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Optimization of Enzymatic Hydrolysis with Cryotin F on Antioxidative Activities for Shrimp Hydrolysate Using Response Surface Methodology

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Abstract

Cryotin F could be used for hydrolyzing shrimp byproducts into bioactive ingredients, which could be used as value-added products. The objective of this study was to investigate the optimum condition for antioxidative activities of the enzymatic hydrolysate produced with Cryotin F using response surface methodology with central composite rotatable design. Shrimp byproducts (shells and heads) were hydrolyzed with Cryotin F. The experimental ranges of the independent variables for 20 experimental runs were 28.2~61.8°C reaction temperature, pH 6~10 and 0.5~5.5% enzyme concentration. The degree of hydrolysis for the reaction products was measured. Their antioxidative activities were measured using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity and Fe-chelating activity. The experimental method with central composite rotatable design was well designed to investigate the optimum condition for biofunctional ingredients with antioxidative activities using Cryotin F because of their high R^2 values of 0.97 and 0.95 for DPPH-scavenging activity and Fe-chelating activity, respectively. Change in enzyme concentration did not significantly affect their antioxidative activities ($p<0.05$). Both DPPH scavenging activity and chelating activity against Fe for the enzyme hydrolysates were more affected by the pH of enzyme hydrolysis than by their action temperature. DPPH-scavenging activity was higher at acidic pH than alkali pH, while chelating activity against Few was inversely affected. Hydrolysate of shrimp byproducts showed high antioxidative activities depending on the treatment condition, so the optimum treatment of enzymatic hydrolysate with Cryotin F and other proteases can be applied to shrimp byproducts (shells) and other protein sources for biofunctional ingredients.

Key words: shrimp byproduct, antioxidative activity, Cryotin F, optimization, response surface methodology

INTRODUCTION

Marine byproducts are a potential source of antioxidant peptides, which may have important implications in human health. Shrimp byproducts consisting of shells and tails have high content of residual protein and hence ideal for preparing bioactive peptides. Shrimp byproducts utilization would not only add value to shrimp industry but could provide a new source of health-promoting antioxidants (1).

Several proteases have been used for preparing protein hydrolysates with many desired properties. Alcalase is a protease which is most commercially used (2). Cryotin F, an enzyme from cod intestine contains several digestive enzymes, but few researchers have studied the properties of hydrolysates prepared from using this enzyme (3).

Response surface methodology has been used to investigate the optimum condition and the effects of the independent variables for several kinds of food processing including extraction, enzyme reaction, chemical reaction and others. One of the commonly used methods is central composite rotatable design (4).

Objective of this study was to investigate optimum conditions for antioxidative activities of shrimp hydrolysate produced with enzyme, Cryotin-F, using surface response methodology with central composite rotatable design.

MATERIALS AND METHODS

Sample preparation and chemicals

Shrimp byproducts, obtained as frozen state from a company in Florida, were washed with tap water. The washed shrimp byproducts were dried in a dry oven and

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ground with a miller. Cryotin F was obtained from North Ltd. (Reykjavik, Iceland). All other chemicals are analytical grade of Sigma Aldrich Chemicals.

Reaction condition

Two grams of dried shrimp powder was added to 20 mL of distilled water and homogenized for 1 min with Biohomogenizer (BioMixer 10810, Biospec Products Inc, Bartlesville, OK, USA). The experimental ranges of the independent variables for 20 experimental runs were 28.2～61.8°C reaction temperature, pH 6.0～10.0 and 0.5～5.5% enzyme condition as shown in Table 1. The reaction time was 20 min and enzyme deactivation was done by heating for 20 min at 90°C before and after enzyme reaction.

Degree of hydrolysis

The degree of hydrolysis of the reaction products were measured by pH-stat method of Holanda and Netto (5). Reaction pH was set to the value which was shown in Table 1 and the reaction was started by injecting the enzyme of the fixed amount. Simultaneously, the fixed pH was set by using 0.1 N NaOH. The added amount of 0.1 N NaOH was used to calculate the degree of hydrolysis of the formula of pH-stat method of Holanda and Netto (5).

DPPH assay of protein hydrolysates

The antioxidative activity against DPPH was measured by using the method of Guerard et al. (6). Residual of reaction solution was separated by using cheese cloth. Further separation was carried out by centrifuging at 2,000 rpm for 10 min at 4°C. Three mL supernatant was mixed with 4.5 mL MeOH and centrifuged. A 3 mL supernatant was used for the antioxidative activity. Sample solution was a mixture of 3 mL supernatant+ 0.150 mL DPPH·MeOH. Ten mg DPPH was solubilized into 50 mL methanol and used. Control was a mixture of 3 mL supernatant and 0.150 mL MeOH. Blank was the mixture of 3 mL MeOH : water (1.5:1) and 0.150 mL DPPH·MeOH. All solutions were covered with aluminum foil to protect from light. The reaction time was 30 min at room temperature. The absorbance was measured at 519 nm and % inhibition for antioxidative activity was calculated as the below formula.

$$\% \text{ inhibition} = \left(\frac{A_{Blank} - [A_{Sample} - A_{Control}]}{A_{Blank}} \right) \times 100$$

Chelating activity against Fe (%)

Chelating activity of protein hydrolysate against Fe is measured with a little modification from the method of Raghavan and Kristinsson (3). The 2.5 mL of protein hydrolysate solution was added into 12.5 mL MeOH. It was centrifuged at 2,000 rpm for 10 min ar 4°C and the supernatant was used. Two mM $FeCl_2$ and 5 mM ferrozine were prepared. To a 3 mL supernatant of sample solution, 0.1 mL $FeCl_2$ and 0.2 mL ferrozine were added, mixed and incubated at room temperature for 30 min. The absorbance of the solution was measured at 561 nm. For blank test, a mixture of 3 mL MeOH, 0.1 mL $FeCl_2$ and 0.2 mL ferrozine was used. For control test, a mixture of 3 mL MeOH, 0.1 mL $FeCl_2$ and 0.2

Table 1. Experimental numbers for three independent variables of central composite rotatable design and their response in terms of antioxidative activities

No	Reaction pH (X_1)	Reaction temp. (°C) (X_2)	Enzyme conc. (%) (X_3)	Degree of hydrolysis (%)	DPPH activity (%)	Chelating activity against Fe (%)
1	-1 (6.8)	-1 (35)	-1 (1.5)	8	82	23
2	1 (9.2)	-1 (35)	-1 (1.5)	11	39	73
3	-1 (6.8)	1 (55)	-1 (1.5)	4	78	34
4	1 (9.2)	1 (55)	-1 (1.5)	12	33	72
5	-1 (6.8)	-1 (35)	1 (4.5)	8	82	35
6	1 (9.2)	-1 (35)	1 (4.5)	21	39	67
7	-1 (6.8)	1 (55)	1 (4.5)	15	83	33
8	1 (9.2)	1 (55)	1 (4.5)	27	55	67
9	-1.682 (6.0)	0 (45)	0 (3.0)	27	92	6
10	1.682 (10.0)	0 (45)	0 (3.0)	24	14	80
11	0 (8.0)	-1.682 (28.2)	0 (3.0)	8	68	46
12	0 (8.0)	1.682 (61.8)	0 (3.0)	8	58	42
13	0 (8.0)	0 (45)	-1.682 (0.5)	4	56	64
14	0 (8.0)	0 (45)	1.682 (5.5)	20	66	54
15	0 (8.0)	0 (45)	0 (3.0)	14	59	52
16	0 (8.0)	0 (45)	0 (3.0)	14	59	58
17	0 (8.0)	0 (45)	0 (3.0)	16	62	64
18	0 (8.0)	0 (45)	0 (3.0)	14	61	53
19	0 (8.0)	0 (45)	0 (3.0)	13	69	60
20	0 (8.0)	0 (45)	0 (3.0)	14	63	57

mL ferrozine was used. Zero value was used with MeOH.

$$\% \text{ Metal chelation} = \left(\frac{A_{Blank} - (A_{Sample} - A_{Control})}{A_{Blank}} \right) \times 100$$

Statistical analysis

All experiments were carried out in triplicate. Regression analysis and analysis of variance (ANOVA) were conducted to examine the statistical significance at the 95% significant level by using SAS software program (Version 8.01, SAS Institute Inc., USA). The three dimensional graph was drawn with Maple 7 software program (Waterloo Maple Inc, Waterloo, Ontario, Canada).

RESULTS AND DISCUSSION

The antioxidant activity, namely DPPH radical scavenging activity and iron chelating ability, of hydrolyzed shrimp byproducts was studied using surface methodology. The concentration of enzyme, Cryotin-F, pH of hydrolysis and reaction temperature were used as independent variables. The degree of hydrolysis (DH), DPPH scavenging activity and iron chelation were dependent variables. The experimental values for three dependent variables on the three independent variables are shown in Table 1. The data was statistically analyzed to investigate the maximum antioxidative activity of the hydrolyzed solution with Cryotin-F.

Response surface methodology for hydrolysate of shrimp byproduct with Cryotin F

Table 1 was statistically analyzed using SAS programs and the results are shown in Table 2. For three dependent variables, there are several points for response surface methodology. Two main methods are 17 and 20 experimental points. In this study, 20 experimental points was tried with 3 more central points from 17 experimental points. The optimum condition for Cryotin F for tilapia hydrolysate has been reported to be 40～50°C, 2～4% enzyme concentration (3). Based on the results, the ranges used for this experiment were pH 6.0～10.0 for reaction pH, 28.2°C～61.8°C for reaction temperature, and 0.5～5.5% for enzyme concentration. The central point was pH 8.0, 45°C and 3.0%. The range of R^2 values for the experiment was 0.86～0.97, showing the proper method of response surface methodology for the optimization of the biofunctional ingredients of enzyme hydrolysate from shrimp byproducts. There are other studies which uses response surface methodology to investigate different variables affecting optimum enzyme hydrolysis. Simpson et al. (7) evaluated hydrolysates of fresh and frozen shrimp with chymotrypsin or trypsin. The hydrolysates prepared with both enzymes had high levels of glycine, proline, arginine, and valine. The optimum conditions for aroma quality using chymotrypsin were obtained with an enzyme to substrate (E : S) ratio of 0.25～0.3% for 2.5 hr at 35°C, while the optimum conditions with trypsin were 0.25～0.3% E : S ratio for 2.5～3.0 hr at 40°C. Cao et al. (8) investigated the optimum condition for protein hydrolysates from the head waste of *Penaensvannamei*, a Chinese sea water shrimp. Substrate concentration at 23% (w/v), pH at 7.85 and temperature at 50°C were found to be the optimal conditions to obtain a high degree of hydrolysis close to 45%. The autolysis reaction was nearly finished in the initial 3 hr.

Degree of hydrolysis of shrimp byproduct with Cryotin F

Fig. 1 is three dimensional figure which shows the effect of reaction pH and reaction temperature at the fixed value of 3% enzyme concentration from the formula (Y_{DH}=251.942292－43.306967×pH+2.526315×

Table 2. Results of response surface methodology on degree of hydrolysis, antioxidative activity on DPPH and chelating activity on Fe of enzyme hydrolysate from shrimp byproduct

Parameter	DF[1)]	Degree of hydrolysis		DPPH-activity		Fe-chelating-activity	
		Estimate[2)]	Pr>|t|[3)]	Estimate	Pr>|t|	Estimate	Pr>|t|
Intercept	1	146.659980	0.0439	211.474971	0.0354	-463.801094	0.0011
pH	1	-43.306967	0.0040	1.395263	0.9318	79.763609	0.0016
Temp	1	1.692179	0.1904	-2.820105	0.1177	5.044075	0.0257
Conc	1	-7.436860	0.3223	-15.403243	0.1459	15.221996	0.2125
pH×pH	1	2.526315	0.0031	-1.837119	0.0662	-3.226933	0.0113
Temp×pH	1	0.041667	0.6957	0.166667	0.2662	-0.135417	0.4326
Temp×Temp	1	-0.026136	0.0181	0.009299	0.4797	-0.040519	0.0211
Conc×pH	1	0.972222	0.1891	0.972222	0.3271	-1.875000	0.1203
Conc×Temp	1	0.133333	0.1384	0.200000	0.1078	-0.091667	0.5048
Conc×Conc	1	-0.543158	0.2219	0.104244	0.8586	0.494763	0.4754

[1)]DF means degree of freedom.
[2)]The values are the estimated value of the statistical result of SAS.
[3)]The probabilities for rejecting the null hypothesis for each parameter are shown in their values.

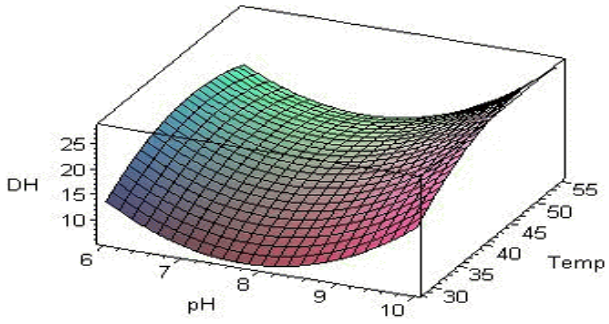

Fig. 1. Relationship between reaction pH and temperature on degree of hydrolysis by hydrolysis of shrimp by-product with 3% enzyme concentration of Croytin F.

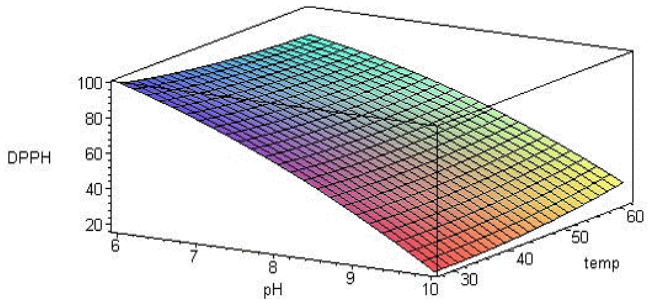

Fig. 2. Relationship between reaction pH and temperature on DPPH scavenging activity by hydrolysis of shrimp by-product with 3% enzyme concentration of Croytin F.

pH × pH − 0.026136 × Temp × Temp) which was obtained from Table 2. As shown in Fig. 1, degree of hydrolysis showed saddle shape, and acidic and alkali solution showed higher degree of hydrolysis. Degree of hydrolysis showed high value in the middle range of 40～45°C in reaction temperature.

Shahidi (9) mentioned that proteins from shellfish may be recovered using a base extraction or enzyme hydrolysis process. Gildberg (10) reported that preparation of fish protein hydrolysates is one of the most important commercial processes, but enzymic methods for accelerated fish ripening and selective tissue degradation are being recognized as interesting alternatives to conventional processing. Bataille and Bataille (11) mentioned that up to 70% of the theoretical protein content of shrimp waste was recovered by a boiling water extraction method. They investigated processing conditions such as time of digestion, volume and concentration of calcium hydroxide to optimize the yield of dry matter recovered on processing the shrimp waste.

Kechaou et al. (12) reported that amino acid quality and composition of enzymatic hydrolysates from cuttlefish (*Sepiaofficinalis*) and sardine (*Sardinapilchardus*) were compared by using the three proteases Protamex, Alcalase, and Flavourzyme by the pH-stat method (24 hr, pH 8.0, 50°C). The total amino acid content differed according to the substrate and the enzyme used. However, regardless of the raw material or the protease used, hydrolysis increased the level of essential amino acids in the hydrolysates. Raghavan and Kristinsson (3) reported that enzymatic hydrolysis decreased the size of tilapia protein hydrolysates and, in general, tilapia protein hydrolysates with low molecular weights were better antioxidants than those with high molecular weights.

Antioxidative activity of hydrolysate of shrimp by-droduct on DPPH

The data from Table 1 was statistically analyzed and the results are presented in Table 3, which shows the DPPH radical scavenging ability of enzyme hydrolysates. The meaningful data can be applied to make the relationship between reaction pH and reaction temperature for antioxidative activity on DPPH. The formula of "$Y_{DPPH} = 166.07658 + 1.395263 \times pH - 2.820105 \times Temp$" is obtained and the relationship is shown in Fig. 2. DPPH-scavenging activity of enzyme hydrolysate from shrimp byproduct was more affected by reaction pH than by reaction temperature, showing higher activity at low pH of hydrolysis.

In a similar experiment, Guerard et al. (6) reported that in the analysis of variance in response surface methodology (RSM), reaction pH and temperature (T) were the two most important factors during hydrolysis ($p<0.001$). They reported that the optimum condition for the hydrolysis conditions (temperature, pH and Alcalase® 2.4 L concentration) was pH 9.7, 66.2°C, enzyme concentration=68.1 Anson units (AU)/kg crude protein to obtain a hydrolysate with strong antioxidant activity measured using DPPH discoloration assay. Raghavan and Kristinsson (3) investigated the antioxidant activities

Table 3. The statistical result for DPPH-scavenging activity of protein hydrolysate of shrimp byproduct

Regression	DF[1)]	Type I sum of squares	R-Square	F value	Pr>F[2)]
Linear	3	6159.519984	0.9278	88.98	<.0001
Quadratic	3	120.187894	0.0181	1.74	0.2226
Crossproduct	3	128.500000	0.0194	1.86	0.2009
Total Model	9	6408.207878	0.9652	30.86	<.0001

[1)]DF means degree of freedom.
[2)]The probabilities for rejecting the null hypothesis for each parameter are shown in their values.

of alkali-treated tilapia protein hydrolysates. Protein isolates obtained using alkali solubilization at pH 11.0 and reprecipitation at pH 5.5 were hydrolyzed using five different enzymes, Cryotin F, Protease A Amano, Protease N Amano, Flavourzyme, and Neutrase, to 7.5, 15, and 25% degrees of hydrolysis (DH). They reported an increase in antioxidant activity of the hydrolysates with an increase in DH. Šližytė et al. (13) reported that the DPPH scavenging activity for antioxidative activity of hydrolysates could be due to the ability to scavenge lipid radicals.

In the further studies on the antioxidative activity of hydrolysates, Rhee et al. (14) isolated 4 antioxidative peptides from Korean rice wine. Among those 4 peptides of Ile-His-His, Val-Val-His(Asn), Leu-Val-Pro, and Leu(Val)-Lys-Arg-Pro, Ile-His-His showed the highest antioxidative activity. Arihara et al. (15) investigated the antioxidative activities of three antioxidative peptides which were isolated from a papain-treated hydrolyzate of pork actomyosin. Their sequences of amino acids for the peptides are Asp-Leu-Tyr-Ala, Ser-Leu- Tyr-Ala and Var-Trp. Based on our studies, further separation and characterization of antioxidative peptides are required.

Chelating activity of hydrolysate of shrimp bydroduct on Fe

The chelating activity of the hydrolysate from shrimp byproduct is shown in Table 1 and the data was statistically applied to obtain Table 2 which shows the significance level of the independent variables for the optimum chelating activity. The data of Table 2 can be applied to make Fig. 3 which shows the relationship between reaction pH and reaction temperature for chelating activity on Fe. Then the formula of "Y_{Fe} = -519.807404 +79.763609 × pH+5.044075 × Temp-3.226933 × pH × pH -0.040519 × Temp × Temp." is obtained and the relationship is shown in Fig. 3. Chelating activity of enzyme hydrolysate against Fe was more affected by reaction pH than by reaction temperature. The chelating activity is increased as pH is increased to alkaline pH.

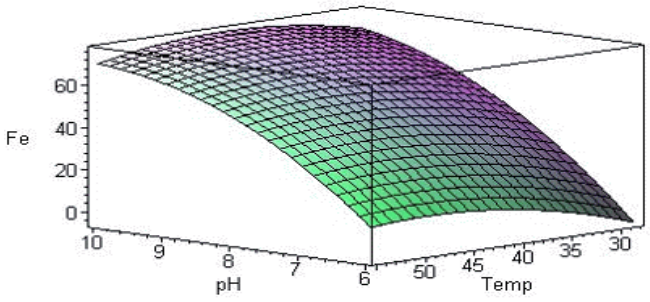

Fig. 3. Relationship between reaction pH and temperature on Fe chelating activity by hydrolysis of shrimp by-product with 3% enzyme concentration of Croytin F.

Raghavan and Kristinsson (3) reported that in an aqueous solution, hydrolysates prepared using Flavourzyme were most effective in chelating ferrous ions. Šližytė et al. (13) reported that the ability of fish protein hydrolysates to inhibit iron induced lipid oxidation was not influenced by time of hydrolysis. The low molecular peptides and amino acids formed during hydrolysis of shrimp byproducts with Cryotin F are expected to be responsible for their antioxidative activity. In addition, shrimp products contain astaxantin, so the amount and activity of phenolic compounds including astaxantin should be considered for the explanation of the total antioxidative activities.

CONCLUSION

Enzyme concentration did not significantly affect their antioxidative activities while reaction pH and reaction temperature affected their antioxidative activities to a significant level of 95%. Antioxidative activity on DPPH showed the 1st order formula, while the others showed the second order formula. Both DPPH-scavenging activity and Fe-chelating ability of enzyme hydrolysates were affected more by pH than by reaction temperatures. DPPH-scavenging activity was higher at acidic pH than alkali pH, while Fe-chelating ability was adversely affected. Shrimp byproduct hydrolysates showed high antioxidative activities depending on treatment condition, so the optimum treatment of enzymatic hydrolysate with Cryotin F and other proteases can be applied to shrimp byproduct and other protein sources to prepare bioactive ingredients. The scale up in the optimum condition is needed to produce the commercially-valuable products. Also, the chemical analyses for identifying the chemical structure of the bioactive components are required.

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REFERENCES

1. Gildberg A, Stenberg E. 2001. A new process for advanced utilisation of shrimp waste. *Process Biochemistry* 36: 809-812.
2. Mizani M, Aminlari M, Khodabandeh M. 2005. An effective method for producing a nutritive protein extract powder from shrimp-head waste. *Food Sci Technol Int* 11: 49-54.
3. Raghavan S, Kristinsson HG. 2008. Antioxidative efficacy

of alkali-treated tilapia protein hydrolysates: A comparative study of five enzymes. *J Agric Food Chem* 56: 1434-1441.
4. Triveni R, Shamala TR, Rastogi NK. 2001. Optimised production and utilisation of exopolysaccharide from *Agrobacterium radiobacter*. *Process Biochemistry* 36: 787-795.
5. Holanda HD, Netto FM. 2006. Recovery of components from shrimp (*Xiphopenaueskroyeri*) processing waste bye nzymatic hydrolysis. *J Food Sci* 71: c298-c303.
6. Guerard F, Sumaya-Martinez MT, Laroque D, Chabeaud A, Dufosse L. 2007. Optimization of free radical scavenging activity by response surface methodology in the hydrolysis of shrimp processing discards. *Process Biochemistry* 42: 1486-1491.
7. Simpson BK, Nayeri G, Yaylayan V, Ashie INA. 1998. Enzymatic hydrolysis of shrimp meat. *Food Chem* 61: 131-138.
8. Cao W, Zhang C, Hong P, Ji H. 2008. Response surface methodology for autolysis parameters optimization of shrimp head and amino acids released during autolysis. *Food Chem* 109: 176-183.
9. Shahidi F. 1995. Extraction of value-added components from shellfish processing discards. *Developments in Food Science* 37: 1427-1439.
10. Gildberg A. 1994. Enzymic processing of marine raw materials. *Process Biochemistry* 28: 1-15.
11. Bataille MP, Bataille PF. 2008. Extraction of proteins from shrimp processing waste. *J Chem Technol Biotechnol* 33: 203-208.
12. Kechaou ES, Dumay J, Donnay-Moreno C, Jaouen P, Gouygou JP, Bergé JP, Amar RB. 2009. Enzymatic hydrolysis of cuttlefish (Sepiaofficinalis) and sardine (Sardinapilchardus) viscera using commercial proteases: Effects on lipid distribution and amino acid composition. *J Biosci Bioeng* 107: 158-164.
13. Šližytė R, Mozuraitytė R, Martínez-Alvarez O, Falch E, Fouchereau-Peron M, Rustad T. 2009. Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (*Gadusmorhua*) backbones. *Process Biochemistry* 44: 668-677.
14. Rhee SJ, Lee CJ, Kim M, Lee C. 2004. Potential antioxidant peptides in rice wine. *Korean J Microbiol Biotechnol* 14: 715-722.
15. Arihara K, Tomita K, Ishikawa S, Itoh M, Akimoto M, Sameshina T. 2005. Antifatigue peptides derived from meat proteins. Japan patent.

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