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Purification and Characterization of Chloramphenicol Acetyltransferase from *Morganella morganii*

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Chloramphenicol acetyltransferase (CAT) was purified to homogeneity from *Morganella morganii* starting with ammonium sulphate fractionation, followed by separation on DEAE-Sephadex A50, and G-100 Sephadex gel filtration. The enzyme was purified 133.3 fold and showed a final specific activity of 60 units/mg protein with a yield of 37%. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme revealed it as a heterotetramer that consists of four subunits with close molecular weights (19.5, 19, 18, and 17.5 kDa). The molecular weight of the native enzyme was calculated to be 78 kDa, as determined by gel filtration, which approximated to that of the four subunits (74 kDa). The enzyme showed a maximum activity at pH 7.8 when incubated at 35°C. A Lineweaver-Burk analysis gave a Km of 5.0 uM and Vmax of 153.8 U/ml. The amino acid composition of the purified enzyme was also determined.

Keywords: Chloramphenicol acetyltransferase, *Morganella morganii*, Purification, Characterization, Amino acid composition

Introduction

Chloramphenicol acetyltransferase (CAT) has been identified in numerous species of bacteria (Shaw, 1967; Murray *et al.*, 1990). This enzyme is involved in the acetylation of the antibiotic chloramphenicol, which uses acetyl-CoA as an acetyl donor to produce chloramphenicol-3-acetate (Murray, Lewendon, Shaw, 1991). It fails to bind to bacterial ribosomes (Burns *et al.*, 1985; Mosher *et al.*, 1990; Shaw and Leslie, 1991), which leads to the resistance of bacteria to that particular antibiotic. Chloramphenicol resistance was shown to be mediated by small plasmids. The expression of the CAT gene was inducible with chloramphenicol in various strains of bacteria (Rogers *et al.*, 1990; Rogers and Lovett, 1990). However, the CAT expression was induced by 2-aminopurine (Kalvakolanu *et al.*, 1991) and mitomycin C (Luethy and Holbrook, 1992), but with an unknown mechanism.

Bacterial resistance to chloramphenicol was demonstrated in various species, such as *Escherichia coli* (Shaw, 1967), several multiple resistant *Staphylococcus epidermidis* (Shaw, Bentley, Sands, 1970), 42 isolates of *Haemophilus influenzae* (Azemun *et al.*, 1981; Powell *et al.*, 1987), *Haemophilus parainfluenzae* and *Haempohilus ducreyi* (Roberts, Actis, Crosa, 1985), *Clostridium difficile* (Wern *et al.*, 1988), and 18 of *Staphylococcus lentus* strains (Schwarz, 1994).

CAT was purified and characterized from various species, including *Staphylococcus epidermidis* (Shaw, Bentley, Sands, 1970), *Haemophilus parainfluenzae* (Shaq, Bouanchaud, Goldstein, 1976), *Staphylococcus faecalis* (Nakagawa, Nitahara, Miramura, 1976), and *Bacteriodes fragilis* (Britz and Wilkinson, 1976). Here, we describe the purification of CAT from *Morganella morganii*, and its characterization including molecular weight, kinetic parameters, and amino acid composition. Factors affecting the activity of purified enzyme are also discussed.

Materials and Methods

Materials Chloramphenicol was kindly provided by Hoechst Orient S.A.S. (Cairo) under a license from Hoechst AG Frankfurt Main-German. DEAE-Sephadex A50, Sephadex G-100, blue dextran 2000, and standard proteins for gel filtration and SDS-gel electrophoresis were purchased from Pharmacia, Uppsala. All other chemicals were of analytical grade.

Microorganisms media and growth conditions Pathogenic bacterial strain *Morganella morganii* was kindly provided by the Bacteriology Department, Faculty of Medicine, Alexandria University, (Egypt). It was identified by the International Microbiological Institute, Bakeham Lane, Egham, Surrey, TW20 9TY (UK). The stock culture was maintained on nutrient agar slants that contained peptone (0.5%), beef extract (0.3%), and agar (2%). The culture was incubated at 30°C for 48 h and stored at 4°C.

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Transfers from single slant cultures 48 h old were taken into 50 ml aliquots of the basal medium that were placed in 250 ml Erlenmeyer flasks to initiate growth. Standard inocula of 2% were taken from the latter liquid culture after growth for 48 h at 30°C on a rotary shaker to start growth in the fermentation flasks.

The basal medium, used for cultivation of the tested bacterial strains, contained peptone (1%), glucose (1%), NaCl (0.3%), $CaCl_2$ (0.001%), $MgCl_2 \cdot 6H_2O$ (0.02%), and KH_2PO_4 (0.005%) with pH 7.8. All of the nutrient solutions were sterilized by autoclaving for 15 min at a pressure of 15/16 $inch^2$ to raise the temperature to 121°C. Chloramphenicol substrate was added after autoclaving and cooling of the medium at a concentration of 0.5 mg/l after a 48-h incubation under aseptic conditions. The flasks were inoculated and incubated on a rotary shaker (250 rpm) at 30 ± 2.0°C for the requisite time. Thereafter, the content of each flask was centrifuged in a cooling centrifuge, whereby the necessary analysis of the bacterial cells and the culture filtrate was made. Each treatment was carried out in triplicate. The results obtained throughout this work were the arithmetic means of at least two experiments.

Preparation of cell-free extract and enzyme assay Bacterial cells (25 g wet weight) were suspended in a final volume of 100 ml 10 mM Tris-HCl buffer (pH 7.8) that contained 0.01 mM β-mercaptoethanol. Cell disruption was achieved by ultrasonication (Labsonic U; B. Braun, Melsungen, Germany) for 3 min in an ice bath at 4°C. It was followed by centrifugation at 15,000 g for 30 min at 4°C to sediment the insoluble material (unbroken cells) and the supernatant (containing cell-free extract) was separated as a crude intracellular enzyme.

An assay of the CAT activity was carried out according to the method described previously (Habeeb, 1972). One unit of activity was defined as the amount of enzyme that is sufficient to acetylate 1 mole of chloramphenicol per minute under standard conditions (Lewendon and Shaw, 1990). The protein concentration was determined by a modified Lowry's method (Ohnishi and Barr, 1978).

Purification of CAT The purification was carried out at 4°C on 100 ml of the crude extract.

Ammonium sulphate fractionation Finely powdered ammonium sulphate was added to a 50% saturation. The mixture was left for 2 h at 4°C and was followed by centrifugation at 8,000 rpm for 20 min at 4°C. The obtained precipitate was dissolved in 100 ml of a standard buffer consisting of 10 mM Tris/HCl (pH 7.8), 0.5 mM β-mercaptoethanol, and 0.2 mM chloramphenicol. The buffered extract was heated at 70°C for 10 min, cooled immediately and centrifuged at 8,000 g for 20 min at 4°C. The resulting supernatant was brought to a 50% saturation with ammonium sulphate, centrifuged and the obtained precipitate dissolved in 10 mM Tris/HCl buffer (pH 7.8). It was dialyzed overnight at 4°C against 10 mM Tris/HCl buffer (pH 7.8) that contained 0.5 mM β-mercaptoethanol and 0.2 mM chloramphenicol. The dialysate was then centrifuged at 8,000 g for 20 min at 4°C to remove the precipitate. The supernatant was used in the subsequent steps.

Ion-exchange chromatography The supernatant (20 ml) was applied to a DEAE-Sephadex A50 column (2.5 × 20 cm) that was pre-equilibrated with a 10 mM Tris/HCl buffer (pH 7.8) that contained 0.5 mM β-mercaptoethanol and 0.2 mM chloramphenicol. It was eluted with a NaCl gradient (0.1-0.5 M) at a flow rate of 1 ml/min. Fractions (5 ml) were collected using a fraction collector (LKB Ultorace) at 4°C. It was assayed for protein at 280 nm, as well as for enzyme activity. Active fractions were pooled and dialyzed (against a 10 mM Tris/HCl buffer-pH 7.8-that contained 0.5 mM β-mercaptoethanol and 0.2 mM chloramphenicol) overnight at 4°C.

Gel filtration The enzyme solution was applied to a Sephadex G-100 column (2.5 × 90 cm) that was pre-equilibrated with a 10 mM Tris/HCl buffer (pH 7.8) containing 0. 5mM β-mercaptoethanol and 0.2 mM chloramphenicol. It was eluted with the same buffer containing 0.2 M NaCl at a flow rate 1 ml/2 min. Active fractions were collected, dialyzed as above, concentrated and lyophilized. This preparation was used in the subsequent step.

Disc PAGE A slab gel electrophoresis was carried out using a 15% polyacrylamide gel (pH 7.8) with a 5% top gel (pH 6.2). After electrophoresis in a Tris-glycine buffer (pH 8.3) at 200 V for 7 h at 70°C, the proteins in the gel were stained with Coomassie brilliant blue R-250 and destained (Stegemann *et al.*, 1978).

Molecular weight determination The molecular weight of CAT was estimated by gel filtration with a Sephadex G-100 column at 4°C that was equilibrated with a 10 mM Tris/HCl buffer (pH 7.8) containing 0.5 mM β-mercaptoethanol, 0.2 mM chloramphenicol and 0.2 M NaCl at a flow rate of 1 ml/2 min. The column was calibrated by using the following proteins under identical conditions: lysozyme (14.3 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (42 kDa), bovine serum albumin (67 kDa) and phosphorylase (97.4 kDa). SDS-PAGE was carried out in 3 mm slab gels of 6% acrylamide in a Tris/borate buffer (pH 7.1) containing 0.1% SDS. Gels were stained with Coomassie brilliant blue R-250 and destained (Stegemann *et al.*, 1978). The previously mentioned protein standards were used.

Determination of amino acid composition This was carried out on the purified enzyme using a Beckman Amino Acid Analyzer (Model 119 GL), according to the previously described method (Speckman *et al.*, 1958).

Results

Purification of CAT The partial purification of the CAT crude extract that was effected by the ammonium sulphate (50%) precipitation showed that most of the enzyme activity was concentrated in the second precipitate. The specific activity of the enzyme was increased from 0.45 U/mg (in the crude extract) to 2.42 U/mg protein. The total protein was decreased from 3,600 mg to 620 mg with activity preservation of 93% and a purification factor of 5.4 fold (Table 1).

Fig. 1 shows the profile of the ammonium sulphate fraction purification on DEAE-Sephadex A50 anion-exchange chromatography. Although this fraction contained different

Table 1. Purification of chloramphenicol acetyltransferase from *Morganella morganii*

Step	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	3600	1620	0.45	100	1.0
$(NH_4)_2$ SO_4 precipitation	620	1500	2.41	93	5.4
Anion exchange chromatography on DEAE-Sephadex	66	660	10.0	41	22.2
Gel filtration chromatography on G-100 Sephadex	10	600	60.0	37	133.3

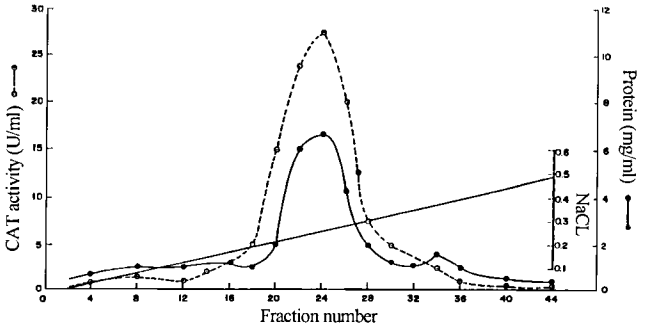

Fig. 1. First DEAE-Sephadex A-50 chromatography of chloramphenicol acetyltransferase. The dialyzed ammonium sulphate precipitate was chromatographed on a DEAE-Sephadex A-50 column by elution with a linear gradient of NaCl. Total protein was monitored at 280 nm and fractions were assayed for CAT activity.

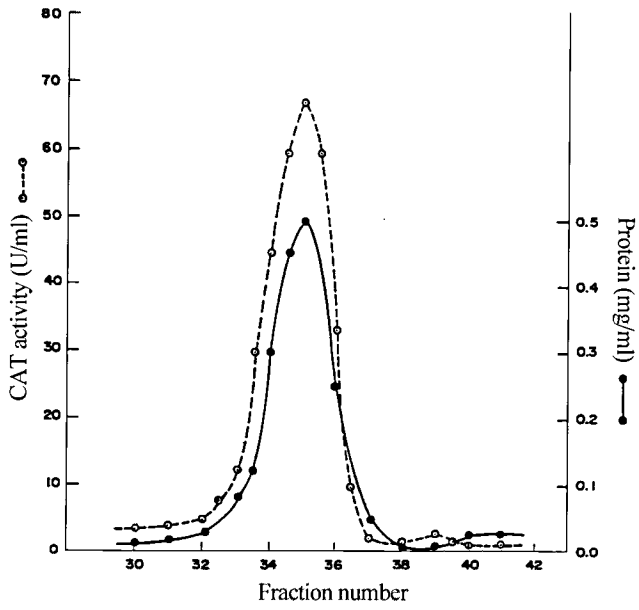

Fig. 2. Second gel filtration of CAT. First, the DEAE-Sephadex collected fraction was applied to Sephadex G-100. Total protein was monitored at 280 nm and the fractions were assayed for CAT activity.

protein molecules, only one band showed activity for CAT. Also, the enzyme was enriched after DEAE as the specific activity was increased to 10 U/mg with a purification factor of 22.2 (Table 1).

The purification of enzyme-rich fractions of the DEAE-Sephadex on the Sephadex G-100 gel filtration is shown in Fig. 2. A sharp distinctive band of CAT activity, which fits with only one protein band, was obtained. Also, the specific activity of the enzyme was further increased to 60 U/mg with a purification factor of 133.3 and a yield of 37% (Table 1).

Disc PAGE of the enzyme preparation from different purification steps showed that the resolved electrophoretic bands were progressively improved from the crude extract to the final step of Sephadex G-100 gel filtration. It revealed only one distinctive band that indicated a pure preparation of CAT (Fig. 3).

Molecular weight of CAT A SDS-polyacrylamide gel electrophoresis showed that the enzyme is a heterotetramer. It consists of four subunits with electrophoretic mobilities: 0.59, 0.61, 0.63 and 0.65 corresponding to molecular weights of 19.5 kDa, 19 kDa, 18 kDa and 17.5 kDa, respectively (Fig. 4). The molecular weight of the native enzyme was calculated to be 78 kDa, as determined by a gel filtration on Sephadex G-100, which is approximately that of the four-subunits together (74 kDa).

Effect of temperature and pH The influence of pH on the activity of CAT was studied using a 0.1 M Tris/HCl buffer of different pH values, ranging from 3.0 to 9.0. The activity of the enzyme increased gradually till pH 7.8, at which maximum activity was observed. At higher pH, the enzyme activity decreased (Fig. 5). The reaction rate of CAT was measured at various temperatures beginning with 25°C in a 0.1 M Tris/HCl buffer (pH 7.8). The reaction rate increased with temperature up to 35°C. At still higher temperatures, the reaction rate declines up to 60°C (Fig. 6). The effect of incubation time on CAT activity was studied in the range of zero to 7 min. (Fig. 7). CAT activity was maximum at zero time and decreased as the time increased.

Kinetic properties of the purified enzyme Lineweaver-Burk analysis gave K_m (Michaelis-Menton constant) of 5.0 μM and a V_{max} value of 153.8 U/ml at optimal pH (pH 7.8) (Fig. 8).

Amino acid composition The amino acid content of CAT,

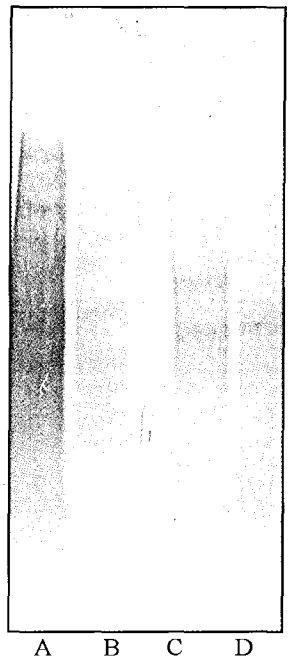

Fig. 3. Polyacrylamide gel electrophoresis of CAT from *Morganella morganii*. Electrophoresis of the enzyme was carried out on a 15% polyacrylamide gel in the absence of SDS and the gel was stained with Coomassie blue R-250. Lane A, crude extract; Lane B, ammonium sulphate fraction; Lane C, DEAE-Sephadex fraction and Lane D, gel filtration G-100 fraction.

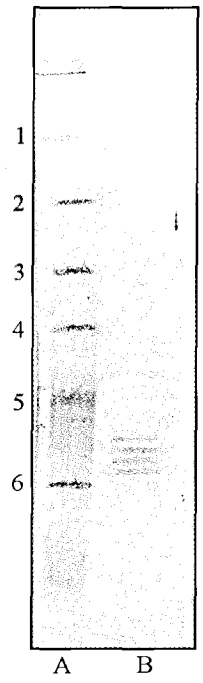

Fig. 4. PAGE-SDS of CAT from *Morganella morganii*. Electrophoresis was carried out on a 6% polyacrylamide containing 0.1% SDS. The gel was stained with Coomassie blue R-250. Lane A included the following standard proteins: 1. phosphorylase (Mr 97.4 kDa), 2. bovine serum albumin (Mr 67 kDa), 3. ovalbumin (Mr 42 kDa), 4. carbonic anhydrase (Mr 31 kDa), 5. soybean trypsin inhibitor (Mr 21.5 kDa) and 6. lysozyme (Mr 14.3 kDa). Lane B contained gel filtration G-100 purified enzyme.

purified from *Morganella morganii*, is shown in Table 2. The enzyme contained high amounts of leucine, lysine, alanine, serine, threonine, and histidine. It was rich in aspartic acid, glycine and glutamic acid.

Discussion

In the present study, we purified CAT to homogeneity from *Morganella morganii*, starting with an ammonium sulphate fractionation, followed by separation on DEAE-Sephadex A50 and G-100 Sephadex gel filtration.

The molecular weight of the purified enzyme was calculated to be approximately 78 kDa, based on a Sephadex G-100 permeation chromatography. A SDS-polyacrylamide electrophoresis showed that the enzyme consists of 4 subunits of close electrophoretic mobility, and a molecular weight of 19.5 kDa, 19.0 kDa, 18.0 kDa and 17.5 kDa, respectively. Assuming that 4 subunits are present in the enzyme in 1 : 1 : 1 : 1 stoichiometry, the experimentally determined molecular weight of the enzyme (74 kDa) is comparable to that calculated from SDS-PAGE (78 kDa). Therefore, it seems that CAT is a heterotetramer protein that is composed of 4 subunits that are close to each other in molecular weight. Different molecular weight values for CAT from different microorganisms have been determined. Previous studies (Schwarz and Cardoso, 1991) showed that the native CAT from *Staphylococcus haemolyticus* had a molecular weight of

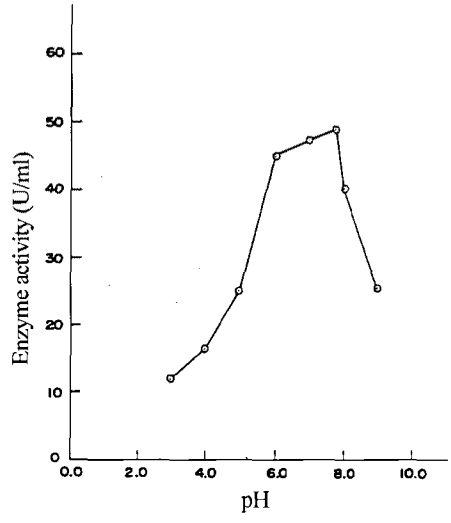

Fig. 5. Effect of pH on CAT activity. This was studied in the range of pH value from 3 to 9 using a 0.1 M Tris/HCl buffer.

70 kDa and consisted of three identical subunits, each with a molecular weight of 23 kDa. Also, other studies (Cardoso and Schwarz, 1992) on CAT variants, encoded by the plasmids PSCS6 and PSCS7 from *Staphylococcus aureus*, showed that

Table 2. Amino acid contents (mole %) of purified CAT

Amino acid	Mole %
Aspartic acid	20.0
Glycine	12.6
Glutamic acid	11.6
Leucine	7.4
Lysine	7.0
Alanine	6.7
Serine	6.1
Threonine	5.3
Histidine	4.9
Proline	4.0
Valine	3.8
Phenylalanine	2.8
Isoleucine	2.7
Arginine	2.5
Tyrosine	2.4
Methionine	0.5

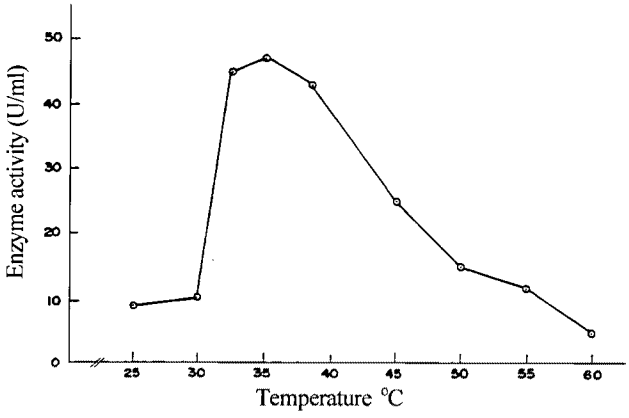

Fig. 6. Effect of temperature on CAT activity. This was studied in the range of 25-60°C in a 0.1 M Tris/HCl buffer, pH 7.8.

both enzymes appeared to be trimers that are composed of three identical subunits, each with a molecular weight of 23 kDa.

Maximum CAT activity occurred when incubated with an optimum substrate concentration at pH 7.8. A similar value of pH was obtained for *Staphylococcus aureus* (Shaw & Brodosky, 1968) and *E. coli* R6/k-10 (Shaw, 1967) CAT activity. However, a higher pH value (8.0) was reported for activity of CAT variants PSCS6 and PSCS7 from *Staphylococcus aureus* (Cardoso and Schwarz, 1992).

The temperature of the reaction was regulated in the range of 25-60°C. Maximum CAT activity was obtained at 35°C. The activity then decreased as the temperature increased. Incubation of CAT at 35°C for different times (zero to 7 min) showed that the activity reached maximum at zero. This decrease in CAT activity with time may be due to the drop in substrate concentration that increases the potential for reverse reactions.

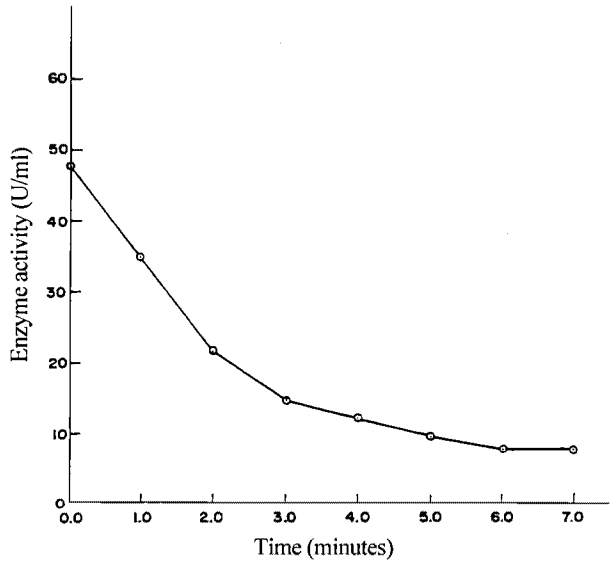

Fig. 7. Effect of incubation time on CAT activity. This was studied in the range of zero to 7 min at 35°C and pH 7.8.

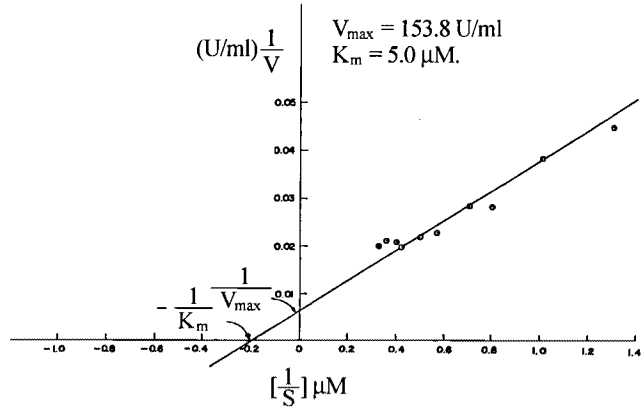

Fig. 8. Determination of K_m and V_{max} of CAT from *Morganella morganii*. A Lineweaver-Burk plot was used to detect the dependence of CAT activity on the chloramphenicol concentration.

Based on a Lineweaver-Burk analysis, the K_m and V_{max} values of CAT from *Morganella morganii* at optimal pH were 5.0 µM and 153.8 U/ml, respectively. This indicates a high affinity of the enzyme to the substrate. A higher K_m value (10.3 µM) for CAT from *E. coli* towards chloramphenicol has been reported (Murray *et al.*, 1988). On the other hand, lower K_m values for chloramphenicol (2.81 µM and 2.5 µM) have been obtained for CAT variants PSCS5 from *Staphylococcus haemolyticus* (Schwarz and Cardoso, 1991) and PSCS6 from *Staphylococcus aureus* (Cardoso and Schwarz, 1992), respectively.

The amino acid composition revealed that CAT from *Morganella morganii* contains a high content of leucine, lysine, alanine, serine, threonine and histidine. It was rich in aspartic acid, glycine and glutamic acid. Lower amounts of proline, valine, phenylalanine, isoleucine, arginine, tyrosine,

and methionine were observed. The content of tryptophane and cysteine could not be determined in the CAT preparation from *Morganella morganii*.

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