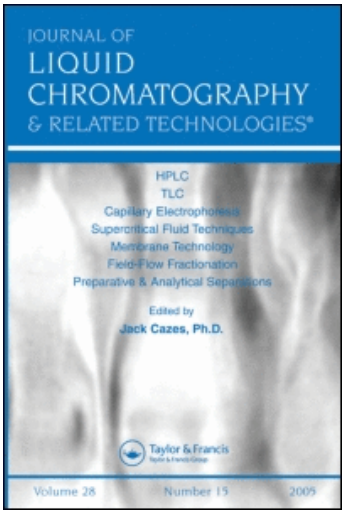

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Karen Kalanik[a]; James W. Webb[a]; Joseph C. Tsang[a]
[a] Chemistry Department, Illinois State University, Bloomington, IL, U.S.A.

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THE USE OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE STUDIES OF PIGMENT COMPONENTS FROM SERRATIA MARCESCENS 08 BEFORE AND AFTER HYDROGEN PEROXIDE OXIDATION

Karen Kalanik, James W. Webb and Joseph C. Tsang*

Chemistry Department,

Illinois State University,

Normal-Bloomington, IL

U.S.A.

ABSTRACT

A method to separate the pigment components of Serratia marcescens 08 by high performance liquid chromatography (HPLC) is described. By maintaining a small but constant amount of concentrated HCl in the mobile phase of 25% ethylene dichloride in methanol on a reverse-phase column (Lichrosorb RP-18), the pigment components were resolved and separated in about 5 min. This method allowed preparative isolation of the individual components for infrared spectroscopic characterization. The interrelationship of the components studied by hydrogen peroxide oxidation of the pigment extract and the HPLC profiles and infrared spectra of the oxidized products as well as those of the individual isolated fractions were investigated. It is suggested that components separated by this method and detected at 272 nm might be related to the parent pigment of prodigiosin.

* To whom all correspondence should be addressed.

INTRODUCTION

Prodigiosin (2-methyl-3n-amyl-6-methoxy-prodigiosene) is the characteristic tripyrrole red pigment commonly produced by Serratia marcescens. It has been suggested that pigments extracted by organic solvents represent a mixture of prodigiosin related components which could be separated by thin-layer or column chromatography into several fractions with antibiotic properties[1]. Even in pigmentless strains, monopyrrole and dipyrrole precursors have been detected and isolated[2,3]. More recently, the availability of more sophisticated instrumentation such as high performance liquid chromatography (HPLC) allows clear demonstration of the considerable heterogeneity in the pigment extracts[4]. In spite of the limited success of the isolation of the mutants which produce these prodigiosin precursors, and the isolation of the precursors themselves, the biosynthetic pathways of prodigiosin remain unclear.

In this communication, we describe a method for the rapid separation of the major components of the pigment extract by reverse-phase HPLC. Successful preparative isolation of the components, along with hydrogen peroxide oxidation of the pigment extract provided the opportunity for us to study the interrelationship of the components. Our results seemed to indicate that for better separation of the components, it was necessary to control the amount of hydrogen chloride in the elution solvent. This factor is important because prodigiosin might occur as the salt of fatty acids[5] in close association with the lipid portion of cell membranes[6]. Our recent observed inhibitory of a cationic antibiotic, polymyxin B, on the pigment formation further supports this new finding[7].

MATERIALS AND METHODS

Serratia marcescens 08 was used in the study. The cells were grown in an enriched medium containing casamino acids, glycerol, sodium chloride and nutrient broth as described previously[8]. They were aerated at room temperature and harvested at the late log phase. The cells were centrifuged at 4 C, and the pigments were extracted by acetone, followed by partition with petroleum

ehter according to the method of Williams, et al.[9] The pigments in the petroleum extract were recovered by rotary evaporation in a stream of nitrogen.

A Spectra-Physics high performance liquid chromatograph (Spectra-Physics, Santa Clara, Calif. U.S.A.) equipped with a 740B pump and a Valco Model VU-6UH Pa-N60 injection valve was used for these separations. A variable wavelength detector (Perkin Elmer LC-55 Spectrophotometer with a Perkin Elmer Model 56 recorder attachment) was used at either 272 nm or 537 nm[10]. A 10 - µl sample loop was used for analytical separations and 50 - µl sample loop was used for preparative collection of the sample fraction components. The columns were a 25 cm x 4.6 mm i.d. stainless steel column packed with Lichrosorb RP-18 of 10 µm particle diameter and a 7 cm x 2.2 mm i.d. stainless precolumn packed with Polygosil 60-D 5 C-18 (Macherey-Nagel). The solvent used was 25% ethylene dichloride (Aldrich, high purity) in methanol (Fisher Certified) at a flow rate of 2 ml/min. To maintain a rapid and reproducible retention time, 10 µl of concentrated HCl were added to 1 liter of mobile phase (10 ppm of concentrated HCl). The HCl had a pronounced effect on the retention time of the major peak of the pigment components (prodigiosin parent peak) (see Results and Discussion). Increasing concentrations of HCl in the mobile phase decreased the retention time of this component (Figure 1). The 10 ppm of concentrated HCl gave the desired retention time of about 5 min for this component.

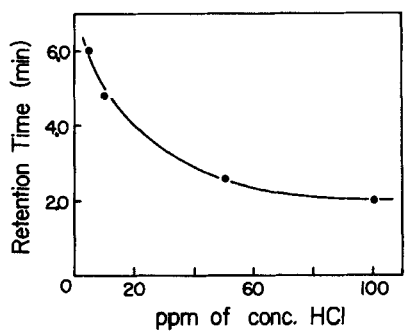

Figure 1. Effect of concentrated HCl on the retention time of prodigiosin component detected at 537 nm.

Oxidation of pigment extract by 30% hydrogen peroxide (Baker) was carried out according to the procedure of Payne, et al.[11] The pigment extract (400 mg) was dissolved in 10 ml of methanol, and enough 0.1 M NaOH was added to give a basic test with litmus paper. The 5 ml of 30% hydrogen peroxide was added and the mixture was stirred at 45 C. At intervals of 1.5 hr, 3.0 hr and 7 days, aliquots of the reaction mixture were taken and the methanol evaporated under nitrogen. Each aliquot was extracted several times with equal volumes of 1M HCl and chloroform; after separation, the chloroform phase was evaporated to dryness for HPLC, Infrared (Perkin Elmer Infrared Spectrophotometer) UV-Visible (Beckman Spectrophotometer Model ACTA VI) spectroscopic analysis.

RESULTS AND DISCUSSION

In an earlier attempt of applying HPLC to separate the pigment components in the chloroform-methanol extracts of S. marcencens, several not well resolved peaks were obtained[4]. There was no mention if the components were fully protonated (red acid form which absorbs at 535-540 nm) or nonprotonated (orange alkaline form which absorbs at 470 nm). Only a single wavelength (546 nm) was used to monitor the separation; therefore, nonpigmented UV-absorbing components escaped detection. In our study, we investigated first the relationship of the retention time of the major components at two wavelengths with ppm of HCl added in the eluting mobile phase. Figure 1 shows the decreasing of retention time with increasing concentration (in ppm) of HCl in the eluting solvent. By maintaining the amount of concentrated HCl at 10 ppm, we were able to ensure the well protonated form to be eluted in about 5 min. Furthermore, by monitoring the detector wavelengths at both 272 nm and 537 nm, it was possible with this system to detect prodigiosin (537 nm) and its metabolites and/or precursors (272 nm), Figure 2 shows the chromatograms of the pigment extract. When detected at 272 nm (Figure 2a), three peaks with retention times 1.6, 3.4 and 5.2 min were obtained. However, at 537 nm (Figure 2e), only one symmetrical peak was obtained. When the three peaks were collected

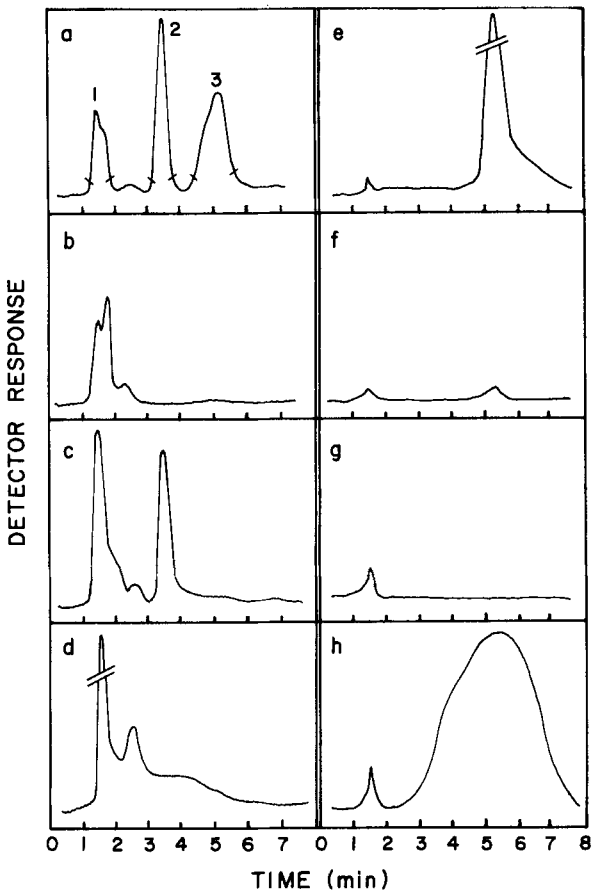

Figure 2. HPLC profiles of pigment extract from Serratia marcescens and its individual isolated components. (a) - (d) detected at 272 nm. (a) original pigment extract; (b) fraction 1; (c) fraction 2; (d) fraction 3; (e) - (h) detected at 537 nm. (e) original pigment extract; (f) fraction 1; (g) fraction 2; fraction 3.

by preparative technique and rechromatographed, fraction 1 (peak 1) showed that it contained only peak 1 (Figure 2b). The chromatograph of fraction 2 showed that it contained peaks 1 and 2 (Figure 2c), while that of fraction 3 showed that it contained peaks 1 and 3 (Figure 2d). When the IR spectra of the original extract and the three collected fractions were compared, it could be noted that they resembled closely those reported by Lynch, et al. as separated by column chromatography[12] and those by Button, et al. as separated by thin-layer chromatography[13]. All fractions except fraction 1 showed clearly defined peaks at 660, 750 and 1,210 cm^{-1}. A group of three peaks occurring at 2,875, 2,950 and 3,040 cm^{-1} was characteristic of the original extract and fraction 1, but to less extent of fractions 2 and 3 which had more pronounced hydrocarbon absorption bands (3,040 cm^{-1}). The absorption of 660 and 750 cm^{-1} probably showed C-H out of plane bending (n-amyl group of prodigiosin) and those at 2,875, 2,950 and 3,040 cm^{-1} indicated the presence of methylene and methyl groups. In all fractions, absorption in the ranges of 1,000 to 1,500 cm^{-1} and 3,000 to 3,100 cm^{-1}, characteristic of the pyrrole ring, was present. Overall, IR spectra of fractions 2 and 3 were almost identical. All collected fractions showed much structural similarity to the original extract with the exception that n-amyl group might be missing in fraction 1. Since fraction 1 was consistently present in the rechromatographed fractions 2 and 3, it is likely that it represents as the precursor or degradation product of fractions 2 and 3.

It has been suggested that prodigiosin could act as an auto-oxidizable electron acceptor[15]. In order to investigate this possibility and to correlate the chemical relationship of the individual isolated fractions, the pigment extract was submitted to hydrogen peroxide oxidation for various periods of time. The reaction products were characterized by HPLC and by IR spectroscopy. Figure 3 shows the chromatograms of the oxidation products. It can be seen that after 1.5 hr, peak 2 disappeared (Figure 3b) while after 3.0 hr, peak 3 disappeared (Figure 3g). As oxidation proceeded, there was an increase of

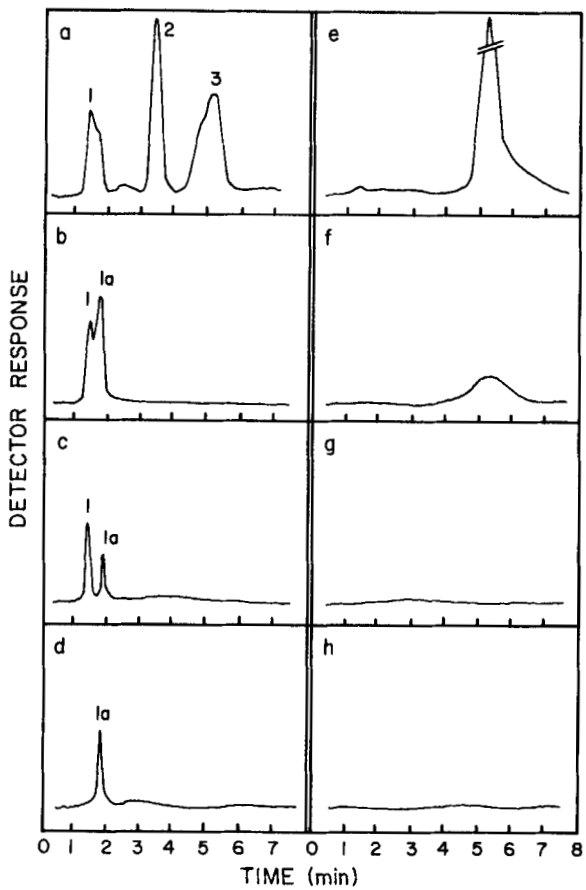

Figure 3. HPLC profiles of hydrogen peroxide oxidized products. (a) - (d) detected at 272 nm. (a) original pigment extract; (b) fraction after 1.5 hr oxidation; (c) fraction after 3.0 hr oxidation; (d) fraction after 7 days oxidation. (e) - (h) detected at 537 nm. (e) fraction after 1.5 hr oxidation; (f) fraction after 3.0 hr oxidation; (g) fraction after 7 days oxidation.

size of peak 1a while peak 1 (retention time 1.5 min) gradually decreased and, finally, completely disappeared at the end of the oxidation period (7 days) (Figure 3d). When the IR spectra of the final oxidation product and the isolated fraction 1 (without oxidation) were compared, they were quite similar, if not identical, to each other.

By maintaining a small but constant amount of concentrated HCl in the mobile phase, we have been able to develop a rapid method to separate the pigment components of S. marcescens 08 by HPLC. Detection at 537 nm or 272 nm enabled us to monitor the eluent for the protonated red form of prodigiosin or the nonpigmented but UV absorbing components, respectively. From the HPLC profiles and IR spectra of the collected fractions as well as those of the hydrogen peroxide oxidized products at various time intervals, we tend to conclude that the components separated by the HPLC method described in this study may be chemically and/or biosynthetically related to the parent pigment, prodigiosin. We intend to apply this method for the characterization of the pigment extracts of other strains of S. marcencens as well as those producing prodigiosin-like pigments.[15]

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