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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.

Key words: amylase, cellulase, chymotrypsin, leaf position, trehalase, trypsin.

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& Iwamoto 1976; Kanekatsu 1978; Eguchi&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 & 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 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 & Ferreira 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 & 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 (Bernfeld 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 the 3rd instar of silkworms are shown in Figures 1–2. The amylase from the 3rd instar showed an optimal condition at pH 10 (Fig. 1A). The activity increased sharply from pH 6 to pH 10 and then decreased dramatically at pH 11. Under the suitable pH, amylase had the highest activity at 50 °C (Fig. 1B), followed by similar activity in broad ranges of 40–45 °C and 55–70 °C. The few isoforms of trehalase were observed under the pH characteristic studies (Fig. 1C). The highest activity was exhibited at pH 6 and pH 11, respectively. The weak acid isoform gave highly desirable characteristics at 45 °C (Fig. 1D). The cellulase was more active in the alkaline condition than in the neutral and acidic conditions, respectively (Fig. 1E). The highest activity was observed under pH 8, followed by the range of pH 9 to 11 (83.08% on average), relative to the other pH levels. Under the chosen pH 8, the cellulase was active in a broad temperature range from 30–75 °C (Fig. 1F). Trypsin displayed maximum activity at pH 10 (Fig. 2A). The relative activity under acidic to neutral conditions was very low, comprising 21.01% on average compared to the alkaline

condition. This enzyme has optimal temperatures at 40 °C and 50 °C, followed by 45 °C (91.53%) (Fig. 2B). Chymotrypsin displayed high activity under a pH range of 8 to 11, and the maximal activity was observed at pH 9 (Fig. 2C). Another peak was also exhibited at pH 5, demonstrating a 1.22-fold decreased activity relative to the peaked pH. The temperature profile of chymotrypsin was exhibited as a ladder type (Fig. 2D). The maximal activity was displayed in a range of 25 °C to 35 °C, followed by 40 °C to 60 °C (85.04% on average) and 65 °C to 75 °C (66.74% on average), respectively.

IVCD and IVPD

IVCD and IVPD were determined by measuring the increase in reducing sugar and liberated reactive amino groups of cleaved peptide in the mulberry leaves, respectively. *In vitro* digestibility of different leaf positions and developmental stages were illustrated in Table 2. The interaction between two factors was not significant. However, IVCD and IVPD were statistically influenced by the developmental stage ($p < 0.001$) and different positions of leaves ($p < 0.05$). The IVCD of the 3rd instar larvae (0.66 ± 0.04 mmol maltose/g) was significant ($p < 0.001$) when compared to the 4th (0.18 ± 0.05 mmol maltose/g) and 5th (0.15 ± 0.02 mmol maltose/g) stages while IVPD was significant higher ($p < 0.001$) in the 4th (9.45 ± 0.46 mmol *DL*-alanine/g) than in the 3rd (7.41 ± 0.57 mmol *DL*-alanine/g) and the 5th (2.92 ± 0.32 mmol *DL*-alanine/g) stages. The highest IVCD of leave position was presented in the third and followed by the second position. However, IVCD was highly improved with the third position ($p < 0.05$). In contrast, the IVPD significantly ($p < 0.05$) increased in the second (7.70 ± 1.09 mmol *DL*-alanine/g) and fourth positions (7.01 ± 1.20 mmol *DL*-alanine/g), when compared to the first position (5.14 ± 0.96 mmol *DL*-alanine/g). The lowest IVPD was found in first position

comparing to third (6.62 ± 1.05 mmol *DL*-alanine/g) and fifth positions (6.51 ± 1.18 mmol *DL*-alanine/g).

DISCUSSION

The findings from the current study indicate that the optimal conditions for amylase were pH 10 and 50 °C which similar to results observed from other studies. Amylase from midgut extracts of 5th instar larvae of cultivars Nistari and Kolar Gold exhibited optimal conditions at pH 9.2 and 60 °C and 8.8 at 60 °C, respectively (Muniv *et al*. 2011). Endogenous amylase from the midgut of *B. mori* is active at pH 9.3, showing similar pattern to porcine pancreatic amylase (Kanekatsu 1978; Abraham *et al.* 1992; Terra & Ferriera 1994). Due to the saccharifying nature of the silkworm's digestive amylase, the optimal pH was found in alkaline because the selection pressure during evolution led to the exclusive presence of alkaline RNQ (Arg, Asn and Gln)-type alpha amylase in the digestive tract of lepidopterans and this enzyme activity was lost in acidic condition (Kanekatsu 1978). Variations in amylase characteristics therefore appears to be affected by taxonomy (species and cultivar), the body part used for enzyme extraction, developmental stage studied and feeding habit. Trehalase activity was mainly located in the midgut epithelial tissue (structure-bound trehalase) in the larval stage of silkworm and maximum activity was achieved at the middle stage of the 5th instar. On the other hand, the activity during pupal to adult development was mainly presented in the midgut contents (soluble trehalase) but with a lack of activity in the epithelium (Yamashita *et al.* 1974). Both forms of insect trehalases are important in energy supply, growth, metamorphosis, stress recovery, chitin synthesis and flight. The optimal conditions for trehalase found in the current study (pH 6 and 45 °C) were similar to conditions reported

in midgut extracts from *B. mori* which were pH 5.5 and 60 °C (Pawar *et al*.2012) and pH 5.4–6.0 (Yanagawa 1971). The ontogenic development of trehalase isoforms in relation to energy reserves and the position of mulberry leaves used for feeding should be of interest for further studies. The optimal pH (pH 8) for assaying cellulase from the current study is well matched with the optimal pH for bacterial proliferation, isolated from the silkworm gut (Anand *et al*.2010). The gut of silkworm contains bacteria that produce the digestive enzyme for the lignocellulose degradation in mulberry leaves (Anand*et al.* 2010). Studies on thermal stability and microbiota community in the gut of silkworms may be important to further understand the utilization of indigestible elements from the mulberry leaves. Most insect trypsin and chymotrypsins have an optimal pH in the range of 8–9 (Terra & Ferriera 1994) which similar in this study. The preference of digestive proteases from the lepidopteran midgut for high alkaline conditions allows these insects to feed on plant material because tannin can bind effectively to protein at acidic pH, reducing efficiency of protein digestion (Panizzi & Parra 2012). The presence of chymotrypsin at pH 5 in the current study may indicate a minor isoform of enzyme in whole gut extract. Low stability under acidic conditions indicates inactive functionality of this isoform for digesting protein along the midgut region.

Feeding silkworms on an artificial diet led to decreased nutritional absorption, energy retention and silk synthesis relative to feeding with mulberry leaves alone (Zhou *et al*. 2008). Screening of the leaf positions using the *in vitro* digestibility technique is needed in order to formulate the mulberry leaf-based diet. In the current study, IVCD and IVPD indicated that the position 1 of mulberry leaves is unsuitable for the three stages of silkworm larva. This position may contain anti-nutritional compounds such as trypsin inhibitors, phytates, oxalates, tannins, polyphenol, lectin and 1-deoxynojirimycin (DNJ),

interfering with enzyme activities from *in vitro* digestibility study (Sudha *et al*.2011). Therefore, the leaves at first position appear to be suitable as an aromatic herbal tea or for use in alternative products, rather than being used for feeding these instar larvae. The decreased IVCD were observed after digesting leaves at positions 4 and 5 because the mature leaves contain large amounts of lignocellulosic materials (cellulose, hemicellulose and lignin), which prevents digestion (Table 1). Based on our *in vitro* digestibility study, an artificial diet for the all developmental stage of instar larvae should include mulberry leaves at either the positions 2 or 3. This finding is in partial agreement with conventional sericulture in Thailand where 3rd–5th instar larvae are fed on the leaf positions 1, 2 and 3 (Thai Agricultural Standard 2010).

IVCD was higher than IVPD in the young instar (3rd), indicating that the young silkworm required nutrients for energy than protein. Therefore, the consumption rate is greatest for carbohydrate and lowest for protein in 1st–3rd instar larvae (Wu & Chen 1988). The highest of IVPD was observed in the 4th instar larvae in this study. During this feeding period, the level of 3-hydroxykynurenine increased, probably due to the metabolism of tryptophan from mulberry leaves in order to prepare the precursors for pigment biosynthesis during the 5th instar stage (Zhou *et al*. 2015). Moreover, this instar had higher DNA, RNA and protein than in 5th instar larvae, indicating that the high rate of conversion and accumulation of protein in these larvae was associated with greater metabolic activity of the tissue (Murthy *et al*. 2014). These findings are in agreement with the higher approximate digestibility found for 4th instar of mulberry pyralid, *Glyphodespyloaris*, fed withmulberry leaves, relative to 5th instar (Oftadeh *et al*. 2014).

CONCLUSIONS

The optimal conditions for studying activity of carbohydrate-digesting enzyme; amylase trehalase and cellulase from the whole body of the 3rd instar were pH 10 at 50 °C, pH 6 at 45 °C and pH 8 at 30 to 75 °C, respectively. Optimal condition of protein-digesting enzymes were pH 10 at 40 °C or 50 °C for trypsin and pH 9 and 25–30 °C for chymotrypsin. The control samples (blank) were run during characteristic studies. The purification of each enzyme and characteristic studies should be conducted. The *in vitro* digestibility indicates the effects of leaf positions on carbohydrate and protein digestibility in the 3rd–5th instar. Since all tissues were extracted from the whole body during this study, further work may use midgut extraction before the application of these findings to sericulture. In addition, increasing the replication for digestibility study may be more appropriate, in order to gain statistically robust results. The preferred leaf positions for the three-instar larvae were positions 2 and 3. Using different ratios of these two positions in order to improve digestibility should be of interest. The current study provides useful nutritional information that could be applied to sericulture farming, as well as for the sustainable development of the sericulture industry.

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Figure captions

Fig. 1. Relative activity of carbohydrate-digesting enzymes in 3rd instar silkworm larvae. The effect of pH at ambient temperature on the activity of amylase (A), trehalase (C) and cellulase (E). The temperature profiles at optimal pH of amylase (B), trehalase (D) and cellulase (F). Data are expressed as mean ± SE (n = 3).

Fig. 2. Relative activity of protein-digesting enzymes in 3rd instar silkworm larvae. The effect of pH at ambient temperature on the activity of trypsin (A) and chymotrypsin (C). The temperature profiles at optimal pH of trypsin (B) and chymotrypsin (D). Data are expressed as mean ± SE (n = 3).

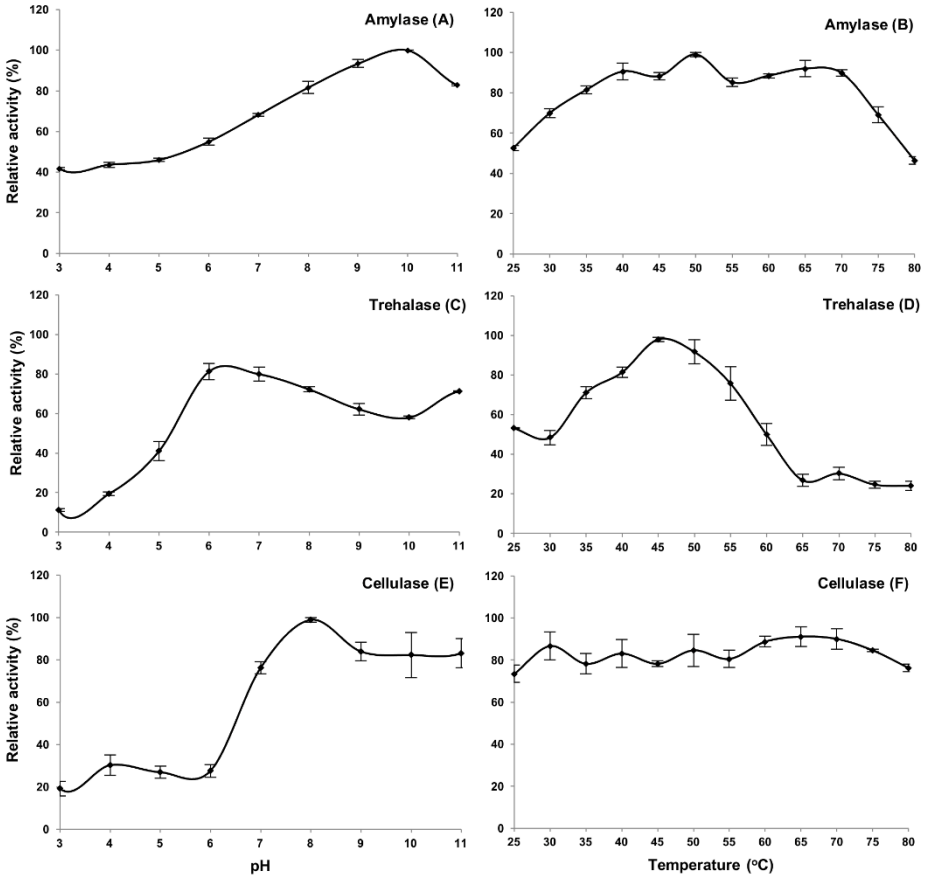


Figure 1

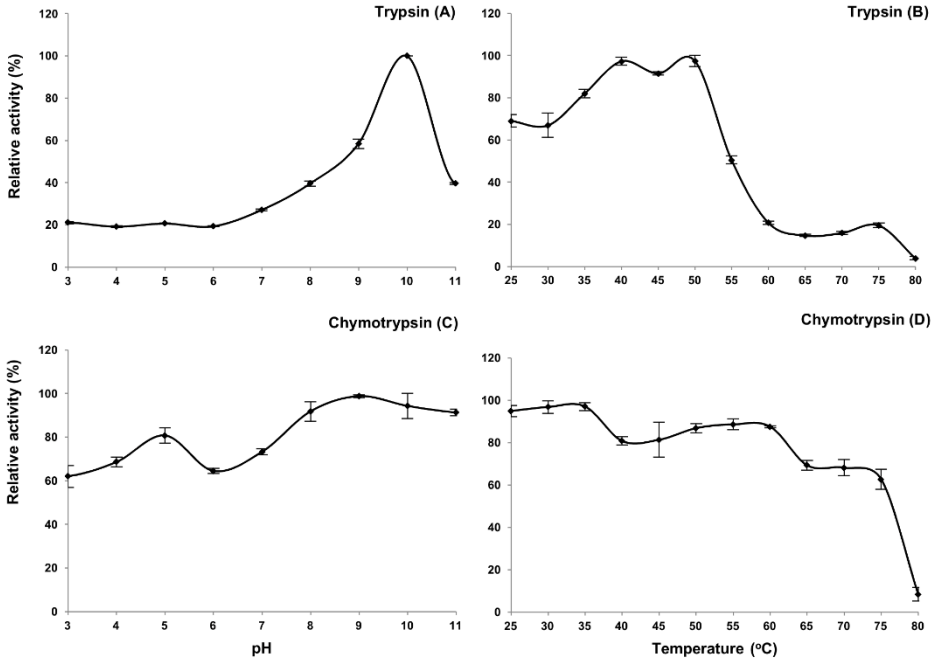


Figure 2

Table 1 The proximate composition of mulberry leaves at positions 1, 2, 3, 4 and 5 used for rearing the 1st, 2nd, 3rd, 4thand 5th instar larvae of silkworms. The samples were harvested from the 2nd (position 1), 3rd (position 2), 4th (position 3),5th–6th (position 4) and 7th–8th (position 5) leaves below the primordia.

Composition	Position of the mulberry leaves				
	1	2	3	4	5
Moisture	6.20 ± 0.01	5.99 ± 0.29	6.73 ± 0.08	7.19 ± 0.15	6.97 ± 0.11
CP (% DM)	25.76 ± 0.01	23.64 ± 0.06	23.18 ± 0.05	22.71 ± 0.17	22.67 ± 0.01
Crude lipid (% DM)	1.34 ± 0.27	1.77 ± 0.11	1.63 ± 0.17	2.82 ± 0.17	3.87 ± 0.13
Crude fibre (% DM)	8.82 ± 0.00	8.94 ± 0.02	8.25 ± 0.15	8.68 ± 0.01	8.72 ± 0.12
ADF (% DM)	14.62 ± 0.13	14.44 ± 0.06	14.35 ± 0.01	17.36 ± 0.05	18.81 ± 0.11
NDF (% DM)	17.46 ± 0.06	14.19 ± 0.07	12.28 ± 0.02	12.46 ± 0.05	13.29 ± 0.18
Ash (% DM)	9.90 ± 0.00	10.39 ± 0.02	10.88 ± 0.10	11.90 ± 0.01	13.02 ± 0.08
NFE (% DM)	47.95 ± 0.27	49.24 ± 0.13	56.03 ± 0.34	53.87 ± 0.01	51.69 ± 0.23
GE (cal/g)	4,067.25 ± 6.72	3,942.69 ± 4.29	3,913 ± 1.36	3,899 ± 12.57	3,921 ± 2.73

CP, crude protein; DM, dry matter; ADF, acid detergent fibre; NDF, neutral detergent fibre; NFE, nitrogen free extract; GE, gross energy.
Values are expressed as mean ± SE from two replicates.

Table 2 *In vitro* carbohydrate (mmol maltose/g) and protein digestibility (mmol *DL*-alanine/g) in different positions of mulberry leaves

using digestive enzymes extracted from the whole body of the 3rd, 4th and 5th instar larvae of the silkworm.

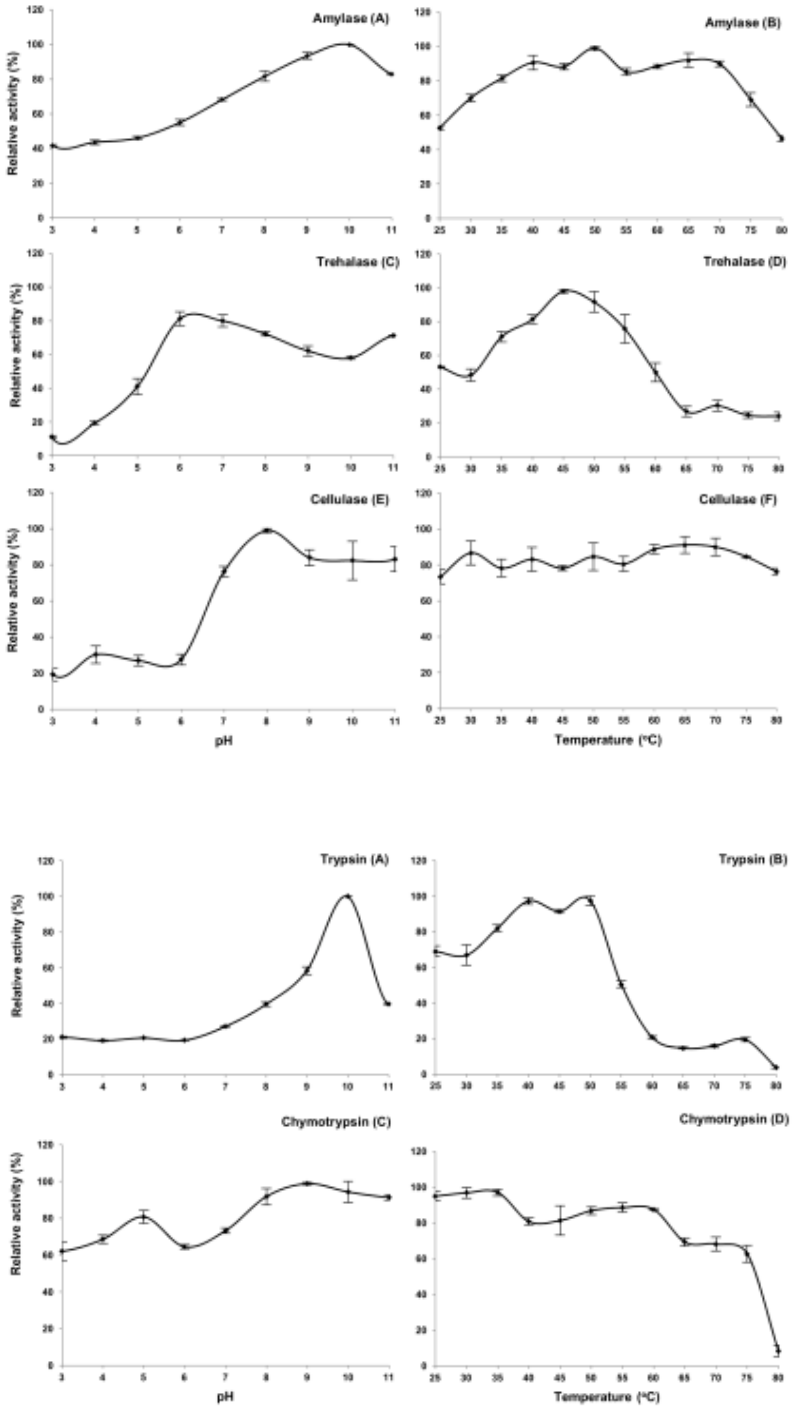
IVD	Stage (S)			Position (P)					*p*-value of the factors		
	3rd	4th	5th	1	2	3	4	5	S	P	S×P
IVCD	0.66±0.04^{b}	0.18±0.05^{a}	0.15±0.02^{a}	0.28±0.06^{a}	0.35±0.11^{ab}	0.46±0.12^{b}	0.26±0.09^{a}	0.29±0.09^{a}	<0.001	0.021	0.239
IVPD	7.41±0.57^{b}	9.45±0.46^{c}	2.93±0.32^{a}	5.14±0.96^{a}	7.70±1.09^{b}	6.62±1.05^{ab}	7.01±1.20^{b}	6.51±1.18^{ab}	<0.001	0.040	0.535

IVD, *in vitro* digestibility; IVCD, *in vitro* carbohydrate digestibility; IVPD, *in vitro* protein digestibility.

Values are expressed as mean ± SE (n = 3).

Significant differences between means of each factor are indicated by different superscripts ($p < 0.05$).

Graphical abstract



Highlights

- Characteristics of whole body enzymes were similar to the enzymes from gut extracts
- Whole body is an alternative enzyme source for digestibility study in silkworm larva
- The leaf positions 2 and 3 were preferred by silkworm instar larvae
- *In vitro* screening supports artificial mulberry leaf-based diet for silkworm larva