A Red, Fluorescent Protein from Silkworm*

Sir:

In the course of the purification of a nuclease from the digestive juice of silkworm larvae, *Bombyx mori* L., we encountered a red surface-colored, fluorescent protein with interesting chemical and physiological properties. As far as we know, this has not been reported in the previous literature.

After 25~40% acetone precipitate of *n*-butanol-treated juice was saved for the purification of nuclease as described previously,1) the supernatant was further added with cold acetone to 50% concentration. The brown-colored precipitate formed was collected by centrifugation, taken up in 0.5 M NaCl-0.04 M sodium carbonate buffer, pH 10.4 (8700 A280 and 475 A605 units from ca. 3 liters digestive juice), and then filtered through Sephadex G-75 gel (3.9×60 cm) which had been adjusted to the same buffer. The red-colored breakthrough, excluded from the gel network, contained the red protein. The pooled fractions were neutralized with 2 M acetic acid, precipitated with (NH₄)₂SO₄ at 30% saturation, taken up in water, dialysed against 0.1 M NaCl until just dissolved, and again filtered through Sephadex G-200 gel (1.9×60 cm). The red protein was again excluded from the gel and emerged as a single, steep peak, well separated from the trailing components. The protein was precipitated with (NH₄)₂SO₄ at 27% saturation, taken up in 0.02 M Tris-HCl, pH 7.2 and dialysed against the same buffer. The dialysate, 254 A₂₈₀ and 21.4 A₆₀₅ units, was then chromatographed on a DEAE-cellulose column (1.9×30 cm) with 1 liter linear gradient of NaCl from 0 to 0.3 M in the same buffer. The red protein appeared at a NaCl concentration of 0.10 M, giving a single, symmetrical peak, with the brown-colored impurities retained on the top of the column. The relevant eluate, 262 to 372 ml, was concentrated by (NH₄)₂SO₄ precipitation at 27% saturation followed by dialysis against

FIG. 1. Ultracentrifugal Pattern of the Purified Red Protein.

The protein concentration was 0.4% in 0.2 M KCl-0.02 M phosphate buffer, pH 7.5. Photographs were taken at 8 min intervals after reaching 56,100 rpm in a Spinco Model E ultracentrifuge. Sedimentation proceeds from right to left.

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an appropriate buffer. Yield, 110 A_{280} and 18.1 A_{605} units.

The protein gave a homogeneous ultracentrifugal pattern, 6.3 s_{20,w} (Fig. 1). Anal. Found: C, 48.08; H, 6.97; N, 15.31%. Amino acid composition (moles amino acid per 100 moles N atom) Found: 0.96, 0.46, 17.2, 2.9, 8.6, 5.0, 3.4, 5.5, 5.7, 4.9, 3.6, 0.72, 7.7, 0.74, 3.8, 4.8, 3.3, 2.8, and 0.96 for Lys, His, (NH_{3}), Arg, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Half-Cys, Val, Met, Ileu, Leu, Tyr, Phe and Try. Nitrogen recovery, 94%. Analysis was made by Mitamura’s analyzer, 500-II on the 6N HCl hydrolysate (110°C, 48 hr). Half-cystine\textsuperscript{2)} and tryptophan\textsuperscript{3)} were determined separately. Figure 2 shows absorption and fluorescence spectra of the protein, each with two maxima—at 278 and 605 m\u00barmu for the former and 335 and 620 m\u00barmu for the latter. A fluorescence at 335 m\u00barmu should be attributed to tryptophan residues. Strong acid or alkali as well as a few minutes’ boiling caused an immediate, irreversible disappearance of the absorption and fluorescence at the longer wavelength. Addition of sodium dithionite also resulted in rapid decolorization of the protein, the extent of which being proportional to the amount of the reagent added. Ascorbic acid and hydroxylamine also decolorized the protein slowly. Overnight incubation of the protein with 5mM urea or 25 \mu g/ml Pronase P (Kaken Kagaku Co., Tokyo), but not trypsin or chymotrypsin, diminished the red color gradually. In view of the seeming resemblance of the spectral and oxidoreduction characteristics of this substance to those of the blue proteins,\textsuperscript{4)} analysis of the protein-bound metals was attempted by means of emission spectrography and atomic absorption spectrophotometry. But the result was negative. The protein was checked for oxidase activity and found negative towards catechol and cresol. Various attempts of reducing (decolorizing) the protein by linking in vitro with electron transfer systems in the homogenate or subcellular fractions from the silkworm midgut, in the presence or absence of NADH, reduced or oxidized cytochrome c and/or succinate, have also been unsuccessful thus far. The protein was, however, found to counteract severely the physiological activity of rice seedlings, Oryza sativa L. That is, when the plants at 8 leaf stage were cut off the roots and the portion of their leaf-sheath was soaked in the protein soln., they withered a few or 48 hr later at the protein concentration of 100 ppm or 10 ppm in water at 22°C. This withering activity of the protein withstood 5 minutes’ boiling. No change was seen in the control plants.

\textsuperscript{4)} See for example S. Kato, Protein Nucleic acid Enzyme, 8, 73 (1963); H. Shiichi and D. P. Hackett, Arch. Biochem. Biophys., 100, 185 (1963).
After all, nothing can be said assuredly at present as to the structural features and physiological function of this red protein. Only it may be an enzyme or an intermediary constituent of oxidoreduction systems in or not in relation to digestion mechanism of the silkworm larvae. Further study is needed to clarify these points.

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