BioMetals 1999, **12**: 83–87

Purification of catechol siderophores by boronate affinity chromatography: Identification of chrysobactin from *Erwinia carotovora* subsp. *carotovora*.

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Received 23 June 1998; accepted for publication 7 August 1998

Catechols are co-planar cis-diols known to form stable, isolable complexes with borate under weakly basic conditions. We exploited this chemistry and developed a boronate affinity chromatography for isolating catechol siderophores. The method was applied to the isolation of chrysobactin, enterobactin, and an unknown catechol siderophore produce by *Erwinia carotovora* subsp. *carotovora* W3C105. Yields of chrysobactin and enterobactin purified by boronate affinity chromatography were at least two-fold greater than those achieved through alternate methods. The unknown catechol produced by *E. carotovora* subsp. *carotovora* W3C105 was isolated by boronate affinity chromatography and shown to be identical to chrysobactin. Boronate affinity chromatography enabled separation of catechol from its rust-colored decomposition products, and simultaneous isolation of catechol and hydroxamate siderophores. Boronate affinity chromatography is a rapid and efficient method for purifying catechol siderophores from bacterial culture supernatants.

Keywords: *Erwinia carotovora*, chrysobactin, catechol, boronate affinity chromatography.

Introduction

Iron is an essential micronutrient for all microorganisms. In the environment iron exists as complex hydrated oxides of Fe(III), many of which are non-stoichiometric and quantitatively insoluble. The low availability of iron in the presence of oxygen and neutral or basic pH limits growth of microorganisms. To obtain and transport the necessary iron, the majority of aerobic and facultative anaerobic bacteria secrete siderophores, which are highly efficient and specific iron chelators (Neilands 1981).

The plant pathogenic bacteria *Erwinia carotovora* and *Erwinia chrysanthemi* produce siderophores of apparently different significance in pathogenesis. *Erwinia chrysanthemi* 3937 produces the catechol siderophore chrysobactin (Cb; N-(N^2-(2,3-dihydroxybenzoyl)-D-lysyl)-L-serine (Figure 1) (Persmark *et al*. 1989), which is required for systemic virulence of *E. chrysanthemi* in its natural host *Saintpaulia ionantha* (African violets) (Enard *et al*. 1988, 1991). In contrast, siderophores produced by the related phytopathogen *Erwinia carotovora* appear dispensable in pathogenicity. *E. carotovora* subsp. *carotovora* W3C105 produces both an unidentified catechol siderophore (Bull *et al.* 1994) and the hydroxamate siderophore aerobactin (Ishimaru & Loper 1992). Although important for *in vitro* growth of *E. carotovora* subsp. *carotovora* W3C105 under iron-limiting conditions, neither catechol nor hydroxamate siderophores impacted potato tuber maceration or aerial stem rot of potato (*Solanum tuberosum* L.) (Bull *et al.* 1996). Nevertheless, siderophore-mediated iron competition is implicated in biological control and population suppression of *E. carotovora* by antagonistic fluorescent *Pseudomonas* spp. (Kloepper *et al*. 1980, 1983). The role of siderophores

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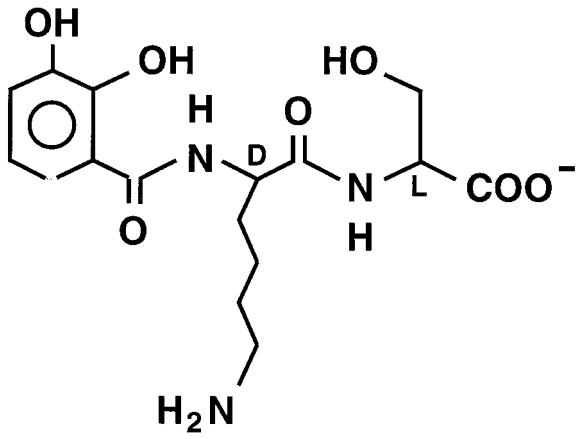

Figure 1. Structure of chrysobactin (Persmark *et al.*, 1989)

in the ecology of soft rot bacteria has yet to be explored (Bull *et al.* 1996).

In our continuing effort to determine the significance of siderophores in soft rot diseases, we sought to isolate and identify the unknown catechol siderophore produced by *Erwinia carotovora* subsp. *carotovora* W3C105. After pursuing several chromatographic methods, it became clear that instability of the unknown catechol hindered purification. We noticed a similar instability and chemical reactivity while isolating enterobactin from *Escherichia coli* AN194 and chrysobactin from *Erwinia chrysanthemi* 3937. Peptide-linked catechol siderophores, like chrysobactin, are generally water soluble at pH values in the range of 4–8. The oxidative decomposition products are Arnow-positive and as soluble as catechol siderophores, making separation without HPLC difficult. Thus we sought to investigate the lability of the molecules and to seek a more general method for the chromatographic isolation of catechol siderophores.

Catechols are co-planar cis-diols known to form stable, isolable complexes with borate under basic conditions (Boeseken 1949). This chemistry has been broadly exploited in the affinity chromatography of other co-planar cis-diols such as carbohydrates and ribonucleotides (Schott *et al.* 1973, Weith *et al.* 1970). Analysis of catecholamines in urine by boronate affinity chromatography also has been reported (Higa *et al*. 1977). Here we describe a simple method based on boronate affinity chromatography for isolating catechol siderophores from bacterial culture supernatants.

Through application of boronate affinity chromatography we isolated chrysobactin from *E. carotovora* subsp. *carotovora* W3C105. We also demonstrate that acidification of catechol-containing solutions prior to chromatography prevents oxidative degradation of catechol, stabilizes it, and increases isolated yields. Portions of this work were reported in an abstract (Barnes & Ishimaru 1993).

Materials and methods

Materials

All glassware was washed in 6N HCl and rinsed in distilled water and ethanol prior to use. All reagents were from Aldrich or Sigma, St Louis, MO, USA, and used without further purification. XAD-4 was from Sigma and cleaned prior to use by washing in hot water for one hour, followed by a continuous wash with methanol for one hour, ethanol for another hour and a final rinse with 25 volumes of distilled water. The boronate affinity gel was Affi-Gel 601 purchased from Bio-Rad Laboratories (Hercules, CA). A comparison sample of synthetic chrysobactin was a gift from J. B. Neilands.

Bacterial strains and growth conditions

Erwinia carotovora subsp. *carotovora* W3C105 and *Escherichia coli* AN194 (Langman *et al.* 1972) were obtained from J. E. Loper. *Erwinia chrysanthemi* 3937 was provided by D. Expert. Strains were grown on Luria-Bertani (LB) agar medium (Sambrook *et al.* 1989) and incubated overnight at 26C (*Erwinia*) or 37C (*E. coli*). Single colonies were added to 2 ml 20 mM phosphate buffer to obtain a slightly turbid suspension. Aliquots of this suspension were added to 5 ml Tris-minimal salts medium (TMS) (Simon & Tessman 1963), and incubated overnight with shaking. Cells were transferred to 50 ml of TMS and grown to late logarithmic phase. Final transfer was made to 500 ml TMS in Wheaton magnetic stir bottles. Incubation was carried out for 36-42 hours at either 26C (*Erwinia*) or 37C (*E. coli*) with vigorous aeration. TMS was supplemented with glucose (10 mM), deferrated casamino acids (0.3%), tryptophan (0.003%), and thiamine (0.002%) as required (Ishimaru & Loper 1992). Casamino acids were deferrated by extraction with 8-hydroxyquinoline, as described previously (Ishimaru & Loper 1992). Addition of 10^{-7} M $FeCl_3$ to TMS was sufficient for establishing iron limiting conditions conducive to siderophore production.

Detection and isolation of catechol siderophores

Catechols were monitored by the Arnow assay (Arnow 1937) at A_{510}; hydroxamates were monitored

by the Csáky assay (Csáky 1948) at A_{520}, and protein/peptide concentrations were determined by the bicinchoninic method (Smith *et al.* 1985) at A_{562}.

Bacterial cultures were centrifuged (6000 x *g*) at 3C for 20 minutes and the supernatant collected. The pooled supernatants of each respective culture were acidified to pH 5.0 with 6N HCl and passed through a XAD-4 column (2.5 x 45 cm) at 500 ml/hour flow rate. The column was washed with one volume of distilled water and the catechol fraction was desorbed with one volume of 1:1 v/v methanol:water, pH 5.0, and collected in 10 ml fractions. Methanol was removed from the pooled catechol-positive fractions by rotary evaporation and the aqueous solution lyophilized.

Boronate affinity chromatography was performed with 5 g of gel (binding capacity of 1.1 mEq/g) and 2,3-dihydroxybenzoic acid (DHBA) as the standard. The gel was poured into a 2.5 x 6 cm column, washed copiously with distilled water and activated by washing with one volume of 10 mM NaOH solution (Figure 2). Excess hydroxide solution was removed with a 0.75 column volume distilled water rinse. The Arnow-positive fraction from the XAD-4 column was taken up in 1–2 ml distilled water and placed on the column slowly (2 ml/hour) to allow equilibration and complex formation. The column was rinsed with one volume of distilled water, pH 7.0–7.25. Catechols were eluted with acidified distilled water (pH 5.0), and collected in 1 ml fractions at a flow rate of 3–5 ml/hour. Catechol-positive fractions were pooled, put through a small XAD-4 column to remove any trace impurities, and lyophilized.

Analysis and spectroscopy

Amino acid composition was obtained from phenyl isocyanate derivatives of acid hydrolyzed samples. Phenylthiohydantoin derivatives were separated on a Waters Picoteq HPLC at Macromolecular Resources, Colorado State University, and quantified by comparison with known standards. Normal phase TLC was performed on 0.25 mm fluorescent silica gel G plates in 90:5:5 v/v methanol:chloroform:acetic acid

Proton (^{1}H) nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AM-500 instrument in the Department of Chemistry, CSU. Samples were run in D_20 using acetone as an internal standard set at 2.225 ppm (Persmark *et al.* 1989). UV-visible spectra were obtained on a Hewlett-Packard 8451A diode array spectrometer. Mass spectrometry was performed on a Fisons VG Quattro-SQ instrument by electro-spray with preconcentration by tandem HPLC.

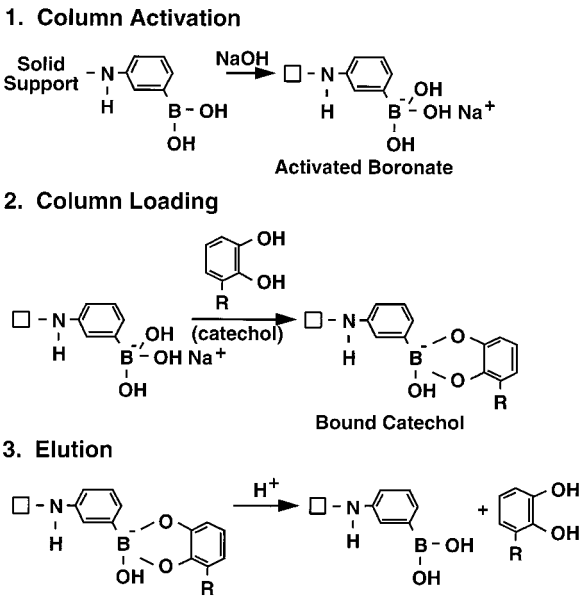

Figure 2. Boronate affinity chromatography method for isolating and purifying catechol siderophores.

Results

Activated boronate formed high affinity complexes with catechol samples at basic pH (Figure 2). Following column activation and column loading with samples concentrated by XAD-4, all catechol(s) present in the XAD-4 sample bound to the boronate affinity gel. Catechol was not detected in the flow-through or wash fractions. In samples from *E. carotovora* subsp. *carotovora* W3C105, the first several fractions collected during column loading and washing (Figure 3: fractions 1–10, pH 7), contained uncomplexed compounds, which tested positive for hydroxamates (Figure 3: peak I). Column elution with a slow flow rate and step gradient of dilute acid, (Figure 3: fractions 11–35, pH 7 – pH 5) resulted in quantitative separation of Arnow positive rust-colored fractions (Figure 3: peak IIa) from clear catechol fractions (Figure 3: peak IIb).

Spectroscopic analysis of the clear catechol fraction (IIb) from *Erwinia carotovora* subsp. *carotovora W3C105* identified the catechol as chrysobactin. Amino acid analysis of the unknown siderophore gave the 1:1 molar ratio of lysine:serine. The ^{1}H NMR spectrum of the unknown was identical to that of synthetic chrysobactin. Mass spectral analysis resulted in $[M+H]^+$ ion peak at 370.1, as expected for chrysobactin. Chrysobactin and the unknown migrated identically in TLC (R_f=0.45).

The yields of catechol were 3.5 mg and 2.5 mg per 500 ml culture of *Erwinia carotovora* subsp. *caro-*

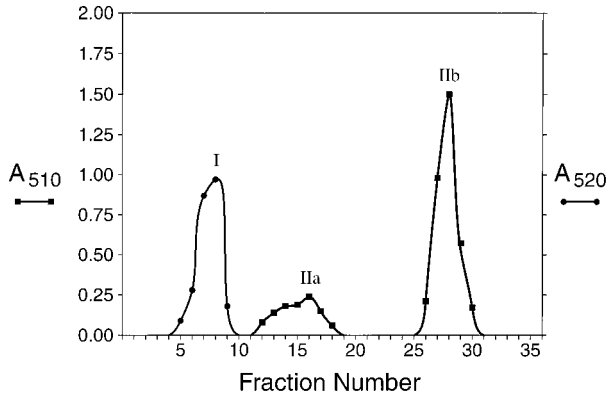

Figure 3. Boronate affinity chromatography elution profile of concentrated supernatants from *E. carotovora* subsp. *carotovora* W3C105. Fractions were monitored for the presence of hydroxamates (Csáky A_{520}) and catechols (Arnow A_{510}): hydroxamate-positive (I); Arnow-positive rust-colored (IIa); clear chrysobactin-containing fraction (IIb). Column loaded and washed at pH 7 (fractions 1–10). Column eluted with a step gradient of dilute acid of pH 7–5 (fractions 11–35).

tovora W3C105 and *Erwinia chrysanthemi* 3937, respectively. Without acidifying the supernatant to pH 5.0 prior to affinity chromatography, only 1 mg/500 ml of chrysobactin was obtained from *E. chrysanthemi*. This indicated an increase in catechol stability and resistance to oxidation at acidic pH. Acidification to pH 3.0 and ethyl acetate extraction of *E. coli* supernatant yielded 2.5 mg Eb/500ml, whereas acidification to pH 5.0 followed by XAD-4 and boronate affinity yielded 7.5 mg Eb/500 ml.

Discussion

We developed a simple affinity chromatography method for purifying catechol siderophores. The method exploits the chemistry of the catechol and is directed at separating catechols from oxidative degradation products omnipresent in culture supernatants. Degradation of catechols is evident as a change in the sample color, from clear to rust, at pH values above 5.0. Deprotonation of the catechol group causes instability and oxidative formation of quinonoid decomposition products (Gerwick *et al.* 1971, Shen *et al.* 1998). The pH of spent culture media is often above 5, which directly affects catechol yield.

Boronate affinity chromatography is a very specific and selective method for isolating catechol siderophores. Use of XAD-2 or XAD-4 has emerged as a general method to concentrate and desalt catechols from crude and semi-pure preparations (Berner *et al.* 1991; Persmark *et al.* 1989). Further chromatographic purification of catechol siderophores has involved Sephadex LH-20 or HPLC. These approaches along with LC-MS for identification and detection of catechols (Berner *et al.* 1991) do not specifically exploit the chemistry of the catechol. Borates at weakly basic pH form stable, 5-membered, anionic complexes with coplanar cis-diols, of which catechols are one major example. The quinonoid decomposition products have a very low affinity for boronic acid, and so are removed cleanly in the weakly basic wash prior to the aqueous acidic elution of the catechol siderophore.

Boronate affinity chromatography obviates the need for growing separate batches of cultures for isolating aerobactin and enterobactin from the same strain. A facile, simultaneous separation of hydroxamate and catechol siderophores occurs with boronate affinity chromatography because hydroxamates do not bind to the column. This can be especially useful when isolating siderophores from strains that produce both enterobactin and aerobactin (Neilands 1981). Enterobactin, an ester-linked catechol siderophore, is commonly isolated by acidifying the sample and then extracting it with ethyl acetate. One drawback of this procedure is the concomitant acid hydrolysis of hydroxamate siderophores, such as aerobactin.

We were somewhat surprised to find that *E. carotovora* subsp. *carotovora* W3C105 produces chrysobactin. Bull *et al.* (1994) detected differences in ability of *E. chrysanthemi* 3937 and *E. carotovora* subsp. *carotovora* W3C105 to crossfeed certain enterobactin indicators. *E. carotovora* subsp *carotovora* W3C105 crossfed *E. coli* AN93 and *Salmonella typhimurium enb-1*, which are deficient in enterobactin synthesis and proficient in DHBA production. In contrast, *E. chrysanthemi* 3937 did not crossfeed *E. coli* AN93 or *Salmonella typhimurium enb-1*. Both *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* W3C105 crossfed the indicators *E. coli* AN193 *and S. typhimurium enb-7,* which are deficient in DHBA production. These findings would be anticipated if *E. carotovora* subsp. *carotovora* produced enterobactin; however, we found no evidence of enterobactin production by *E. carotovora* subsp. *carotovora W3C105*. Furthermore, there were no additional catechol siderophores detected that could account for the differences in crossfeeding ability. We hypothesize that additional compounds influence the crossfeeding of *E. coli* AN93 and *Salmonella typhimurium enb-1* by *E. carotovora* subsp. *carotovora* W3C105 and

E. chrysanthemi. Studies on siderophore uptake and genetic complementation with catechol-deficient mutants of *E. chrysanthemi* and *E. carotovora* subsp. *carotovora* will provide insights into the apparent differences between these two chrysobactin-producing species.

Acknowledgments

We thank J. B. Neilands for the generous gift of synthetic chrysobactin, and J. E. Loper and D. Expert for providing bacterial strains and manuscripts for our review. We also thank A. Van Buren for technical assistance, C. Wassell for figure preparations, and G. J. Feistner for manuscript review. This work was supported in part by Colorado Agricultural Experiment Station and by the Colorado Potato Administrative Committee.

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