Isolation Of Total RNA Silkworm (Bombyx Mori L.) Larvae (LEPIDOPTERA:BOMBYCIDAE) C301 On Their Several Body Tissues

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ABSTRACT: Bombyx mori (Lepidoptera:Bombycidae) a group of insect has high economy value. Continually domestication by human leads to the insect get loss of its productivity and more sensitive to high temperature especially while its cultivated in tropical climate. The temperature effect HSP gene of Bombyx mori larvae that responsible for maintaining the body from temperature stress. The gene was obtained by isolation total RNA of larval 5th instar from several organs such as head, silk gland, cuticle layer and and rectum using Trizol reagent. Results showed that the total RNA was found on larval head, silk gland and rectum, whereas, clumping of calcium carbonate occurred on cuticle layer. Based on analytical and RNA quantitative quality using Nanospectrophotometer UV-vis indicate that RNA total on larval cuticle was not detected because of undisolved RNA total in DEPC water and ddH_2O.

Key word: Bombyx mori, cuticle layer, rectum, silk gland

1. INTRODUCTION

Temperature in the environment influence growth, development, reproduction and distribution of organisms. Each organism has biological adaptation in extreme temperature (Hochachka dan Somero 2002; Bergant dan Trdan, 2006; Bhattacharjee 2008; Kumar dan Tripathy 2009). Whereas, Hong dan Vierling (2000) reported that each organism has thermotolerance and adaptive response on certain temperature. Bombyx mori, domestificated insect and sensitive to environment especially in tropical climate. High temperature reduced physiological activities of silk worm. Generally, at the first larvae development high temperaure prolong life span and determine cocoon character. However, the fluctuation and wide range temperature inhibit larvae development (Regniere et al., 2012). High temperature during stage end of development accelerate their growth and shortened lifespan. In the other side, low temperature inhibit the growth of larvae and prolong their lifespan (Rahmathulla et al., 2004). Optimum temperature for of silkworm growth between 20^{o}C - 28^{o}C and maximum productivity occurred at 23^{o}C - 28 ^{o}C. High temperature (> 30^{o}C) affect on their health, and temperature < 20^{o}C inhibit physiological first instar growth as indicated by the larvae become weak and sensitive to diseases (Sam-Eun 1998). Introducing high quality of larvae from temperate to tropical climate is not solution to improve productivity of silk worm.

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Interaction between genetic and environment determine quality of larvae such as body weight, disease resistant and fecundity (Jakaria et al., 2001). Noor (1996) reported that Previous study by Pal et al., (2014) reported that maintaining temperature at 24ºC reduced larvae mortality and increase pupa weight and cocoon. The optimun temperature for larvae cultivation range between 25 and 26°C (Hussain et al., 2011; Khan 2014). High larva performance affected by interaction between genetic and environment. Rahmathulla 2012 stated that high extreme environment influence genotype expression. Population with different characters controlled by genes such as morphology, and physiology expreesed as phenotypic plasticity required by population to adapt to their environment. RNA is unstable molecule and hort halftime after it extracted from cells or tissues (Brooks 1998). Therefore, specific treatment to isolate RNA are required to prevent degradation (Kojima and Ozawa 2002; Buckingham and Flaws 2007). There are several RNA molecules found in cell such as ribosomal (rRNA) (80% -90%), messenger RNA (mRNA) (2,5% -5%) dan transfer RNA (tRNA) (Buckingham and Flaws 2007). Isolation of RNA from cells and tissues are the first procedure for gene expression analysis (Chomczynski and Sacchi 2006). RNA is biomolecule of nucleotides chain. Each nucleotide consists of nitrogen base, sugar and phosphate. Compare to DNA, RNA molecule is single strand, ribose sugar and uracil base. Biomolecule extraction of DNA, RNA and protein are essential for molecular biology (Wink 2006). Isolation of RNA molecule particularly RNAse activity of single RNA strand is fundamental step for expression analysis (RT-PCR, northern blotting) cDNA construction and gene cloning. Steps of RNA isolation consists of RNA handling, sample preparation, quality and quantity analysis, and purification. RNAse, an enzyme that has a function to degrade RNA. High temperature degrade RNA. Unstability of RNA particularly is caused by RNAse in all tissues of bacteria and fungi in environment (Brooks 1998; Buckingham and Flaws 2007). The use of strong denaturant was use to isolate total RNA to inhibit endogen RNAse (Doyle 1996). Based on size and sequence, eukaryotic cells consist of four variation of ribosomal RNA such as 28S, 18S, 5.8S, and 5S (Mitra 2003).

2. MATERIALS AND METHODS

TRIzol® Reagent 1mL containing mono-phasic of phenol dan guanidine isothiocyanate was used to isolate RNA. Tissues were homogenized and lysed by adding chloroform 0,2 mL followed by centrifugation12000 g for 15 minute. The pellet was suspended by adding 500 µL isopropyl alcohol, washed by 1 mL 75% ethanol (in DEPC-water) and dissolved in RNAse with free water. Larvae of Bombyx mori larvae 5th instar were obtained from Center of Natural Silkworm Candiroto Temanggung Center Java. Their eggs were hatched in wrapped black clothed for 10 days (room temperatures). The larvae were maintained 15 days (room temperatures) from the first instar to 5th instar. The larval were feed Morus leaves (Morus sp.). Total RNA was isolated after the larvae maintained for 15 days (5th instar). The isolation was conducted from parts of their body such as head, silk gland, cuticle layer and rectum.

2.1. Eggs Hatching and Cultivating of Bombyx mori Larvae

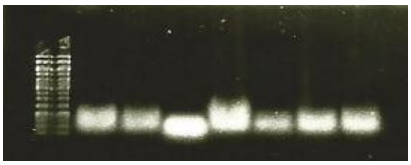
About 100 eggs were hatched by incubating in the hatch box for 10 days at ambient temperature (28-30^{o}C) until 5th. After 4 days of incubation was signed by the presence blue spot on the eggs, the eggs were covered by black paper for 6. days. After 10 days of incubation, the young larvae were removed to cultivating box. The 1st instar larval were desinfected using mixed calcium carbonate ($CaCO_3$) and calcium hypochloride ($Ca(ClO)_2$) (95:5). The larvae were feeded 3 times a day by shredded fresh Morus alba leaves for 15 days. Instar period was signed by moulting. After 4 days, 90% of the larvae stop eating. The stop eating larvae then were sprinkled by calcium carbonate powder. At the end of each instar, some of the larvae were picked and removed to another place to reduce their density. Maintaing during larvae development were coducted regularky to prevent pest and diseases. Cleaning process was conducted once at 1st and 2nd instars at larval age 4 and 8 days respectively. Whereas, instar 3rd to 5th instars the cleaning was conducted twice after second feeding before moulting. The larvae were desinfected after moulting and before the second feeding. After the age larvae (9-15 days) or at instar 3rd to 5th instars larvae were feed daily by intact leaves and their branch that put crosslinked in the larvae nest.

2.2. Total RNA Isolation

RNA was isolated from larvae head, silk gland and rectum. About 5 g of the tissues were homogenized in mortar using liquid nitrogen. The powdered homogenized was placed in eppendorf tube that contain 1 mL Trizol. The mixed was homogenized and incubated at ambient temperature (28-30^{0}C) for 5 minute. About 2 mL of chloroform was added, mixed gently and incubated for 3 minute. The mixture was vortexed and centrifuged 12000 g for 15 minute at 4^{o}C. The supernatant was pippeted and add with 500µl isoprophyl alcohol in eppendorf then incubated at ambient temperature for 10 minute. The mixture was centrifuged 12000 g, 4^{o}C, 10 minute. The pellet was rinsed by 1 mL 75% ethanol then centrifuged. The pellet (RNA total) was dried using vaccum dryer and resuspended in d_2H_2O. The quality and quantity of RNA were determined using UV nanospectrophotometer, wavelength 260 and 280 nm respectively. The whole RNA total was analyzed using agarose gel 1% in 1xTAE buffer. The RNA stained with EtBr (0,5 µg/mL) and visualized in GelDoc UV transilluminator (Labquip). The RNA then dissolved in DEPC-water or ddH_2O and incubated at 55^{o}C for 10 minute.

3. RESULTS AND DISCUSSION

RNA from body tissues of silkworm (Bombyx mori) to obtain the RNA total was succesfully isolated. Based on UV nanospectrophotometer measurement the ratio OD260/OD280 total RNA that isolated showed pure RNA with no protein contamination. Electrophoresis of total RNA for the whole analysis showed the presence of two dominant RNA strands. Both of the strands are ribosomal RNA (rRNA) 28S dan 18S. Electrophoresis of total RNA isolated from some body parts larvae of Bombyx mori was shown in Figure 1.



M 1 2 3 4 5 6 7

Figure 1. *Total RNA body parts of Bombyx mori larvae, M = marker; 1-3 = head; 3-5 = silkgland; 6-7 = rectum*

Result showed that high intact of total RNA isolated can be used to as template for total cDNA sinthesis. Total quantity of RNA isolated from several body parts of instar V silkworm larvae as shown in Table 1.

Table 1. *RNA total several body patrs isolated from several body parts of 5th silkworm Bombyx mori larvae*

Body tissues	RNA total (ng/µL)	Absorbance (nm)	
		260/280	260/230
Head	+	+	+
Silk gland	+	+	+
Cuticle layer	-	-	-
Rectum	+	+	+

(-) not found

Table 1 showed that RNA total was found on head, silk gland and rectum. On cuticle layer contain white clump that not suspended either in DEPC water or ddH_2O. We suggested that the clump was salt that suspended by isopropanol. Salt is contaminant during nucleic acid purification Therefore, isopropanol used as the first presipitate and to restore RNA (Sambrook and Russel 2001). Several separation methods and purification to obtain RNA are required to eliminate salts (Smarason and Smith 2003). Total RNA is liquid phase in acid condition, while DNA and protein in intephase or organic phase. Insect head is a center of food and anterior sensory. The organ consists of brain, eyes and ocelli complex, antenae and mouth. Some head segments are immobile or relatively mobile. Based on the mobility 5 segments contribute for head formation, 3 posterior segment performed gnathocephalon called appendage gnathal located around mouth for food absorption. The others segmental ganglia

performed subesophagial on brain ganglia. Intercalary segment antenae homolog to rod segment performed proencephalon (Brusca and Brusca 2003; Snodgrass 1935; Weber, 1966). Head development as a basic to determine evolution level and ontogeny (Posnien et al., 2010). The head of Bombyx mori contain active prothoracicotropic hormone for pupa development. Structure of the hormone consists of two unidentical peptide chain (A and B chain) similar to insulin as found in vertebrate animals. Chain A consist of 20 amino acid residu. Chain B contain four microheterogeneous peptide, two of them contain 28 residu and the others 26 residu, 4K-PTTH-II homolog (40%) to human insulin, and similar to pig relaxin in cistein carboxyl residu terminal of A chain in amino acid residu pyroglutamic-B chain terminal. Identical distribution of six residu cistein showed that 4K-PTTH-ll similar to insulin (Nagasawa et al., 1986). According to Suzuki et al. (1983), Nagasawa et al. (1984), Kataoka et al. (1991) hormones prothoracicotropic (PPTH), Eclocion (EH), diapause (DH) and melanization reddish and coloration hormone (MRCH) present in larva head of silkworm. Silk protein was synthesized by silk gland of larva cells and stored in lumen gland and converted into filament. Mondal et al., (2007) reported that silk filament has thin and long structure, light, soft with high affinity, absorbance and thermotolerance. Protein of the filamen contain collagen, elastine, keratine and sporgin (Komatsu 1980). The outer layer of filament consists of cericine that non suluble incold water but dissolved in small fraction in hot water (Gulrajani 1988). Protein in the inner layer of filament is fibroin. Filament of silkworm excreted by spineret (Shimizu 2000) in liquid form and become harder while in contact to the air (Borgohain 2015). Besides cericin (70-80%), fibroin (20-30%) and carbohidrate (1,2-1,6%), raw silk filament contain lipid and wax (0,4-0,8%), inorganic salts (0,7%) and coloring agent (0,02%) (Mondal et al., 2007). Silk synthesis was controlled by stored amino acids of degenerated intestine and integumen (Naguchi et al., 1974). Amino acid particularly glycine, alanine, serine and tyrosin were synthesized by silk gland and transamination process (Prudhomme et al., 1985). The biosynthesis involved in amino acid production, ribosomes, mRNA and tRNA (Kamili and Masoodi, 2000). Messenger RNA of silkworm contain repeated 60-75% polypeptides fibroin molecules with the sequence GGX-GCY-GGX-GCY-GGX- [UCZ (atau AGCU) -GGX- (GCY -GGX) 2] 8-UCZ (or AGCU) -GGX-GCY-GCY-GGX-UACU in which X, Y and Z present on each ribonucleotide (Suzuki and Brown 1972). Total RNA isolation of silk gland of Bombyx mori can be used to detect protein synthesis degradation and development pupae level. The genes such as Ser2, Ser3, fib-L, P25, dan CYP450 involved in protein metabolism (Li et al., 2015). Gene box in silk gland controls sericine-1 expression (Tsubota et al. 2016). In addition, total RNA isolation can be isolated from larvae mesenteron (Yu et al., 2015; Ito et al., 2016). Wu et al. (2016) reported that analysis genome ncRNAs of larvae comprehensively essentials for study of genetic and evolution. The development of larvae molecular mark, essential for linkage map and strain fingerprint for breeding (Nagaraju and Goldsmith 2002). Total RNA of larvae mesenteron tissue from gastrointestinal tract to rectum at the first instar to the end of instar V was isolated by Mahesha et al. (2013) and Gao et al. (2016). Rectum of Lepidoptera consists of anterior and posterior that spread to integument to form anus. At the anterior contain cryptonephric rectal covered by simple separate intine and canal layer (Henson 1937; Srivastava 1959; Ramsay 1976; MacGown dan Sikorowski 1982; Azuma et al., 2012). Hindgut larva part of cryptonephric system for electrolite balance, and feses formation (Reinecke et al., 1973; Chapman 1998; Azuma et al., 2012; ; Vessaro-Silva et al., 2014). Mayurama and Azuma (2015) reported that total RNA isolated in mesenteron of gastrointestinal tract used for aquarin expression on columnar cells. Whereas total RNA of Bombyx mori mesenteron was isolated to indentify cDNA code for aminopeptidase (Yassin et al. 2010). Cuticule, protective layer consists of chitin, protein and pigment (Wu et al., 2016). Koga et al., (1992) reported that chitinase is one of enzymes in integument of B. mori. The role of the enzyme is to release cuticle layer by hydrolysis chitin integument. Isolation of total RNA from tissue contain chinase required specific treatment (Smarason and Smith 2003; Buckingham and Flaws 2007). Total RNA isolated from Bombyx mori cuticle using RNeasy Midi kit (Qiagen, Inc.) was conducted by Abdel-Banat dan Koga (2016). Trizol solution used to isolate total RNA adipose tissue cuticle layer of Bombyx mori (Li et al., 2016 ; Gao et al., 2016). Total RNA body tissues of 5th instars Bombyx mori presented on Table 2.

Table 2. *The quantity of total RNA isolated from several parts of the body Larval silk (Bombyx mori) 5th instar with UV - Vis anospektrofotometer*

Body organ	RNA total (ng/µL)	Absorbance	
		λ260/280	λ260/230
Head	1656	1.899	1,340
Head	2704	1.825	1.275
Silk gland	1210	1.796	1.010
Cuticle layer	-	-	-
Rectum	1196	1.880	1.907

(-) not found

Total RNA was found head, silk gland and rectum. No RNA was found on cuticle layer. The formation of white undissolved clump on the layer suggested salts that suspended by isopropanol. High total RNA was found on each with absorbance level from 1.796 to 1.899 on λ260/280 and from 1.010 to 1.907 on λ260/230. The result showed that isolation of total RNA using trizol reagent are specific for these organs.

CONCLUSION

Total RNA isolation using Trizol reagent, was found only in the head, silk gland and rectum while the cuticle going clotting lime and from the analysis of the quality and quantity of RNA by UV nano spectrophotometer total RNA was not detected .

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