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Enhancing Extraction Yield of Chlorella Extract by Enzyme Treatment

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An efficient production method of chlorella extract was developed by enzymatic treatment using cell lytic and proteolytic enzymes. The suitable dosage of Tunicase, a cell lytic enzyme, was found to be 1.0% (w/w). Proteolytic enzymes were screened to obtain high chlorella growth factor (CGF) index, which indicates crude CGF content and solid recovery. Among the seven tested proteases, Esperase, whose optimal dosage was 1.0% (w/w), was selected. By co-treatment using optimal dosages of Tunicase and Esperase, the highest CGF index and solid recovery were obtained. The CGF index and solid recovery of co-treatment were remarkably enhanced by 250 (4.36 → 15.21) and 220% (12.65% → 40.15%), respectively, than those of the non-treated extracts.

Key words: *chlorella extract, enzymatic hydrolysis, solid recovery*

Chlorella vulgaris, one of the unicellular green algae, has been found to contain highly nutritious substances exerting various beneficial biological effects. In particular, the hot-water extract of *C. vulgaris* is known to be a new type of biological response modifier, which exhibits various immunostimulant activities including the augmentation of resistance against bacteria and virus infections and the suppression of tumor growth [Konishi *et al.*, 1985; Hasegawa *et al.*, 1994; Hasegawa *et al.*, 1997]. When the hot-water extract was given subcutaneously to mice or orally to rats that had been rendered neutropenic by the cyclophosphamide treatments, an accelerated recovery of the neutrophils and the restoration of protection against infection with *E. coli* were observed [Konishi *et al.*, 1990; Hasegawa *et al.*, 1990]. This extract contained a chlorella growth factor (CGF) as well as a variety of substances including amino acids, peptides, proteins, vitamins, sugars, and nucleic acids [Han *et al.*, 2002; Hidaka *et al.*, 2004]. CGF, the component peculiar to chlorella extract, was a water-soluble nucleotide-peptide complex containing sulfur and showed a molecular mass of 5~10 kDa [Han *et al.*, 2002]. Recently, a great deal of attention has been focused on the chlorella extract as a food additive due to its various physiological functions. The chlorella extract is generally obtained *via* a simple extraction using hot water [Hasegawa *et al.*, 1994; Han *et al.*, 2002]. Despite its simplicity, this process has some disadvantages such as low extraction yield and loss of the active ingredients. However, little effort has been made to develop an efficient production process of the chlorella extract. The present approach is an attempt to develop an efficient production method of the chlorella extract. To increase the extraction efficacy, enzyme treatment was performed in combination with a simple extraction method. The strategy of the enzyme treatment involved lysing of the chlorella cell wall by the cell lytic enzyme, followed by the hydrolysis of the chlorella protein for the release of CGF using the proteolytic enzyme. In the hydrolysis of the chlorella cell wall and protein, effects of the enzyme treatments during the hydrolysis of chlorella cell wall and protein on the CGF content and the extraction yield were investigated.

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Materials and Methods

Materials. The dried *C. vulgaris* powder was purchased from Yaeyama Shokusan Co. Ltd. (Okinawa, Japan). Tunicase FN (Daiwa Kasei K.K., Osaka, Japan) was used in the lysis of the cell wall. The commercial proteases, Alcalase, Esperase, Flavourzyme, Kojizyme, Neutrase, and Protamex, were obtained from Novozymes (Bagsvaerd, Denmark), and Collupulin MG was a product of DSM Corp. (Heerlen, Netherlands).

Enzymatic hydrolysis of chlorella. Dried chlorella powder was suspended in 100 mL of distilled water at a concentration of 2% (w/w). The reaction pH was adjusted

to pH 8.0 for the cell lytic enzyme treatment, and then to the optimal pH of each proteolytic enzyme with 1 N NaOH or HCl. Enzyme hydrolysis was divided into two steps. In the first step, Tunicase was added. The cell wall lysis was carried out at 40°C for 3 h. In the second step, proteolytic enzyme was used. The hydrolysis was performed at the optimal conditions for 3 h. In all cases, the enzyme dosage was measured based on the chlorella powder weight; Tunicase and proteolytic enzyme were added as enzyme/chlorella ratio of 0-2.5% (w/w), respectively. After all enzymatic treatments, the reaction suspension was boiled for 20 min and centrifuged, and the supernatant was used for analysis.

Analytical methods. The extraction yield was expressed as solid recovery and calculated as ratio weight of the lyophilized extract to the weight of the chlorella powder. Although the content of crude CGF is generally determined using a spectrophotometer at the absorbance at 260 nm in the related industries [Han *et al.*, 2002], in this work dimensionless CGF index was defined as the total absorbance of the crude extracted CGF per 1 g chlorella powder and calculated according to the following equation:

$$\text{CGF index} = A \times D \times W_1/W_2,$$

where A is the A_{260} of extract solution supernatant, D is dilution factor, W_1 and W_2 are weights of the lyophilized extract and chlorella powder, respectively.

Results and Discussion

Effect of cell lytic enzyme treatment. Effects of the cell lytic enzyme treatment on the CGF content in the chlorella extract and solid recovery of the chlorella extract were examined. The crude CGF content of the chlorella extract was expressed as CGF index in this study. Tunicase, originating from *Arthrobacter* sp. ATCC 21712, which shows the cell lytic activity at pH 8.0, was used. To obtain the optimum concentration of the cell lytic enzyme, chlorella suspension (2%) was treated with Tunicase at different concentrations (0-2.5%), on a solid weight basis. Hydrolysis at 40°C for 3 h and hot-water extraction resulted in the increased CGF index and solid recovery as the Tunicase dosage increased, then reached plateau at 0.5 and 1.0% treatments, respectively (Fig. 1). At 1% Tunicase, the CGF index and solid recovery were notably improved in comparison to those obtained without the enzyme treatment. Therefore, the suitable dosage of Tunicase was found to be 1.0%. The increase of solid recovery and CGF index must have resulted from the release of the intracellular components including the CGF complex obtained by the cell lysis. Changes in the CGF index and solid recovery with the reaction time at 1.0% Tunicase indicated that CGF index scarcely changed, whereas the solid recovery increased with increasing reaction time. This result is in accordance with the report that treatment of the cell lytic enzyme was moderately

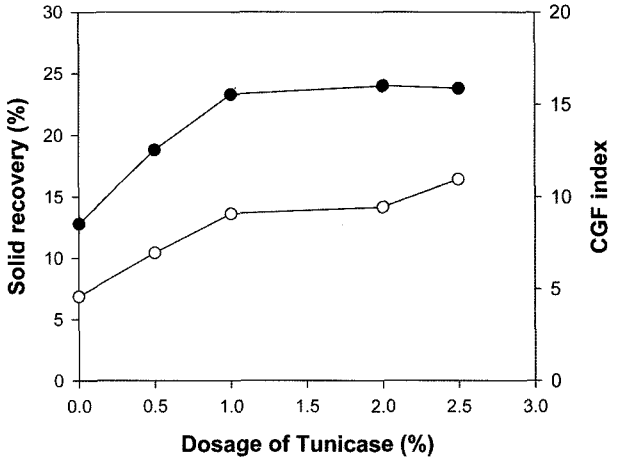

Fig. 1. Effect of dosage of Tunicase on solid recovery (●) and chlorella growth factor (CGF) index (○). Reaction conditions: temperature; 40°C, pH 8.0, reaction time; 3 h.

Table 1. Effect of proteolytic enzyme treatment on the production of chlorella extract

Commercial name	Optimal conditions		CGF index[1)]	Solid recovery[1)] (%)
	pH	Temp. (°C)		
DSM Corp. (Netherlands)				
Collupulin	5.0~7.0	50~70	11.65	22.3
Novozymes (Denmark)				
Alcalase	6.5~8.5	60	16.30	21.8
Esperase	7.5~10.0	50~70	16.88	38.8
Flavourzyme	5.0~7.0	50	13.65	15.0
Kojizyme	5.5~6.5	50	14.00	22.0
Neutrase	5.5~7.5	45~55	13.43	33.0
Protamex	6.0	50	13.98	36.5

[1)]Calculation methods for the CGF index and solid recovery are described in Materials and Methods.

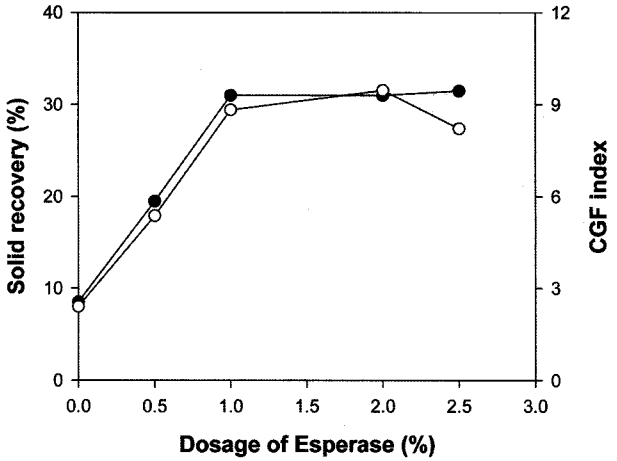

Fig. 2. Effect of dosage of Esperase on solid recovery (●) and chlorella growth factor (CGF) index (○). Reaction conditions: temperature; 50°C, pH 8.0, reaction time; 3 h.

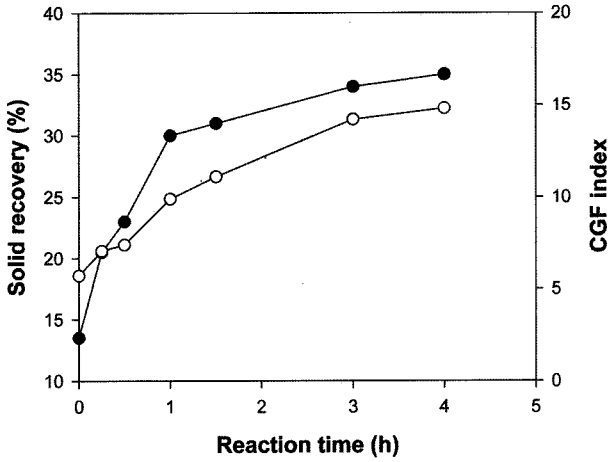

Fig. 3. Typical time course of solid recovery (●) and chlorella growth factor (CGF) index (○) during Tunicase and Esperase co-treatment. Reaction conditions: enzyme dosage, 1% Tunicase and 1% Esperase; temperature, 50°C, pH 8.0.

effective in the production of the yeast extract [Kim *et al.*, 2001].

Effect of proteolytic enzyme treatment. To choose the suitable proteolytic enzyme, chlorella suspension hydrolyzed with Tunicase was treated with commercially available proteases at 2% concentration, on a solid weight basis. Based on the CGF index and solid recovery of each protease, Esperase was selected for the hydrolyzation of the chlorella protein (Table 1). The CGF index and solid recovery with different concentration of Esperase added at 0-2.5% (on a solid weight basis) are shown in Fig. 2. Treatment for 3 h showed CGF index and solid recovery were strongly depended on the Esperase concentration in the range of 0-1%. In 1% Esperase treatment, the solid recovery and CGF index increased to 265 and 268%, respectively, in comparison with those of non-Esperase treatment. The suitable dosage of Esperase was found to be 1.0%. The changes in CGF index and solid recovery of 1.0% Esperase with the reaction time indicated that both CGF index and solid recovery significantly increased with the increasing reaction time (data not shown). The CGF index and solid recovery were improved by 55 and 108%, respectively, after 3 h hydrolysis. This result indicated that the protease treatment degraded the chlorella protein, which in turn increased the solubility of the protein, in accordance with the findings of other works on yeast cells and hemoglobin [Chae *et al.*, 2001 and In *et al.*, 2002]. In particular, enhancement of the CGF index was due to the hydrolysis of the protein part in the nucleotide-peptide complex (crude CGF) and an increase in the solubility of the effective component such as heme-iron enriched peptide produced from hemoglobin [In *et al.*, 2003].

Effect of enzyme treatment sequence. To find the best enzyme treatment sequence, the chlorella suspension was treated with different sequences of the cell lytic enzyme

Table 2. Effect of enzyme treatment methods on production of chlorella extract

Enzyme treatments	Reaction conditions	CGF index[1)]	Solid recovery[1)] (%)
No treatment	Extraction (boiling, 20 min)	4.36 ± 0.18	12.65 ± 0.50
Single-step hydrolysis (T[2)] 1%)	Hydrolysis (pH 8.0, 40°C, 3 h) → Extraction (boiling, 20 min)	9.17 ± 0.54	24.50 ± 0.71
Single-step hydrolysis (E[3)] 1%)	Hydrolysis (pH 8.0, 50°C, 3 h) → Extraction (boiling, 20 min)	7.71 ± 1.56	28.00 ± 4.24
Two-step hydrolysis (T 1% → E 1%)	Hydrolysis [pH 8.0, (40°C, 3 hr → 50°C, 3 h)] → Extraction (boiling, 20 min)	13.10 ± 1.05	38.8 ± 2.10
Single-step hydrolysis (T 1% + E 1%)	Hydrolysis [pH 8.0, 50°C, 3 h] → Extraction (boiling, 20 min)	15.21 ± 1.40	40.15 ± 4.45

[1)]Calculation methods for the CGF index and solid recovery are described in Materials and Methods.
[2)]T: Tunicase.
[3)]E: Esperase.

and protease. Enzymatic hydrolysis was carried out by either two-step reaction or a simultaneous reaction with Tunicase and Esperase. The former was conducted in two steps: the first treatment with Tunicase, followed by the second hydrolysis with Esperase, and the latter was the co-treatment with Tunicase and Esperase. The changes of solid recovery and CGF index with reaction time for the simultaneous treatment of Tunicase and Esperase are presented in Fig. 3. The CGF index and solid recovery at various Tunicase and Esperase treatment conditions including the single treatment of each enzyme are summarized in Table 2. The single-step simultaneous hydrolysis with Tunicase and Esperase was the most effective enzyme treatment method. The highest CGF index of 15.21 and solid recovery of 40.15% was obtained by the co-treatment with Tunicase and Esperase. CGF index and solid recovery of the co-treatment were remarkably enhanced to around 250 (4.36 → 15.21) and 220% (12.65% → 40.15%), respectively, than those obtained from non-treatment. The increase in the solid recovery by the enzyme treatment was very similar to the other works on defatted soy [Chae *et al.*, 1997] and animal by-products from food processing [Chae and In, 2004]. In conclusion, usage of the cell lytic enzyme and proteolytic enzyme and co-treatment of these enzymes were effective for the production of chlorella extract.

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References

Chae HJ, In MJ, and Kim MH (1997) Optimization of enzymatic treatment for the production of hydrolyzed vegetable protein. *Korean J Food Sci Technol* **29**, 1125-1130.

Chae HJ, Joo H, and In MJ (2001) Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. *Bioresource Technol* **76**, 253-258.

Chae HJ and In MJ (2004) Study on utilization of animal by-products from food processing by enzyme treatment. *J Korean Soc Appl Biol Chem* **47**, 197-201.

Han JG, Kang GG, Kim JK, and Kim SH (2002) The present status and future of Chlorella. *Food Sci Ind* **6**, 64-69.

Hasegawa T, Yoshikai Y, Okuda M, and Nomoto K (1990) Accelerated restoration of the leukocyte number and augmented resistance against *Escherichia coli* in cyclophosphamide-treated rats orally administrated with a hot water extract of *Chlorella vulgaris*. *Int J Immunopharmacol* **12**, 883-891.

Hasegawa T, Okuda M, Nomoto K, and Yoshikai Y (1994) Augmentation of the resistance against *Listeria monocytogenes* by oral administration of a hot water extract of *Chlorella vulgaris* in mice. *Immunopharmacol Immunotoxicol* **16**, 191-202.

Hasegawa T, Kimura Y, Hiromatsu K, Kobayashi N, Yamada A, Makino M, Okuda M, Sano T, Nomoto K, and Yoshikai Y (1997) Effect of hot extract of *Chlorella vulgaris* on cytokine expression patterns in mice with murine acquired immunodeficiency syndrome after infection with *Listeria monocytogenes*. *Immunopharmacology* **35**, 273-282.

Hidaka S, Okamoto Y, and Arita M (2004) A hot water extract of *Chlorella pyrenoidosa* reduces body weight and serum lipids in ovariectomized rats. *Phytother Res* **18**, 164-168.

In MJ, Chae HJ, and Oh NS (2002) Process development for heme-enriched peptide by enzymatic hydrolysis of hemoglobin. *Bioresource Technol* **84**, 63-68.

In MJ, Kim DC, Chae HJ, and Oh NS (2003) Effects of degree of hydrolysis and pH on the solubility of heme-iron enriched peptide in hemoglobin hydrolysate. *Biosci Biotechnol Biochem* **67**, 356-367.

Kim DC, Chae HJ, Oh NS, and In MJ (2001) Effect of cell lytic enzyme on the production of yeast extract. *J Korean Soc Appl Biol Chem* **44**, 273-275.

Konishi F, Tanaka K, Himeno K, Taniguchi K, and Nomoto K (1985) Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CV): resistance to Meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. *Cancer Immunol Immunother* **19**, 73-78.

Konishi F, Tanaka K, Kumanoto S, Hasegawa T, Okuda M, Yano I, Yoshikai Y, and Nomoto K (1990) Enhanced resistance against *Escherichia coli* by subcutaneous administration of the hot-water extract of *Chlorella vulgaris* in cyclophosphamide-treated mice. *Cancer Immunol Immunother* **32**, 1-7.