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Screening of Gelatinolytic Enzyme Producing Bacteria for Production of Hydrolysate with Antioxidative Activity

Samart Sai-Ut, Soottawat Benjakul [+] and Punnanee Sumpavapon

Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

Abstract. Over 500 different bacterial strains were isolated from 30 samples e.g., fish, containers and equipment from fish dock by swabbing technique. From the primary screening, twenty-five isolates capable of producing gelatinolytic enzymes higher than 5 U/mg were selected. Those possessed different morphologies. Based on activity tested at 45 ℃, five isolates (D10, G02, H11, K12, and S13) were finally selected. Gelatinolytic activity ranged from 45.58 to 61.88 U/mg. When gelatin hydrolysates were produced by the enzyme from the selected isolates, DH of 4.70-6.22% was obtained. Hydrolysate exhibited varying antioxidative activities when tested by DPPH and ABTS radical scavenging assays. Among all hydrolysates, that produced from strain K12 showed the highest DPPH and ABTS radical scavenging activities (2.43 ± 0.12 and 133.70 ± 0.91 μmole TE/g protein). Gelatinolytic enzymes from selected isolates were able to hydrolyze gelatin, thereby producing antioxidative peptides, which could be used as natural antioxidant or functional food.

Keywords: Antioxidative activity, Bacteria, Gelatinolytic enzyme, Gelatin hydrolysate, Screening

1. Introduction

Microbial proteases represent one of the largest classes of industrial enzymes, accounting 40% of the total worldwide sale of enzymes [1]. Microbes have been known as a good source of enzymes having the numerous characteristics. They have broad biochemical diversity with the rapid growth and the limited space is required for cultivation [2]. Although a variety of proteolytic fungi and bacteria have been isolated, only a few of them provide high activity with commercial success. Additionally, hydrolytic activity and specificity toward substrate is one of primary factors, in which processor or user need to consider [3].

Generally, protease showed the low hydrolytic activity toward collagen and gelatin. However, the microorganisms isolated from fish processing plant or fish dock, where the collagen or gelatins are available, may possess collagenolytic or gelatinolytic activities. As a whole, protease obtained from those microbes can be comparable to those available in the market. Those gelatinolytic enzymes can be used as the novel protease capable of hydrolyzing gelatin, particularly from fish origin. Nowadays, hydrolysate with bioactivity, especially antioxidative activity, has gained increasing attention for consumer. Therefore, this investigation aimed to isolate microorganisms with gelatinolytic activity from fish docks and to determine antioxidative activity of resulting hydrolysate produced by selected strains.

2. Material and Methods

2.1. Isolation and Screening for Gelatinolytic Enzyme Producing Bacteria from Fish Dock

About 500 different bacterial strains were isolated from fish docks of Songkhla, Thailand during March and May, 2012. Samples were collected by swabbing the surfaces of fish, containers and equipments in fish dock, Songkhla, Thailand. The collected samples were transported to the Department of Food Technology,

+Corresponding author: Tel.: + 66 7428 6334; fax: +66 7455 8866.
E-mail address: soottawat.b@psu.ac.th.

Prince of Songkla University, Hat Yai, Thailand. Upon arrival, the samples (1 mL) were mixed with 9 mL of sterile diluents containing 0.85% NaCl. Microbes in those samples were isolated using spread plate and streaking methods on nutrient agar (Merck, Darmstadt, Germany). The obtained isolates were cultured in nutrient broth (NB) for 18 h at 37 ℃. Culture broths were centrifuged at 10,000 ×g and at 4 ℃ for 15 min using a refrigerated centrifuge (Avanti® J-E, Beckman Coulter, Palo Alto, CA, USA). The supernatants were determined for gelatinolytic activity.

To measure gelatinolytic activity for screening, the supernatant was assayed using fish gelatin as a substrate as per the method of McLaughlin and Weiss [4]. A reaction mixture was 50 mM Tris-HCl (pH 7.5) containing 0.36 mm $CaCl_2$, and 5 mg/mL of fish gelatin. Reaction mixture was incubated at 37 ℃ for 30 min. Activity was also assayed at 45 ℃ for enzymes produced by the selected strains. To initiate reaction, 0.1 mL of supernatant was added. Reaction was stopped by submerging the reaction mixture in water bath at 90 ℃ for 10 min (Memmert, Schwabach, Germany). The α-amino acid content in the mixture was determined according to the method of Benjakul and Morrissey [5]. One unit (U) of gelatinolytic activity was defined as the amount of enzyme which released 1 μmole of α-amino acid per min under the specified condition. Protein concentration was determined by the Lowry method [6] using bovine serum albumin as a standard.

2.2. Production of Gelatin Hydrolysate

Gelatin hydrolysates with antioxidant activity were prepared. Gelatin (1 g) was dissolved in 100 mL of distilled water. The pH of mixture was adjusted to 7.5 with 1 N NaOH. The hydrolysis reaction was started by the addition 1 mL of the cell-free supernatant from selected strains into 10 mL gelatin solution. After 1 h of hydrolysis at 45 ℃, the enzyme was inactivated by heating at 90 ℃ for 15 min in a water bath. The mixture was referred to as 'gelatin hydrolysate' and was determined for DH and antioxidative activities.

2.3. Determination of α-amino Acids and DH

To diluted gelatin hydrolysate samples (125 μL), 2.0 mL of 0.2 M phosphate buffer, pH 8.2 and 1.0 mL of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50 ℃ for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino acid was expressed in terms of L-leucine. DH was calculated as follows [5]:

$$DH = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where L_t is the amount of α-amino acid in hydrolysate at time t. L_0 is the amount of α-amino acid in the original gelatin. L_{max} is total α-amino acid in the original gelatin obtained after acid hydrolysis (6 M HCl at 100 ℃ for 24 h).

2.4. Determination of Antioxidative Activities

2.4.1. DPPH Radical Scavenging Activity

DPPH radical scavenging activity was determined according to the method of Khantaphant and Benjakul [7]. Sample solution (1.5 mL) was added with 1.5 mL of 0.1 mM 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in 95% ethanol. The mixture was allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve (0–60 μM) and expressed as μmole Trolox equivalents (TE)/g protein.

2.4.2 ABTS Radical Scavenging Activity

ABTS radical scavenging activity was determined as described by Khantaphant and Benjakul [7]. ABTS radical ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution (7.4 mM 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)) with 2.6 mM potassium persulfate at the ratio of 1:1 (v/v). The mixture was allowed to react in dark for 12 h at room temperature. Prior to assay, $ABTS^{\bullet+}$ solution was diluted with methanol to obtain an absorbance of 1.1 (± 0.02) at 734 nm. To initiate the reaction, 150 μL of

sample was mixed with 2.85 mL of $ABTS^{\bullet+}$ solution. The absorbance was then read at 734 nm after 2 h dark incubation at room temperature. ABTS radical scavenging activity was expressed as μmole TE/g protein.

3. Results and Discussion

3.1. Isolation and Screening of Gelatinolytic Enzyme Producing Bacteria

About five-hundred isolates were obtained from 30 samples collected from surfaces of fish, containers, floor and fish processing equipments including cutting board, knife, glove, etc. from the docks of Songkla. These cultures were screened for their ability to produce an extracellular gelatinolytic enzyme. Twenty-five isolates showed hydrolytic activity towards fish gelatin (5.16 to 41 U/mg protein), when tested at 37 ℃ (Table I). The unique habitats in marine environment provide microbes with novel physiological and metabolic capabilities for survival and a great potential for the production of metabolites, not found in terrestrial environments [8]. Those microbes had varying morphologies, suggesting the differences in species.

Table I: Specific activity[1] and colony morphology of selected isolates

No.	Code	Specific activity (U/mg)	Colony morphology		
			Form	Elevation	Margin
1	A02	8.64 ±0.56	Irregular	Flat	Filamentous
2	A15	6.47 ±1.59	Punctiform	Umbonate	Entire
3	B08	5.16 ±0.89	Circular	Flat	Entire
4	C12	6.38 ±1.14	Circular	Flat	Filamentous
5	C13	16.14 ±0.62	Irregular	Flat	Labate
6	D10	19.04 ±1.14	Circular	Umbonate	Labate
7	F01	5.48 ±0.48	Filamentous	Flat	Filamentous
8	F04	10.03 ±0.32	Irregular	Flat	Labate
9	F08	6.78 ±0.79	Irregular	Flat	Labate
10	G01	7.95 ±1.49	Irregular	Flat	Filamentous
11	G02	27.54 ±0.38	Circular	Umbonate	Curled
12	H11	32.97 ±1.37	Punctiform	Flat	Curled
13	I11	13.88 ±1.23	Filamentous	Flat	Filamentous
14	K03	7.03 ±0.85	Circular	Convex	Filamentous
15	K05	6.40 ±0.69	Circular	Flat	Labate
16	K06	8.46 ±0.53	Filamentous	Flat	Filamentous
17	K12	41.97 ±2.12	Irregular	Flat	Filamentous
18	O02	20.29 ±0.78	Irregular	Flat	Undulate
19	S02	7.23 ±0.33	Filamentous	Flat	Filamentous
20	S13	26.17 ±0.31	Filamentous	Flat	Filamentous
21	V09	15.89 ±0.32	Irregular	Flat	Filamentous
22	W03	8.22 ±0.70	Irregular	Flat	Labate
23	X08	12.56 ±1.09	Punctiform	Raised	Entire
24	Y01	5.49 ±1.00	Circular	Flat	Erose
25	Z04	6.75 ±1.19	Circular	Flat	Entire

[1] Specific activity was determined at 37 ℃

3.2. Antioxidative Activity of Gelatin Hydrolysate Produced from Selected Bacteria Enzyme

Five isolate were selected as the potential gelatinolytic enzyme producers. When their enzymes were tested at 45 ℃, a temperature used for gelatin hydrolysis, strain D10 showed the highest specific activity, while strain K12 and S13 had the lowest specific activity ($P < 0.05$). DH of gelatin hydrolysate was in accordance with the activity of enzyme used, which varied among strains.

DPPH and ABTS radical scavenging activities of gelatin hydrolysates prepared using enzyme from six isolates are shown in Table II. In general, the increases in radical scavenging activities were found in all hydrolysates, compared with gelatin. The results indicated that antioxidative peptides were produced during

the hydrolysis. The highest DPPH radical scavenging activity was found in gelatin hydrolysate prepared using enzyme from the strain K12 (2.43 μmole TE/g protein).

For ABTS radical scavenging activity, hydrolysate produced using enzyme from strain K12 also exhibited the highest ABTS radical scavenging activity (133.70 μmole TE/g protein). Gelatin hydrolysates from bigeye snapper skin with DH ranging from 5% to 25% prepared using Alcalase had the increased ABTS scavenging activity with increasing DH [9].

DPPH and ABTS radical scavenging activities are based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals, by converting it to the non-radical species [10] and [11]. The results suggested that gelatin hydrolysates had the ability to scavenge free radicals, thereby preventing oxidation via a chain breaking reaction. Gelatin hydrolysate prepared using enzyme from selected isolates might serve as a potential source of natural antioxidant to prevent lipid oxidation in various foods.

4. Conclusion

Gelatin hydrolysate with antioxidative activity could be produced using gelatinolytic enzyme from microbes isolated from fish dock. Five isolates were promising producers of gelatinolytic enzymes. Gelatin hydrolysate prepared using enzyme from selected isolates exhibited antioxidative activity, but their activity varied, depending on enzyme activity.

Table II: Specific activity, DH and antioxidative activities of gelatin hydrolysate prepared using gelatinolytic enzyme from selected isolates

Code	**Specific activity[1] (U/mg)**	**%DH**	**DPPH radical scavenging (μmole TE/g protein)**	**ABTS radical scavenging (μmole TE/g protein)**
Control	-	-	0.69 ± 0.25^{d}	118.48 ± 0.56^{d}
D10	61.88 ± 5.35^{a}	6.22 ± 0.26^{a}	1.93 ± 0.23^{b}	131.19 ± 0.88^{b}
G02	55.07 ± 2.12^{b}	5.84 ± 0.06^{b}	1.58 ± 0.11^{c}	125.47 ± 0.63^{c}
H11	56.98 ± 2.29^{b}	5.89 ± 0.07^{b}	1.81 ± 0.14^{bc}	127.67 ± 0.64^{c}
K12	46.43 ± 1.21^{c}	4.70 ± 0.03^{d}	2.43 ± 0.12^{a}	133.70 ± 0.91^{a}
S13	45.58 ± 1.60^{c}	5.04 ± 0.06^{c}	1.85 ± 0.15^{bc}	127.10 ± 0.83^{c}

[1] Specific activity was determined at 45 °C

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