Ferrorosamine A from *Erwinia rhapontici*

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**Abstract.** The pink pigment of *Erwinia rhapontici* has been shown to be the known ferrous complex of proferrorosamine A, previously isolated from *Pseudomonas* species. The identification was based primarily on electronic absorption (visible), mass spectrometric, and nuclear magnetic resonance data. A purification procedure different from those hitherto reported was developed that in the mg range allows the straightforward isolation of both ferrorosamine A and proferrorosamine A as triethylammonium salts suitable for molecular weight determination by field desorption-mass spectrometry. *E. rhapontici* is pathogenic to various plants and is, therefore, likely to produce one or more unspecific toxic compounds. Proferrorosamine A, the complexation capacity of which can probably cause an iron deficiency in plants, may well be such a factor.

**Cultivation.** The culture medium consisted of: 1) a buffer solution containing 5 g K$_2$HPO$_4$ · 3 H$_2$O, 0.5 g MgSO$_4$ · 7 H$_2$O, and 5 g (NH$_4$)$_2$SO$_4$ in one liter H$_2$O. The pH was adjusted to 6.3 with 10% H$_2$SO$_4$ to give a clear solution. Solid media contained additional 2% agar. 2) Ten g/l sodium gluconate or mannitol as carbon source; for ferrorosamine A production, 50 mg/l ferric citrate was added, and for proferrorosamine A production, 2 g/l asparagine was added. All chemicals were obtained either from Merck or EGA.

**Extraction and purification.** After centrifugation the medium was extracted by a cation exchange column (Dowex AG 50WX8, 20–50 mesh, H$^+$-form, Serva). A column 6 cm in diameter and 44 cm in length was used for the culture centrifugate of each fermenter. The resin was washed with 51 of water and then eluted with 10% aqueous NH$_3$. The pH was adjusted to 6.3 with 10% H$_2$SO$_4$ to give a clear solution. Solid media contained additional 2% agar. Ten g/l sodium gluconate or mannitol as carbon source; for ferrorosamine A production, 50 mg/l ferric citrate was added, and for proferrorosamine A production, 2 g/l asparagine was added. All chemicals were obtained either from Merck or EGA.

**Extraction and purification.** The extraction medium was extracted by a cation exchange column (Dowex AG 50WX8, 20–50 mesh, H$^+$-form, Serva). A column 6 cm in diameter and 44 cm in length was used for the culture centrifugate of each fermenter. The resin was washed with 51 of water and then eluted with 10% aqueous NH$_3$. The pH was adjusted to 6.3 with 10% H$_2$SO$_4$. The combined eluate (1 l) was concentrated to 200 ml under vacuum (Biichi) not exceeding 35°C. Protein and salts were precipitated with 500 ml of methanol and the supernatant was evaporated to dryness under vacuum and the buffer decomposed.
Fig. 1. Production of proferrorosamine A by *Erwinia rhapontici* (followed by absorption measurement at 556 nm after transformation to ferrorosamine A) in relation to the oxygen content of the culture medium.

Instrumental analyses. Electronic absorption spectra were determined on a Beckmann spectrometer model 25. Mass spectra were obtained on a Varian MAT 731 mass spectrometer connected to a Finnigan-MAT SS 200 data system. Field desorption emitters were generated by the high-temperature mode using naphthalene as a carbon source [9]. Desorption of ferrorosamine A was achieved best when an equal amount of mannitol was added to the pigment (10 µg each) with an emitter heating of 7–10 mA. NMR-spectra were recorded with a Bruker WM 300 instrument. Optical activity was found to be $[\alpha]_D^{23} = +17^\circ$ on a Perkin Elmer Polarimeter 241.

Results

Because growth factors are not required by *E. rhapontici* [1], a very simple synthetic medium could be used for cultivation, thus facilitating the pigment purification procedure. Studies at different temperatures showed that pigment production decreased with increasing temperature and failed at 36°C, the growth rate then also being drastically reduced. Such a temperature dependence is likewise reported for *Pseudomonas GH* [11]. To achieve better yields and easier purification, Helbling [2] as well as Shiman [11] found it more suitable to avoid direct formation of ferrorosamine A and to isolate the propigment instead. But if one omits iron from the gluconate or mannitol medium, *E. rhapontici* neither produces the pigment nor the propigment. Previous studies [7] have shown that under iron deficiency the production of proferrorosamine A by *Pseudomonas roseus fluorescens* can be stimulated by asparagine. We found this to be true for *E. rhapontici* as well.

The transfer to submerged cultures first failed. The oxygen demand turned out to be crucial, the propigment yield increasing with the partial pressure of oxygen in the medium (Fig. 1). Because the 21 test cultures were not thermostated, part of the increase in yield might be effected by the cold as a result of vaporization, which decreased the temperature in the most vigorously aerated culture from 24°C (ambient temperature) to 21°C. It is important to note that in contrast to the results obtained by Helbling with *P. roseus fluorescens*, no reduction in yield was observed when the oxygen partial pressure was increased beyond 70%. In the gluconate/asparagine medium, lacking iron but thoroughly aerated, propigment production of *E. rhapontici* was consistent.

To isolate and purify ferrorosamine A and its propigment for unambiguous spectroscopic identification, we found neither the procedure of Shiman nor that of Helbling entirely satisfactory. Helbling concentrated the culture filtrate in the rotatory evaporator, which for larger batches is very time consuming, because he was interested not only in proferrorosamine A but also in other bacterial metabolites. The repeated extraction/crystallization procedure of Shiman seemed suitable only for amounts of propigment in the gram range. But as already pointed out by the latter author, proferrorosamine can very easily be extracted from the medium by means of a cation exchanger. We combined this concentration step with anion-exchange chromatography to yield with comparatively little effort both ferrorosamine A and its propigment in the form of their triethylammonium (TEA) salts.

The proferrorosamine A TEA salt was very suitable for molecular weight determination by field desorption-mass spectrometry (FD-MS), which showed the quasi-molecular ion $[M + H]^+$ of the TEA-salt at m/z 292 and that of the amino acid at m/z...