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The effects on *in vitro* digestibility from different developmental stages of silkworm larvae, *Bombyx mori* (Lepidoptera: Bombycidae) and position of mulberry leaves, *Morus alba* (Rosales: Moraceae)

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ABSTRACT

Mulberry leaves (*Morus alba* var. Buriram 60) at the positions 1, 2, 3, 4 and 5 were harvested from 2nd, 3rd, 4th, 5th–6th and 7th–8th below the primordia, respectively; to evaluate on *in vitro* digestibility of carbohydrate (IVCD) and protein (IVPD) by using the crude enzyme extract from whole body of 3rd to 5th instar larva of mulberry silkworm (*Bombyx mori*). The crude enzymes were extracted from the whole body of larva against the reviewed data of gut extract from the previous studies. The optimal temperature and pH was similar between whole body and gut extracts, indicating the possible use of whole body for *in vitro* digestibility study. There was no statistical interaction between leaf position and developmental stage of larva. In all stages of larva, the leaf positions 2 and 3 were higher in IVCD than in the other positions ($p < 0.05$), whereas the lowest IVPD was found in position 1 ($p < 0.05$). The IVCD was highest in 3rd instar larva ($p < 0.001$) while the decrease trend of IVPD was observed in 4th, 3rd and 5th stages ($p < 0.001$), respectively. Based on the digestibility values, the preferred leaf positions for the mulberry silkworm instar larvae (3rd–5th) were leaf positions 2 and 3. This *in vitro* screening of the leaf supports the development of an artificial mulberry leaf-based diet for *B. mori* used in sericulture.

Introduction

The mulberry silkworm, *Bombyx mori* (Lepidoptera: Bombycidae), is a very important part of the sericulture industry due to its high productive performance. The silkworm is not only an economically important insect in the sericulture and global textile industries, but also as a new animal model for screening insecticides before the usage (Shen et al., 2011; Wang et al., 2011). The quality of mulberry leaves for feeding the silkworms is a key factor, contributing to 38% of the overall success of cocoon production which affected by environmental condition (Deka et al., 2011).

Digestive enzymes play a vital role in the metabolism of food in animals which breakdown the complex form of nutrients present in the food into simple forms for absorption and utilization. The digestive enzymes in the midgut of *B. mori*, which include amylase, trehalase, cellulase and proteases, have been studied by various scientists (Yamashita et al., 1974; Eguchi and Iwamoto, 1976; Kanekatsu, 1978; Eguchi and Kuriyama, 1983; Abraham et al., 1992; Anand et al., 2010). Protein and carbohydrates are the main components of artificial silkworm diets, so our understanding of nutrient utilization can be expanded by studying the activities of digestive enzymes. These enzymes are located in various insect body parts, including salivary glands, the haemolymph, the gut (midgut and hindgut), fat body cell and thoracic muscles and epithelial cells in the midgut (Yamashita et al., 1974; Asadi et al., 2010; Lokesh et al., 2012; Pawar et al., 2012; Savithri and Rajitha, 2014). However, only the salivary glands and midgut enzymes are involved in food digestion, while the other organs and their enzymes play a role in cellular metabolism. These characteristics of

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digestive enzymes respond to the gut environment and vary among insect species and type of diet (Anand et al., 2010).

Several studies have reported the optimal conditions for digestive enzymes in specific organs of *B. mori*. (Yanagawa, 1971; Yamashita et al., 1974; Kanekatsu, 1978; Abraham et al., 1992; Terra and Ferriera, 1994; Anand et al., 2010). However, little information is available on the digestive enzymes in whole body. Recently, Tabatabaei *et al.* (2011) reported that digestive amylase activity in the larval stage of carob moth, *Ectomyeloisceratonidae* (Lepidoptera: Pyralidae), was similar for extracts from the whole body and from the midgut, indicating the whole body as an alternative source of enzyme since providing the high amount of extracted volume for biochemical studies. The aims of this study were to characterize the main digestive enzymes from the whole body of silkworms (amylase, trehalase, cellulase, trypsin and chymotrypsin) and to perform *in vitro* digestibility screening of mulberry leaves for silkworm instars. Only the 3rd instar silkworm was chosen for this pilot study due to the fact that digestive enzymes, metabolic profiles and weight gain exhibit significant changes during young silkworm stages (Shankar et al., 2015; Zhou et al., 2015). *In vitro* digestibility of carbohydrate (IVCD) and protein (IVPD) of mulberry leaves, *Morus alba* (Rosales: Moraceae) using the crude enzyme extract from whole body of 3rd to 5th instar larva of mulberry silkworm was also examined in this study.

The basic findings from this work could be applied to further studies of silkworm physiology, biochemistry and nutrition. Establishing the digestibility of an artificial mulberry leaf-based diet for 3rd to 5th instar stages was an additional goal of the *in vitro* experiment.

Materials and methods

Preparation of mulberry leaves and chemical composition

The positions 1, 2, 3, 4 and 5 were harvested from 2nd, 3rd, 4th, 5th–6th and 7th–8th fresh mulberry leaves (*M. alba* var. Buriram 60) below the primordia, respectively. The leaves were manually cleaned of dirt and foreign material before being cut into three parts. The cleaned leaves were dried at 50 °C for 24 h using a hot air oven (FED 115, Binder, Tuttlingen, Germany), milled to obtain a fine powder and sieved through a 0.125 mm mesh. For *in vitro* study, the leaves were cleaned, cut, freeze-dried by freeze dryer (Coolsafe 110–4, Labogene, Lynge, Denmark) for 24 h, milled, sieved, and kept in an auto-desiccator cabinet (SanplaDrykeeper, Sanplatec, Osaka, Japan) prior to the *in vitro* digestibility test with the crude enzymes from the 3rd, 4th and 5th instar larvae. The chemical composition of the leaf samples, including their moisture, crude protein (CP), crude lipid, ash, crude fibre, acid detergent fibre (ADF) and neutral detergent fibre (NDF), were determined according to the methods proposed by AOAC (2000). Gross energy (GE, cal/g) was determined using a bomb calorimeter (CAL2K, Digital Data System (Pty). Ltd., Gauteng, South Africa). Nitrogen free extract (NFE, %) was calculated as 100 – (CP + crude lipid + crude fibre + ash). The measurements were carried out in two replicates and all values are expressed as the percentage of dry matter (DM).

Digestive enzyme extraction

The 3rd, 4th and 5th instar larvae of the Thai silkworm (*B. mori* strain Nang-noi) were obtained from the Queen Sirikit Centre, Nakhon Ratchasima, Thailand. The larvae were starved for 24 h prior to sampling in order to prevent metabolic flexibility induced by food intake. The whole body of the larva was extracted in 200 mM phosphate buffer (pH 8) (1:2 *w/v*) using a micro-homogenizer (TH 02, Omni International, Marietta, USA). The homogenate was centrifuged at 15,000 × *g* for 30 min at 4 °C. The supernatant was collected as the crude enzymes and was kept at − 80 °C until it was used for studying enzyme activity and *in vitro* digestibility. The digestive enzymes from 4th and 5th instar larvae were also extracted as described above, but these enzymes were used for screening digestibility only.

Characterization of digestive enzyme activity

The crude extract enzyme from 3rd instar larva was selected to study on characterization of digestive enzyme activity. The effect of pH on the digestive enzyme activity was assayed at ambient temperature. The activity of amylase (EC 3.2.1.1) was assayed according to Areekijseree et al. (2004) using soluble starch as the substrate. Trehalase activity (EC 3.2.1.28) was assayed based on Gaikwad and Bhawane (2015) using trehalose as the substrate. Cellulase activity (EC 3.2.1.4) was assayed according to Vatanparast et al. (2014) using carboxymethyl cellulose (CMC) as the substrate. The products from these three enzymes were stained using 1% dinitrosalicylic acid (DNS) and measured using a spectrophotometer at 540 nm (Bernfled, 1955) against linear range of standards (maltose, glucose and glucose for the three enzymes, respectively). The activity of trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) was assayed according to the method described by Rungruangsak-Torrissen et al. (2006) using *N*-benzoyl-*L*-Arg-*p*-nitroanilide (BAPNA) and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPNA) as substrates, respectively. The product of both enzymes were measured spectrophotometrically at 410 nm against linear range of *p*-nitroanilide. For the temperature study, the assays were conducted in the range of 25–80 °C using the chosen pH. Blank samples were run concurrently against the real samples when the crude enzyme volume was replaced by its extraction buffer at specified conditions. The activity of the observed digestive enzymes was expressed as relative activity (%).

In vitro carbohydrate and protein digestibility

The digestibility study was performed according to the method described in Sansuwan et al. (2017). The reaction mixtures contained 5 mg dried leaf, 10 ml phosphate buffer (pH 8.2) and 125 µl crude enzyme extract. This cocktail was incubated at 30 °C under 200 rpm for 24 h. The real samples were run simultaneously against the blank samples when the extraction buffer was replaced by equal volume of its enzyme. The IVCD and IVPD were expressed as mmol maltose/g and mmol *DL*-alanine/g, respectively.

Statistical analysis

The experiment was performed using a completely randomized factorial designs. Two-way ANOVA was used to evaluate the effects of leaf position (1–5) and *in vitro* digestibility in the 3rd, 4th and 5th instar larvae (Fixed factors) which Duncan's Multiples Range Test (DMRT) was used as post-hoc analysis and statistical significance was accepted at $p < 0.05$. The interaction between the factors was evaluated. Results were expressed as mean ± SE (standard error; $n = 3$). All analyses were done in R-statistic package Rcmdr (R Development Core Team, 2008).

Results

Proximate chemical composition of mulberry leaves

The average chemical composition of mulberry leaves from different positions is shown in Table 1. The lignocellulosic materials (ADF) was increased in a position dependent manner while the opposite trend was observed for NDF content (which includes, hemicellulose and cellulose as the major components).

Digestive enzymes characterization of the 3rd instar larvae

The optimal conditions for studying activity of digestive enzymes in