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QUANTITATIVE DETERMINATION OF 2-METHYL-3-AMYLPYRROLE, A PRECURSOR OF PRODIGIOSIN††

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ABSTRACT

Prodigiosin, a characteristic red antibiotic pigment produced by *Serratia marcescens*, is synthesized by the enzymatic coupling of two precursors, 2-methyl-3-amylpyrrole (MAP) and 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC). When MAP is replaced by 2,4-dimethyl-3-ethylpyrrole (DEP), a prodigiosin analog can be synthesized. Although MAP and MBC have been extracted from the corresponding mutants producing a single precursor and analyzed by high-performance liquid chromatography (HPLC), the extraction procedures are tedious and time consuming. In this paper we report a single-step quantitative method of determining the amount of MAP in the culture medium of strain WF. A standard curve was constructed with DEP, extracted MBC from mutant 9-3-3, and condensing enzyme from mutant 9-3-3. The absorbance of the prodigiosin analog formed between DEP and MBC per μg of DEP was calculated. The amount of MAP in the culture medium of mutant WF used in the syntrophic formation of prodigiosin was determined according to the standard curve and expressed as μg of DEP. This method was applied to determine the effect of metal ions on MAP synthesis.

INTRODUCTION

Prodigiosin is a characteristic red pigment antibiotic synthesized by *Serratia marcescens*. The biosynthetic process of prodigiosin in *S. marcescens* is quite complex.

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It is influenced by various factors, such as temperature (Hussain Qadri and Williams, 1972; Morrison, 1966), amount of oxygen (Heinemann et al., 1970), composition of the growth media (Williams and Hussain Qadri, 1979), detergents (Feng et al., 1982), and certain antibiotics (Tsang and Sheung, 1980; Williams and Gott, 1964; Williams and Hassain Qadri, 1979). A bifurcated pathway was proposed for prodigiosin formation (Franklin and Snow, 1975; Morrison, 1966). Two precursors, a monopyrrole, 2-mythyl-3-amylpyrrole (MAP) and a bipyrrole, 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC) are synthesized separately, then enzymatically condensed to form the pigment (Williams and Hussain Qadri, 1979). It was shown by high-performance liquid chromatography (HPLC) that the reduction in pigment synthesis in the presence of polymyxin B, a membrane active antibiotic, was partly due to a reduction in MAP synthesis (Tsang and Feng, 1983). It was the first time that the effect on pigment synthesis at the level of precursor synthesis was reported. However, the extraction procedures were tedious and time consuming. Therefore, the objective of this study was to find an alternative method to HPLC to quantitate MAP in *S. marcescens*.

Syntrophic pigment synthesis serves as the basis of MAP quantitation in the culture medium of strain WF, a mutant producing MAP but not MBC. Syntrophic pigment synthesis involves the cross-feeding of precursors for pigment formation when either one or both of the precursors are supplied to the cells containing the condensing enzyme. When 2,4-dimethyl-3-ethylpyrrole (DEP) was replaced by MAP, a syntrophic pigment analog was formed. Since DEP can be purchased, it was a logical choice to use DEP to construct a standard curve.

It has been reported that Zn(II) was the most inhibitory first transition metal ion on pigment formation in the wild-type strain 08 (Furman et al., 1984). Therefore, this method was applied in the study of the effect of Zn(II) on MAP synthesis in *S. marcescens* strain WF.

MATERIALS AND METHODS

Chemicals

$Zn(OAc)_2.2H_2O$ was used in this study (Fisher CP. Pittsburg, Pennsylvania, U.S.A.). 2,4-dimethyl-3-ethylpyrrole (DEP) was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A. MBC was extracted from the growth medium of mutant 9-3-3 according to the method previously described (Feng et al., 1982).

Bacterial Strains and Growth Conditions

S. marcescens mutant WF and mutant 9-3-3 were used in this study. These nonpigmented mutants were kindly supplied by Professor R.P. Williams, Baylor College of Medicine, Houston, Texas. Mutant WF can only synthesize MAP while mutant 9-3-3 can only synthesize MBC (Silverman and Munoz, 1973).

Bactopeptone (0.5%)-Glycerol (1%) Broth (PGB) (Difco, Detroit, Michigan) was used for bacterial cultivation. PGB was found to be an excellent medium for the synthesis of prodigiosin and its precursors (Williams and Hussain Qadri, 1979). Bacterial cells were grown in a 125 ml Erlenmeyer flask containing 30 ml of broth in the presence or absence of Zn(II) in a shaker bath at 100 rpm at 25°C. The concentration of Zn(II) used was 15.63 μg/ml, the maximum concentration which allows growth to occur in its presence (Furman et al., 1984). After 24 hr growth, the bac-

terial culture was centrifuged at 10,000 rpm in a Sorvall SS-3 centrifuge to separate the cells from the growth media.

Syntrophic Pigment Synthesis

After 9-3-3 and WF cultures were centrifuged, the supernatants were shown to contain MBC and MAP, respectively. Both the WF and 9-3-3 mutants, and the wild-type strain 08, produce the enzyme required for condensation to occur in the terminal step of prodigiosin synthesis. Mutant 9-3-3 cells were chosen for the source of the enzyme since they do not produce MAP, the compound to be measured.

Syntrophic pigment synthesis was used to estimate the amount of MAP in WF cultures before its extraction for HPLC analysis (Feng et al., 1982). When isolated MBC and condensing enzyme from 9-3-3 cells were supplied in excess to aliquots of WF supernatant containing MAP, prodigiosin was formed. The standard curve was constructed with different amounts of DEP along with excess MBC and condensing enzyme to form a syntrophic pigment analog.

Extraction and Spectrophotometric Measurements

The prodigiosin and prodigiosin analog produced were extracted with a 1% acetic acid/methanol solution. The amounts of these pigments synthesized were determined spectrophotometrically at 534 nm (Williams et al., 1956) with a Beckman Model 35 spectrophotometer. The absorbance of prodigiosin produced was used as an indirect measurement of MAP content in the sample.

Cellular Protein Content Determination

The cellular protein contents of the cultures were determined by the method of Lowry et al. (1951) after the cells were hydrolyzed overnight with NaOH.

RESULTS AND DISCUSSION

Figure 1 shows the DEP standard curve which is linear in the range of 1 to 4 μg of DEP. The slope factor for the standard curve was expressed as absorbance units/μg DEP. Figure 2 represents the amount of pigment produced when MAP was provided by the WF culture. The addition of supernatant containing MAP to excess MBC and condensing enzyme produced the syntrophic pigment which was extracted and monitored at 534 nm. A linear relationship between various amounts of supernatant and the absorbance of the pigment formed was established. In this experiment the slope of this line was shown to be 0.772 absorbance units/ml supernatant. From the standard curve, it was possible to convert the amount of MAP in terms of DEP. The same approach was used when the culture was grown in the presence of 15.63 μg/ml Zn(II) (Figure 3). In this case the slope of the line was shown to be 0.266 absorbance units/ml supernatant. In comparison with the slope factor in the absence of Zn(II), a decrease in the slope indicated a reduction in MAP synthesis. In order to monitor the growth of the organism under these conditions, a measurement of the cellular protein content was determined. When the protein content was compared in the presence and absence of Zn(II), there was an average reduction of approximately 16% in cultures containing Zn(II). From the slope factors of the curves (Figures 2 and 3), it could be shown that the synthesis of MAP was reduced by almost 70% by the presence of Zn(II) in the growth medium. When the protein content was taken into account, there was approximately a 64% reduction in MAP expressed

as μg DEP/mg protein. This decrease was due to a reduction in protein synthesis in cultures containing Zn(II).

These results indicate that this method can be used successfully in quantitating the amount of MAP in WF cultures. It is a rapid method in comparison with that employing the use of HPLC. We plan to use this method to study the effect of other transition metal ions on the synthesis of prodigiosin and its precursors.

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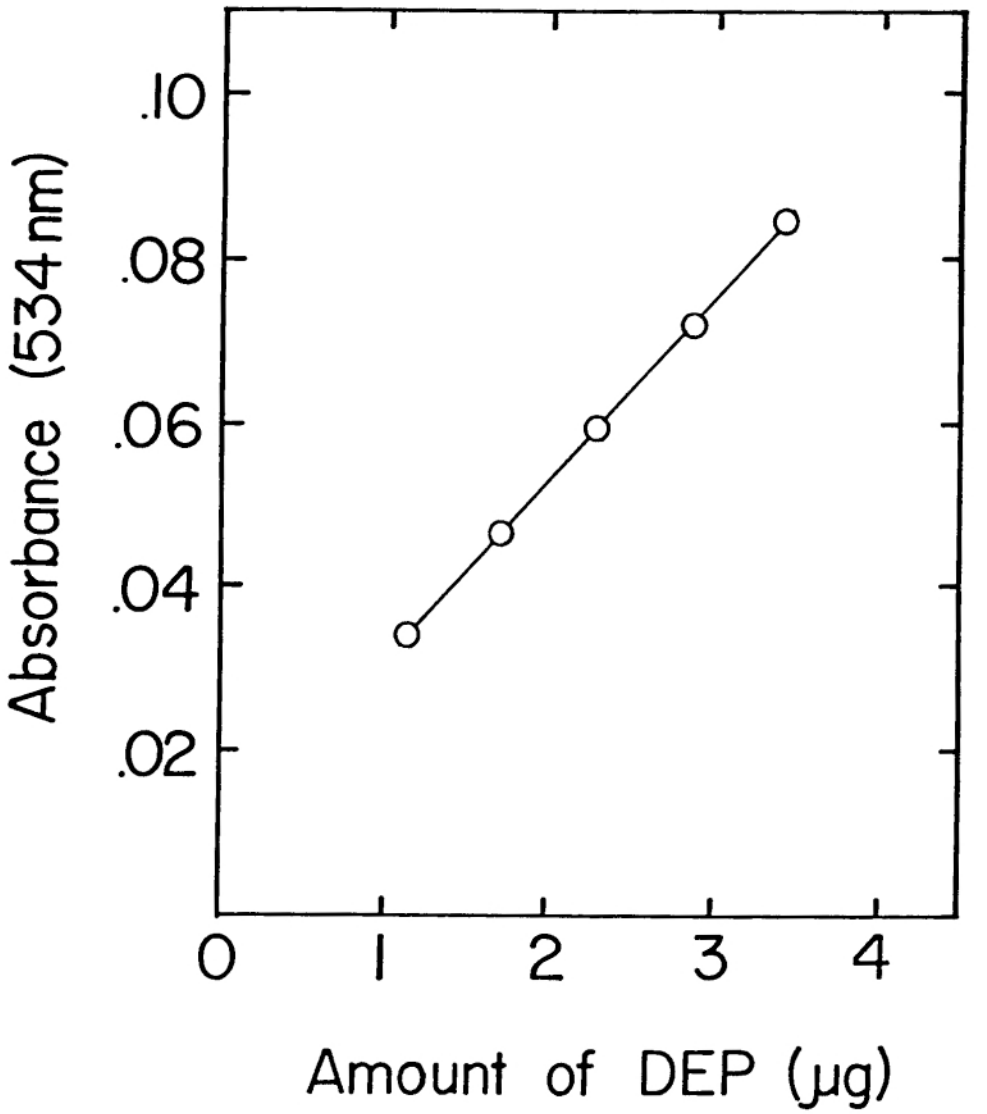

Fig. 1. Standard curve of prodigiosin analog synthesis with MBC and DEP. Amount of analog synthesized corresponds to its maximum absorption at 534 nm.

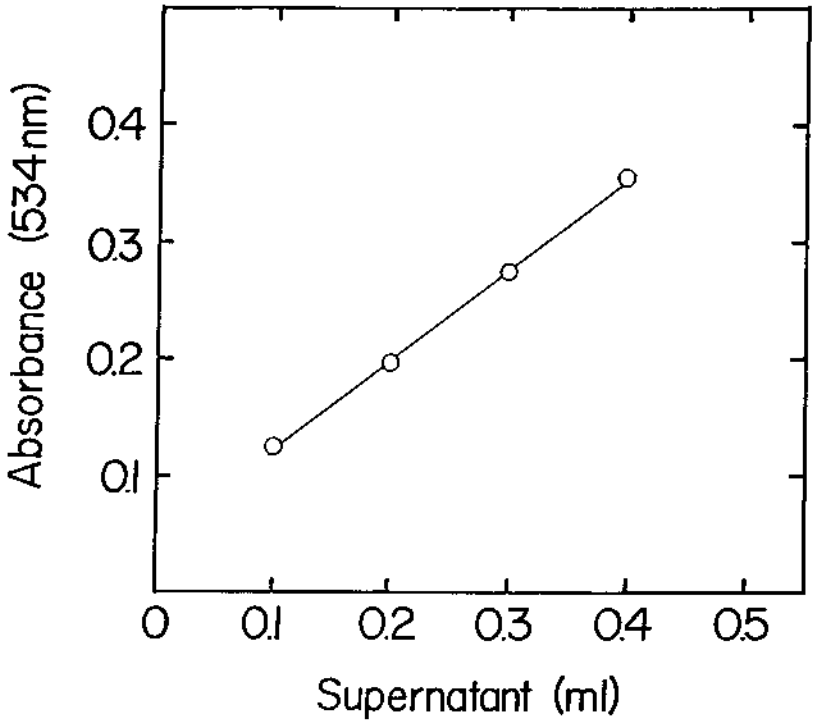

Fig. 2. The amount of MAP in the culture medium of mutant WF was measured by the absorbance at 534 nm of the prodigiosin formed.

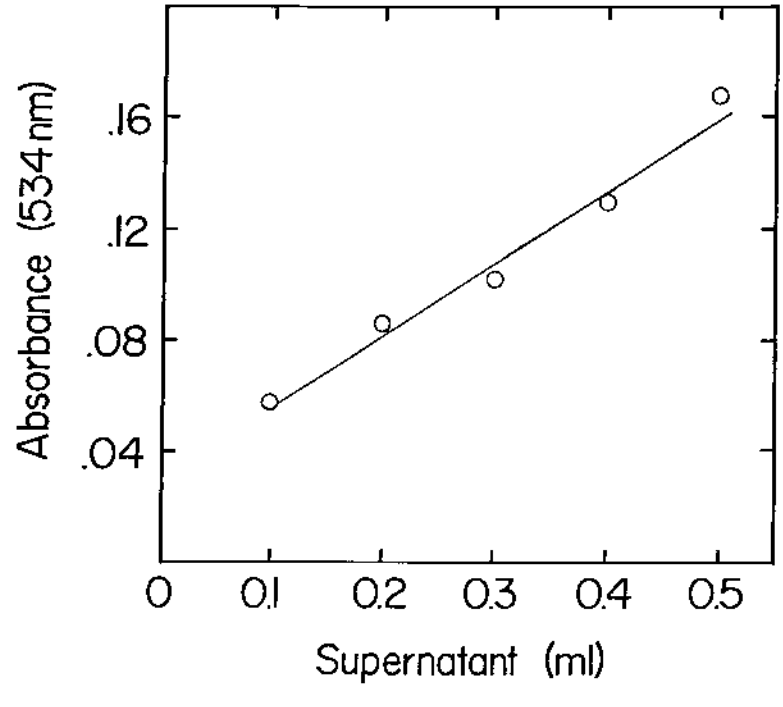

Fig. 3. The amount of MAP in the culture medium of mutant WF in the presence of Zn(II) was measured by the absorbance at 534 nm of the prodigiosin formed.