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Dedicated to the memory of
Professor Mircea D. Banciu (1941–2005)

FLUORESCENT CONJUGATES OF CASEIN AND OVALBUMIN WITH 4,7-DIPHENYL-1,2,5-OXADIAZOLO[3,4-c]PYRIDINE-6-CARBOXYLIC ACID: PREPARATION AND ANALYSIS

Mihaela Camelia BĂLAŞU* and Angela POPESCU

Department of Organic Chemistry, "Politehnica" University of Bucharest,
Splaiul Independentei 313, 060042 - Bucharest, Romania

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Fluorescent conjugates are widely used in biology and medicine. We used for this study hen ovalbumin and bovin casein. The conjugation reaction of proteins with 4,7-diphenyl-1,2,5-oxadiazolo[3,4-c]pyridine-6-carboxylic acid (DOPCA) was performed with diciclohexylcarbodiimide (DCC) and N-hydroxymaleimide (NHM). Fluorescent conjugates were separated by gel chromatography and organic solvent precipitation. Purified fluorescent conjugates were subsequently analysed by fluorimetry and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These analyses showed that the tested conjugation reaction yielded fluorescent conjugates at thiol groups. The strongest emission was obtained with the ovalbumin conjugate. The limits of detection by electrophoresis in presence of detergent for both protein conjugates are also reported.

Proteins conjugated with fluorescent dyes at the thiol functional groups are principally used for probing biological structure, function and interactions.[1] Fluorescent dyes are also useful to facilitate analytical assay of thiol-containing proteins in chromatographic and electrophoretic separation.[1]

Ovalbumin is the most abundant protein in the avian egg white and belongs the serpins, a group of structurally related proteins (SERine Proteinase Inhibitors).[2,3] There are six cysteine (Cys) residues in the single polypeptide chain of hen ovalbumin. The structural and functional characteristics of ovalbumin are significantly influenced by the state of Cys sulfhydryl groups.[4]

Caseins are the most abundant proteins in milk. Milk caseins form a nonhomogenous mixture of proteins with specific micellar structures. This mixture contains three groups of proteins: α-casein (αS1- and αS2-), β-casein and κ-casein.[5] γ-Casein has been identified as the proteolytic fragment of β-casein.[6] Caseins have fewer Cys residues than the ovalbumin. αS1-Casein and β-casein contain one Cys residue while αS2-casein contains three Cys residues.

In this work we used commercially available proteins: hen ovalbumin and Hammersten casein (a casein mixture consisting of αS1-casein 39%, αS2-casein 10%, β-casein 37% and γ-casein 13%). 4,7-Diphenyl-1,2,5-oxadiazolo[3,4-*c*]pyridine-6-carboxylic acid (DOPCA) is a fluorescent compound which was used by us in other research to obtain the succinimidil DOPCA. We used this compound to fluorescently label the bovine serum albumin.[7] DOPCA was previously reported as intermediate in the synthesis of fluorescent compounds used in electronic devices.[8]

Preparation of fluorescent conjugates

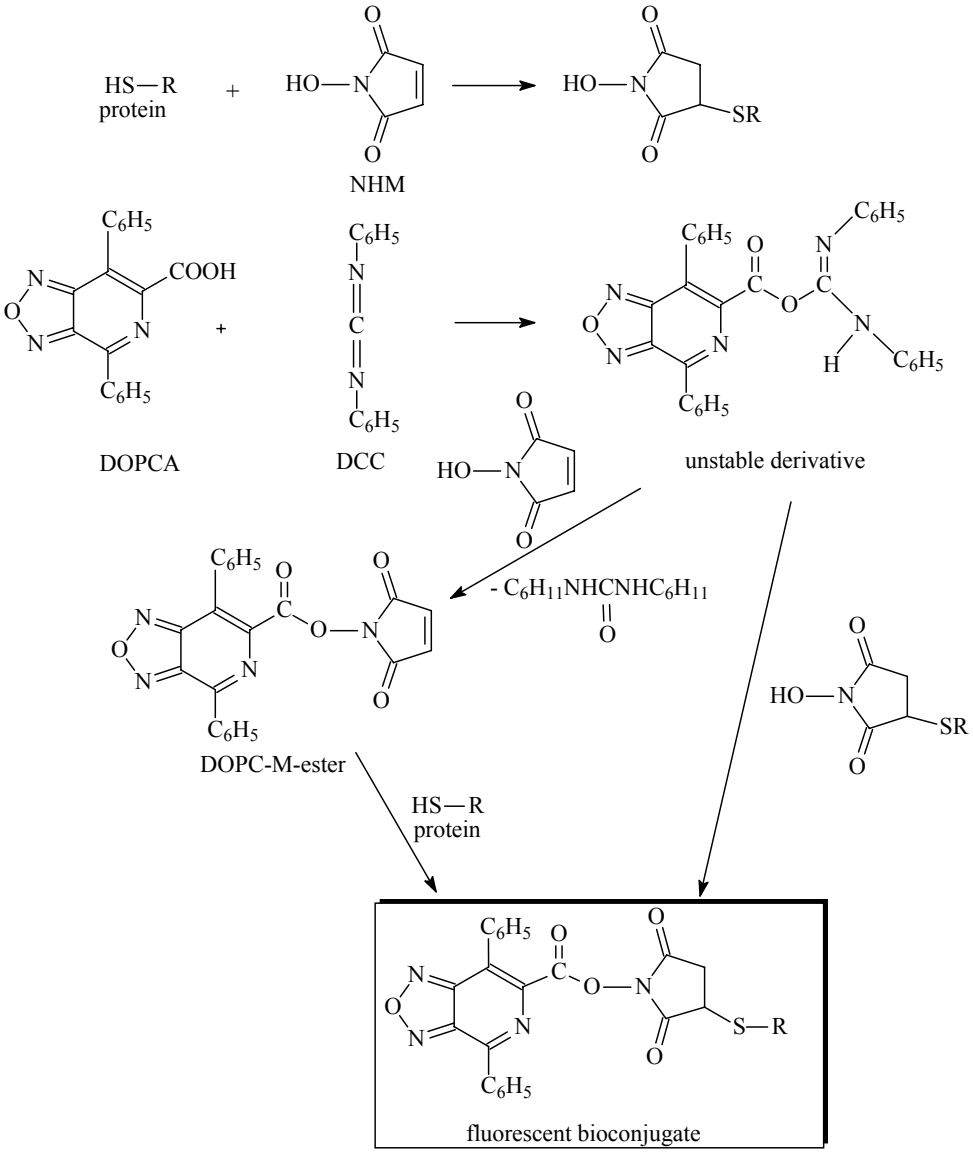
The preparation of bioconjugates were began with the reaction of protein thiol groups with the maleimidil derivative of fluorescent compound. This reaction involves addition of the thiol across the double

*Corresponding author: e-mail mc_balasu@chim.upb.ro

bond of the maleimide to yield a stable thioether in the pH range 6.5-8.[9] This pH range is very important because some reactions can compete significantly with the thiol modification, particularly above pH 8.[9,10]

DOPCA was prepared from commercial reagents.[7,8] The maleimidil derivative of DOPCA was generated by the use of carbodiimide reaction of dicyclohexylcarbodiimide (DCC) and N-hydroxymaleimide (NHM). (Scheme 1) The reaction between N-hydroxymaleimidil derivative of protein and the activated DOPCA could be other possible reaction pathway in our reaction conditions (Scheme 1). A concentrated stock solution of the reactive DOPCA was prepared in DMSO and was imediately added in small aliquots to the protein in phosphate-buffered saline solution. Reaction was performed under nitrogen at room temperature for two hours. The protein solutions, free of oxygen, were previously treated with tris-(2-carboxyethyl)phosphine to specifically reduce the disulfide bonds of proteins.[11] Upon completion of reaction mercaptoethanol was added to block the excess of reactive species.

The bioconjugates were purified from the reaction mixtures by gel chromatography on Sephadex G-50[12] followed by acetone precipitation.[13] The chromatographic fractions were subjected to UV-VIS spectrophotometric and fluorescence analysis. The protein sediments obtained by acetone precipitation were solubilised with electrophoresis loading buffer and migrated in SDS- polyacrylamide gel.



Scheme 1 – Preparation of protein conjugates by reactions of DOPCA with DCC and NHM.

Analysis of fluorescent conjugates

The fluorescent bioconjugates separated by chromatography were analyzed by UV-VIS spectrometry and fluorimetry. Fractions containing proteins were evaluated by recording the absorbance at λ = 280nm. Absorbance data correlated with fluorescence data recorded from the same fractions led to the identification of fractions containing the purified fluorescent bioconjugates. The richest fraction of ovalbumin conjugate (fraction 10, Figure 1) showed a stronger fluorescence signal than the richest fraction of casein conjugate (fraction 15, Figure 1). Conjugated casein eluted more slowly from the Sephadex G-50 column than did the conjugated ovalbumin (Figure 1), similar to the free proteins (data not shown). Fluorescence data are in agreement with the Cys content of the proteins.

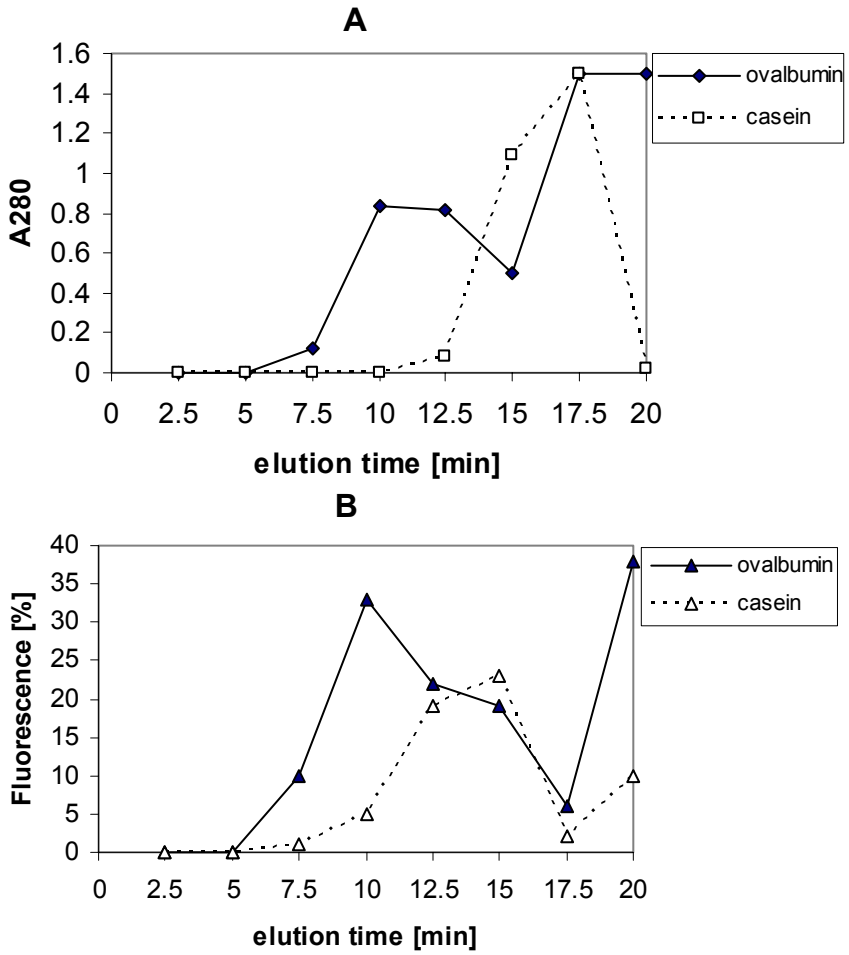


Fig. 1 – Sephadex G-50 chromatography of protein conjugates
A) A280 ; B) Fluorescence (excitation : 340-380nm, emission: 515-525nm).

After acetone precipitation and re-solubilization of the precipitated proteins we obtained samples that were subjected to SDS-polyacrylamide gel electrophoresis[14]. (Figure 2) Exposure of the gel to UV-light in a ChemDocSystem revealed some strong fluorescent bands and some slightly fluorescent bands. The bands corresponding to the theoretical molecular weights of the proteins used, i.e., 42750 (hen ovalbumin) and 25200 (24529, αS1- casein; 26019, αS2-casein; 25107, β-casein) observed by Commassie blue staining suggested that the slightly fluorescent bands were protein conjugates. Samples with various quantities of proteins (ovalbumin 10-80 µg/lane and casein 4-40µg/lane) were separated by SDS-PAGE. The fluorescent albumin conjugate was detected in the lane loaded with 10 µg protein, whereas the casein fluorescent conjugate was detected only in the lane loaded with 40 µg protein.

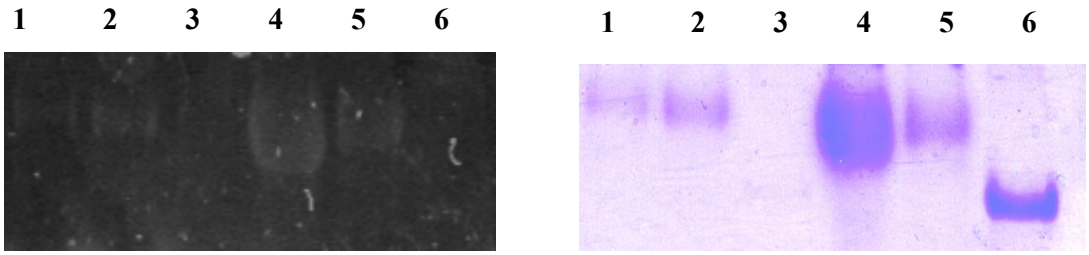


Fig. 2 – Evidence of proteins after gel electrophoresis by: A) Trans UV illumination at 302 nm; B) Comassie blue staining. In the figure are shown: ovalbumin and casein reaction mixtures (ovalbumin: lane1-10µg, lane 2-20µg and casein lane 3- 4µg); ovalbumin and casein reaction mixtures precipitated (ovalbumin: lane 4-80µg, lane 5-40µg and casein lane 6- 40µg).

In conclusion the present study demonstrates that:

1) fluorescent conjugates of ovalbumin and casein could be obtained with reaction mixture of: DOPCA, NHM and DCC;

2) fluorescent ovalbumin conjugate displayed a stronger fluorescence intensity than the fluorescent casein conjugate, as shown by the fluorescence analysis of the chromatographically purified conjugates and by the electrophoretic analysis of the precipitated conjugates. This result is in agreement with the higher Cys content of ovalbumin.

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