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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR S-ADENOSYL-L-METHIONINE:MACROCIN O-METHYLTRANSFERASE

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SUMMARY

A high-performance liquid chromatographic (HPLC) procedure was developed to assay S-adenosyl-L-methionine:macrocin O-methyltransferase. This enzyme catalyzes the rate-limiting terminal reaction of tylosin biosynthesis in *Streptomyces fradiae*. HPLC analysis was improved by resin treatment of cell-free extracts to remove endogenous tylosin and related compounds. Relomycin was selected as an internal standard and the enzymatic reaction conditions were optimized. The reaction mixture was extracted with ethyl acetate to recover the substrate, product and the internal standard. Efficient separation of the macrolide antibiotics was provided by ion-pair reversed-phase HPLC. An average relomycin recovery was 90%. The O-methyltransferase activity could be routinely and reproducibly determined by monitoring tylosin formation at 285 nm.

INTRODUCTION

With the recent elucidation of the biosynthetic pathway from tylactone to tylosin in *Streptomyces fradiae*[1,2], it became possible to study key metabolic enzymes for the regulation of tylosin biosynthesis. Insight into enzyme control or substrate deficiency of a rate-limiting metabolic step in tylosin biosynthesis is both academically and industrially interesting. For example, enzyme control might possibly be exerted at the level of gene expression and post-translational modification of the gene product. It is not known, however, whether the structural genes for tylosin biosynthesis are regulated by induction, repression or derepression nor whether they are regulated coordinately or independently. Also, little information is available on post-translational regulation of tylosin biosynthesis *i.e.*, ligand activation and inhibition, covalent modification and protein catabolism.

To initiate an enzymological study of the regulation of tylosin biosynthesis, S-adenosyl-L-methionine:macrocin O-methyltransferase (macrocin O-methyltransferase) was selected for investigation based on several observations which, as listed below, suggest that the enzyme catalyzing the terminal biosynthetic reaction (Fig. 1) is rate-limiting. Significant amounts of macrocin, the substrate of the enzyme, ac-

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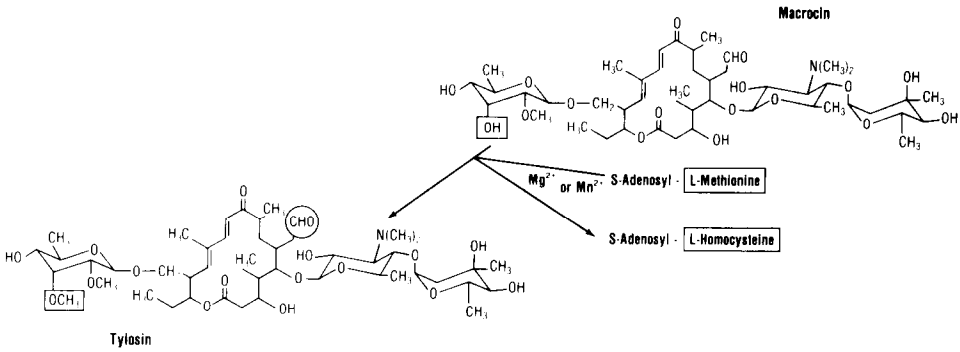

Fig. 1. S-Adenosyl-L-methionine:macrocin O-methyltransferase mediated conversion of macrocin to tylosin. Chemical groups which are involved in the O-methyltransferase reaction are enclosed in squares. Relomycin is derived from tylosin by reduction of the circled CHO to CH_2OH.

cumulated under various fermentation conditions of *S. fradiae*. Positive correlation for tylosin production and the enzyme activity was observed in *S. fradiae* strains which produced normal and elevated levels of tylosin[3,4]. Also, the enzyme was inhibited by the substrate, product and other tylosin-like compounds[3].

The O-methyltransferase activity was assayed previously[3] by counting amyl-acetate extractable [^{14}C]tylosin, which was formed by transfer of a radioactive methyl group from S-adenosyl-L-methione (SAM) to macrocin. While the radiochemical procedure was adequate for comparative analysis of the enzyme activity in cell-free extracts of various *S. fradiae* strains, it was not designed for routine estimation of product purity. An alternative approach for the enzyme assay was provided by the observation that tylosin and structurally related compounds could be separated by HPLC[5,6]. Addition of 1-pentanesulfonic acid, a pairing agent, to mobile phases of reversed-phase HPLC columns improved separation of the compounds[7]. The assay method, as developed from both the radiochemical and the HPLC procedures for monitoring product formation was rapid, impurities were detectable and the method was highly reproducible in determining the activity of the O-methyltransferase in resin-treated extracts of *S. fradiae*. Furthermore, expression of two or more structural genes for the post-tylactone biosynthetic pathway can be simultaneously analyzed in a single *S. fradiae* extract by measuring substrate disappearance by HPLC.

EXPERIMENTAL

Materials

Highly purified macrocin, relomycin and tylosin were obtained from the Analytical Development Department, Eli Lilly (Indianapolis, IN, U.S.A.). S-Adenosyl-L-methionine was purchased from Sigma (St. Louis, MO, U.S.A.). Duolite non-functional polystyrene resin ES865 was obtained from Diamond Shamrock (Redwood City, CA, U.S.A.). 1-Pentanesulfonic acid sodium salt was purchased from Eastman-Kodak (Rochester, NY, U.S.A.). HPLC-grade tetrahydrofuran was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). HPLC-grade acetonitrile was purchased from MCB Manufacturing Chemists (Cincinnati, OH, U.S.A.).

Preparation of resin-treated extracts

A tylosin-producing strain of *Streptomyces fradiae* was grown under conditions as described previously[8]. Cells from idiophase cultures were washed three times with 100 m*M* potassium phosphate buffer, pH 7.8, and disrupted by ultrasonic treatment[3]. Ultrasonic extracts were centrifuged at 40,000 *g* for 30 min. The supernatant fractions, designated as cell-free extracts, were then stirred with 10% (v/v) Duolite resin ES865 for 10 min and passed through glass wool to separate the extracts from the resin. Unless specified, the resin-treated extracts were used in all analyses which involved macrocin O-methyltransferase.

Ion-pair reversed-phase HPLC

HPLC components including system controller, data module, Model 6000A pump, Model 710B Waters intelligent sample processor and Model 450 variable-wavelength detector, all were purchased from Waters Assoc. (Milford, MA, U.S.A.). Reversed-phase Zorbax® C_8 columns (15 cm × 4.6 mm, 6 μm particle size) were obtained from DuPont (Wilmington, DE, U.S.A.). Macrocin, relomycin and tylosin were separated using a mobile phase of 65% ion-pair solution, 20% acetonitrile and 15% tetrahydrofuran. The ion-pair solution was prepared with 0.025% 1-pentanesulfonic acid and 1% (v/v) acetic acid in water. The compounds were monitored at a wavelength of 285 nm. The concentrations of mobile-phase components were changed slightly as required for maintenance of desirable separation efficiency. The flow-rate was maintained at 2 ml/min. Peak shapes and column efficiencies were improved by maintaining the column temperature at 55°C[7].

RESULTS

Sample preparation

Cell washing and resin treatment. Tylosin and structurally related compounds, which were shown to be inhibitory to the O-methyltransferase[3], could be efficiently removed by a combination of cell washing and resin treatment of cell-free extracts. Tylosin concentrations (40 ± 10 μM) in cell-free extracts decreased by about 80% when cell washing was increased from one to three times; such decrease was accompanied by an approximately 30% increase in the specific enzyme activity. The resin treatment removed 70% of the remaining tylosin (10 μM) and macrocin (2 μM) from cell-free extracts (Fig. 2) and further increased the specific enzyme activity by 25%. Relomycin, the internal standard for routine enzyme analysis, was not detectable in resin-treated extracts (Fig. 2).

Optimization of enzymatic reaction conditions. The O-methyltransferase activity was analyzed in a total reaction volume of 0.5 ml according to the conditions as previously described[3]. A linear increase in the enzyme activity was observed with increasing protein concentrations of resin-treated extracts. The values of K_m for macrocin and SAM, as determined by the double-reciprocal plot of Lineweaver and Burk[9], were 33 μM and 29 μM, respectively. The enzyme activity was greatly stimulated by manganese sulfate, magnesium sulfate or cobalt chloride and to a lesser degree by iron(II) sulfate or zinc sulfate (Table I). A linear increase in tylosin formation was observed for the first 10 min of reaction time at 25°C, after which a slower rate ensued presumably due to enzyme denaturation and tylosin inhibition.

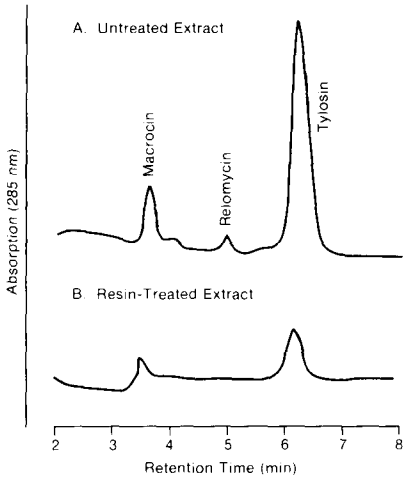

Fig. 2. HPLC analysis of macrocin, relomycin and tylosin from untreated extract (A) and resin-treated extract (B).

The enzyme was unstable; about 30% activity remained after 3 h at 4°C. It was very stable for 3 h at 4°C in the presence of 10% ethanol. No protective effect was conferred on the enzyme by potassium chloride, reducing agents (dithiothreitol and β-mercaptoethanol), or some common co-factors (NAD^+, NADH, $NADP^+$, NADPH, FMN and FAD) of bacterial enzymes. Maximal enzyme activities were observed between 20 and 200 m*M* of potassium phosphate buffer, pH 7.2. The optimal pH, as determined in 100 m*M* potassium phosphate and Tris buffers, was from 7.5 to 8.0. Even though the specific enzyme activity at an optimal pH of 7.8 was greater in the Tris buffer than in the potassium phosphate buffer, Tris buffer was not favored for routine enzyme analysis because of serious degradation of the substrate, product and internal standard. The enzyme activity was routinely determined at 25°C (room temperature).

Selection of relomycin as internal standard. Relomycin, 23-(demycinosyloxy)

TABLE I

METAL ION REQUIREMENT OF MACROCIN O-METHYLTRANSFERASE

Assayed with 1.22 mg protein of a resin-treated *S. fradiae* extract as described in the text.

Metal ion added (22 mM)	*Activity (nmoles tylosin formed/10 min)*
None	0.70
$CaCl_2$	0.06
$CoCl_2$	3.02
$CuSO_4$	0.00
$FeSO_4$	1.61
$MgSO_4$	3.42
$MnSO_4$	3.49
$ZnSO_4$	1.30

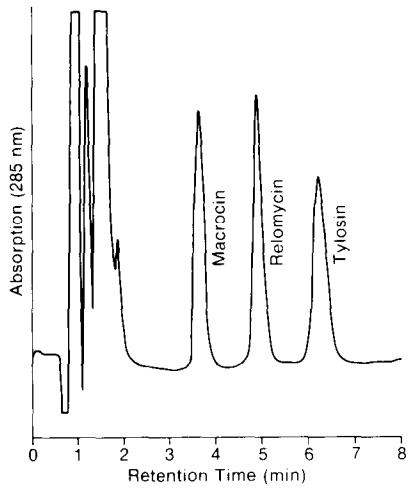

Fig. 3. HPLC separation of standard macrocin, relomycin and tylosin at 0.7 nmole.

tylosin and tylactone[10] were compared for suitability as an internal standard for quantitative HPLC analysis of macrocin and tylosin after extraction by ethyl acetate from enzymatic reaction mixtures. Tylactone was unfavorably partitioned to the mobile phase from ethyl acetate, and 23-(demycinosyloxy) tylosin was eluted at approximately two retention times relative to that of tylosin (Fig. 3). Relomycin appeared ideal to serve as the internal standard for two reasons. First, it is structurally very similar to tylosin (Fig. 1) and it was well separated from macrocin and tylosin (Fig. 3). Second, resin treatment of cell-free extracts removed relomycin to an undetectable level and, under the reaction conditions as described below, no relomycin was generated in the reaction mixture (Fig. 4).

Solvent extraction of macrocin, relomycin and tylosin. Five organic solvents

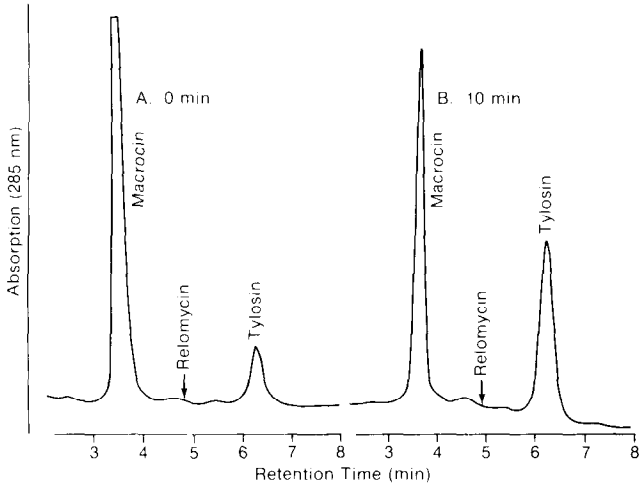

Fig. 4. HPLC analysis of macrocin and tylosin at 0 min (A) and 10 min (B) of enzymatic reaction in the absence of external relomycin.

were compared for extraction efficiency, compound stability, solvent volatility and availability of HPLC-grade solvent. Ethyl acetate was more suitable than amyl acetate, methylene chloride, chloroform, and *n*-butanol as the extraction solvent for the three compounds. Several variables were evaluated to determine the optimal conditions for the recovery of the substrate, product and internal standard from the enzymatic reaction mixtures. The most significant variable for recovery improvement was pH. Trichloroacetic acid was used instead of Formalin[3] to interrupt enzymatic reactions, since the latter reagent interfered with HPLC separation of the three compounds. Protein denaturation by addition of trichloroacetic acid to pH 2.6 ± 0.2, followed by pH adjustment with sodium hydroxide to 12.9 ± 0.2, appeared to increase ethyl acetate extractable macrocin and tylosin approximately four-fold, while it did not cause detectable hydrolysis of the substrate, product and internal standard under the reaction conditions. In addition to the pH optimization, extraction at 60°C with vigorous shaking for 10 min and separation of organic and aqueous layers by low-speed centrifugation (*i.e.*, 300 *g*) also contributed to recovery improvement. Prior to the optimization, the relomycin recovery varied significantly; a typical relomycin recovery was less than 20%. Under the optimized conditions as described here, an average relomycin recovery was 90%. The high relomycin recovery was accompanied by reliable reproducibility with an average deviation of approximately 3% for duplicate tylosin analyses.

HPLC analysis

The UV response for macrocin, tylosin and relomycin was linear for a range of 0.2 to 2.0 nmoles. An injection volume of at least 5 μl was required for reproducible quantitation of the three compounds, which were well separated within 7 min (Fig. 3).

A routine HPLC assay procedure for macrocin O-methyltransferase

The optimal conditions which were derived from the sample preparation improvement as described in the preceding sections were routinely used in determining the activity of the O-methyltransferase. A total reaction volume of 0.5 ml contained

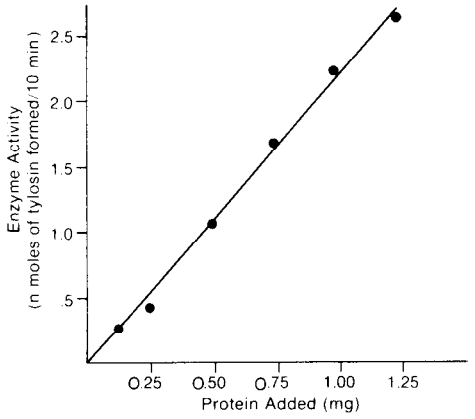

Fig. 5. Linearity of the O-methyltransferase activity with protein concentration, analyzed with a resin-treated *S. fradiae* extract under optimized conditions as described in the text.

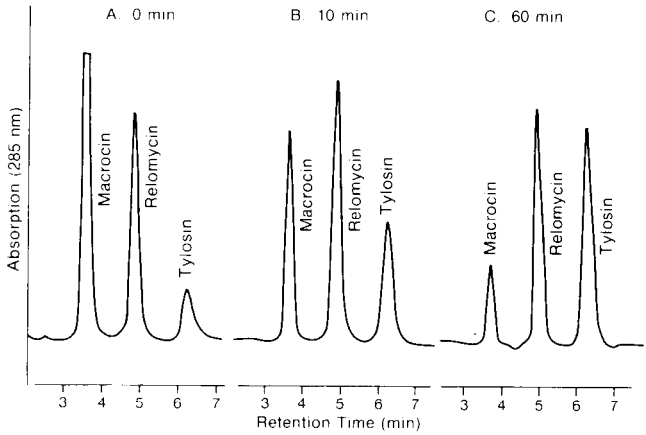

Fig. 6. HPLC analysis of macrocin and tylosin at 0 min (A), 10 min (B) and 60 min (C) of enzymatic reaction with relomycin as the internal standard.

11.2 nmoles of macrocin, 30 nmoles of SAM, 10 μmoles of magnesium sulfate and 0.10–1.25 mg protein of a resin-treated extract (Fig. 5) in 100 m*M* potassium phosphate buffer, pH 7.8, in the presence of 10% ethanol. The enzymatic reaction was initiated by addition of SAM. The reaction mixture was incubated at 25°C for 10 min. A control was an identical mixture with zero incubation time. Both samples and controls were run in duplicate. Trichloroacetic acid was added to reaction mixtures to pH 2.6 for interruption of enzymatic reaction, which was followed by addition of 5.6 nmoles of relomycin as the internal standard. Sodium hydroxide was added to pH 12.9 prior to solvent extraction. Macrocin, relomycin and tylosin were extracted from the reaction mixture into 1 ml of ethyl acetate by vigorous shaking at 60°C for 10 min. The ethyl acetate layer was separated from the aqueous layer by centrifugation at 300 *g* for 5 min, and a portion (*i.e.*, 125 μl) of the organic layer was injected into the reversed-phase Zorbax C_8 column without further treatment.

The activity of macrocin O-methyltransferase was determined by monitoring tylosin formation at a wavelength of 285 nm, using relomycin as the internal standard (Fig. 6A and B). One activity unit was defined as the amount of the enzyme that catalyzed formation of 1 nmole tylosin under the assay conditions as described. The specific activity was determined as nmoles of tylosin formed per min per mg protein. Protein contents were determined by the method of Lowry *et al.*[11], using bovine serum albumin (fraction V) as the standard[8].

Application of the assay procedure

Stoichiometric conversion of macrocin to tylosin. The stoichiometric ratio of tylosin formation to macrocin disappearance was analyzed with resin-treated *S. fradiae* extracts under a variety of reaction conditions. A stoichiometric ratio of 1:1 was clearly established with variation in extract amount, macrocin and SAM concentrations, and reaction time (Table II). Approximately 65% of total macrocin was converted to tylosin within 1 h (Fig. 6A and C); the incomplete conversion was presumably caused by product inhibition of and macrocin binding to the O-methyltransferase.

TABLE II

MACROCIN O-METHYLTRANSFERASE CATALYZED CONVERSION OF MACROCIN TO TYLOSIN

Determined with different resin-treated *S. fradiae* extracts under reaction conditions as specified in the table and described in the text.

Reaction conditions				*Stoichiometric analysis*		
Macrocin added (nmoles)	*SAM added (nmoles)*	*Protein added (mg)*	*Incubation time (min)*	*Macrocin disappearance (nmoles)*	*Tylosin formation (nmoles)*	*Tylosin formation–macrocin disappearance*
2.8	4.2	0.3	15	0.547	0.500	0.91
2.8	4.2	0.4	15	0.533	0.667	1.25
2.8	8.4	0.5	30	0.667	0.653	0.98
2.8	8.4	0.5	60	1.007	0.920	0.91
2.8	8.4	0.5	90	1.560	1.780	1.14
2.8	4.2	0.5	120	0.980	1.087	1.11
2.8	8.4	0.5	120	1.460	1.593	1.09
5.6	30.0	1.3	10	2.096	2.240	1.07
5.6	30.0	1.3	30	3.448	3.720	1.08
5.6	30.0	1.3	60	3.656	3.528	0.96
5.6	30.0	1.3	120	3.768	3.288	0.87
8.4	30.0	1.3	10	2.392	2.504	1.05
8.4	30.0	1.3	30	5.256	5.136	0.98
8.4	30.0	1.3	60	4.904	5.424	1.11
8.4	30.0	1.3	120	5.608	5.400	0.96
						Average:1.03

DISCUSSION

Resin treatment of *S. fradiae* cell-free extracts removed tylosin and related compounds and invariably led to a significant increase in the specific activity of macrocin O-methyltransferase. This observation is consistent with both the previous finding[3] and our recent data that the enzyme was susceptible to inhibition by tylosin, relomycin and macrocin but not by tylactone. It remains to be determined whether tylosin or a structurally related metabolite of tylosin biosynthesis is the *in vivo* regulator of the O-methyltransferase activity. Residual tylosin and related compounds in resin-treated extracts appeared not to inhibit the O-methyltransferase and a linear activity increase was observed with increasing protein concentrations (Fig. 5).

The stoichiometric ratio of 1:1 for the conversion of macrocin to tylosin provides two conclusions. First, the O-methyltransferase catalyzed conversion of macrocin to tylosin (Fig. 1) is the only metabolic route for macrocin transformation in operation under our *in vitro* conditions. Second, the activity of the O-methyltransferase can be determined by either tylosin formation or macrocin disappearance. The activity analysis by tylosin formation was highly reproducible with a typical deviation of 3% for duplicate assays. In contrast, the activity analysis by macrocin disappearance appeared more suitable for high-activity than low-activity extracts, since the recovery of the substrate varied up to 10% depending on the amount of the extract

used in the assay. That the variation appeared to be caused by specific macrocin binding to the O-methyltransferase in the active-enzyme extracts was supported by our observation that no such variation was observed in the extracts after enzyme inactivation by storage at 4°C for one week or in extracts from a mutant lacking the O-methyltransferase activity[8].

Chromatographic analysis of the assay products was inherently diagnostic of operational deviations. For example, detection of hydrolytic products might indicate prolonged exposure of macrocin and tylosin at a low pH. Also, abnormally low relomycin recoveries could be suggestive of alkaline conditions leading to macrolide degradation.

The optimal reaction conditions for the O-methyltransferase as derived from both the radiochemical[3] and the HPLC assays, were mutually agreeable in general, indicating that either assay was usable for the enzyme. It is expected, however, that the HPLC assay can be modified to analyze, by demethylmacrocin disappearance, SAM:demethylmacrocin O-methyltransferase, which catalyzes the penultimate metabolic reaction of tylosin biosynthesis[1,2]. Thus, coordinate or independent regulation of the two O-methyltransferases can become amenable to enzymological experimentation. Since all metabolites of tylosin biosynthesis subsequent to tylactone contain the UV-absorbing tylactone structure[2], other steps in the pathway may be examined by HPLC.

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