ISOLATION AND PROPERTIES OF A YELLOW-GREEN FLUORESCENT PEPTIDE FROM AZOTOBACTER MEDIUM[1]

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The presence of yellow-green pigments in cultures of Azotobacter has been observed by many investigators since first reported by Beijerinck (1901) in his description of Azotobacter agile. Johnstone (1955, 1957) observed the fluorescence of the media in ultraviolet light and reported some fluorimeter measurements on media from different species. Johnstone and Fishbein (1956) used fluorescence of the culture as an aid in species classification.

Previous experiments have shown that yellow-green pigment production and fluorescence accompany a low level of iron in liquid cultures (Wilson and Knight, 1952; Johnstone and Fishbein, 1956; Johnstone et al., 1959). Johnstone et al. (1959) established that the fluorescence is not contributed by riboflavin but by water-soluble, thermostable pigments. Their attempt to fractionate the medium by continuous paper electrophoresis yielded a white and a yellow fluorescing fraction.

Since iron is involved in nitrogen fixation and other electron transfer systems and there is a correlation between the occurrence of fluorescent material and the availability of iron in the medium, we have attempted the isolation and characterization of the pigments. We wish to report that the principal yellow compound is a peptide with an attached yellow-green

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fluorescent chromophore, and that the peptide chain contains the uncommon amino acids homoserine and β-hydroxyaspartic acid. The procedure for the isolation of this compound in a highly purified form, a partial characterization, and some of its properties are summarized. The procedures used will be described in detail elsewhere.

CULTURE AND ISOLATION

The Azotobacter agilis (A. vinelandii) used in these experiments was the strain O originally obtained from the University of Wisconsin and maintained in liquid culture as previously described (Bulen, 1961). For pigment isolation 6 liter cultures were grown for 16 hr in iron-deficient medium with high aeration. After removal of the cells, fluorescent material was adsorbed on charcoal and eluted from a charcoal-Celite column with aqueous acetone. The eluate was concentrated and impurities removed by acidification and centrifugation. The supernatant solution was fractionated on a Dowex 50 column by gradient elution, using water and increasing concentrations of NH_4OH to 0.5 M. In addition to the yellow-green fluorescent compound, a yellow fluorescent, a blue-white fluorescent, and three blue fluorescent compounds were separated. The yellow-green fluorescent fraction was further purified on a Dowex 1 column in the acetate form by elution with dilute acetic acid. The desired fraction was concentrated and the yellow-green fluorescent compound precipitated with ethanol, washed first with absolute ethanol, then with ether and dried in air. The compound was obtained as a bright yellow powder in yields averaging ca. 9 mg/liter of medium.

The purification was followed by observing the absorption spectra of the different fractions, and the relative amount of yellow-green fluorescent material was determined from the absorption at 380 mμ in 0.1 N HCl. The

final product was free of the light absorbing contaminants observed in spectra of crude or partially purified fractions. Fluorescence spectra indicated the removal of fluorescent contaminants. Paper chromatographic examination with a number of solvent systems, although accompanied by spot elongation or tailing in some, gave no indication of multiple fluorescent spots. No reaction was obtained at the fluorescent spots or elsewhere when developed chromatograms were treated with aniline phthalate, alcoholic $AlCl_3$, or diazotized sulfanilic acid. A deepening of the yellow color and a quenching of fluorescence followed treatment with Ehrlich's reagent. Prior to hydrolysis, no ninhydrin reacting material was evident.

PROPERTIES AND CHARACTERIZATION

The purified compound is readily soluble in water but insoluble in all organic solvents tested.

The absorption spectra of aqueous solutions determined with a Cary recording spectrophotometer at different pH values are presented in Figs. 1 and 2. At all three pH values the compound exhibits two maxima, both of which shift to longer wavelengths with increasing pH. The specific absorption coefficient of the air dried sample in 0.1 N HCl at the 380 mμ maximum is 15.5 cm^2/mg.

At alkaline pH values the compound is subject to photodecomposition. The dashed curve in Fig. 2 shows the spectral change accompanying exposure of the compound in 0.1 N KOH to 1 hr illumination with a tungsten lamp.

The activation and fluorescence spectra in 0.1 N HCl determined with the Aminco-Bowman spectrophotofluorometer are reproduced in Fig. 3. The fluorescent maximum occurs at 488 mμ and only minor shifts accompany an increase in pH. The relative fluorescent intensity, however, is increased ca. three-fold in phosphate at pH 7 and ca. two-fold in 0.1 N KOH.

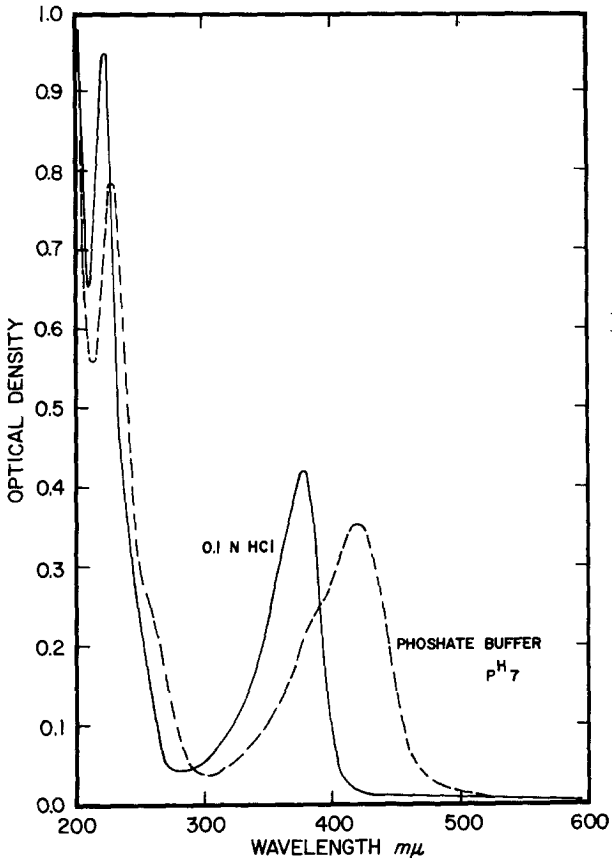

Fig. 1. Absorption spectra of yellow-green fluorescent peptide in 0.1 N HCl and in phosphate buffer pH 7. Concentration 26.5 μg/ml.

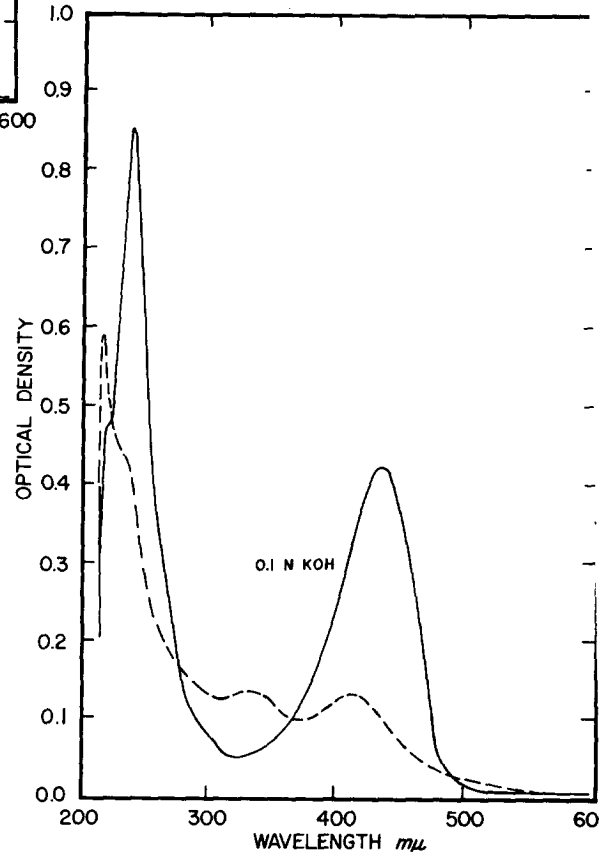

Fig. 2. Absorption spectra of yellow-green fluorescent peptide in 0.1 N KOH before and after photodecomposition. Concentration 26.5 μg/ml.

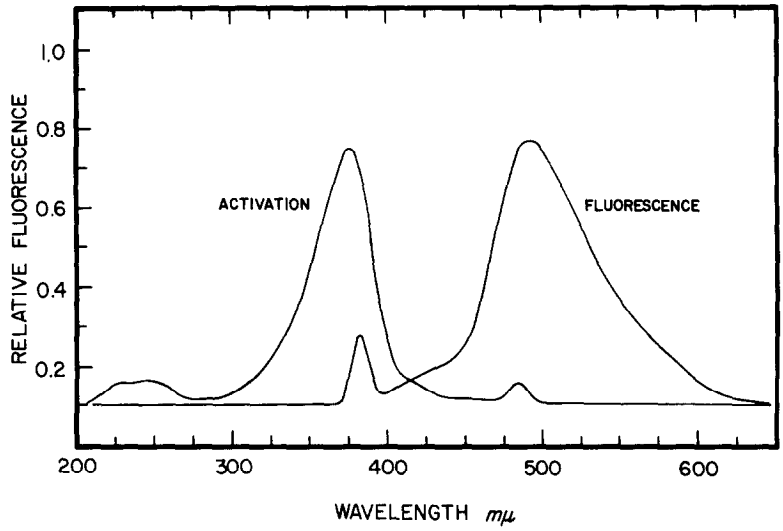

Fig. 3. Activation and fluorescence spectra of yellow-green fluorescent peptide in 0.1 N HCl. Concentration 3 μg/ml. Slit arrangement No. 3; meter multiplier setting 0.03; sensitivity 50.

Acid hydrolysis followed by two-dimensional chromatography and treatment with ninhydrin revealed the presence of one minor and six major amino acids. These have been identified from elution volumes on the Beckman/Spinco Model 120 amino acid analyzer, by co-chromatography in different two-dimensional solvent systems, and by the use of selective chemical and polychromatic reagents on two-dimensional chromatograms. Serine, homoserine, glycine, citrulline, aspartic acid and β-hydroxy-aspartic acid were identified and appear to be present at mole ratios of 2, 2, 1, 1, 1, and 1 respectively. The minor amino acid was identified as ornithine and is probably formed from citrulline during acid hydrolysis. Based on recoveries from the amino acid analyzer, the compound is ca. 62% peptide. The hydrolyzate gave a negative test for phosphate with the Fiske and Subba Row (1925) procedure.

A small amount of the chromophore was obtained in crystalline form as orange clusters of wedge shaped plates when the hydrolyzed material,

after removal of HCl, was allowed to stand in citrate buffer pH 2. 2. The washed crystals were used for examination of the absorption, fluorescence, and activation spectra of the chromophore in 0. 1 N HCl. Spectra are nearly identical to those shown for the parent peptide except in the 210 mμ region where the peptide structure absorbs. Thus an attached chromophore which is stable to acid hydrolysis and has a relatively low solubility in water is responsible for the absorption and fluorescence properties of the yellow-green fluorescent peptide. The structure of this chromophore is now under investigation in our laboratory.

The failure of previous investigators to isolate the yellow pigment from Azotobacter medium may have resulted from the non-realization of its peptide nature and the photodecomposition occurring at alkaline pH values. With the availability of the pure material, the biochemical origin and function, if any, of this compound can be investigated. As a working hypothesis we consider the compound to be normally associated with an iron-protein and to be released into the medium when the coordinating iron atom is unavailable. Alternatively it could arise from a biosynthetic pathway that is interrupted by iron deficiency.

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