activity disappears in the liquid silk at the beginning of spinning and reappears in the cocoon[4,5]. At present, the physiological significance of this enzyme in the cocoon and the mode of its incorporation into silk proteins remain unexplained. This paper reports on the localization of the enzyme in the silk fibres and also on the presence of other disaccharidases in the cocoon of *B. mori*.

*Materials and methods.* *B. mori* (bivoltine race L×KA) larvae were reared on fresh mulberry leaves at 27 °C. Mature 5th instar larvae were allowed to spin in paper cups and whole cocoons were used as enzyme source. In another experiment the silk fibres were collected at intervals of 24 h for 3 days and were used for assaying enzyme activity.

The enzyme activity was assayed by adding 100 mg of silk fibres to 5 ml of 0.04 M buffer solution containing 2% of a sugar (sucrose, maltose, lactose, cellobiose or trehalose, obtained from Sigma Chemical Company, USA). The buffers used included phosphate buffer, pH 6.5 for invertase and pH 6.0 for trehalase and acetate buffer, pH 4.5 for cellobiase and maltase and pH 4.0 for lactase. After incubating for 1 h at 37 °C, the reaction mixture was placed in a boiling water bath for 6 min. The amount of glucose present in the supernatant was determined by 'Glucostat Special', obtained from Worthington Biochemical Corporation, New Jersey, USA. When acetate buffer was used, the reaction mixture was brought to neutral pH using 1 N NaOH before terminating the reaction by boiling.

*Results and discussion.* Enzymes capable of hydrolyzing sucrose, trehalose, cellobiose and lactose were detected in the cocoon of the silkworm *B. mori*. Sucrase activity was found to be the highest as compared to the other hydrolases, which were present only in trace amounts. The cocoon was devoid of maltose hydrolyzing enzyme (table 1). The sucrase activity present in the silk fibres that were spun on different days is shown in table 2. Enzyme activity could be detected in the silk fibres spun during the first 24 h. Further studies with the silk fibres produced on the first day revealed that the sucrase was present only in the outer layer of the cocoon.

The presence of sucrose hydrolyzing enzyme in the whole cocoon of *B. mori* and *P. ricini* has been reported[4,5]. The present studies reveal that the sucrose hydrolyzing enzyme is localized in the outermost layer of the cocoon. The silk gland of *B. mori* is divided into 3 segments. The posterior segment synthesizes fibroin and exports it to the middle segment where it is stored until the larva starts spinning. The middle segment of the silk gland synthesizes a number of digestive enzymes apart from serosin during the feeding stage. Since fibroin starts accumulating at the middle segment 2–3 days before the larva starts spinning, it is likely that at least part of the enzymes secreted during this period get entrapped in the fibroin. Fibroin has been shown to be synthesized by the mature larvae for a period of 3 days after the onset of spinning[6]. Since the middle segment ceases to secrete saccharidases at the post-feeding stage, the silk fibres formed at the latter period are devoid of enzyme activity. The absence of maltase activity in the cocoon suggests that the sucrose hydrolysing enzyme of the cocoon is not $\alpha$-glucosidase but probably a $\beta$-fructosidase.

Table 1. Disaccharide hydrolyzing enzymes of *B. mori* cocoon

| Disaccharide | μmole glucose formed/h/g cocoon |
|---|---|
| Sucrose | 80 |
| Lactose | 14 |
| Maltose | 0 |
| Cellobiose | 13 |
| Trehalose | 17 |

Table 2. Sucrase activity in silk spun on different days

| Day | μmole glucose formed/h/g cocoon |
|---|---|
| 1 | 212 |
| 2 | 0 |
| 3 | 0 |

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## The role of the cysteinyl and one of the tryptophyl residues in the neurotoxic action of suberitine

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*Summary.* The selective photooxidation of the single cysteinyl residue and one of the 3 tryptophyl residues of suberitine has been performed by irradiation using crystal violet and proflavine respectively as photosensitizers. Crystal violet and the protein form a 1:1 complex with a consequent partial inhibition of the neurotoxic activity. The latter is completely abolished by specific photooxidation of cysteine which is probably involved in the active site of the protein. The modification of the tryptophyl residue induces a large loss of the activity as a consequence of a photoinduced extensive denaturation of suberitine.

We have previously described[1] some physico-chemical properties of suberitine, the toxic principle of the marine sponge *Suberites domuncula*[2]. Amino acid analysis and spectroscopic studies showed that this protein contains a single cysteinyl residue and one largely solvent-accessible tryptophyl side chain per protein molecule.

To shed further light on the structure-function relationships in suberitine, we investigated the role performed by the aforementioned amino acid residues in conformational stability and biological activity. The specific modification of cysteine and tryptophan was achieved by the dye-sensitized photooxidation technique which, by a proper

control of the reaction conditions, leads to selective and localized chemical modifications of proteins[3].

*Materials and methods.* All reagents were commercial products of analytical grade. Urea (Merck), proflavine hemisulphate (British Drug Houses), and crystal violet (Merck) were used. Only freshly prepared solutions of these compounds were used.

The purification of suberitine and the determination of the toxic activity were performed as described previously[2].

Absorption spectra were recorded by a Perkin Elmer 576 spectrophotometer. Fluorescence emission spectra were recorded at 15 °C in the ratio mode by a Perkin Elmer MPF 4 spectrophotofluorimeter[4].

Irradiations were performed at 20 °C with a 1250 W halogen lamp (Osram). Details of the experimental arrangement have been described elsewhere[5]. In 1 set of experiments, the irradiated solution (2.1 ml) contained 0.015 mM suberitine and 0.018 mM crystal violet in 0.15 M sodium acetate, pH 7.6. A chemical filter (80% aqueous acetone) was used to eliminate wavelengths below 330 nm.

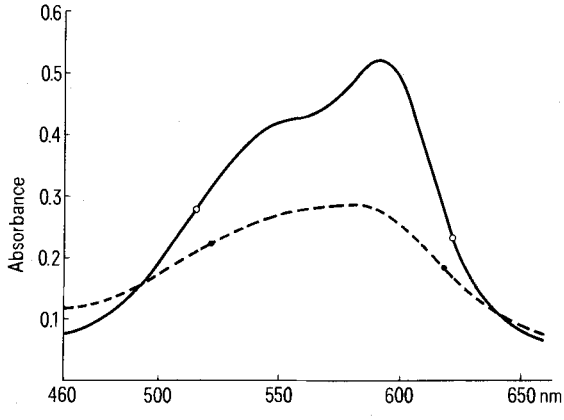


Fig. 1. Optical absorption spectrum of 0.018 mM crystal violet (———) and of 0.018 mM crystal violet added to 0.015 mM suberitine (- - - -) in 0.15 M sodium acetate solution at pH 7.6.

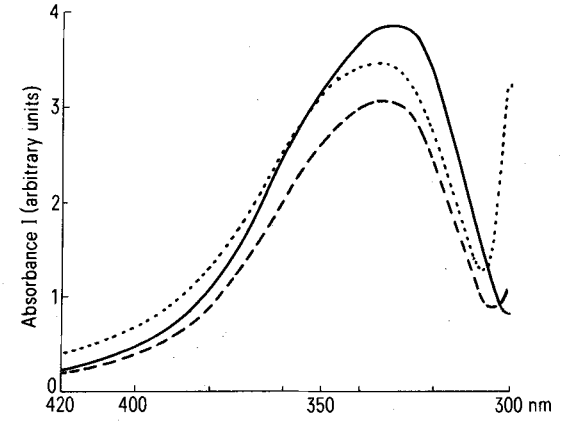


Fig. 2. Fluorescence emission spectrum of native suberitine (———), suberitine added to equimolar crystal violet (- - - -), and suberitine irradiated for 1 min in the presence of equimolar crystal violet (....). The experimental conditions were those specified for figure 1. The spectra were excited at 295 nm and were recorded at room temperature using excitation and emission slits of 7 nm and 8 nm, respectively. I is the absorbance expressed in arbitrary units.

100 µl were taken at fixed times for biological activity measurements. A 30-min-irradiated solution was treated with acetone to precipitate the protein which was subjected to the standard amino acid analysis on a LKB 4101 instrument. For conformational studies, 6 M urea was added to crystal violet-suberitine samples, either unirradiated or 1 min irradiated. As a control, an equimolar suberitine solution devoid of crystal violet was also investigated. The 295 nm-excited fluorescence emission was followed over several hours.

In another set of experiments, the irradiated solution (3 ml) contained 0.029 mM suberitine and 0.272 mM proflavine in 0.15 M sodium acetate, pH 7.6 and a cut-off filter at 430 nm (80% nitrobenzene in acetone) was used. At fixed times, 100 µl-aliquots were diluted to 2 ml with 0.15 M sodium acetate, pH 7.6 for both the bioassay and the conformational studies after the addition of 6 M urea. Unirradiated suberitine solutions, both in the presence and in the absence of proflavine, were used as controls.

*Results.* The interaction of crystal violet and suberitine. The addition of 0.015 mM suberitine to an equimolar crystal violet solution perturbs the absorption spectrum of the dye (fig. 1), suggesting the occurrence of a ground state complex between crystal violet and suberitine. This hypothesis is supported by the quenching of the protein fluorescence in the presence of crystal violet (fig. 2). Spectrophotometric titration at 590 nm indicates that the stoichiometry of the complex is 1:1. Dye binding probably occurs at the active site of suberitine, since the presence of crystal violet reduces the toxic efficiency of the protein to about 50%. Triphenylmethane derivatives inhibit other sulphydryl-containing enzymes[6].

Crystal violet-sensitized photooxidation. Crystal violet sensitizes the selective photooxidation of cysteinyl residues to cysteic acid in protein molecules[6-9].

Under our conditions, the amino acid compositions of unirradiated and 30-min-irradiated suberitine are identical. Since cysteine is destroyed during acid hydrolysis, the determination of this amino acid was performed by the 5-5′-dithio-bis (2-nitrobenzoic acid) method[10]. Unirradiated protein showed the presence of 1-SH group per protein molecule; on the contrary, 30-min-irradiated protein gives no reaction, indicating complete photoinduced modification of the single -SH group. In parallel the biological activity falls to about 10% after 1 min, and the zero after

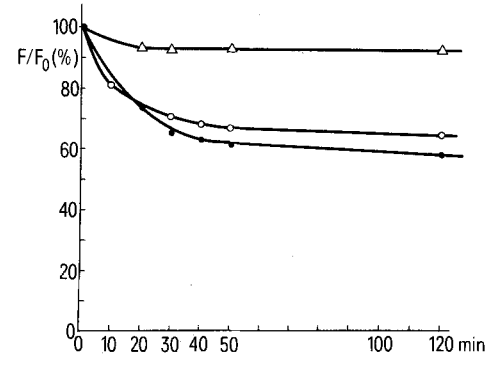


Fig. 3. Time-dependency of the decrease of the fluorescence emission intensity of suberitine upon addition of 6 M urea: ●——●, unirradiated suberitine, ○——○ unirradiated suberitine added to equimolar crystal violet, △——△ 1-min-irradiated suberitine in the presence of equimolar crystal violet. The fluorescence was excited at 295 nm and observed at 330 nm. $F_0$, fluorescence intensity at zero time; F, fluorescence intensity at time t.

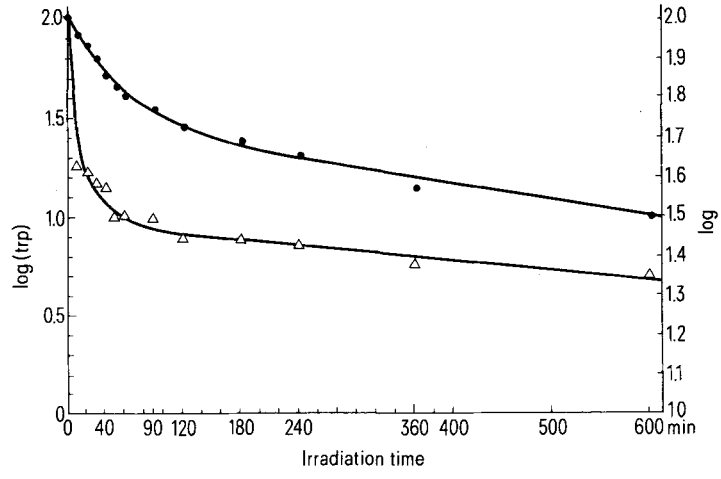


Fig. 4. Time-course of the decrease in the tryptophan content (●——●) and biological activity (△——△) of suberitine when irradiated with visible light in the presence of proflavine as the photosensitizer. All iradiations were carried out at pH 7.6 in the presence of 0.15 M sodium acetate. Data are expressed as the log of percentage of the residual tryptophan concentration and the log of the biological activity.

30 min irradiation (the data are referred to the toxic activity displayed by the unirradiated crystal violet-suberitine complex). Since UV-absorption spectrum of suberitine is unchanged after 30 min exposure to light, the aromatic residues are insensitive to crystal violet-sensitized photooxidation. Analogously, irradiation does not affect the shape of the fluorescence emission spectrum of suberitine; however, the emission intensity reverts essentially to the values typical of crystal violet-free suberitine (see fig. 1), thus indicating that after modification of the thiol group, crystal violet is no longer bound to suberitine.

Urea induces a time-dependent quenching of tryptophan emission (fig. 3) in native suberitine probably as a consequence of a shift into a polar environment of buried indole side chains[11]. Crystal violet exerts very little effect on the rate and magnitude of the denaturation process. On the other hand, only a small time-dependent quenching of the tryptophan emission is noticed for 1-min-irradiated suberitine, suggesting that the protein is unfolded after irradiation, before the addition of 6 M urea.

Proflavine-sensitized photooxidation. On proflavine-sensitized photooxidation of suberitine a semilogarithmic plot[12] of the amount of recovered tryptophan (estimated according to Genov and Jori[13]) as a function of the irradiation time (fig. 4) shows the presence of 1 fast-reacting and 2 slow-reacting tryptophyl residues. The most readily photooxidizable tryptophan performs a major role for the toxic action of suberitine, since its modification reduces the activity to about 13% (fig. 4). This preferential modification of 1 tryptophan is not due to binding of proflavine to suberitine, since the presence of the dye brings about no change in the spectroscopic and biological properties of the protein.

Suberitine samples irradiated for 20, 40 and 600 sec display about the same sensitivity to 6 M urea; after 12-h incubation, the residual fluorescence intensity is about 90% that of the original one suggesting that suberitine is extensively denatured after the modification of 1 tryptophyl residue.

*Discussion.* Our results imply that the single cysteinyl and one of the 3 tryptophyl residues of suberitine are essential for the biological function and for the conformational stability of the protein.

Probably, the cysteine residue is located within the suberitine active site since it undergoes a specific photooxidation sensitized by active site-bound crystal violet; photosensitized oxidation of dye-protein complexes is usually restricted[14] within a narrow spatial range surrounding the bound photosensitizer. However, it is not clear from our data whether the loss of toxic activity of crystal violet-irradiated suberitine is a consequence of the chemical modification of the thiol moiety per se or of the consequent extensive alteration of protein conformation, as suggested by the comparative studies concerning the effect of urea on native and photooxidized suberitine.

Similar conclusions can be drawn concerning the role of the fast-reacting tryptophyl residue. The photomodification of this amino acid, which involves a disruption of the pyrrole portion of the indole ring[15], greatly depresses, but does not completely eliminate, the biological activity of suberitine. Further photooxidation of the 2 less accessible tryptophyl side chains brings about only a slight further decrease of the residual activity. Therefore, it is likely that the reduced toxicity of suberitine modified at the single tryptophan is a consequence of the concomitant alteration of the native 3-dimensional organization of the protein. This is clearly demonstrated by the urea-induced denaturation studies on unirradiated and photooxidized suberitine.

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