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Spectroscopic Study of Porphyrin Production and Accumulation in Culture of *Propionibacterium acnes*

M. Idrish Miah
Department of Physics, Bangladesh Institute of Technology,
Chittagong, Chittagong-4349, Bangladesh

Abstract: The Gram-positive skin bacterium *Propionibacterium acnes* was grown semi-anaerobically on Eagle's medium for 16 days at pH 6.7. Porphyrins were produced and accumulated in culture-grown *P. acnes*. Fluorescence spectroscopy was used to identify and quantify the amount of porphyrins produced in culture during the growth of the cells. Free base protoporphyrin IX (PPIX) as well as both free base coproporphyrin III (CPIII) and metallocoproporphyrin III (MCPIII) were identified in the culture. The CPIII was the dominant porphyrin species present in the early cultured-cells, however, the production and accumulation of PPIX was found to increase linearly with the age of the culture, which could be used in estimating the age of the culture of *P. acnes*. This investigation might help to understand efficient photodynamic therapy (PDT) of *P. acnes*.

Key words: Pectroscopy, cell culture, porphyrin production, *Propionibacterium acnes*, acne vulgaris, PDT

Introduction

The skin bacterium *Propionibacterium acnes* (*P. acnes*), implicated in the skin disease acne vulgaris (Holland *et al.*, 1981 and Bogorad, 1979), produces endogenous porphyrins (Melø *et al.*, 1982; Kjeldstad *et al.*, 1986 and Kessel and Dougherty, 1983) which fluoresce on Wood's light examination (Johnsson *et al.*, 1987). This fluorescence can be attributed to the production of different porphyrins during the biosynthesis of heme (Cornelius *et al.*, 1967). In the heme biosynthesis pathway, uro-porphyrinogen III is converted to coproporphyrinogen III, the precursor of protoporphyrin IX (PPIX), which is the penultimate compound. Metallation by ferrochelatase, i.e., incorporation of Fe^{+2} into PPIX, yields heme (Jordan, 1990; Dailey, 1990 and Bogorad, 1979). Porphyrinogens are colourless, metabolic active tetrapyrroles. When oxidized they become porphyrins, which are photosensitizing agents and can induce cell damages after irradiation (Kjeldstad *et al.*, 1986; Kessel *et al.*, 1983 and Melø *et al.*, 1985). Endogenous porphyrins might therefore take part in the photo destruction of *P. acnes* (Melø *et al.*, 1985). Irradiation of *P. acnes* with blue light leads to photo excitation of bacterial porphyrins, singlet oxygen formation by energy transfer from excited triplet-state porphyrins and eventually the bacterial damage (Arakane *et al.*, 1996).
The presence of coproporphyrin III (CPIII) and PPIX in culture-grown *P. acnes* have been reported (Melø *et al.*, 1982 and Kjeldstad *et al.*, 1984). In addition to this, Kjeldstad *et al.* (1984) have demonstrated that

the production of porphyrins in *P. acnes* varies with pH on the growth medium. The pH may differ according to the metabolic substances of *P. acnes* that could influence the activity of the enzymes involved in the biosynthesis of heme (Bogorad, 1979). It has been proposed that *in vivo* fluorescence spectroscopy can be used to identify and quantify porphyrins in cell culture (Melø *et al.*, 1982 and Kjeldstad *et al.* 1984). In earlier studies (Melø *et al.*, 1982 and Kjeldstad *et al.*, 1984), the identification and explanation of the fluorescing substance at 612nm in *P. acnes* was unclear because several porphyrins could show emission in this spectral region. The fluorescence at 580nm was also poorly understood. Since the efficacy of photo destruction of *P. acnes* depends on the amount of photosensitizing porphyrins present in the cell system (Kennedy *et al.*, 1992), the study of production and accumulation of porphyrins in the culture is of great importance. The aim of the present study was to identify and quantify the amount of porphyrins produced and accumulated in culture of *P. acnes* growing at semi-anaerobic conditions in view mainly of developing a technique of finding periods for sampling the cell population for efficient PDT treatment.

Materials and Methods

Propionibacterium acnes, serotype 2 (CN 6278), was grown on blood agar plates made from phosphate buffered (pH = 6.7) Eagle's medium. During the growth, plates were kept in a semi-anaerobic atmosphere (2% O_2) at 37^{0}C in darkness. Before experiments the cells were harvested and suspended in phosphate buffer saline (PBS) with a pH 6.7. For the pH-titration measurements, a 2-days old culture with different pH values in range 4.5-7.5 was used. pH changes were brought about by adding small amounts of HCl. The pK value of the cell system was 5.8. The pH of the cell suspension was measured by a pH-meter (pH electrode, Philips, C14/02). The fluorescence emission spectra were recorded on a spectrofluorometer (Perkin-Elmer Luminescence Spectrometer LS 50B) immediately after transfer of bacteria to PBS. In order to correct for varying cell densities, the fluorescence intensities were divided by the optical densities of the suspensions. The optical density was measured by a spectrophotometer (Shimadzu, Shimadzu Mectem, Japan).

Results and Discussion

The fluorescence spectra from the cell samples of 2, 4 and 7 days old cultures of *P. acnes* grown on Eagle's medium at pH 6.7 are shown in (Fig 2). A peak emission at 612nm was observed from young cells in early culture of 2 days old, while the maximum emission from 7 days old cell suspension was observed at 635nm. From a 4 days old cell suspension two major peaks (primary maximum at 612nm and secondary maximum at 635nm) overlapping the maxima at 612nm and 635nm for 2 days and 7 days old culture, respectively, were obtained. The fluorescence spectrum with an emission maximum at 612nm from the 2 days old culture was due to a porphyrin located outside the cells because the same spectrum was obtained from supernatant of the cell suspensions. The spectrum from the 7 days old culture was mainly due to PPIX free base in hydrophobic environments bound in lipid phase or to proteins (Lamola *et al.*, 1981 and Schwartz *et al.*, 1980), since the emission maximum was at 635nm. The spectra say that the young

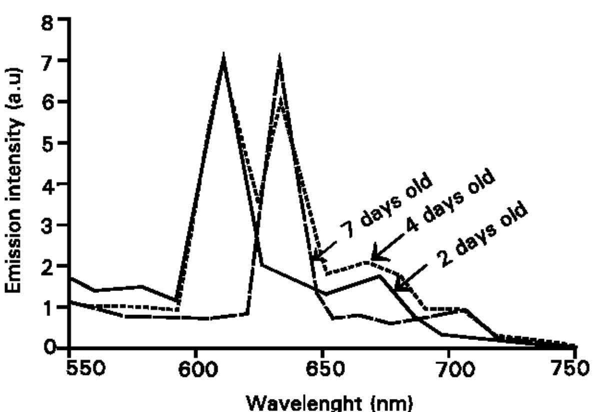

Fig. 1: Fluorescence spectra from cell suspensions of a 2, 4 and 7 days old culture of *P. acnes*. The cells were grown semi-anaerobically on Eagle's medium (with pH 6.7) at 37°C in darkness.

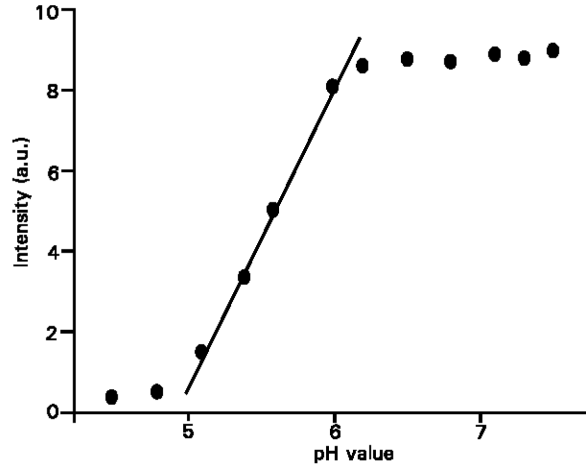

Fig. 2: Results of a pH titration of a 2 days old cell suspension. The pH values correspond to the peak values at 612nm. The fitting shows that a pH-transition between about 5 and 6 has been occurred.

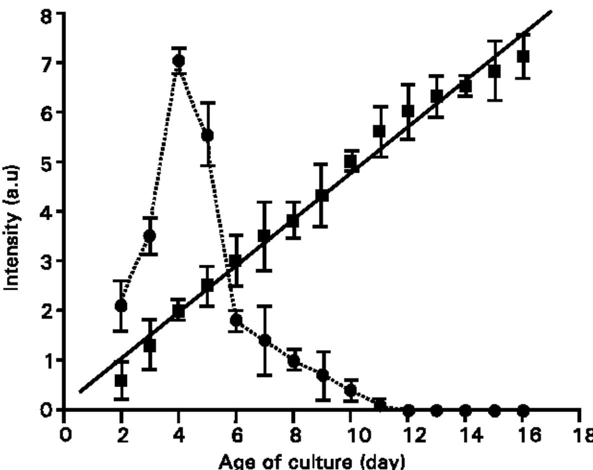

Fig. 3: Production and accumulation of coproporphyrin III (CPIII) and protoporphyrin IX (PPIX), measured as the fluorescence intensities at 612 and 635nm, respectively, as a function of the age of culture of *P. acnes* during growth. Bars show standard errors from four experiments taking cell suspension from four cultures under the same conditions. A linear curve (solid line) is fitted to the data for the production and accumulation of protoporphyrin IX, which shows that the amount of protoporphyrin IX increases linearly with the age of culture of *P. acnes*. In order to correct for varying cell densities, the fluorescence intensities were divided by the optical density of the suspensions.

cells in early culture contains mainly CPIII, and PPIX is the dominant porphyrin species in old cultures (Fig. 1).

An experiment for identifying porphyrin forms with a fluorescence maximum at 612nm was reported elsewhere (Dempsey *et al.*, 1961), where CPIII and PPIX were incorporated into anionic detergent micelles (sodium dodecyl sulphate, SDS) which solubilized both the free base and the monobase forms of the porphyrin. In that system there was a rapid exchange of both porphyrin forms between the water and the lipid phase. The relative amount of the free base to the monobase in the lipid phase, where both these forms of the porphyrin were fluorescent, was found to depend upon the pH of the water phase (Dempsey *et al.*, 1961). The result of the Micelle experiment reported by Dempsey *et al.* (1961) says that an emission with a maximum at 612nm may either be due to CPIII free base or to PPIX monobase, since only these species have fluorescence maxima at approximately this wavelength. In order to distinguish between CPIII free base and PPIX monobase forms, a pH titration experiment was performed.

A pH titration of 2 days old culture (Fig. 2) shows that a change in the base form of the present porphyrin was taken place in the pH range about 5-6 (see the fitting), where transition between free

bases and monobases are known to take place. This observation excludes the PPIX monobase mentioned above as a candidate responsible for the *P. acnes* bacterial fluorescence at 612nm, since no monobase-dibase transition occurs in this pH interval as was not observed in micelles by Dempsey *et al.* (1961). The observed 612nm- fluorescence from the *P. acnes* must therefore be due to the free base form of CPIII. As seen in the figure (Fig. 1) as well as found in our earlier studies (Kjeldstad *et al.*, 1984), a third fluorescent component emitting maximally at 580nm was obtained. The intensity of this component and that of CPIII was changed in an antiparallel way when the suspensions were kept in darkness, which indicates that the substance fluorescent at 580nm is a derivative of CPIII. Since some metalloporphyrins have fluorescence maxima in this region (Lamola, 1982), the fluorescence obtained at 580nm might be due to metal containing CPIII or metallocoproporphyrin III (MCPIII). The observed fluorescence from the *P. acnes* might thus be due to free base PPIX and to both free base CPIII and MCPIII. The insertion of a metal ion in the center of the porphyrin molecule may explain the changes in the fluorescence properties of CPIII during darkness. The incorporated metal ion increases the probability for intersystem crossing and hence photooxydation and removes the degeneracy in the energy levels which explains the shift in the emission maximum from 612 to 580nm. There was also a leakage of synthesized CPIII out of the cells and the free base form was bound to SDS-like structures in the cell surroundings. The free base fluorescence was disappeared when the pH was lowered as in anionic detergent systems, as found in the Micelle experiment (Dempsey *et al.*, 1961), but the monobase fluorescence was not appeared, which indicates that the monobase remains in the water phase. The change in the free base fluorescence properties at that pH may be due to the ionization of the carboxylic acid side chain of the porphyrin. The production and accumulation of CPIII and PPIX were measured as the fluorescence intensities at 612 and 635nm, respectively, in a 16-days long culture during the growth of *P. acnes*. The amounts of CPIII and PPIX as a function of the age of the culture are shown in Fig. 3. In the early culture, the production and accumulation of CPIII and PPIX were found to increase with the age of culture, with higher production rate of CPIII than that of PPIX. This means from the point of view of cell line that in the lag phase and early log phase CPIII was predominantly produced in culture of *P. acnes*. The production and accumulation of CPIII was decreased after reaching its maximum value around 4 days. The production and accumulation of PPIX was found to increase linearly with the age of the culture of *P. acnes* during growth (Fig. 3). During the log phase, the cell proliferation rate was high and hence PPIX accumulation occurred. This model may be used as an estimation of age of the culture of *P. acnes* during growth under semi-anaerobic conditions for sampling the cell population for any physicochemical treatment with them. Porphyrin-induced photo destruction of the pilosebaceous follicles results from reaction with singlet oxygen generated by energy transfer from excited triplet-state porphyrins produced by *P. acnes*. Since CPIII is partially soluble in water and PPIX is a nonpolar substance, it is found that PPIX is more toxic to lipophilic structures, whereas CPIII, which is less hydrophobic, is more toxic to water-soluble structures (Sandberg *et al.*, 1981). It can thus be considered that the production of PPIX by *P. acnes* is more likely to lead to the development of inflammatory lesions than the production of polar porphyrins. This suggests that the analysis of PPIX in the cell system is more important than that of other porphyrins in any

cytotoxic or comedogenic effect studies. It has been established that the efficacy of photo damage of *P. acnes* depends on the amount of photosensitizing porphyrins present in the cell system (Melø *et al.*, 1985 and Kennedy *et al.*, 1992), which indicates that the exact knowledge about porphyrins and PPIX content in *P. acnes* is required in the PDT treatment for them. The present investigations for porphyrin identification and production and accumulation in *P. acnes* may help to understand pathophysiology of acne vulgaris, one of the commonest skin conditions to affect the humans, with 70% of adolescents developing acne and widen the treatment options for acne patients.

In conclusion, *P. acnes* contained mainly CPIII, with peak fluorescence at 612nm, and PPIX, with fluorescence maximum at 635nm, as the dominant porphyrin species. The PPIX was accumulated inside the cells and CPIII, both free base and metal containing, outside the cells. In the lag and early exponential phases, CPIII was predominantly produced. The production and accumulation of PPIX in *P. acnes* was found to increase linearly with the age of the culture during growth of the cells, which may be used for estimating the age of the culture of *P. acnes*.

Exposure of *P. acnes* with light in the blue region around 415nm could result in photodynamic stimulation of porphyrins stored in the bacteria, singlet oxygen production and bacterial killing (Arakane *et al.*, 1996). Porphyrins produced by *P. acnes* have thus been considered to have cytotoxic and comedogenic effect, which may be relevant to the occurrence of inflammation in acne patients. Thus the analysis of these porphyrins may be helpful in understanding the pathophysiology of acne vulgaris and may widen the treatment options to include PDT.

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