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Process Optimization for the Production of *Philosamia ricini* (Eri Silk) Pupae Hydrolysate

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ABSTRACT

The method for protein hydrolysate production from Eri silk pupae (*Philosamia ricini*), waste from silk reeling process, was investigated. The appropriate process started by blending fresh pupa into fine particles and water was added to adjust final protein concentration to 4.6%. The pH was adjusted to 7.5 to accommodate the enzyme activity. Commercial enzyme Alcalase was added to 0.5% and the process was carried out at 50°C for 120 min with stirring. The protein hydrolysate from silk pupa was freeze-dried, ground into fine powder and analysed for compositions. The maximum degree of hydrolysis (DH) resulted from this condition was 73.27% with nitrogen recovery 62.82%. The hydrolysate product was water soluble and was rich in essential amino acids.

Key words: silk hydrolysate, Eri silk pupae, production, optimization

INTRODUCTION

In recent years, natural products have grown from a niche segment to one of the fastest-growing categories in personal care with market value 2,000 million US dollars per year. Cosmetics are the key products, and ingredients with specific properties are extremely needed, especially protein. Different properties of protein are obtained from different molecular weights. For example, better film forming quality is obtained when high molecular weight protein is applied in the products, such as hair shampoo or conditioner, whereas low molecular weight protein makes the products, such as skin moisturizer, with better moisture maintenance (Challoner *et al*., 1997). Silk protein or silk protein hydrolysate are one of such ingredients which can improve skin look and cover skin problem (Kato *et al*., 1998; Yamada *et al*., 2001; Chang-Kee *et al*., 2002; Hu *et al*., 2005). They can be obtained by either chemical or enzymic hydrolysis and considered for applications as a functional material for various preparations. However, Otterburn (1989) reported that the strong chemical hydrolysis condition affected resulting protein hydrolysate by converting cysteine and serine amino acids into dehydroalanine and eventually into lysinealanine which showed to be toxic.

The by-products of manufacturing silk include the unusable parts of the pupa and cocoon. Cocoon can be processed to make Dupion silk, or re-processed into flow-silk and spun-silk yarns. Cocoon could be partially dissolved using lithium bromide aqueous solution and further processed to obtain silk fibroin membrane as immobilisation matrix (Liu *et al*., 1995a; Liu *et al*., 1995b). Hydrolysed silk contains silk peptides with

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average molecular weight 1,000 daltons which are water-soluble and well compatible with surfactant. The resulting fibroin film that is shining could be easily coated onto human hair and skin. Anonymous (1998) chemically hydrolysed silk fibroin into dipeptides and tripeptides which were water soluble and homogeneously compatible with the water-based cosmetic products. Park *et al.* (2002) reported digestion of fibroin protein by acid hydrolysis and bacterial serine protease into peptides with average sizes in range of 500-10,000 daltons. The pupae can be sold for fertilizer, biogas (Viswanath and Nand, 1994), feed stuff (Nandeesha *et al.*, 1990; Rangacharyulu *et al.*, 2003) and other agricultural purposes. Furthermore, Yang (2002) also reported that silkworm pupas have been used as Chinese traditional medicines since ancient time. Pharmacological studies show that silkworm pupas are alimental for increasing immunity, protecting the liver and preventing cancer. Proximate analysis of pupa showed that it contains 55–60% protein, 25–30% lipid, 4.96% fiber, and other substances, e.g. hormones, trace elements and vitamins, thus indicating that it could be a good protein source-for various purposes (Yang *et al.*, 2002; Rangacharyulu *et al.*, 2003).

One approach to upgrade silk pupa by-products was use of proteases by choosing the enzyme type and hydrolysis conditions to obtain the hydrolysed silk pupa protein product. Therefore, the main purpose of this research was to optimize the enzymatic hydrolysis condition suitable for silk pupa protein hydrolysate which could be further applied as protein source for cosmetics.

MATERIALS AND METHODS

1. Preparation and analysis of raw material and products

The *Philosamia ricini* (Eri silk) pupa was used in this study. It was ground into fine size using Vita mix® and then stored in sealed plastic bottle at temperature -20°C until use. Raw material and all products were sampled for proximate analysis; moisture, ash, protein, lipid and microbiological properties as described in the AOAC (2000). Protein patterns of hydrolysate were accessed by 12% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using 12% separating gel and 4% stacking gel, followed by Coomassie staining according to the manufacturer instruction (Pharmacia: EPS 300). Total amino acid was analysed according to AccQ. Tag Amino acid analysis method (1993).

2. Enzymatic hydrolysis of silk pupae

The production of a hydrolysate was performed as follows (adapted from Chang-Kee *et al.*, 2002): ground silk pupa were mixed with distilled water to obtain optimal protein content with final volume of 100 g. Before subjecting to enzymatically hydrolysis, the suspension was adjusted to suitable pH with 4 N NaOH. The reaction mixture was incubated at appropriate temperature with continuous stirring for a period of time. The pH of reaction was controlled by addition of 4 N NaOH whose volume was recorded for calculation of % degree of hydrolysis (%DH). %DH is defined as the percentage of peptide bonds cleaved during the hydrolysis reaction (Adler-Nissen, 1982).

$$\% \text{ DH} = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100$$

wherein B = Volume of NaOH (ml) used in hydrolysis reaction

N_b = NaOH concentration (N)

MP = Protein content (N x 6.25) (gram)

α = Degree of dissociation of the

$$\alpha\text{-NH group} = \frac{10^{pH-pK}}{1+10^{pH-pK}}$$

pK = Average pK of α-NH group liberated during the hydrolysis and significantly depending on

temperature

h_{to} = Total number of peptide bonds in a given protein (meqv/g protein)

The enzymatic reaction was terminated by heating the hydrolysate to 100°C for 15 min. The cell debris was removed by centrifugation at 8,000 rpm for 15 min at 4°C and pH was adjusted to 7.0 with 1 N HCl. The resulting silk hydrolysate was subjected to freeze-drying for overnight to obtain water-soluble silk powder which was afterwards ground into 60-mesh powder.

Selection of commercial protease used for hydrolysis of silk pupae

The experiment was carried out following the previous procedure using Completely Randomized Design (CRD) and different commercial protease enzymes as 4 treatments, indicated as 1) control (without enzyme), 2) Alcalase 2.4L FG (55-70 °C, 6.5-8.5, Novozymes A/S, Denmark), 3) Neutrase 0.8L (45-55 °C, 5.5-7.5, Novozymes A/S, Denmark), and 4) Flavourzyme 500L (45-60 °C, 5.0-7.0, Novozymes A/S, Denmark) with the ratio of protein:enzyme = 6:0.5. Each experiment was performed in triplicate. The analysis was done using Analysis of Variance or ANOVA and Duncan's Mew Multiple Range Test (DMRT). The enzyme with highest DH was selected as the most appropriate enzyme and used for further experiments.

Optimisation for concentration of substrate, enzyme and hydrolysis period for hydrolysis of silk pupae

The experiment was carried out at pH 9.5 and 60 °C using Central Composite Design (CCD) with 3 factors and 5 levels ($-\alpha$, -1, 0, 1, α). The first factor was substrate concentration varying from 4.6, 6, 8, 10, and 11.4%. The second factor was enzyme concentration ranging from 0, 0.2, 0.5, 0.8, and 1%. The last factor was hydrolysis period from 70, 90, 120, 150, and 170 min. Each experiment was performed in triplicate. The analysis was done using Response Surface Methodology (RSM) with Quadratic Model. The condition with highest % nitrogen recovery (NR) (Bjorn *et al*., 2002) was selected as the most appropriate enzyme and was used for further experiments.

$$\text{nitrogen recovery (\%)} = \frac{\text{total nitrogen in hydrolysis} \times \text{hydrolysate weight}}{\text{total nitrogen in raw material} \times \text{raw material weight}} \times 100$$

Optimisation for pH and temperature for hydrolysis of silk pupae

The experiment was carried out at optimal condition obtained from previous section using Central Composite Design (CCD) with 2 factors and 5 levels ($-\alpha$, -1, 0, 1, α). The first factor was reaction pH varying from 7.0, 7.5, 8.5, 9.5 and 10.0. The second factor was temperature ranging from 48, 50, 55, 60 and 62 °C. Each experiment was performed in triplicate. The analysis was done using Response Surface Methodology (RSM) with Quadratic Model. The condition with highest %nitrogen recovery (NR) and %hydrolysis was selected as the most appropriate enzyme.

RESULTS AND DICUSSION

1. Preparation and analysis of raw material

Analysis of silk pupa from *P. ricini* (Eri Silk) showed high moisture content as 74.66%, indicating liquid as its main component, 18.44% protein, 4.24% lipid and 1.58% ash as shown in Table 1. Comparison with *Bombyx mori* and *Attacus ricinii* pupae showed similarity as the main components were also moisture (65.13% and 70.14%, respectively) with protein and ash contents of 11.99 and 0.79%, respectively for *B. mori* and 15.97 and 1.36%, respectively for *A. ricinii* (Mishra *et al*., 2003). However, the lipid

Table 1 Chemical composition of silk worm.

	% on fresh weight			
	Eri silk cocoon[1/]	Eri silk pupa[1/]	*Bombyx mori* pupa[2/]	*Attacus ricinii* pupa[2/]
Moisture	4.26±0.14	74.66±0.42	65.13	70.14
Ash	3.22±0.03	1.58±0.03	0.79	1.36
Protein	96.32±0.30	18.44±0.24	11.99	15.97
Lipid	1.55±0.02	4.24±0.01	20.10	11.09

[1/] Laboratory analysis

[2/] Mishra *et al*. (2003)

Each experiment was performed in triplicate.

content (20.10%) in *B. mori*, which was domestic strain, was much higher than those in *P. ricini* (4.24%) and *A. ricinii* (11.09%), which were both wild strains. The protein content, even though, was not as high as that in Eri silk cocoon, 96.32%, it still was rather high and then could be applied as protein sources for either cosmetics or foods.

2. Enzymatic hydrolysis of silk pupae

Selection of commercial protease enzymes used for hydrolysis of silk pupae

The protein hydrolysis reaction using enzyme as catalyst is very efficient, specific and can be carried out under mild conditions so that the nutritional quality of the amino acids is maintained (Adler-Nissen, 1982). For example, protein hydrolysis using alkaline solution not only destroyed some essential amino acids (tryptophane, cysteine or serine), but it also resulted in change of amino acid structure from *L*-form into *D*-form which human beings were not able to consume (Hall and Ahmad, 1992). As a result, enzymatic catalysis is chosen for the experiments. Table 2 shows DH of hydrolysates prepared with different protease enzymes. DH means the percentage of enzymatically hydrolysed peptide bonds compared with the original peptide bonds in raw material representing a number of peptide bonds those being degraded during the reaction. In case that DH is high, it indicates high level of protein hydrolysis into smaller peptides and free amino acids (Adler-Nissen, 1982). According to the results, it demonstrated that the highest DH (52.39%) was obtained from Alcalase while the Neutrase and Flavourzyme gave 39.29% and 23.96%, respectively. Furthermore, the reaction without any protease enzymes at pH 7.0, 50 °C and at pH 9.5, 55 °C gave lower DH (13.93% and 15.78%, respectively) caused by the protein auto-hydrolysis of silk pupa. Therefore, the protease enzyme was actually needed to enhance hydrolysis reaction.

Table 2 Effect of different commercial protease enzymes on % degree of hydrolysis (DH).

Tr.	Enzyme	Enzyme (%v/w)	Substrate (% protein)	Time (min)	pH	Temp (°C)	DH (%)
1	No enzyme	0	6	90	7.0	50	13.93 d
2	No enzyme	0	6	90	9.5	55	15.78 d
3	Alcalase 2.4 L FG	0.5	6	90	9.5	55	52.39 a
4	Neutrase 0.8 L	0.5	6	90	7.0	50	39.29 b
5	Flavourzyme 500 L	0.5	6	90	7.0	50	23.96 c

Means in the same row with different letters are significantly different at 95% level ($p \leq 0.05$) as determined by Duncan's multiple range test

Figure 1 shows the sizes of Eri pupa protein without and with the enzymatic hydrolysis. It indicated that the reaction without enzyme showed two prominent bands (82 and 40 kDa) which were similar to that before the hydrolysis reaction (data not shown). In contrast, after hydrolysis, the prominent bands appeared not only at 82 kDa but also at 31 kDa with smear area between 7-16 kDa. This suggested that parts of pupa protein were hydrolysed into smaller peptides by added enzymes. However, the hydrolysis patterns obtained from 3 commercial enzymes were rather different. The ones from Neutrase and Flavourzyme were very similar and gave hydrolysate with majority size around 82 and 31 kDa and minority size below 31 kDa whereas the one from Alcalase did not show the 82-kDa band at all but instead it demonstrated smear area below size 7 kDa. These results were consistent with DH mentioned above in Table 2. Therefore, it could be concluded that the three protease enzymes possessed significantly different hydrolysis ability to pupa protein and Alcalase was the most efficient protease enzyme for hydrolysis of pupa protein with DH of 52.39%. Thus, the Alcalase was selected for the next experiments.

Optimisation for concentration of substrate, Alcalase, and hydrolysis period for hydrolysis of silk pupae

Figure 2 shows protein sizes of Eri pupa hydrolysate from different hydrolysis reactions as monitored by 12% SDS-PAGE. Ratios between protein substrate and Alcalase used in different hydrolysis reactions were indicated as Eri pupa protein:Alcalase. The difference between reactions without and with enzyme was shown in lanes 2 and 3, respectively. The prominent bands around 75-100 kDa in lane 2 disappeared after enzymatic hydrolysis in lane 3. Furthermore, when the enzymatic reaction was prolonged from 60 to 120 min (lanes 3 and 4, respectively), the obvious protein bands around 10-25 kDa also disappeared indicating the effect of hydrolysis period. The effect of enzyme concentration on hydrolysis reaction was also demonstrated in lanes 5 and 6;

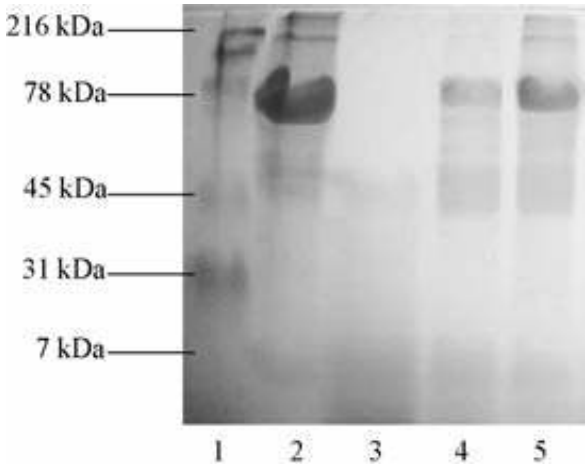

Figure 1 Protein size of Eri pupa hydrolysate as monitored by 12% SDS-PAGE. Lane 1, protein molecular weight marker; (Kaleidoscope Prestained Standards, Bio-Rad, USA) which comprise myosin (216 kDa), BSA (78 kDa), carbonic anhydrase (45 kDa), soybean trypsin inhibitor (32 kDa) and aprotinin (7 kDa) Lane 2, Eri pupa hydrolysate hydrolyzed without enzyme; Lane 3, Eri pupa hydrolysate hydrolyzed with Alcalase; Lane 4, Eri pupa hydrolysate hydrolyzed with Nutrase; Lane 5, Eri pupa hydrolysate hydrolyzed with Flavozyme.

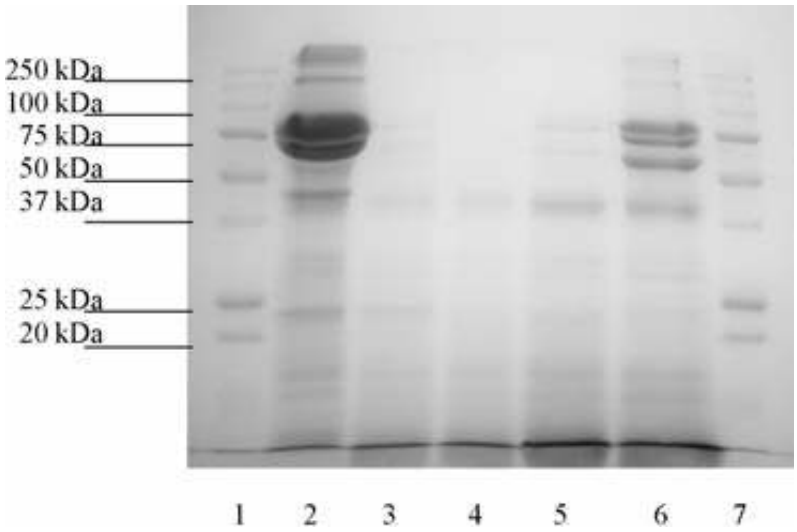

Figure 2 Protein size of Eri pupa hydrolysate as monitored by 12% SDS-PAGE. Lanes 1 and 7, protein molecular weight marker; (Precision Plus Protein™ Standards, Bio-Rad, USA) which contains 7 highly purified recombinant proteins in molecular masses from 20 to 250 kDa; Lane 2, Eri pupa protein:Alcalase 4.6:0 for 60 min; Lane 3, Eri pupa protein:Alcalase 4.6:0.5 for 60 min; Lane 4, Eri pupa protein:Alcalase 4.6:0.5 for 120 min; Lane 5, Eri pupa protein:Alcalase 10.0:1.0 for 120 min; Lane 6, Eri pupa protein:Alcalase 10.0:0.5 for 120 min.

the more enzyme used in the reaction, the more powerful protein hydrolysis was.

Figures 3 and 4 demonstrates the Response Surface Curves indicating the effects of substrate concentration, Alcalase concentration and hydrolysis period on DH and %nitrogen recovery, respectively. It was shown that DH and nitrogen recovery were slightly influenced by a period of hydrolysis (70-170 minutes) whereas they were strongly affected by the concentrations of substrate and Alcalase. DH and %nitrogen recovery tended to decrease as the substrate was increased (4.6–11.4% protein). This was relative to the ratio between substrate and enzyme in the reaction. The results also indicated that protein substrate concentrations provided saturation conditions and thus slightly decreasing hydrolysis reaction occurred. In most foods with a protein concentration of 4% or higher, substrate saturation is expected (Mutilangi *et al.*, 1995). In contrast, DH and %nitrogen recovery were raised as more enzyme was added (0-1%). This indicated that more enzymes in the reaction could effectively bind to more substrates and then yielding more products. As also shown in the tannery fleshing protein hydrolysis reaction experimented by Raju *et al.* (1997) that the enzyme concentrations played a very vital role in addition to other factors. In conclusion, the superimposed response surface curve between DH and %nitrogen recovery (Figure 5) demonstrated that the most optimum hydrolysis condition for Eri pupa protein was 4.6% protein, 0.5% Alcalase and 120 minutes to obtain the highest nitrogen recovery 76.98%.

Optimisation for pH and temperature for hydrolysis of silk pupae by Alcalase

Table 3 demonstrates the effect of pH and temperature of hydrolysis reaction on DH and %nitrogen recovery. However, when the results were statistically analysed using multiple regression in order to obtain the regression

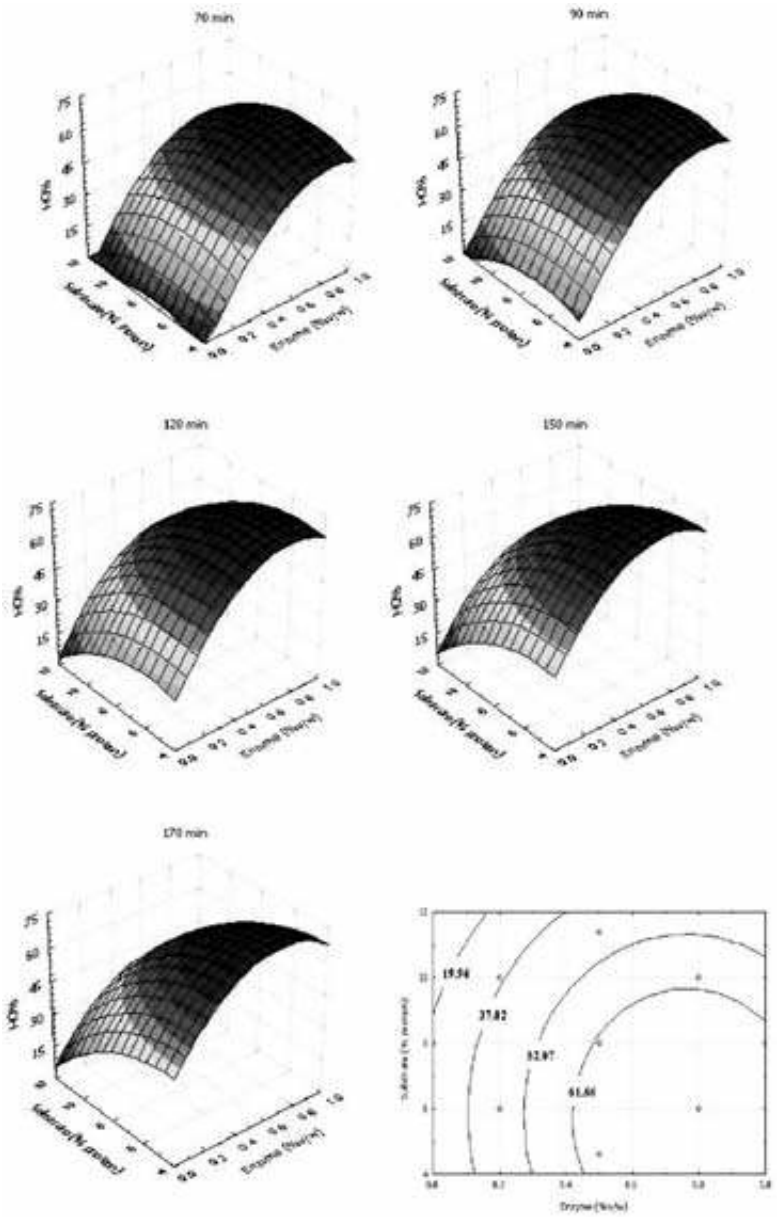

Figure 3 Effect of substrate concentration, enzyme concentration and hydrolysis period on degree of hydrolysis (DH) as shown by the Response Surface Curve.

coefficients for the second order polynomial equation, the results indicated that the proposed model possessed no significant (p>0.05). It could be concluded that the range of pH and temperature used for hydrolysis reaction did not directly affect DH and %nitrogen recovery. Therefore, the reaction with pH 7.5 and temperature 50°C was selected as the optimal pH and temperature due to that this condition consumed the lowest energy.

Analysis of products

The Eri pupa hydrolysate powder contained 4.63% moisture, 11.47% total nitrogen, 1.70% lipid, 11.97% ash, total plate count, yeast

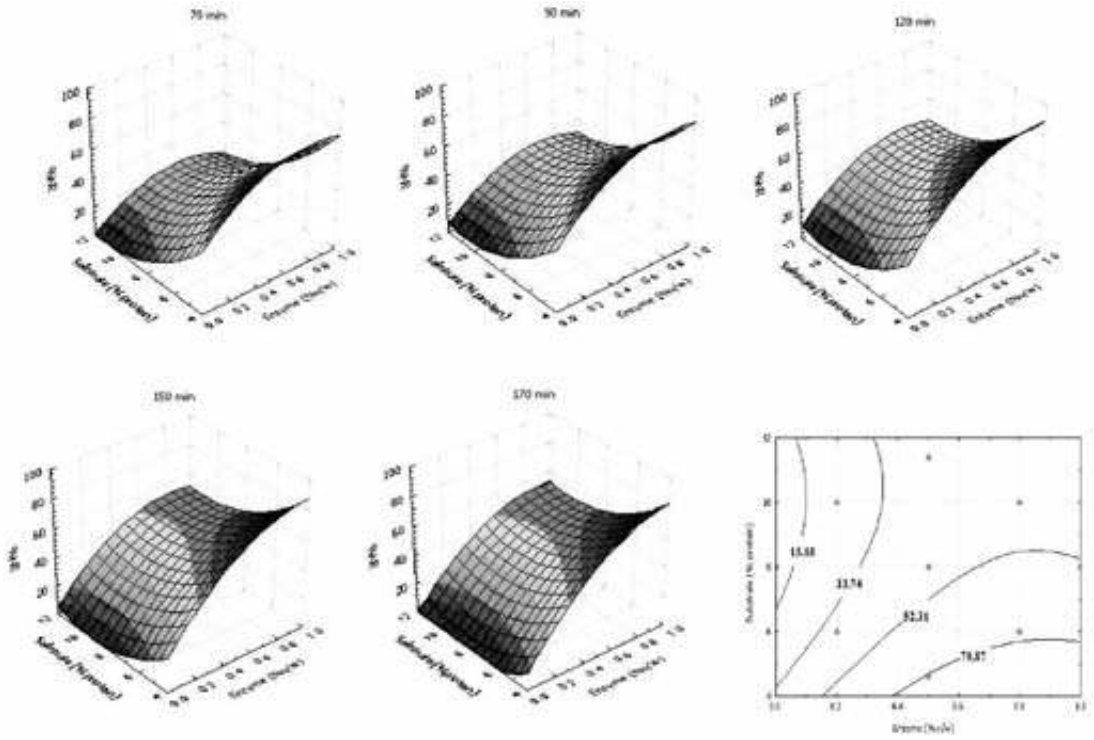

Figure 4 Effect of substrate concentration, enzyme concentration and hydrolysis period on %nitrogen recovery as shown by the Response Surface Curve.

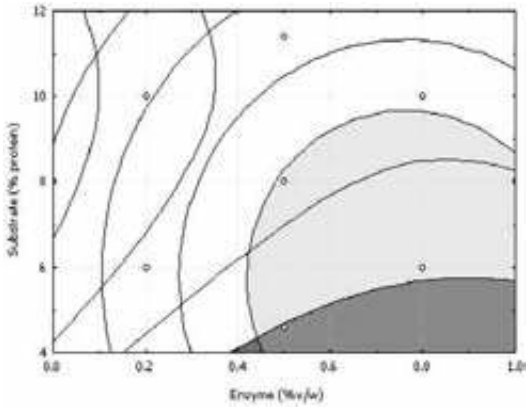

Figure 5 Effect of substrate concentration, enzyme concentration and hydrolysis period on degree of hydrolysis (DH) and %nitrogen recovery as shown by the Superimposed Response Surface Curve

and mold < 100 CFU/g with 100% water solubility. Comparison with the commercial products, "Promois®", demonstrated similarity in protein contents with less moisture and more ash contents as shown in Table 5.

Table 6 shows the amounts of free and total amino acids in the products. It demonstrated that the sum of free amino acids was 16.40% out of 54.21% total amino acids with tyrosine, histidine, phenylalanine, leucine and arginine as main free amino acids (1.77, 1.57, 1.39, 1.33 and 1.32%, respectively). This implied that enzymatic hydrolysis of pupa protein resulted in hydrolysate containing not only free amino acids but also water-soluble short-chain peptides. Furthermore, Table 7 shows types and amounts of amino acids present in the product compared with those in raw material and the results indicated similarity in

Table 3 Effect of different reaction pH and temperatures on %degree of hydrolysis (DH) and %nitrogen recovery (NR) (under the reaction condition %protein:enzyme = 4.6:0.5 for 120 minutes).

Tr.	Independent variance		Dependent variance	
	pH	Temperature (°C)	DH(%)	NR(%)
1	7.5	50	73.27	62.26
2	9.5	50	67.29	64.45
3	8.5	55	72.73	72.38
4	7.5	60	76.02	51.20
5	9.5	60	70.88	81.95
6	7.0	55	73.31	59.68
7	8.5	55	75.05	78.44
8	10.0	55	70.71	81.84
9	8.5	48	64.29	89.28
10	8.5	55	77.54	68.00
11	8.5	62	79.13	54.06

Table 4 Mathematical model of Eri silk protein hydrolysis by multiple regression for %degree of hydrolysis (DH) and %nitrogen recovery (NR).

Dependent variable	Mathematical model	R^2	*p*-value
% DH (y_1)	$-242.958 + 19.752x_1 + 8.102x_2 - 1.402x_1^2 - 0.07067x_2^2 - 0.042x_1x_2$	0.753	0.123^{ns}
% NR (y_2)	$108.235 - 41.029x_1 + 4.614x_2 - 1.749x_1^2 - 0.159x_2^2 - 1.428x_1x_2$	0.737	0.141^{ns}

x_1 = pH; x_2 = Temperature (°C); ns = non-significant ($p \leq 0.05$)

Table 5 Chemical and biological properties of products

Properties	Eri pupa hydrolysate powder	Commercial products "Promois®"	
		SILK-1000P[1/]	SERICIN-P[2/]
Moisture (%)	4.63±0.15	10.0	12.0
Total nitrogen (%)	11.47±0.21	13.0	13.0
Lipid (%)	1.62±0.01	not determined	not determined
Ash (%)	11.97±0.03	3.0	4.6
Total plate count (CFU/g)	< 100	not determined	not determined
Yeast and mold (CFU/g)	< 100	not determined	not determined

[1/] obtained from fibroin hydrolysis

[2/] obtained from sericin hydrolysis

Each experiment was performed in triplicate

amino acids profiles between the two samples. Interestingly, loss of cysteine and methionine was observed in the hydrolysate. This could be due to the adjustment of pH and heat treatment which is known to denature some amino acids—in particular, cysteine and methionine (Sarwar, 1999).

Table 6 Comparison of free and total amino acids present in the product.

Amino acids(g/100 g sample)	Eri pupa hydrolysate powder	
	Free amino acids	Total amino acids
Aspartic acid + Asparagine	0.76	5.23
Serine	1.27	4.02
Glutamic acid + Glutamine	1.20	6.77
Glycine	0.39	2.52
Histidine	1.57	2.47
Arginine	1.32	3.80
Threonine	0.86	3.45
Alanine	1.01	2.94
Proline	0.40	2.85
Cysteine	0.16	Not determined
Tyrosine	1.77	3.60
Valine	0.98	3.26
Methionine	0.57	Not determined
Lysine	1.04	3.83
Isoleucine	0.69	2.57
Leucine	1.33	3.82
Phenylalanine	1.39	3.08
Total	16.70	54.21

Table 7 Comparison of the products and raw material in terms of types and amounts of amino acids.

Amino acids(%mol)	Eri pupa raw material	Eri pupa hydrolysate powder
Aspartic acid + Asparagine	8.51	9.29
Serine	6.82	9.04
Glutamic acid + Glutamine	10.90	10.95
Glycine	9.27	7.93
Histidine	3.16	3.76
Arginine	5.24	5.16
Threonine	4.23	6.84
Alanine	11.06	7.80
Proline	4.49	5.85
Cysteine	0.51	Not determined
Tyrosine	4.47	4.70
Valine	6.51	6.58
Methionine	2.31	Not determined
Lysine	6.35	6.19
Isoleucine	4.79	4.63
Leucine	7.70	6.88
Phenylalanine	3.69	4.41
Total	100	100

CONCLUSION

Proximate analysis revealed 18.44% high protein in Eri Silk pupa suitable for protein source in various applications. DH reached maximum to 79% to obtain most soluble nitrogen when hydrolysis condition was the best optimized. However, the condition with a slightly lower DH, 76%, was selected as the condition for hydrolysate production due to the economic reason. The resulting protein hydrolysates were shown to be well solubilised. Characterisation of resulting hydrolysate also showed similarity in properties to the commercial silk hydrolysate. Even though they were originally from different parts of silk, one from silk pupa and another from silk sericin, their properties could be comparable for substitution. Therefore, it is advantageous to consider scaling up the production process for industrial purpose, as well as incorporation such abundantly available inexpensive protein ingredients as one of substitutes to cosmetic or food industries in the future.

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