J.Serb.Chem.Soc. 68(4–5)243–248(2003)
JSCS – 3039

UDC 547.962.4-035.57+547.567:66-96
Original scientific paper

Chemical modification of β-lactoglobulin by quinones*

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(Received 31 December 2002)

Abstract: The avarone/avarol quinone/hydroquinone couple, as well as their derivatives show considerable antitumor activity. In this work, covalent modifications of β-lactoglobulin, isolated from cow milk, by avarone, its model compound 2-*tert*-butyl-1,4-benzoquinone, and several of their alkylthio derivatives were studied. The techniques applied for assaying the modifications were: UV/VIS spectrophotometry, SDS PAGE and isoelectrofocusing. The results of the SDS PAGE suggest that polymerisation of the protein occurs. The shift of the pI of the protein upon modification toward lower values indicates that lysine amino groups are the principal site of the reaction of β-lactoglobulin with the quinones.

Keywords: avarone, quinone, β-lactoglobulin, covalent modification.

INTRODUCTION

Avarol/avarone is a sesquiterpenoid hydroquinone/quinone couple with a pronounced antitumor activity.[1–4] Two mechanisms of action of quinones can be considered in the cell: generation of oxygen radicals[5] and alkylation of cellular nucleophiles. Based on studies of the chemical reactivity and biological activity of avarone **I**, its model compounds, and many of their derivatives, we suggested that the addition of cellular nucleophiles to avarone and its derivatives is important for biological activity.[6]

In order to test this hypothesis, it is necessary to show that biomolecules undergo chemical reactions with avarone and its derivatives with the formation of covalent bonds. In this work, covalent modifications of β-lactoglobulin (β-LG), a protein of 18 kDa isolated from cow milk, by quinones avarone, 2-*tert*-butyl-1,4-benzoquinone **II** and several alkylthio-derivatives were studied. This protein was chosen because it is readily available, and can be isolated in a very pure state.

* Dedicated to Professor Miroslav J. Gašić on the occasion of his 70th birthday.

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EXPERIMENTAL

Avarol was isolated from the marine sponge *Dysidea avara*, and oxidised to avarone with silver oxide.[7] Alkylthio-derivatives of avarone and its model compound 2-*tert*-butyl-1,4-benzoquinone were synthesized by nucleophilic addition of thiols to quinones.[8]

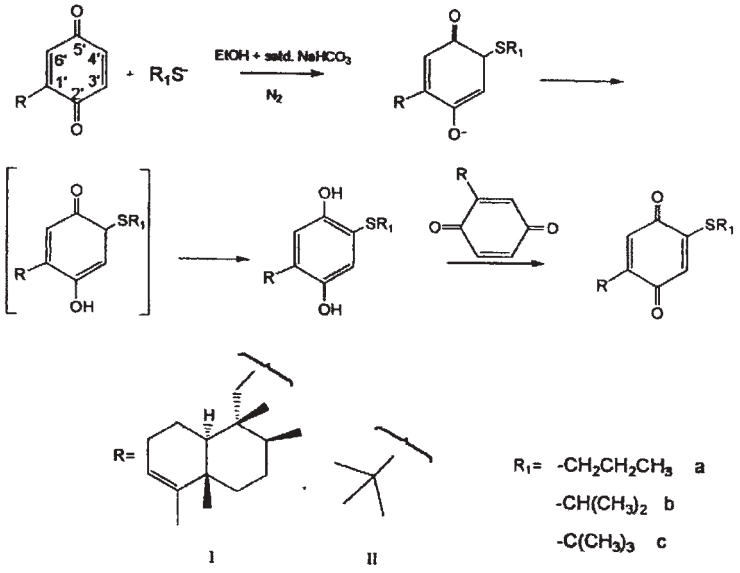
β-Lactoglobulin was isolated from cow milk by a standard procedure.[9] The obtained protein solution was concentrated by ultrafiltration and then lyophilized. From 2L of cow milk, 976 mg of pure β-lactoglobulin were obtained.

Modification of β-lactoglobulin were performed in 20 % EtOH containing 50 mM $NaHCO_3$. The concentration of the protein was 5 mg/mL and the concentration of the quinones was 2 mg/mL. The final volume was 1.2 mL. Upon modification, the protein was desalted on a Sephadex G-50 Medium column (length 6 cm and diameter 9 mm). The protein eluted in the void volume.

The chemical modification of β-lactoglobulin was assayed by UV/VIS spectrophotometry (Beckman DU-50 spectrophotometer), SDS PAGE[10] and isoelectrofocusing.[11]

RESULTS AND DISCUSSION

The alkylthio-derivatives of **I** and **II** were obtained by nucleophilic addition of thiols to the quinones (Schemes 1 and 2).[8]

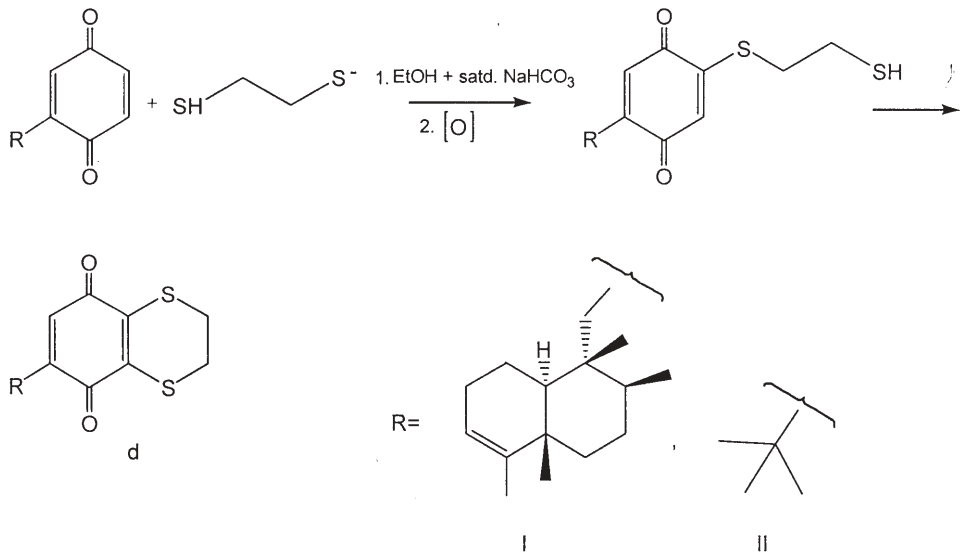


Scheme 1.

β-Lactoglobulin was modified in 20 % ethanol at a moderately basic pH. The modifications of the protein were visible immediately. The colour of the reaction mixture changed immediately after mixing the quinones with the protein.

Spectral changes were visible for all the tested compounds, except for the ethylenedithio-derivatives of the quinones (Fig. 1).

The protein modification was further investigated by SDS PAGE (Fig. 2). The derivatives 2-*tert*-butyl-5-isopropylthio-1,4-benzoquinone **IIb**, 2-*tert*-butyl-5-propylthio-1,4-benzoquinone **IIa** and 4'-*tert*-butylthioavarone **Ic** modified the protein and gave very sharp bands



Scheme 2.

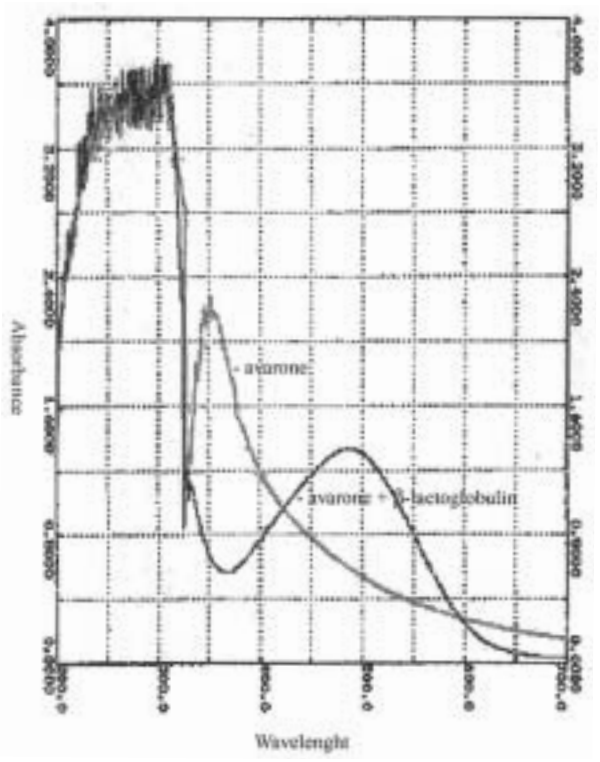


Fig. 1. UV/VIS spectra of avarone and of a mixture of β-lactoglobulin and avarone.

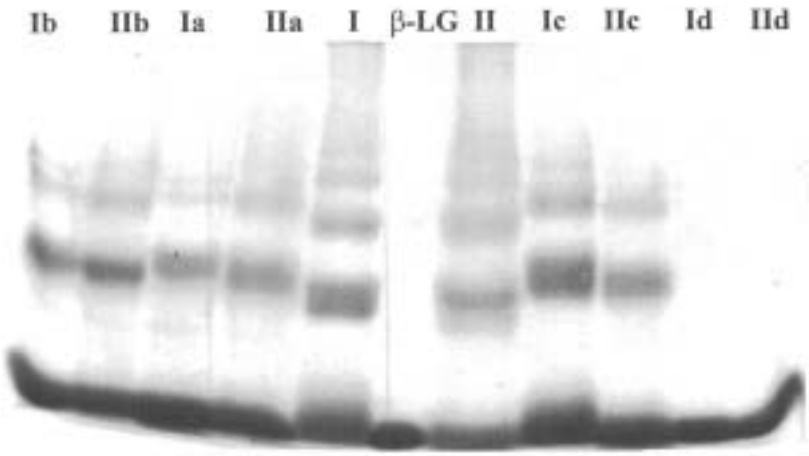


Fig. 2. SDS PAGE electrophoregram of β-lactoglobulin upon modification with the quinones.

of 36 kDa and 64 kDa. The derivatives 4'-isopropylthioavarone **Ib**, 4'-propylthioavarone **Ia** and 2-*tert*-butyl-5-*tert*-butylthio-1,4-benzoquinone **IIc** gave a sharp band of 36 kDa and a weak band of 64 kDa. The most prominent changes were observed for the unsubstituted compounds **I** and **II** (bands of 36kDa, 64 kDa, 128 kDa). The derivatives 3',4'-ethylendithioavarone **Id** and 2-*tert*-butyl-5,6-ethylendithio-1,4-benzoquinone **IId** did not modify

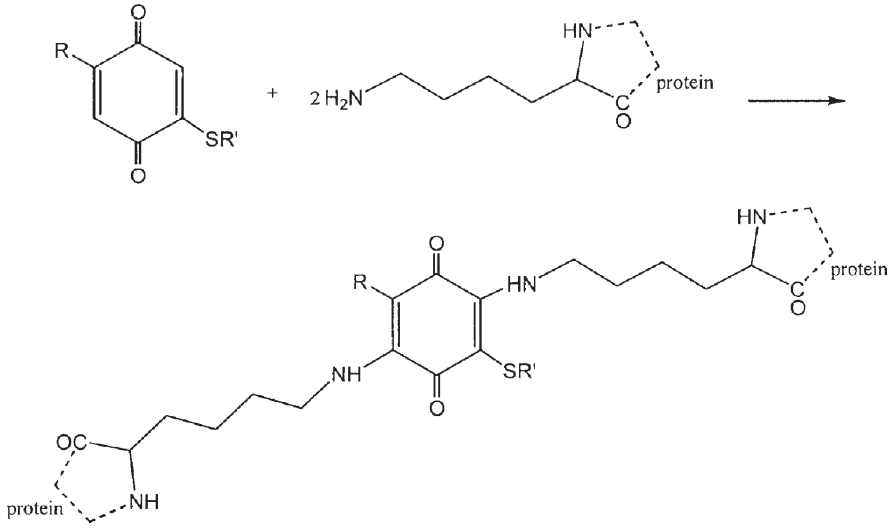


Fig. 3. Cross-linking of the protein molecules by quinones.

the protein. These results suggest that polymerisation of the protein occurs. The most likely reaction is the Michael addition of the lysine NH_2-groups to the quinone nucleus (Fig. 3).

As expected, derivatives **Id** and **IId** were not shown to modify β-lactoglobulin. With these derivatives Michael addition is possible only at the least reacrive position of the quinone nucleus adjacent to the alkyl group.

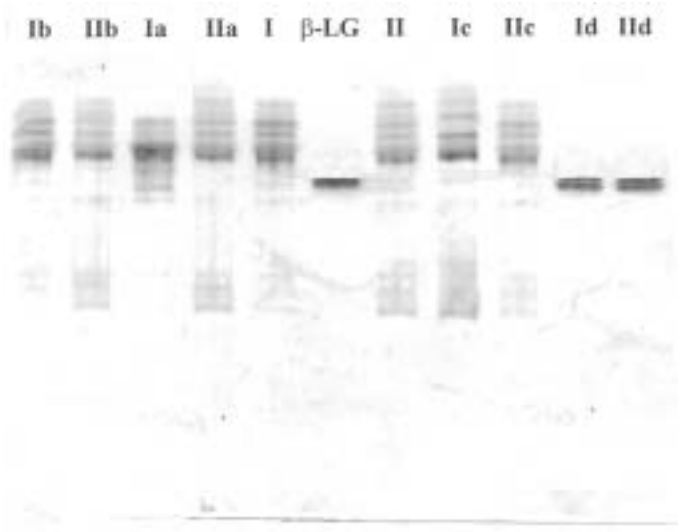


Fig. 4. Isoelectrofocusing gel.

β-Lactoglobulin contains four disulphide bonds[12] and one cysteine residue, which is not reactive because it is situated in the interior of the molecule. On the other hand, the protein contains 15 reactive ε-amino groups of lysine residues.[13] In order to ascertain whether the ε-amino groups of lysine were the target of modification, the system was studied by isoelectrofocusing (Fig. 4). The shift of the pI of the protein (pI = 5.4) upon modification toward lower values (5.1; 4.5; 4.3; 4.1; 3.9), as determined by isoelectrofocusing, indicates that the lysine amino groups of the protein are indeed the principal sites of the reaction with the quinones.

CONCLUSION

In conclusion, these results show that avarone, its derivatives and model systems are capable of covalent modification of β-lactoglobulin by reaction with the lysine amino groups. The results provide evidence that the biological activity of avarone is, at least in part, caused by its reaction with cellular nucleophiles. This conclusion is strongly supported by preliminary results of the biological activity of avarone and its derivatives, which show that derivative **Id**, which does not modify β-lactoglobulin, has a much weaker biological activity than either avarone or its monosubstituted alkylthio-derivatives.[14]

Acknowledgements. This work was partially supported by the Ministry for Science, Technolgies and Development of the Republic of Serbia (Project No. 1586).

ИЗВОД

ХЕМИЈСКЕ МОДИФИКАЦИЈЕ β-ЛАКТОГЛОБУЛИНА ХИНОНИМА

ИРЕНА НОВАКОВИЋ[1], ЗОРАН ВУЈЧИЋ[2], ТАТЈАНА БОЖИЋ[2], НАТАША БОЖИЋ[2], НЕНАД МИЛОСАВИЋ[2] и ДУШАН СЛАДИЋ[2]

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Хинонско/хидрохинонски пар аварон/аваролa и њихови деривати показују значајну антитуморску активност. У овом раду проучаване су ковалентне модификације β-лактоглобулина, изолованог из крављег млека, авароном, његовим модел-једињењем 2-*tert*-бутил-1,4-бензохиноном и њиховим алкилтио-дериватима. За испитивање модификација коришћене су UV/VIS спектрофотометрија, SDS PAGE и изоелектрофокусирање. Резултат SDS PAGE указује да се протеин полимеризује. Померање pI вредности протеина након модификације ка нижим вредностима показује да су амино групе лизина главна места реакције β-лактоглобулина са хинонима.

(Примљено 31. децембра 2002)

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