

Effect of estradiol-17 β on cell area, lumen area and trehalase activity of posterior silk gland of *Bombyx mori* L.

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Estradiol-17 β (E₂) at the dose of 1 μ g/g caused an increase in cell area, lumen area and the total (cell + lumen) area of posterior silk gland (PSG) in *Bombyx mori* indicating that exogenously applied estradiol-17 β has a regulatory influence on silk gland activity. A dose-dependent variation in trehalase activity of PSG was found on the 5th day after topical administration of estradiol on 1st and 2nd day of the fifth larval instar. Of all the doses of E₂ used, 1 μ g/g dose had maximum stimulatory effect on trehalase activity. Co-administration of each of a specific receptor antagonist for estradiol, the ICI-182780 and a protein biosynthetic blocker, cycloheximide with E₂ suppressed the E₂-induced increase in silk gland activity. The results suggest some specific metabolic action of E₂ on silk gland and offer a promising way for future investigations regarding the physiological significance of vertebrate steroids in insects.

The endocrine system in insect functions as an important link between the environment and various physiological and developmental events. The insect hormones can be categorised into two main classes: (1) the true hormones, viz., the juvenile hormone and ecdysteroids, produced in epithelial hormonal glands, and (2) the neuropeptides, synthesised in neurosecretory cells¹. However the discovery of vertebrate peptide and steroid hormones in various insect species including *Bombyx mori*, and the evidence for its possible involvement in their physiology indicate that there exist a varied degree of complexity in insect endocrine system²⁻¹⁵. In the present study an attempt has been made to understand whether estradiol-17 β (E₂), a vertebrate female sex steroid, exerts any effect on cell area, lumen area and the trehalase activity of posterior silk gland (PSG), destined for the synthesis of silk fibroin in *B. mori*. In insects, the enzyme trehalase is metabolically very important, because their energy needs are in large part supported by glucose generated by this enzyme activity on haemolymph trehalose¹⁶. It is distributed at various levels of activity in practically every tissues of insect and among them the midgut and flight muscles show a considerable level of activity¹⁶. The fluctuation of

trehalose content and trehalase activity accompanying the growth of middle and posterior silk glands of the silkworm and its regulation by KK-42 has been studied¹⁷. Thus the alterations in the trehalase activity of the posterior silk gland may be used as a good index to judge the role of estradiol-17 β on the growth and development of silk gland. Besides, the changes in the cell area, lumen area and the total (cell + lumen) area of PSG have been determined for studying the estradiol action on silk gland. The specific antiestrogenic compound, ICI-182780 and a protein synthesis blocker, cycloheximide have been used alone or simultaneously with estradiol-17 β to demonstrate the counteraction of estradiol – induced changes in the silk gland activity by these compounds. ICI-182780 has been found to be a specific inhibitor of estrogen action and shows excellent growth inhibitory effects in both cell and animal models of human breast cancer¹⁸. The absence of agonistic activity of ICI has made this steroidal antiestrogen superior to tamoxifen in suppressing tumour cell growth¹⁹. Thus, in the present study, ICI-182780 has been used to understand the specificity of estradiol action on silk gland of *B. mori*.

Materials and Methods

Animals—Fifth instar larvae of *Bombyx mori* L., strain Nistari (an indigenous multivoltine strain) were used. The larvae were fed exclusively on fresh

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mulberry leaves and reared in a 12:12 hr L:D cycle at 25 $\pm$ 1 $^{\circ}$ C with 70-90% RH.

Treatment of animals—Freshly ecdysed fifth instar larvae of *Bombyx mori* L. of about same body weight (0.389 ± 0.018 g) were separated from the stock rearing tray and were divided at random in different groups for experiments. The estradiol-17 β (Sigma Chemical Co., USA) was dissolved in acetone at a stock concentration of 1 mg/ml. Different doses of estradiol-17 β (E₂) (0.10, 0.25, 0.50, 1, 2, 4 μ g/g body weight, or otherwise as mentioned in the text) were applied topically in 5 μ l of acetone with the help of Hamilton syringe over the dorsal surface along the dorsal vessel on 1st and 2nd day of the fifth instar larvae. ICI-182780 (a gift from Dr. A E Wakeling, Zeneca Pharmaceuticals, UK), a specific and potent antiestrogenic compound, at the dose of 1 μ g/g, and cycloheximide (Sigma Chemical Co., USA), at the dose of 0.5 μ g/g, were applied topically in 5 μ l of acetone alone or simultaneously with estradiol 17 β . The control larvae were treated with equal volume of vehicle (5 μ l of acetone) only.

Measurement of cell area, lumen area and total (cell + lumen) area of posterior silk gland—The thin sections (5-7 μ m) of the posterior silk glands (PSGs) of 5th day of fifth larval instar were made and stained with haematoxylin and eosin adopting the usual procedures. The cell area, lumen area and the total (cell + lumen) area of PSG of the control and treated (1 μ g/g E₂) larvae were measured from the histological slides with the help of a camera lucida. The images were projected over a 1 mm² graph paper and the areas were thus calculated. The sections were viewed through microscope at a magnification of 150.

Determination of trehalase activity—The trehalase (EC 3.2.1.28, 1, α -D-glucopyranosyl- α -D-glucopyranoside 1-gluco-hydrolase) activity was measured by monitoring the release of glucose from the trehalose substrate according to the method of Dahlqvist²⁰ with some modifications. The liberated glucose was measured by o-toluidine method of Stroev and Makarova²¹.

The enzyme activity was measured from the mitochondrial, cytosolic and microsomal fractions of the posterior part of the silk glands (PSGs), taken from the 5th day of the fifth larval instar. The procedure of homogenisation and subcellular fractionations of PSG were done as described by Keshan and Ray²². For measuring the enzyme activity, 250 μ l of enzyme fraction (50-100 μ g

protein) and 85 μ l of 98 mM trehalose (final concentration is 25 mM trehalose) in 25 mM GP buffer [25 mM phosphate buffer, pH 6, containing 5% (v/v) glycerol], pH 6 were incubated for 1 hr at 37 $^{\circ}$ C with vigorous shaking. The reaction was then stopped by adding 165 μ l of 30% TCA (final concentration is 10% TCA) in cold. It was then centrifuged and from the supernatant the liberated glucose was measured by o-toluidine method. The absorbance was then measured at 620 nm and the enzyme activity was expressed as nmoles of glucose released/min/mg protein.

Determination of protein—Protein content was determined by the method of Lowry *et al.*²³ using bovine serum albumin (Sigma Chemical Company, USA) as standard.

Statistical analysis—Student's 't'-test was performed for analysis of data, and $P < 0.05$ was considered as significant.

Results

Effect of estradiol-17 β on cell area, lumen area and total (cell + lumen) area of posterior silk gland—Sections of posterior silk gland (PSG) from the control and E₂-treated groups were subjected to camera lucida drawing over 1mm² graph paper for calculation of cell area, lumen area and the total (cell + lumen) area. The control silkworm showed a cell area of 2059.0 ± 46.24 sq.mm, a lumen area of 795.14 ± 25.79 sq.mm. and a total (cell + lumen) area of 2854.16 ± 68.16 sq.mm. Sections of PSG of E₂-treated silkworms showed a rise in the cell area, lumen area and the total (cell + lumen) area by 38% (2850.0 ± 102.19 sq.mm., $P < 0.001$), 62% (1290.60 ± 135.22 sq.mm., $P < 0.01$), and 42% (4040.60 ± 223.18 sq.mm., $P < 0.001$) over the respective control values (Table 1 and Fig.1).

Effect of estradiol-17 β on trehalase activity of posterior silk gland—Trehalase catalyses a specific irreversible reaction for conversion of trehalose to glucose for further metabolism. The trehalase activity of posterior silk gland (PSG), when expressed as the specific activity, was found to be highest in the mitochondrial fraction ($P < 0.001$) in comparison to that in respective microsomal and cytosolic fractions of the silk gland cells (Table 2). Therefore, for successive studies the trehalase activity of the mitochondrial fraction of the PSG cells was taken into account.

Of all the doses of estradiol-17 β (E₂, 0.1, 0.25, 0.5, 1, 2 and 4 μ g/g) examined, the doses up to 1 μ g/g caused a significant increase ($P < 0.05$ -0.001) in the trehalase activity of PSG in comparison to the control value (Fig. 2). Higher dose of E₂ (4 μ g/g) showed a significant decrease ($P < 0.02$) in the enzyme activity in comparison to control. E₂ at 2 μ g/g dose could not

alter the enzyme activity in comparison to control. A dose-response effect was observed between 0.1 and 0.5 (rise, $P < 0.01$), 1 and 2 (fall, $P < 0.001$), and 2 and 4 (further fall, $P < 0.02$) μ g/g dose of E₂ (Fig. 2).

*Effect of ICI-182780 and cycloheximide on estradiol-17 β -induced activity of posterior silk gland—*ICI-182780 (1 μ g/g) and cycloheximide (0.5 μ g/g)

Table 1—Effect of estradiol-17 β (E₂), ICI-182780 (ICI) and E₂ + ICI* on the cell area, lumen area and total (cell + lumen) area of posterior silk gland on 5th day of the fifth larval instar of *B. mori*.

[Values expressed as sq. mm. are mean $\pm$ SE from 10-15 randomly chosen sections of different silk glands.]

	Cell area	Lumen area	Total (cell + lumen) area
Control	2059.0 $\pm$ 46.24	795.14 $\pm$ 25.79	2854.16 $\pm$ 68.16
E ₂ (1 μ g/g)	2850.0 $\pm$ 102.19 ^a	1290.60 $\pm$ 135.22 ^b	4040.60 $\pm$ 223.18 ^a
ICI (1 μ g/g)	2143.0 $\pm$ 137.46 ^{NS}	816.75 $\pm$ 113.69 ^{NS}	2959.75 $\pm$ 239.25 ^{NS}
E ₂ (1 μ g/g) + ICI (1 μ g/g)	2163.8 $\pm$ 59.14 ^{NS}	869.80 $\pm$ 53.12 ^{NS}	3033.6 $\pm$ 109.73 ^{NS}

*The treatments were made on 1st and 2nd day of fifth instar larvae.

P values: ^a<0.001; ^b< 0.01; NS: non-significant

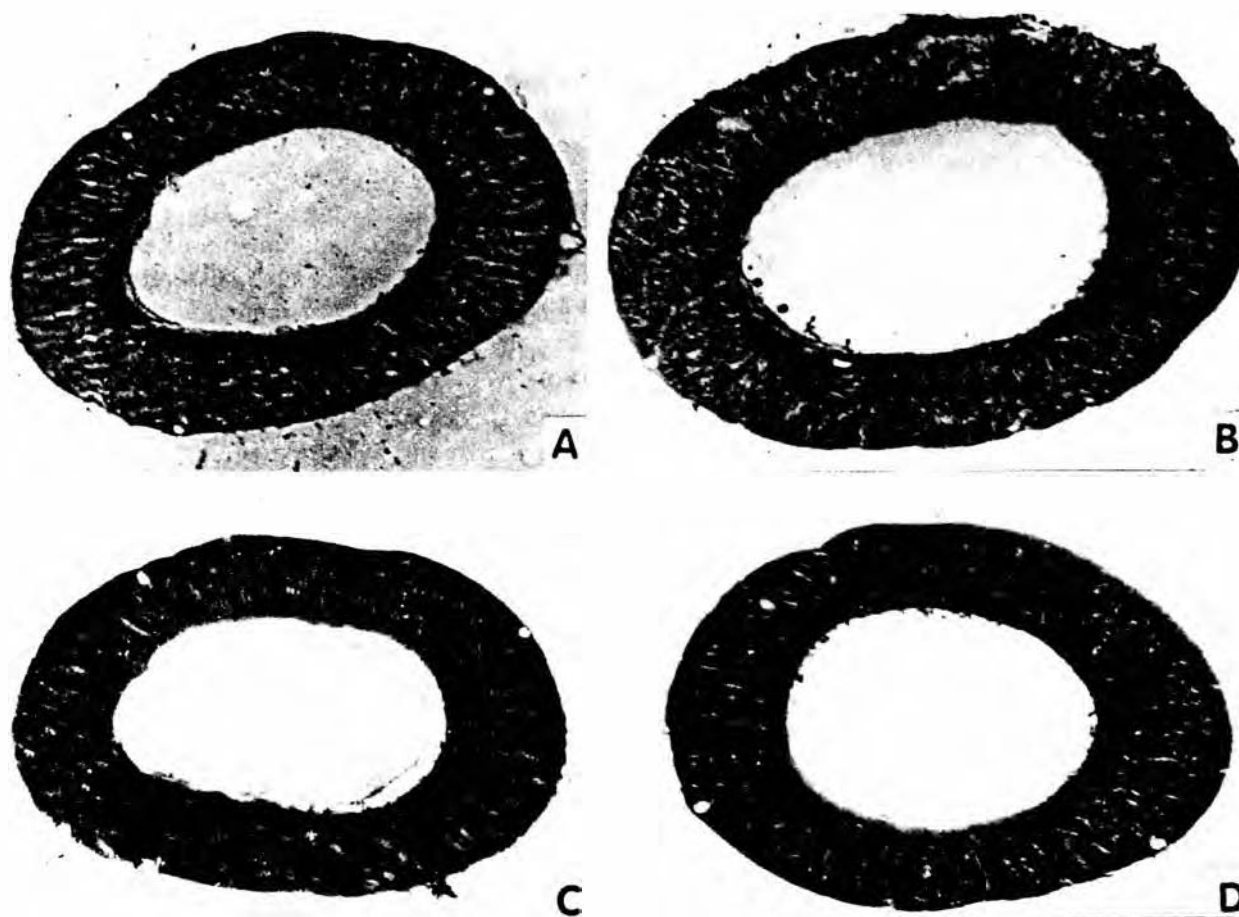


Fig. 1—Microphotographs of sections of posterior silk gland of control (A), estradiol-17 β (E₂)-treated (B), ICI-182780 (ICI)-treated (C) and (E₂ + ICI)-treated (D) larvae of 5th day of the fifth instar larvae of *B. mori*. 150 X.

were used alone or simultaneously with estradiol-17 β (E₂, 1 μ g/g body weight) for studying the nature of action of estradiol-17 β on silk gland. ICI-182780 or cycloheximide did not inhibit the silk gland activity when it was treated alone, but it suppressed the E₂-induced silk gland activity when it was treated simultaneously with E₂. ICI-182780 counteracted ($P < 0.001$) the E₂-induced increase in trehalase activity (Fig. 3), and the cell area, lumen area and total (cell + lumen) area of PSG (Table 1 and Fig. 1) when used simultaneously with E₂. An almost total suppression of the E₂-induced rise in trehalase activity was also found ($P < 0.001$, Fig. 3) by the treatment of cycloheximide along with E₂.

Discussion

The present results has clearly demonstrated that estradiol-17 β has a specific action on trehalase activity and the cellular and lumen area of posterior silk gland. In the present study the acetone has been used as a vehicle for the topical application of the hormone because it seems to be the most suitable solvent, as it has a high penetrating capacity and

causes little damage to the insect's body²⁴. In *B. mori*, topical mode of the hormone treatment is the most widely used method and has been successfully demonstrated for observing the effects of insect hormones on silk gland function²⁵⁻²⁹.

Table 2—Trehalase activity in subcellular fractions of posterior silk gland (PSG) on 5th day of the fifth instar larvae of *Bombyx mori*.

[Values are mean $\pm$ SE of 10-15 pooled determinations of the enzyme activity in 3 different sets of experiments. Each pool consists of PSGs from three silkworms.]

Fractions of PSG	Trehalase activity (nmoles glucose/min/mg)
(A) Mitochondrial	1.265 $\pm$ 0.075
(B) Cytosolic	0.538 $\pm$ 0.060
(C) Microsomal	0.580 $\pm$ 0.034

't'-test probability differences, P -values; NS=non-significant

$P < 0.001$	NS
A-B	B-C
A-C	

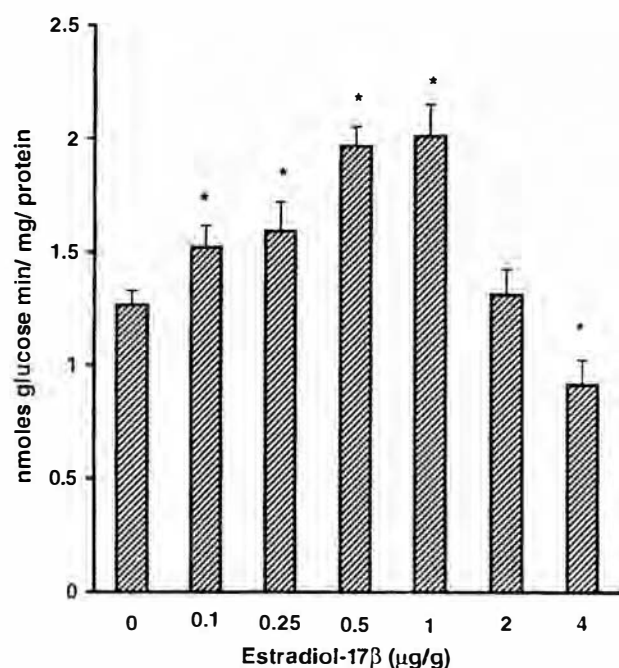


Fig. 2—Effect of estradiol-17 β (E₂) on trehalase activity of posterior silk gland on 5th day of the fifth instar larvae of *B. mori*. Data represent mean $\pm$ SE of 10-15 pooled determinations of the enzyme activity in 3 different sets of experiments. Each pool consists of glands from three silkworms. The vertical bars on the columns denote standard error. Asterisks denote significant differences ($P < 0.05 - < 0.001$) in comparison to the control value.

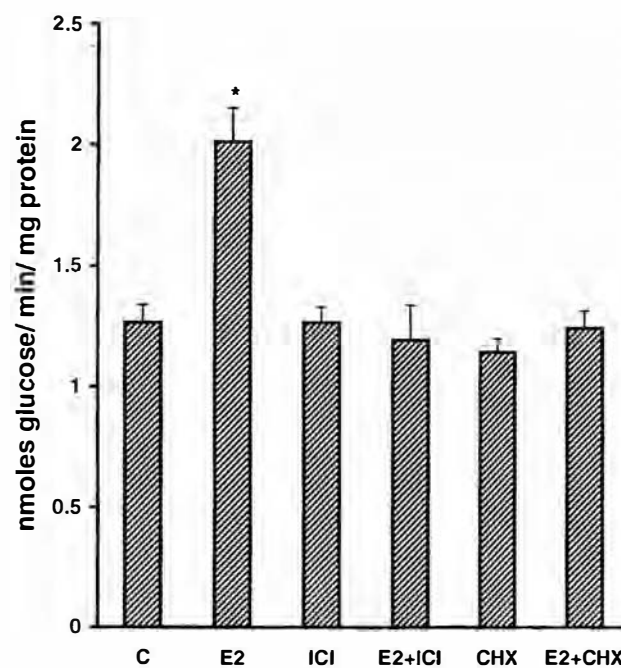


Fig. 3—Effect of estradiol-17 β (E₂), ICI-182780 (ICI), E₂ + ICI, cycloheximide (CHX) and E₂ + CHX on trehalase activity of posterior silk gland on 5th day of the fifth instar larvae of *B. mori*. Data represent mean $\pm$ SE of 10-15 pooled determinations of the enzyme activity in 3 different sets of experiments. Each pool consists of glands from three silkworms. The vertical bars on the columns denote standard error. Asterisk denotes significant differences ($P < 0.001$) in comparison to the control value.

The effect of administration of the exogenous hormone in the insect's body, particularly in *B. mori*, is very much dependent on the time of hormone treatment, and on the developmental status of the experimental animals. In the present experiment, the E₂ treatment was made on the 1st and 2nd day of the fifth larval instar keeping in view that the sensitivity of the silk gland to the exogenous hormone remains higher during the preparatory phase of the silk gland³⁰. This period of treatment also coincided with the phase of DNA synthesis and the beginning of RNA and protein accumulation in silk gland³¹. Keshan³² demonstrated that the highest response of silk gland to E₂ treatment was found on the 5th day, which was reflected on wet weight of total silk gland, a very good index to judge the growth and cellular activity of silk gland. Thus in the present study the 5th day of fifth larval instar was chosen to demonstrate the effect of estradiol-17 β on silk gland of *B. mori*.

The estradiol-17 β at 1 μ g/g dose caused an increase of cell area, lumen area, and total (cell + lumen) area of posterior silk gland. This indicates that the exogenously applied estradiol-17 β (1 μ g/g) has a regulatory influence on silk gland activity. It appears that the lumen area and cellular area of the PSG increases to accommodate increased silk protein content in the lumen. Keshan and Ray³³ found that estradiol at 1 μ g/g dose resulted in a significant enhancement in cocoon shell weight, silk filament length per cocoon, and the reelability of the cocoons: 7.7% ($P < 0.001$), 19.6% ($P < 0.02$), and 11% ($P < 0.02$) respectively. By contrast, the denier per unit silk filament was decreased by 9% ($P < 0.01$) in the E₂ treated group³³. This measurement indicated a finer silk fibre. Thus in the present study the increased cellular and lumen area of PSG after E₂ administration indicates stimulated secretory activities of the silk gland along with its increased growth.

In the present study, the highest activity of trehalase was observed in mitochondrial fraction of PSG. The mitochondrial nature of trehalase in flight muscle of higher Diptera and Hymenoptera is well known^{34,35} and this enzyme has been suggested as an important flux regulating enzyme³⁶. As the mitochondrial fraction showed the highest activity of trehalase, in our successive experiments the enzyme activity was studied using the mitochondrial fraction only, to show the nature of E₂-responsiveness.

The increase in trehalase activity after the treatment of estradiol-17 β at lower dose, indicated an

increased trehalose utilisation by the PSG cells. Yaginuma and Happ³⁷ reported that there occurs a dose-dependent increase in trehalase activity in BAGs (bean shaped accessory reproductive glands) of mealworm beetle (*Tenebrio molitor*) when BAGs from 0- and 2-day pupae were exposed to 20-HE. Bombyxin caused a reduction in concentration of the major haemolymph sugar, the trehalose, by elevating the activity of trehalase in the midgut and muscle, and thus induced hypotrehalosemia³⁸. Thus it seems that in the present experiment the E₂-induced increase in trehalase activity is responsible for regulation of trehalose accumulation and its utilisation by PSG cells and serves as an indispensable substrate for energy production and biosynthesis of macromolecules. The regulation of trehalose accumulation in the silk gland by modulating the trehalase activity, after the treatment of methoprene, has been reported³⁹. The importance of the trehalase enzyme in the life of *Bombyx* larvae is evident from the fact that the treatment of a trehalase inhibitor, validoxylamine A (VAA), at early stage of *Bombyx* larvae causes a reduction in overall activities of larvae, possibly by preventing the utilisation of the energy source, whereas the treatment at later stage of last instar gives a lethal effect⁴⁰. Thus, it may be presumed that the enhanced trehalase activity in PSG after the treatment of estradiol-17 β is responsible for maintaining the E₂-induced increase in physiological activities of the silk gland by making the glucose available to them for increased energy production and/or its utilisation in the intermediary metabolic pathway.

The biphasic nature of action of estradiol-17 β on silk gland trehalase activity may be due to graded activation of the hormone responsive mechanism operating within the silk gland as a function of availability of the hormone depending upon doses of E₂ administered. A relatively less increase, unchanged level or a decrease in the silk gland activity at higher doses of the estradiol-17 β , in comparison to the control, showed relatively less intensity of the hormonal action. This could be due to the adaptation of the hormone responsive mechanism in the form of receptor loss, desensitisation, etc. at higher doses of hormone administered. From studies in vertebrates, it has been found that the excess hormone can lead to desensitisation of the target cell, both at the level of hormone binding to receptor and at post-receptor steps⁴¹. Thummel⁴² proposed a simple model to explain the temporal pattern of expression of genes

arose from ecdysone-dose-response studies using cultured larval organs. According to him, the BR-C (broad complex), EcR and E74B promoters are most sensitive to ecdysone, responding at a critical threshold concentration of about 2×10^{-9} M. In contrast, approximately 10-fold higher hormone concentration are required to repress EcR and E74B and induce E74A transcription. This model emphasizes that hormone pulses of different concentration can have different regulatory consequences. Thus the dose-dependent variation in silk gland activity after the treatment of estradiol-17 β indicates possible receptor-mediated hormonal action of E₂ associated with physiological responses. The inhibition of silk gland activity by high dose of E₂ may also be due to the induction of catabolic pathway, which led to estradiol inactivation. In *Manduca sexta*, the administration of high level of 20-HE or its agonist caused insect to respond by switching on an enzyme of ecdysteroid inactivation⁴³.

The inhibition of this E₂-induced increase in trehalase activity by cycloheximide indicated that there occurred an increase in the synthesis of enzyme protein following estradiol treatment, suggesting that E₂ action on silk gland occurred primarily at the nuclear level. The counteraction of E₂-induced increase in trehalase activity and the cell and lumen area of PSG by ICI-182780 suggested that E₂ action may be mediated by its putative receptor on silk gland. Wakeling *et al.*¹⁸ observed that the relative binding affinity of ICI-182780 for rat uterine estrogens receptor is 0.89, compared to 1 of estradiol. Although some controversies exist concerning the mechanism of action of ICI-182780⁴⁴, there is universal agreement that it functions by competitive binding to the nuclear estrogen receptor and inhibits the activation by the estrogen response element⁴⁵. The antiestrogenicity of ICI compound may also be due to a loss of receptor protein either by inhibition of its synthesis and/or its increased degradation^{46,47}. Whatever may be the mechanism of antiestrogenicity of ICI compound, it is clear that it is a highly potent and specific inhibitor of estrogen action. This, in turn, strengthened the present data to interpret that the estradiol action on silk gland may be mediated through its putative receptor and that its action as observed was specific. Thus, the present results clearly indicate that estradiol alters specifically the trehalase activity, and the cellular and lumen area of the posterior silk gland of *B. mori*.

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