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Review

Induction, development and regulation of trimolters: Great progress in the domesticated silkworm (*Bombyx mori* L.)

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As the only truly agricultural and great economic domesticated insect, the silkworm is not only value as a model for genetics and rmolecular biology research, but it also has great value for new silk materials. Induction and regulation of trimolter induced by anti-juvenile hormone have shown vast potential to be significant patterns in silkworm research, especially after the further studies on the regulation mechanism of trimolters. This paper reviews the great progress of the induction technique of trimolter silkworms, particularly focusing on its cocoon quality, cocoon filament and the production of advanced silk materials. This review also presents the control technology of fine filament size and the regulatory mechanism of trimolters during the developmental stages.

Key words: Silkworm, trimolter, chemical induction, development, filament control, regulatory mechanism.

INTRODUCTION

The mulberry silkworm, *Bombyx mori* L., an important agricultural economic insect, has been extensively treated as the traditional domesticated animal to produce silk for more than 5000 years in lots of countries, such as China, India and Thailand (Xiang, 1995). It is also one of the best model systems, through its known developmental and physiological characters, for lepidopteron biological studies for decades in the area of genetics, genomics and proteomics (Zhou et al., 2008). Silkworm has also been served as a significant bioreactor to produce recombinant proteins (Tamura et al., 2000; Tomita et al., 2003). Additionally, the commercial value and scientific significance of the trimolter silkworms induced by anti-juvenile hormone have made it the subject of intensive studies to yield advanced silk materials since the 20th century. Furthermore, the fields of induction and regulation of trimolter cocoon have been rapidly developed, with particular progress being made after the important discovery for the production of fine filament silk. This review will concentrate on the main and attractive progresses of chemical induction, development, filament control and regulatory mechanism in the area of induced trimolter silkworms and silk-reeling industry.

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Abbreviations: PTTH, Prothoracicotropic hormone; **JH,** juvenile hormone.

CHEMICAL INDUCTION OF TRIMOLTERS

Relations of three biological hormones

It is well known that domestic strains of silkworms are excellent test animals for studying insect endocrinology because of their easy rearing, handling and genetic homogeneity. The growth, development and metamorphosis of insect (silkworm) are controlled by prothoracicotropic hormone (PTTH) produced from secretion cell of brain nerve, ecdysteroids produced from prothoracic gland and juvenile hormone (JH) produced from corpus allatum (CA) (Xiang et al., 2005). PTTH acts as stimulation to prothoracic gland to secrete ecdysteroids, while JH is another hormone which is secreted by corpus allatum and transported into hemolymph lymph. PTTH and JH control the growth, development and metamorphosis of insect (silkworm) directly (Xu and Xu, 2001). Juvenile hormone analogue (JHA) is suggested to induce larval ecdysis through activation of the brain and maintenance of the responsiveness of the prothoracic gland (PG) to PTTH (Mitsuoka et al., 2001). Three synthetic compounds, dl-C18-Cecropia JH and its two isomeric analogs induced a larval-larval molt when injected or topically applied to allatectomized 4th instar silkworms (Ohtaki et al., 1971).

Functions of juvenile hormone

Research and application of Juvenile hormone (JH) in silkworms have attracted many scientists and farmers over the past decades because synchronization of larval stages in silkworms is rather easier than in other insects. JH is a type of compound with the chemical structure of half terpene and half alkene, and its biosynthesis has different pathways intrinsic to different insects (Halarnkar and Schooley, 1990; Kethidi et al., 2006). JH has several biological functions, such as keeping the shape and properties of larvae, promoting gonadial maturity and imaginal diapause and production of insect pheromone (Wyatt and Davey, 1996; Wilson, 2004). The moultinism and voltinism of silkworm were closely related to JH titer in its hemolymph (Kamimura and Kiuchi, 1998; Kamimura et al., 2003). And JH titer in hemolymph of silkworm was maintained and balanced together by means of biosynthesis and metabolism (Gilbert et al., 2000; Li et al., 2003, 2004).

Anti-juvenile hormones and trimolters

Anti-juvenile hormones (JHs) and their analogues, a group of physiological active substances belong to imidazole compounds, which have significant action to induce insect precocious metamorphosis. The discovery of insect anti-JHs in plants by Bowers et al. (1976) stimulated the chemical approaches in the field of anti-JH research and several anti-JHs have been discovered, although many practical chemicals for pest control have not yet been developed. In sericulture and silk research fields, these compounds are thus considered more useful to control silkworm moultinism because the action of precocious metamorphosis induced by anti-JHs can be expected to produce advanced or novel silk materials. Due to the important and attractive actions of these imidazoles, many anti-JHs, such as SM-1, Jinlu trimolter inducer, and KK-42, KK-22, Kang-20 and YA_{20}, SSP-11 and SD-III, and their biochemical functions have been studied and applied to induce trimolter silkworms in recent 30 years.

Lu and Li (1987) firstly introduced and successfully applied compound SM-1 which had the function of anti-JH to induce the tetramolter silkworms into trimolters. In addition, based on this experimental study and the needs of producing new silk materials in the fields of sericulture and silk-reeling industry, Lu (1993) reported that they found another new anti-JH compound named Jinlu trimolter inducer, which was also used successfully to produce fine filament cocoon silk, and the induction ratio of trimolters was 100% under the suitable concentration. Further, a lot of anti-JHs were demonstrated to be effective in the induction of precocious metamorphosis in silkworms, some of the main approaches involved in exploring the trimolter inducers are listed as follows.

KK-42

When an imidazole derivative compound KK-42 (1-benzyl-5-[(E)2,6-dimethyl-1,5-heptadienyl]imidazole) was applied to the 3rd instar larvae of the silkworm, 100% underwent precocious metamorphosis at the end of the 4th instar, and then, the researcher, Kuwano (1985), concluded that changes in JH levels during the 3rd larval instar could modify the secretion pattern of prothoracic glands and that during the next larval instar, very low ecdysteroid levels during the early stages of the presumptive last (4th) larval instar were directly related to precocious metamorphosis. Likewise, KK-42 was considered to be a type of anti-JH because the precocious effect induced by KK-42 could be all counteracted by juvenile hormone analogue (Gu and Chow, 1997).

KK-22

Imidazole compound KK-22 (1-Citronellyl-5-phenyl imidazole) was applied to the diets of 4th instar silkworm larvae, and its anti-JH activity was determined by the induction ratio of precocious metamorphosis. Larvae were more sensitive at the earlier stages of 4th instar to KK-22 and there were no significant differences in induction of precocious pupation among the administration methods, topical application, oral ingestion and feeding on diet. However, anti-JH activity of KK-22 vanished when methoprene, a JH analogue, was applied immediately after KK-22 treatment (Asano et al., 1986). Furthermore, successive application with KK-22 prevented the 3rd instar larvae from molting into the 4th instar, most 3rd instar larvae in feeding periods prolonged two days or more and had 70% or more increased body weight at 3rd molting time experienced precocious pupation at the subsequent 4th instar (Asano et al., 1986). Additionally, the activity of KK-22 to the 3rd silkworm was investigated by feeding treatment with artificial diet for successive days of the 3rd instar period. Drastic precocious metamorphosis was induced in response to the doses of KK-22, 100% at 50 to 200 ppm, 95% at 25 ppm, and 45% at 12.5 ppm in the diet (Asano, 1984).

Kang-20

Earlier researchers found that imidazole compounds had different physiological activities under the different conditions. Yamashita (1987) confirmed that the secretion of ecdysteroids of 4th instar of silkworm could be obviously restrained and the silkworms would undergo precocious metamorphosis if they were treated with anti-JH Kang-20 at the early stage of the 4th instar. Furthermore, Lin et al. (1991) reported that trimolter silkworms were efficiently induced from tetramolters by feeding Kang-20 to the 4th instar larvae and could spin smaller cocoons. However, silkworms could not spin cocoons at the current instar when they were treated with Kang-20 at the 3rd instar (Zhuang, 1988). The results indicated that trimolters might need a lowest critical period of development to be precocious metamorphosis, and Kang-20 had the same physiological action as JHA under the special condition (Lin et al., 1991).

YA_{20}

Zhuang et al. (1992) investigated the effect of trimolters induced by anti-JH, YA_{20}. There was low trimolter efficiency with YA_{20} in the 1^{st} instar. Subsequent investigation showed that, in the 2^{nd}～4^{th} instars, YA_{20} could induce the trimolter high efficiently (100%) in anytime of the 24th hour ago for 48 hours (300ppm).

SSP-11

Kiuchi et al. (1985) analyzed the induction effect of trimolters induced by imidazole compound triflumizole, SSP-11 (E-4-chloro-a, a, a-trifluoro-N-[(C_1H-imidazole-1-yl)-2-propryethy-lidene]-O-toluidine), the results indicated that the whole instars shortened for 2.5 to 4 days when the silkworms were treated at the 3rd and 4th instars.

SD-III

Tan et al. (1992) reported the biological activity of anti-JH SD-III, it is a juvenile hormone antagonist which exerted strong anti-JH and anti-ecdysteroid actions on silkworms, and 95% of trimolter silkworms were induced with the application of 300 ppm SD- III by body surface spraying. The results suggested that the most appropriate time for application was 24 h after the post-molt feeding of the 3rd instar.

DEVELOPMENT OF TRIMOLTERIZATION

Physical properties of trimolter cocoon

Currently, almost all trimolter silkworm inducers, including SM-1, Jinlu trimolter inducer, KK-42, KK-22, Kang-20, YA_{20}, SSP-11 and SD-Ⅲ, have been involved in the innovation projects of silk materials. Moreover, these projects have involved nearly all physical properties of the trimolter cocoons, including the length of cocoon silk, filament size, reelability, neatness and cleanness defect, cocoon weight, cocoon shell weight and cocoon shell percentage. Lu and Xiao (1997) reported that the length of cocoon silk of genetic trimolter and induced trimolter (by Jinlu trimolter inducer) were 1142 and 1240m, and the filament size of them were 2.367 and 2.155dtex (1D/denier =1.1dtex), respectively. And, the reelability, neatness and cleanness defect of trimolter cocoons were better than tetramolter cocoons. The cocoon of induced trimolter has significant physical properties compared with the tetramolter and genetic trimolter.

Kataoka et al. (1985) investigated the physical properties of cocoon filaments of trimolter silkworms. The trimolter cocoons had a filament size ranging from 3 to 0.8D (denier). Relationship between body weight of the silkworm and cocoon weight and between cocoon weight and filament size shows linearity with a positive correlation. Another test of induced trimolters demonstrated that the cocoons were smaller than the tetramolter ones, filament size of smaller cocoon was finer and the filament strength was higher in general. And the cocoon filament size was 1.04D and the raw silk size deviation was low (U%: 4.59%). The strength of the raw silk was 30% higher in comparison to the raw silk of the authorized and silkworm race Asahi × Tokai (Tsubouchi et al., 1997).

Kanda et al. (1985) synthesized anti-JH, KK-42 and examined its effect on the induction of precocious trimolters. Almost all of the treated larvae became trimolters when the 24-h-old 4th instar larvae were treated with the test solution of 0.5 μg/μl. And the cocoon weight, cocoon shell weight and cocoon shell percentage were 0.9 g, 13 cg, and 14%, respectively. The size of the cocoon filament was 1.2D, and the length of the filament was 800 m, the neatness defect was 95 points, and the cleanness defect was 93 points. Examination of the physical properties indicated that the raw silk made from trimolters was characterized by high dry and wet strengths, knot strength and young's modulus in spite of their fine denier. Moreover, the silk fabric was softer and its crease recovery was slightly higher than those of the control.

Yoshida et al. (1989) studied the trimolter cocoons treated with anti-JH active substances and found that the tensile strength of the treated silk was 1.45 g/D vs. 1.37 g/D for the untreated silk. The stiffness and compressive resilience of silk treated with anti-JH were greater than those of the untreated silk, and the crystallinity of the treated silk was greater than that of the untreated silk. The average Ca content of silk treated with anti-JH was 1.85 mg/g, vs. 1.64 mg/g for the untreated silk, which indicated that the increase in tensile strength and crystallinity of the treated silk was due to the increase of Ca content of the silk.

Liu et al. (1987) compared the reelability of trimolter cocoons induced by SM-1(the 4th instar and the 3rd instar) and the physical properties of their filament as well as the tetramolting ones used as controls. The results show that the average size of cocoon filament of above three type silkworms were 1.29, 2.04 and 2.59D, respectively, while the coefficient of variation of size of cocoon filament were 9, 12 and 19%, respectively. The thinner the size of cocoon filament was, the stronger the young's modulus and intensity were, and its reelability percentage is obviously superior to that of tetramolting cocoons.

Commercial value and advanced silk materials

Trimolter silkworm has marked advantages in sericulture and the silk-reeling industry. There were many reports focusing on the trimolters induced by anti-JHs originated in plants or biological synthesis to produce fine filament size cocoons. It had significant value to develop new or advanced silk materials, which could be reeled into raw silk with superior quality and fine filament size by using anti-JHs at the preliminary stages of the 2nd, 3rd and 4th instars of silkworms. Li et al. (1991) produced the induced trimolters and successfully obtained the fine filament size cocoons which could be reeled into 4A～5A rank raw silk, and the main indexes of silk quality were as follows, deviation 0.64D, cleanness defect 97.35 points, neatness defect 94 points, strength 4.11 g/D, elongation 20% and cohesion 96 points. Therefore, it was the ideal materials for producing top grade silk clothing which had the characteristics of enough strength, lighter and thinner and crease-resist properties.

Lin et al. (1994) reported the pilot test of the induced trimolters by spraying inducer on the silkworm body on a large scale in the countryside, and the induction ratio of trimolters was 95%, and they gained high economic value. Lu et al. (1994) found the good measure to improve the silk production of the trimolter cocoons induced by Jinlu inducer, the cocoon content and cocoon shell could be increased 5 and 10%, respectively, and the silk kept the original characteristics of fine filament size by using inducer again at the 5th day after the first feeding after molting stage in the 4th instar. While Tan et al. (1992) observed the biological activity of anti-JH SD-Ⅲ, the development of trimolters was uniform and the living activity of pupae, productivity of silk per 50 kg mulberry leaves, the percentage of silk per cocoon and the quality of raw cocoon were better than those of normal silkworms. The size of the raw silk was fine and the comprehensive variance was low, which could be used to reel high quality raw silk of 5A (13/15D).

The effects of anti-JH agent, KK-42 and biosynthesis of silk proteins of silkworm were studied in a popular bivoltine silkworm hybrid, Xinhang (Chinese) × Keming (Japanese). Administration of KK-42 on the 1st day of the 4th instar larvae induced 100% trimolters, and the total larval duration was shortened by four days. The impact of KK-42 on enhanced incorporation rate of radioactive glycine, cocoon weight, RNA content, and fibroin content was significant compared to control, which leads to superior quality of silk (Miao and Bharathi, 2009). While Zhuang et al. (1992) reported the treatment results with anti-JH analogue, YA20, in silkworms, and confirmed that trimolter cocoons had better reelability, little size deviation of cocoon filament, better strength of raw silk, elongation and cohesion. Moreover, it could produce 4 to 6A high quality silk with fine size skein.

Miyajima et al. (2001) induced two Japanese tetramolter silkworm races, Hakugin and Honobono, into trimolter by the imidazole compound, triflumizole (SSP-11) and obtained super thin cocoon filaments. The higher concentration of 280 ppm increased the percentage of trimolter up to approximately 100%. Cocoon filament sizes of the trimolters induced by the 3rd instar treatment in Hakugin and Honobono were 1.36 and 1.72D, and those induced by the 4th instar treatment were 0.99 and 1.03D, respectively. In addition, the deviation of a filament size in cocoon of these trimolter was remarkably small. The results showed that these super thin cocoon filaments would be very useful as new silk materials.

CONTROL OF COCOON FILAMENT

Cocoon production and filament size were originally controlled by using bioactive substances, anti-JH analogues in silkworm strains in the early years of 1980s. As such, the cocoon filaments were smaller than those of the controls. Despite the application of many anti-JHs in sericulture and silk-reeling industry and the achievement of many of the new silk material project's declared aims, these successes are only the first step towards an entire understanding of the control of trimolter cocoon filament.

Intermediate control

When the 3rd instar larvae were treated with the solution for the same duration, all larvae molted into 4th instar but the duration of the 3rd instar was prolonged by 1～2 days. Their feeding period was prolonged by three days as compared with the above anti-JH treated trimolter larvae without any further treatment during the 4th instar. They spun larger cocoons than those from trimolter larvae treated during the 4th instar period. However, the cocoon filaments were of an intermediate size between that of the controls and that of the trimolters treated during the 4th instar period (Akai et al., 1984). In addition, subsequent investigators, Kiuchi et al. (1985) evaluated the other characters of trimolters induced by administration of the juvenile hormone antagonist during the 1st 48 hour of the 1st, 2nd, 3rd, or 4th larval stages, the induction percentages were 51, 88, 100, and 93%, respectively. Cocoon and cocoon shell weights of trimolters induced by 4th instar treatment were 50 and 33% of those of the controls, respectively, and those induced by the 1st, 2nd and 3rd instar treatments were intermediate between those of the controls and the 4th instar treatment group.

Treatment of JH and anti-JH

In 1986, Akai et al. (1986) studied the conditions of formation of undersized cocoons from trimolterization, the size and fine structure characteristics of the cocoon filament, and the possible control of the size of filament by anti-JH treatments. The cocoon size and the size of cocoon filament were smaller than those of the control (tetramolter). The results revealed that the range of the size of the cocoon filaments could be widely controlled by the treatment of JH and anti-JH.

PRODUCTION OF SUPER FINE DENIER SILK

Li et al. (2009) applied anti-JH, SM-1, to induce trimolter silkworms into bimolters in the 2nd or 3rd instar which had undergone regular rearing up to anti-JH treatment. This preliminary test showed that consecutive treatment with 800 mg/L anti-JH for 2 days had the best induction effect. Under this treatment, all trimolter larvae could be induced into bimolters. The obtained bimolter larvae could spin cocoons of which the finest filament had a size of only 0.4 dtex, with an average size of 0.7～0.9 dtex. And, the diameter of this ultrafine silk filament was less than 10 micrometer. Wu et al. (2012) studied the induction effects of trimolters induced by Jinlu trimolter inducer and the cocoon quality differences of six different silkworm races. The quality of cocoon and silk were different because of silkworm races and treatment periods, and there was significant difference between the 3 instar and 4 instar treatment groups, and the sizes of spring and autumn cocoon filament were 1.199 dtex (1.079 D) and 0.892 dtex (0.803 D).

REGULATORY MECHANISM OF TRIMOLTERS

The technical achievements of the past decades have driven the rapid improvement of trimolter induction and have enabled the regulatory mechanism of cocoon filament size to be further discussed in many ways. Induction technologies act like a new tool in sericulture, while regulatory mechanism in silkworm is the dynamic and attractive work in the area of insect life science. Some significant proposed theories of mechanism of induction trimolters are reviewed as follows.

Silk gland and hormones

Miao et al. (2001) treated the 4th instar larvae of the silkworm with anti-JH, Jinlu inducer, and the conversion ratio of tetramolters into trimolters was 100%, and they found many neurosecretory granules of the brain transferred to the cells of the corpora allata, but there was little endoplasmic reticulum. In the prothoracic gland, the micropile edge was clear and there were large nucleoli, mitochondria and endoplasmic reticulum. The investigation revealed that the anti-JH inhibited the secretion of corpora allata and initiated the activity of prothoracic gland. Another experiment confirmed that anti-JH, Jinlu inducer, could decrease the weight of silk gland, yet the content of RNA per silk gland showed the same trend with the contrast. Also, the results showed that anti-JH treated in the 3rd instar would shorten the duration of silkworm, and enhance the healthiness and the cocoon shell rate (Miao et al., 1996).

Theory of JH titer in the hemolymph

In order to study the mechanism of action of the imidazole compound KK-42 which acts as a trimolter inducer, the synthesis of JH by isolated silkworm corpora allata (CA) was determined *in vitro* by means of a short-term radiochemical assay. The ecdysone titers in the hemolymph were determined according to the method of radioimmunoassay. Combining the culture of the prothoracic glands (PG) and brains with the radioimmunoassay of ecdysone (MH), the secretory kinetics of PTTH in the brains after treatment with KK-42 was determined successfully. Together with other experiments, a new theory about the mechanism of KK-42 action is proposed. The result of the study on the synthetic activities of CA and the CA incubated with KK-42 in vitro indicated that the target organ of KK-42 was CA. When KK-42 was applied in the early stage of the 4th instar, the synthetic activity of CA was decreased. Accordingly, it informed to the brain so that the PTTH secretion was also decreased. The latter action delayed the increase of MH titer in the hemolymph. It was the co-action of this delayed MH peak and relatively lowers JH titer that induced the precocious pupation and the trimoltism of the silkworm (Wu et al., 1991).

Anti-JH and posterior silk gland

Lin et al. (1991) induced a tetramolter strain of silkworm efficiently into trimolters by feeding Kang-20 to the larvae during the first 48 h of the 4th instar. RNA polymerase Ⅰ and Ⅱ activities in the posterior silk gland of trimolters were determined. They observed that polymerase activities transcribed on hot-denatured calf thymus DNA and endogenous DNA rose rapidly after Kang-20 treatment and reached a maximum level on the 4th day of the instar. The enzyme stimulation was in good agreement with the increase of RNA contents in the posterior silk gland.

Hemolymph protein profile analysis

Kawaguchi et al. (1994) induced two types of artificial trimolters named A and B by excising the corpora allata from the normal tetramolter silkworms at the periods before and after the third molt, respectively. By using polyacrylamide gel electrophoresis (PAGE), these trimolters were analyzed for the banding patterns of hemolymph proteins at their final (fourth) instar. A feature resembling that of the last (fifth) instar of the normal tetramolter, that is, with an increasing difference in content of the female-specific protein, was found to occur. However, this was preceded by a feature of the penultimate instar of the normal tetramolter, that is, with a low content of hemolymph proteins without sexual differences. Wang et al. (2012) treated polyvoltine silkworm race, P50, with Jinlu trimolter inducer in the 4th instar, and the hemolymph proteins were extracted from the different stages of the female silkworms, and these proteins were isolated and identified based on 1DE-LC-MS technology. Differential protein brands related to development and regulation were detected, and 82 matchable candidate proteins were obtained by MS analysis and database retrieval. And, 26 protein compositions were identified by eliminating the redundant candidate proteins.

Silk fibroin synthesis

The regulatory mechanism of SM-l on silk fibroin synthesis in trimolter silkworm was studied. Researchers thought that SM-1 might regulate and control the synthesis of silk protein by affecting the synthesis of the nucleinic acid in the latter silk gland of silkworm; SM-1 might impact indirectly on the genes which synthesize the silk proteins and promote the genes start early to copy, transcribe and translate into silk protein (Shen et al., 1990).

CONCLUSION

All the exploration and techniques mentioned so far have revolutionized the ability to characterize the innovation in sericulture and silk-reeling industry, especially in silk textile fields. Additionally, the mechanism of trimolter has moved the focus of traditional silk production towards the advanced silk materials researches and has catalyzed the emergence of life science of special insect, which focuses on the relations between the bioactive substance and trimolter silk with fine filament size. However, silkworm induction is still in the developing stages with research focusing on a variety of fields, such as physiology, biochemistry, molecular biology, proteome and sex regulation. And, to our knowledge, there exist little reports focusing on the regulatory mechanism of trimolters on the protein level, this work to analyze the differential expression of the sex-related fat body proteins and hemolymph proteins during the larvae-pupae developmental stages of the trimolter silkworms will not only help researchers to understand the regulatory mechanism of trimolters, but will also serve as method for studying protein synthesis and expression by using two-dimensional gel electrophoresis followed by mass spectrometry identification and bio-informatics. Furthermore, new technologies developed in the life science will provide new insight into the traditional sericulture and silk-reeling industry to further study the advanced silk materials.

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