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Optimization of the production of *Momordica charantia* L. Var. *abbreviata* Ser. protein hydrolysates with hypoglycemic effect using Alcalase

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ABSTRACT

Momordica charantia L. Var. *abbreviata* Ser. protein was hydrolyzed using six different proteases. The results showed Alcalase 2.4L to have the best hydrolyzing capacity, followed by Pancreatin. In addition, Alcalase hydrolysate had stronger hypoglycemic effect than that of Pancreatin hydrolysate at the same dose. Alcalase was chosen to produce *M. charantia* L. Var. *abbreviata* Ser. protein hydrolysates (MCPHs) with hypoglycemic effect. Response surface methodology (RSM) was applied to optimize the hydrolysis conditions using Alcalase. Model equation was proposed with regard to the effect of enzyme/substrate ratio, pH and temperature. The optimum values for enzyme/substrate ratio, pH and temperature were found to be 2.37%, 9.2 and 57 °C respectively.

1. Introduction

Momordica charantia L. Var. *abbreviata* Ser. (MCV) belongs to a short-fruited group of the Cucurbitaceace family. The size of its fruits is only about one-fifth of the commonly seen *M. charantia*. Derived from a wild plant, it was domesticated and improved by Jiangsu High-quality Farm Product Development Center, China and has been successively planted over large areas. It is a popular vegetable in Chinese cooking. In addition, it is often used to prepare health beverages and foods. At present, scientific information reported on chemical and biological properties of MCV remains limited. Zhao (2005) reported crude saponins in MCV could significantly lower the blood glucose level in alloxan-induced diabetic mice. A previous study (Yuan, Gu, Tang, & Wasswa, 2008) showed the filtrate of MCV aqueous extract through ultrafiltration (UF) membrane with 10 kDa molecular weight cut-off (MWCO) had significant hypoglycemic effect in alloxan-induced diabetic mice, while the retentate obtained after UF had no effect on the blood glucose level in diabetic mice. The retentate is rich in protein, making it one of the promising sources of vegetable proteins. Enzymatic hydrolysis has been widely applied to improve and upgrade the functional and nutritional properties of food proteins. Various physiological activities have been detected in the hydrolysates derived from the proteolytic hydrolysis of many food proteins, such as antimicrobial, immunomodulatory, antihypertensive, antioxidant, opioide and mineral binding (Korhonen & Pihlanto, 2003).

Although different types of synthetic oral hypoglycemic agents and insulin are available for the treatment of diabetes mellitus (DM), insulin cannot be taken orally and the synthetic agents can produce serious side effects and toxicity (Akhtar & Iqbal, 1991; Tolman & Chandramouli, 2003; Yamamoto, Nakajima, Yamazaki, & Yokoi, 2001). Therefore, search for safe and effective agents has continued to be an important area of active research. There is a growing interest to identify natural antidiabetic agents in medicinal plants, including several species of the same genus in Cucurbitaceace family such as *M. charantia* (Ahmed, Lakhani, Gillett, John, & Raza, 2001; Cakici et al., 1994) and *Momordica cymbalaria* (Rao, Kesavulu, & Apparao, 2001, 2003). Most of these studies focused on the hypoglycemic fractions directly extracted from natural plants, such as aqueous extracts (Chaturvedi, George, Milinganyo, & Tripathi, 2004; Mahomoodally, Gurib–Fakim, & Subratty, 2007; Raza, Ahmed, John, & Sharma, 2000), saponions (Matsuda et al., 1998; Ng, Wong, Li, & Yeung, 1986) and peptides (Khanna, Jain, Panagariya, & Dixit, 1981; Nag, Medicherla, & Sharma, 2000). However, little information exists about the hypoglycemic effect of protein hydrolysates in the plants.

In the present study, the hydrolyzing effect of six proteases on MCV protein was compared and Alcalase was chosen to produce MCV protein hydrolysates with hypoglycemic effect. The hydrolysis conditions using Alcalase were optimized by RSM.

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2. Materials and methods

2.1. Materials and chemicals

Fresh fruits of MCV were collected in July 2006 locally and authenticated by Jiangsu Academy of Agricultural Sciences. Neutrase 0.8L, Alcalase 2.4L, Trypsin and Protamex were obtained from Novo Enzymes (Bagsvaerd, Denmark). Pancreatin and Papain were supplied by Deyang Biochemical Products Company (Sichuan, China). Glibenclamide was purchased from Tianjin Pacific Pharmaceutical Co., Ltd. (Tianjin, China). Alloxan was purchased from Sigma Co. (St. Louis, MO, USA). The other chemicals and reagents used were of food or analytical grade quality.

2.2. Preparation of protein isolates

Fresh MCV fruits were washed thoroughly. The fruits were sliced into approximately 1.5 in. pieces. To 2 kg of the sliced fruits, 4 L of distilled water was added, blended and then stirred for 8 h at 25°C. This process was repeated three times. The resultant slurry was centrifuged at 4000*g* for 20 min. The supernatant was passed through a microfiltration (MF) membrane having a pore size of 0.2 m (Tianjin Motian Membrane Engineering and Technology Co., Ltd., Tianjin, China). The filtrate was subsequently passed through a UF membrane with 10 kDa MWCO (Tianjin Motian Membrane Engineering and Technology Co., Ltd., Tianjin, China) to remove compounds whose molecular weight (MW) was less than 10 kDa (MC1). The retentate obtained after UF was adjusted to pH 4.0 with 0.5 M HCl and centrifuged at 8000*g* for 20 min at 4°C. The precipitates were washed three times with distilled water (pH 4.0), dispersed in a small amount of distilled water, and adjusted to pH 7.0 with 0.1 M NaOH. The dispersed product was then freeze-dried.

2.3. Hydrolysis of the protein by different proteases

MCV protein was dissolved as a 5% (w/v) solution in 40 ml distilled water. The pH was adjusted using 0.3 M NaOH according to the hydrolysis conditions for each enzyme (see Table 1). The suspension was incubated for 30 min at optimum temperature, depending on the enzyme used, and continuously stirred.

Six proteases (Alcalase 2.4L, Papain, Protamex, Trypsin, Pancreatin and Neutrase) were used under the optimum conditions (Table 1). The reaction was initiated by the addition of the enzyme to give a final enzyme/substrate ratio (*E*/*S*) of 2:100 (w/w). The pH of the mixture was kept constant by continuous addition of 0.2 M NaOH. After 4 h hydrolysis, the proteases were inactivated by heating at 95 °C for 15 min. The reaction mixture was then centrifuged at 3000*g* for 20 min to remove insoluble substrate fragments. The supernatant was concentrated, freeze-dried and stored at −20 °C until required further analysis. The hydrolysates obtained were analyzed for protein recovery (Kjeldahl $N \times 6.25$).

Table 1
Conditions and protein recovery of the hydrolysis of MCV protein with different proteinase

Proteases	*T* (°C)	pH	Protein recovery (%, w/w)
Trypsin	37	8.0	64.23
Pancreatin	37	8.0	74.22
Neutrase	50	7.0	45.22
Papain	50	7.0	45.33
Protamex	45	7.0	52.46
Alcalase 2.4L	60	8.0	80.33

2.4. Determination of degree of hydrolysis

Degree of hydrolysis (DH) is the percentage ratio between the number of peptide bonds cleaved (h) and the total number of peptide bonds in the substrate studied (h_{tot}). The DH was determined based on the consumption of base (NaOH) by the pH-stat method (Adler-Nissen, 1986). The percent DH was calculated by the following equation:

$$\mathrm{DH}(\%) = \frac{BN_b}{M_p \alpha h_{tot}} \times 100$$

where B is the base consumption in ml; N_b is base normality; α is the average degree of dissociation of the α-NH2 groups in the protein substrate; M_p is the mass (g) of the protein ($N \times 6.25$), and h_{tot} is the total number of peptide bonds available for proteolytic hydrolysis (7.8 m equiv/g).

2.5. Hypoglycemic effect

2.5.1. Animals

Male Kunming mice weighing 25–30 g were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All mice were maintained in plastic cages under standard environmental conditions of temperature, relative humidity and dark/light cycle. Mice were fed on a standard chow diet and water ad libitum. The mice were used for experimentation after a 6-day acclimatization period. All the experiments were done during daytime. The guidelines for the care of animals were strictly followed throughout the study.

2.5.2. Experimental induction of diabetes in mice

After an 18 h fasting, mice were intraperitoneally injected with ice cold alloxan monohydrate (Sigma chemicals, St. Louis, MO, USA) freshly dissolved in distilled water (2%) at a dose of 200 mg/kg bw (Zheng, 1999). After one week, the fasting blood glucose (FBG) level of mice was measured and only mice with FBG level of between 13 mmol/l and 20 mmol/l were used for the experiments.

2.5.3. Estimation of blood glucose level

Blood samples were collected from the mice tail tip and blood glucose levels (BGL) were estimated using an electronic glucometer (Roche Diagnostics GmbH, Mannheim, Germany).

2.5.4. Hypoglycemic effect in alloxan-induced diabetic mice

Normal and diabetic mice were randomly divided into groups of 12 mice each. After an overnight fast of the mice, the FBG level (0 h) was measured. Then distilled water, samples tested and a reference drug (glibenclamide) were orally administered to the different groups. BGL was determined at 0, 2 and 4 h after administration.

2.6. Optimization of enzymatic hydrolysis

Response surface methodology (RSM) was used to optimize the hydrolysis conditions of MCV protein. Three factors (*E*/*S*, pH and temperature) and the levels at which they were employed are presented in Tables 2a and 2b. The levels were adopted and coded −1, 0 and +1.

The response DH was analyzed using the SAS (Statistical Analysis System Institute Inc., 1989, Cary, NC, USA) program. A quadratic polynomial regression model was assumed for predicting the responses. The model proposed for the response is given below

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2$$

Table 2a
Independent variables and their levels in optimization of hydrolysis for the MCV protein

Independent variables	Symbol	Coded variable levels		
		−1	0	1
[*E*]/[*S*] (%, w/w)	X_1	1	2	3
pH	X_2	8	9	10
T (°C)	X_3	50	55	60

Table 2b
RSM test design and values for the response variable, degree of hydrolysis

Run	Independent variables[a]			Response[b]
	X_1	X_2	X_3	*Y*
1	−1	−1	0	12.62
2	−1	1	0	12.95
3	1	−1	0	12.38
4	1	1	0	13.25
5	0	−1	−1	10.38
6	0	−1	1	12.05
7	0	1	−1	11.96
8	0	1	1	13.49
9	−1	0	−1	11.81
10	1	0	−1	12.65
11	−1	0	1	13.03
12	1	0	1	14.21
13	0	0	0	14.50
14	0	0	0	14.57
15	0	0	0	14.57

[a] Independent variables X_1, X_2 and X_3 represent enzyme/substrate ratio (*E*/*S*), pH and temperature (*T*), respectively.
[b] Response *Y* represents % Degree of Hydrolysis (DH).

where X_1, X_2 and X_3 are the independent variables. The model goodness-of-fit was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). The response surface and contour plots were developed using the fitted full quadratic polynomial equations, obtained by holding one of the independent variables at a constant value and changing the levels of the other two variables.

2.7. Statistical analysis

All data were expressed as mean ± SD for all experiments and a two-tailed student's *t*-test was used to calculate the significant difference between groups and among group means. A *p*-value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Selection of enzyme for hydrolysis of MCV protein

Different researchers had various criteria to select suitable enzyme used for hydrolysis of protein (Baek & Cadwallader, 1995; Kristinsson & Rasco, 2000a; Simpson, Nayeri, Yaylayan, & Ashie, 1998; Xia, Wang, & Xu, 2007). In this study, the selection criterion was the ability of enzyme to reach a high degree of hydrolysis at a low concentration and hypoglycemic effect of hydrolysates.

Although many factors affected the hydrolysis yield, the type of enzyme used had the greatest effect on the yield and properties of the final product. The hydrolysis of MCV protein with different enzymes proceeded at a rapid rate during the initial 30 min and then slowed down thereafter (Fig. 1), indicating that maximum cleavage of peptides occurred within 30 min of hydrolysis. Similar results have been reported in fish protein (Kristinsson & Rasco, 2000b) and whey protein (Mutilangi, Panyam, & Kilara, 1995). Among the

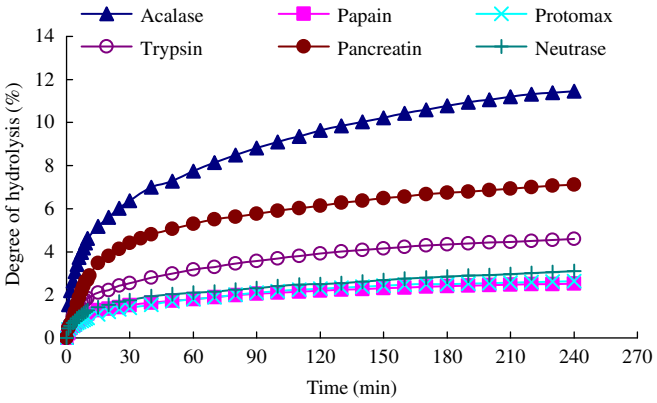

Fig. 1. Enzymatic hydrolysis of MCV protein with different proteases at enzyme/substrate ratio of 2:100 (w/w) and MCV protein concentrations at 5%.

proteinases used, Alcalase 2.4L exhibited the highest values in terms of DH and protein yield (Table 1), followed by Pancreatin. High protein recovery by Alcalase and its low cost may provide an incentive for using it in commercial operations. However, taking into account the purpose of hydrolysis which was to produce hydrolysates with hypoglycemic effect, the hypoglycemic effect of protein hydrolysates prepared by Alcalase and Pancreatin was evaluated in order to have a choice for the most suitable proteinase.

The physiological function of protein hydrolysates is related to various factors, including the protein source and the enzyme used (Fairclough, Hearty, Silk, & Clark, 1980; Keohane, Grimble, Brown, Spiller, & Silk, 1985). The hypoglycemic effect of Alcalase and Pancreatin MCV protein hydrolysates was determined in alloxan-induced diabetic mice (Table 3). The results showed both Alcalase and Pancreatin MCV protein hydrolysates significantly lowered the BGL in diabetic mice, while the native MCV protein had no effect on the BGL in diabetic mice, indicating that it was peptide(s) produced during hydrolysis that caused the hypoglycemic effect. Moreso, Alcalase based hydrolysates had a stronger hypoglycemic effect than Pancreatin based hydrolysates at the same dose. Alcalase based MCV protein hydrolysates produced a reduction in BGL of 46.15% and 52.59% after 2 and 4 h of oral administration, respectively. Pancreatin based MCV protein hydrolysates produced a reduction in BGL of 46.15% and 52.59% after 2 and 4 h of oral administration, respectively. Therefore, Alcalase was chosen to produce MCV protein hydrolysates with hypoglycemic effect.

3.2. Optimization of enzymatic hydrolysis

RSM was used to optimize protein hydrolysis using Alcalase. The response surfaces were used to study the effects of various parameters on Alcalase hydrolysis. Table 2b shows the responses of the dependent variable degree of hydrolysis. The application of RSM yielded the following model equation:

$$Y = 14.53 + 0.51X_1 + 0.78X_2 + 0.75X_3 - 0.64X_1^2 - 1.59X_2^2 - 0.97X_3^2 - 0.37X_1X_2 + 0.09X_1X_3 - 0.04X_2X_3$$

The results of the analysis of variance (ANOVA) for DH, shown in Table 4, demonstrate that the statistical model is significant at a 99% confidence level ($p < 0.01$). The total determination coefficient, $R^2 = 0.9988$ implies that the regression models explained the reaction well. The model was considered adequate with satisfactory R^2 values (>0.85). As the test of lack of fit hypothesis was not significant ($p > 0.05$) in model equation, the model was fitted to the DH data. A good fit means that the generated models adequately explained the data variation and significantly represented the actual relationships between the reaction parameters.

Table 3
Effect of Alcalase and Pancreatin hydrolysates on blood glucose level in diabetic mice

Group	Dosage (mg/kg)	Blood glucose level (mmol/l)		
		0 h	2 h	4 h
Normal control	–	4.23 ± 0.70	4.39 ± 0.66	4.23 ± 0.57
Diabetic control	–	16.13 ± 2.99	15.51 ± 2.93	15.32 ± 2.93
MCV protein	100	15.42 ± 3.21	16.21 ± 28	15.42 ± .99
Alcalase hydrolysate	100	15.31 ± 3.28	9.23 ± 1.86[a]	8.62 ± 1.49[b]
Pancreatin hydrolysate	100	15.53 ± 3.20	10.42 ± 1.75[a]	9.72 ± 1.62[a]
Glibenclamide	20	15.73 ± 3.16	8.54 ± 1.79[a]	8.02 ± 1.37[b]

Values are given as mean ± SD for 12 mice in each group.
[a] $P < 0.01$, compared with initial value.
[b] $P < 0.001$, compared with initial value.

Table 4
ANOVA results of DH as affected by enzyme/substrate ratio, pH and temperature during optimization experiments using Alcalase

Source	Degree of freedom	Sum of squares	Mean square	*F*-value	*p*-value
Model	9	24.74	2.75	475.61	<.0001
Linear	3	11.39	3.80	656.68	<.0001
Quadratic	3	12.79	4.26	737.47	<.0001
Interactions	3	0.57	0.19	32.68	0.0010
Residual	2	0.0026	0.0013		
Lack of fit	3	0.0263	0.0088	6.74	0.1319
Pure error	5	0.0289	0.0058		
R^2		0.9988			

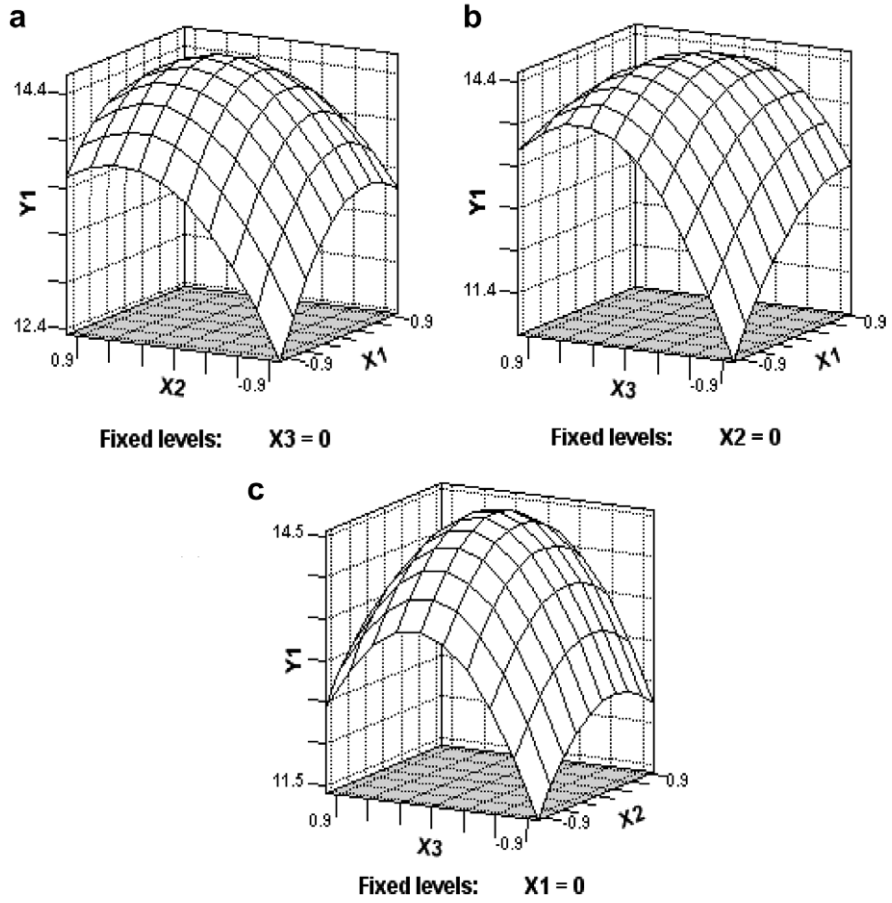

Fig. 2. Response surface plots for degree of hydrolysis as a function of (a) enzyme/substrate ratio and pH (b) enzyme/substrate ratio and temperature (c) pH and temperature. Other factors were set at their optimal values.

Three-dimension response surface plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent one. The results of DH affected by *E*/*S*, pH and temperature are shown in Fig. 2. The independent variables and maximum predicted values from the figures correspond with the optimum values of the dependent variables (responses) obtained by the equations. The optimum conditions for *E*/*S*, pH and temperature obtained using RSM with regard to DH were 2.37, 9.2, and 57 °C, respectively.

4. Conclusions

Compared with Pancreatin based MCV protein hydrolysates, Alcalase based MCV protein hydrolysates provided a higher DH, protein recovery and hypoglycemic activity. The optimum conditions for *E*/*S*, pH and temperature obtained using RSM with regard to DH were 2.37, 9.2, and 57 °C respectively. Future studies will focus on the purification of hypoglycemic peptide(s) from the hydrolysates. The hydrolysates provide a versatile supply of the benefits of MCV proteins and can be incorporated as a supplement in health-care food, drugs and/or combined with other hypoglycemic drugs.

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