed at a much higher rate than those with the longer glutaryl or α-amino adipyl side chains. The lower rate for the *N*-acetyl 7-ACA may be due to the fact that it has two acetyl groups competing for binding at the active site. The cephalosporins with side chains containing bulky ring systems were also hydrolyzed at a lower rate. The enzyme exhibited Michaelis-Menten kinetics with cephalosporin C.

TABLE 2. Effect of increasing ester chain length on esterase activity[a]

Length of ester	Relative activity (%)
Acetate C_2	100
Propionate C_3	34
Butyrate C_4	5
Caproate C_6	0
Caprylate C_8	0
Caprate C_{10}	0
Laurate C_{12}	0
Myristate C_{14}	0
Palmitate C_{16}	0
Stearate C_{18}	0

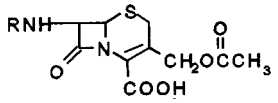
[a] The enzyme was incubated with *p*-nitrophenyl esters ranging in carbon chain length from C:2 to C:18 in 100 mM potassium phosphate (pH 6.5)–2% acetonitrile. Enzyme activity was monitored spectrophotometrically as described in Materials and Methods.

From double reciprocal plots, the K_m for hydrolysis of cephalosporin C was found to be 51.8 mM with a corresponding V_{max} of 7.9 μmol/min/mg. The reaction products, desacetyl cephalosporin C and acetate, did not inhibit the reaction to any appreciable extent. Five international units of enzyme completely hydrolyzed a 1.0% solution of cephalosporin C within 30 min at 30°C with no side products observed by HPLC.

The esterase was found to be active in a pH range of 4.5 to 7 with optimal activity at a pH of 6.0 with both *p*-nitrophenyl acetate and cephalosporin C. *p*-Nitrophenyl acetate was used for most of the biochemical characterizations because of its greater stability under a wide range of pH and temperature. The optimal temperature for the reaction was 25°C. The enzyme was unstable when incubated at temperatures above 25°C (Fig. 3), with rapid inactivation observed between 30 and 45°C. The enzyme also became unstable during deglycosylation with endoglycosidase H. Concomitant loss of activity was observed with the appearance of the lower-molecular-weight deglycosylated enzyme. The enzyme lost over half of its activity compared to the control (which contained no glycosidase enzyme). This indicates that the sugar moieties may play a role in stabilizing the native structure of the enzyme.

B-type carboxyl ester hydrolases are typically nonspecific esterases with an active-site serine (11). The amino acid sequence about the active site has been found to be Gly-X-Ser-X-Gly in many mammalian carboxylesterases, amidases, and proteases (8). To further determine if serine is involved in catalysis, the enzyme was incubated with several serine-modifying mechanism-based inhibitors (Table 4). The results strongly suggest the presence of an active-site serine for the *Rhodosporidium* enzyme. Phenylmethylsulfonyl fluoride, 3,4-dichloroisocoumarin (DCI), and dimethyl phosphite all inhibited the enzyme. The histidine-modifying reagent diethylpyrocarbonate essentially inactivated the enzyme. This data coupled with the proposed inactivation of chymotrypsin by DCI in which the DCI acylates both the active-site serine and histidine to form diacylated inactive enzyme (7) suggests a role for histidine in the hydrolysis of substrate. Histidine has previously been proposed to be present at the active site of carboxylesterases (11). Sulfhydryl-modifying agents iodoacetamide and *N*-ethylmaleimide had little or no effect on the activity of the enzyme, although slight activation was observed with β-mercaptoethanol and dithiothreitol. The presence or absence of metal ions also had little or no effect on the enzyme, although slight inhibition was observed with EDTA. This may be due to

TABLE 3. Relative rates of esterase activity against cephem substrates[a]



Substrate	Relative Rate (%)
R = —H	100
—CCH$_3$	51
—CCH$_2$Cl	105
—CCHCl$_2$	108
—CCH$_2$Br	114
—CCH$_2$I	103
—CCH$_2$CH$_2$CH$_2$COOH	105
—CCH$_2$CH$_2$CH$_2$CHCOOH (NH$_2$)	68
—CCH$_2$CH$_2$CH$_2$CHCOOH (NHCCH$_3$)	42
—CCH$_2$—C$_6$H$_5$	17
—CCH$_2$—C$_6$H$_4$—CH$_3$	68
—CCH$_2$—C$_6$H$_4$—OCH$_3$	41
—CCH$_2$—C$_4$H$_3$S	34

[a] The reaction mixtures contained the substrates at 10 mg/ml, esterase, and 100 mM potassium phosphate (pH 6.5). The mixtures were assayed by HPLC as described in Materials and Methods.

the interaction between the enzyme and EDTA that is also responsible for the release of the enzyme from the cell.

Comparison of the synthetic ability of cephalosporin esterases. The cephalosporin esterase from *R. toruloides* can acetylate desacetyl cephalosporins under mild conditions in the presence of a donor such as isopropenyl acetate with yields of greater than 75% (19). The synthetic ability of the esterase from *R. toruloides* was compared with that of two cephalosporin esterases, one from *B. subtilis* ATCC 6633 (12) and another from *B. subtilis* ATCC 9466. A variety of acetyl donors were tested to determine the best yield of 7-ACA from desacetyl 7-ACA (data not shown). The esterases differed in their preference of acetyl donor, with the esterases from *B. subtilis* giving the best product yields with ethylene glycol diacetate as acetyl donor while the esterase from *R. toruloides* gave high yields with isopropenyl acetate as donor. Overall product yields are

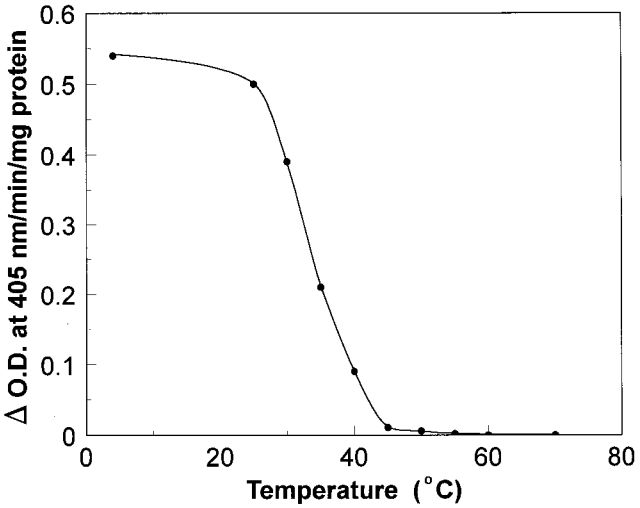


FIG. 3. Thermal stability of esterase. Enzyme was incubated at various temperatures for 15 min and then immediately placed on ice. Activity was then assayed as described in Materials and Methods. O.D., optical density.

given in Table 5. The cephalosporin esterase from *R. toruloides* clearly gave the best synthetic yields under conditions that were optimal for both enzymatic activity and stability of the cephalosporin nucleus.

DISCUSSION

In this study, we have described the purification of cephalosporin esterase from *R. toruloides* ATCC 10657 and its biochemical and physical properties. The industrial applications of this enzyme are twofold: the enzyme can deacetylate various cephalosporins with high yields and negligible side products, and in addition, this enzyme can specifically O acetylate cephalosporins with high yields in the presence of an acetyl donor. This particular quality is of considerable interest because current published chemical methods of O acetylation require blocking groups for any reactive amino group and conditions often lead to the lactonization of the cephalosporin, resulting in a loss of product (19).

A comparison of three well-characterized cephalosporin esterases (1, 12, 17) from *B. subtilis* and *R. toruloides* shows

TABLE 4. Effect of enzyme modulators on esterase activity[a]

Effector	Relative activity (%)
None	100
β-Mercaptoethanol	137
Dithiothreitol	115
Iodoacetamide	104
N-Ethylmaleimide	85
Phenylmethylsulfonyl fluoride	53
DCI	43
Dimethyl phosphite	51
Diethylpyrocarbonate	7
$MgCl_2$	100
$MnCl_2$	97
$ZnCl_2$	93
$CaCl_2$	95
EDTA	82

[a] Enzyme was incubated in the presence of 10 mM reagent for 15 min at 25°C. The reaction mixture was then diluted 100-fold into assay mixture and assayed with *p*-nitrophenyl acetate as described in Materials and Methods.

TABLE 5. Synthesis of 7-ACA from desacetyl 7-ACA[a]

Esterase source	Acetyl donor	Yield (%)
R. toruloides ATCC 10657	Isopropenyl acetate	79
B. subtilis ATCC 6633	Ethylene glycol diacetate	26
B. subtilis ATCC 9466	Ethylene glycol diacetate	54

[a] Reactions were carried out as described in Materials and Methods. The reaction time for *R. toruloides* and *B. subtilis* ATCC 9466 was 200 min, and the time for *B. subtilis* ATCC 6633 was 48 h.

striking differences between the bacterial enzymes and that from *R. toruloides*. The molecular weights and subunit compositions of the enzymes differ considerably. The enzyme from *R. toruloides* is monomeric, with a molecular weight of 80,000, whereas the *B. subtilis* enzymes are multimeric, with molecular masses ranging from 150 to 280 kDa (17). The high pH (8.0 to 8.5) and temperature (40 to 55°C) optima for the *B. subtilis* enzymes (17) are in a range in which nonenzymatic hydrolysis and breakdown of cephalosporin are significant. The *R. toruloides* enzyme, on the other hand, is optimally active at lower temperature (25°C) and pH (6.0), where the cephalosporins are more stable. The *R. toruloides* enzyme is superior in its synthetic ability, distinguishing it from the bacterial enzymes. The K_m and V_{max} values of the enzymes toward cephalosporin C are summarized in Table 6 (1, 12, 17). There are slight differences in the kinetic parameters of the four enzymes. The K_m values are all relatively high for both the synthetic (data not shown) and hydrolytic reactions, in the millimolar range, suggesting that cephalosporin C is not the natural substrate for these enzymes. The V_{max} values for hydrolysis are similar for the three enzymes, with the *R. toruloides* enzyme having the highest V_{max}. The rates for the synthetic reaction are several orders of magnitude less than that for the hydrolytic reaction (data not shown).

The amino acid sequence of the esterase was compared with those available through the National Center for Biotechnology Information's BLAST search. As expected, some sequence similarity was found with eukaryotic lipases and esterases, particularly around the putative active-site serine (G-X-S-X-G). The greatest similarity was found with lipases from the yeasts *C. rugosa* and *C. cylindracea* and the filamentous fungi *Geotrichum candidum* and *Galactomyces geotrichum*. However, no similarities were observed with other cephalosporin esterases in the database. No other eukaryotic cephalosporin esterases were present in the database, and the eukaryotic carboxylesterases represented showed much less sequence relatedness than the lipases listed above, making this unique among the class of cephalosporin esterases.

TABLE 6. Comparison of the kinetic properties of the cephalosporin C esterase from *R. toruloides* and those from several strains of *B. subtilis*

Esterase source	K_m (cephalosporin C) (mM)	V_{max} (cephalosporin C) (μmol/min/mg)
R. toruloides ATCC 10657	52	7.90
B. subtilis ATCC 6633	~5	ND[a]
B. subtilis WRRL-B-58	15	1.08
B. subtilis SHS0133	24	1.38

[a] ND, not determined.

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REFERENCES

1. **Abbott, B. J., and D. Fukuda.** 1975. Physical properties and kinetic behavior of a cephalosporin acetyl esterase produced by *Bacillus subtilis*. Appl. Microbiol. **30:**413–419.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.
3. **Davis, B.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. **121:**404–427.
4. **Demain, A. L., R. B. Walton, J. F. Newkirk, and I. M. Miller.** 1963. Microbial degradation of cephalosporin C. Nature (London) **199:**909–910.
5. **Elder, J. H., and S. Alexander.** 1982. *endo*-β-*N*-Acetylglucosamidase F: endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high-mannose and complex glycoproteins. Proc. Natl. Acad. Sci. USA **79:**4540–4544.
6. **Fujisawa, Y., H. Shirafuji, and T. Kanzaki.** 1975. Deacetylcephalosporin C formation by cephalosporin C acetylhydrolase of *Cephalosporium acremonium* mutant. Agric. Biol. Chem. **39:**1303–1309.
7. **Harper, J. W., K. Hemmi, and J. C. Powers.** 1985. Reaction of serine proteases with substituted isocoumarins: discovery of 3,4-dichloroisocoumarin, a new general mechanism based serine protease inhibitor. Biochemistry **24:**1831–1841.
8. **Heyman, E.** 1980. Carboxylesterases and amidases, p. 291–323. *In* W. B. Jakoby (ed.), Enzymatic basis of detoxification, vol. 2. Academic Press, New York, N.Y.
9. **Huber, F. M., R. H. Baltz, and P. G. Caltrider.** 1968. Formation of desacetylcephalosporin C in cephalosporin C fermentation. Appl. Microbiol. **16:** 1011–1014.
10. **Jeffery, J. D., E. P. Abraham, and G. G. F. Newton.** 1961. Deacetylcephalosporin C. Biochem. J. **81:**591–596.
11. **Kirsch, K.** 1971. Carboxylic ester hydrolases, p. 43–69. *In* P. D. Boyer (ed.), The enzymes, vol. 5. Academic Press, London, United Kingdom.
12. **Konecny, J., and W. Voser.** 1977. Effects of carrier morphology and buffer diffusion on the expression of enzymatic activity. Biochim. Biophys. Acta **485:**367–378.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
14. **O'Callaghan, C. H., and P. W. Muggleton.** 1963. The formation of metabolites from cephalosporin compounds. Biochem. J. **89:**304–308.
15. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
16. **Smith, A., and P. J. Bailey (Glaxo Group).** August 1985. U.S. patent 4,533,632.
17. **Takimoto, A., K. Mitsushima, S. Yagi, and T. Sonoyama.** 1994. Purification, characterization and partial amino acid sequences of a novel cephalosporin-C deacetylase from *Bacillus subtilis*. J. Ferment. Bioeng. **77:**17–22.
18. **Trimble, R. B., and F. Maley.** 1984. Optimizing hydrolysis of *N*-linked high-mannose oligosaccharides by *endo*-β-*N*-acetylglucosamidase H. Anal. Biochem. **141:**515–522.
19. **Usher, J. J., G. Romancik, M. Politino, and D. A. Lowe (Bristol-Myers Squibb).** April 1996. U.S. patent 5,512,454.
20. **Von Heijne, G.** 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **133:**17–21.