Structural Analysis of Antioxidative Peptides from Soybean β-Conglycinin

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Protease hydrolyses of a soybean protein, β-conglycinin (7S protein), yielded antioxidative activity against the peroxidation of linoleic acid in an aqueous system at pH 7.0. Six antioxidative peptides were isolated from the hydrolysate prepared with protease S by size exclusion chromatography and reversed-phase HPLC. The amino acid sequences of the peptides were determined using a gas-phase protein sequencer and electron spray mass spectrometry. The peptides were composed of 5–16 amino acid residues, including hydrophobic amino acids, valine or leucine, at the N-terminal positions, and proline, histidine, or tyrosine in the sequences.

Keywords: *Antioxidative peptide; soybean protein; β-conglycinin*

INTRODUCTION

Various physiological activities have been detected in the hydrolysates derived from the proteolytic hydrolysis of many food proteins. In the case of soybean protein, a hydrolysate obtained with pepsin produced immune responses in rats (Yamauchi and Suetsuna, 1993). An antioxidative effect was also observed in a soybean protein hydrolysate (Yamaguchi et al., 1975). The protein was hydrolyzed with 6 N HCl at 100 °C or with protease. The antioxidative activity reached a maximum after a short period of hydrolysis and then decreased in both hydrolysis methods. The antioxidative peptides were fractionated by size exclusion chromatography; however, no structural information has been available so far.

In this study, we examined the antioxidative effects of enzymatic hydrolysates of a soybean protein, β-conglycinin, with five different proteases. Six antioxidative peptides were isolated from the hydrolysate obtained with protease S, and their amino acid sequences were determined.

MATERIALS AND METHODS

Materials. Soybean β-conglycinin was prepared from *Glycine max* using the method of Iwabuchi and Yamauchi (1987). Protease M from *Aspergillus oryzae*, protease N from *Bacillus subtilis*, protease P from *Aspergillus melleus*, and protease S from *Bacillus* sp. were obtained from Amano Seiyaku Co. (Nagoya, Japan). Although the substrate specificities of these proteases have not been investigated thoroughly, proteases N and S are composed of endopeptidases, and proteases M and P are known to contain both endopeptidases and exopeptidases. Pepsin from porcine gastric mucosa was purchased from Merck Co. (Darmstadt, Germany). Linoleic acid (~99%) was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade from Nakarai Chemicals Co. (Kyoto, Japan) or Wako Junyaku Co. (Osaka, Japan).

Enzymatic Hydrolysis. β-Conglycinin (1.0 g) was dissolved in 33 mL of distilled water. Protease (30 mg) was added to the protein solution after the pH was properly adjusted. Enzymatic hydrolyses were performed at pH 3.0 and 50 °C for protease M, at pH 7.0 and 55 °C for protease N, at pH 8.0 and 45 °C for protease P, at pH 8.0 and 70 °C for protease S, and at pH 2.0 and 37 °C for pepsin. Optimal conditions were adapted as the manufacturer recommended. After digestion, hydrolysates were heated in boiling water for 3 min to inactivate proteases. The hydrolysates were neutralized and centrifuged (10 min at 20000*g*). The supernatants were lyophilized and stored in a desiccator at room temperature until use. The degree of hydrolysis (DH) of hydrolysates was determined using the *o*-phthaldialdehyde method (Church et al., 1983).

Measurement of Antioxidative Activity. Samples dissolved in 1.5 mL of 0.1 M phosphate buffer (pH 7.0) and 1.0 mL of 50 mM linoleic acid in ethanol (99.5%) were mixed in test tubes (5-mL volume). The tubes were sealed tightly with silicon rubber caps and kept at 60 °C in the dark. At regular intervals, aliquots of the reaction mixtures were withdrawn with a microsyringe for measurement of the oxidation using the ferric thiocyanate method (Mitsuda et al., 1966) with a slight modification. To 50 μL of the reaction mixture were added 2.35 mL of 75% ethanol, 50 μL of 30% ammonium thiocyanate, and 50 μL of 20 mM ferrous chloride solution in 3.5% HCl. After 3 min, absorbance of the colored solution at 500 nm was measured in a 1-cm cuvette with a Jasco Model Ubest 30 spectrophotometer. The number of days taken to attain an absorbance of 0.3 was defined as the induction period. The induction period refers to the relative antioxidative activity of the samples.

Size Exclusion Chromatography. The lyophilized hydrolysate (200 mg) was dissolved in 50 mM acetic acid and fractionated by size exclusion chromatography on a Sephadex G-25 column (1.5 × 85 cm) equilibrated and eluted with the same solvent. Fractions of 4 mL were collected at a flow rate of 20 mL/h. The absorbance at 280 nm and antioxidative activity of all fractions were measured. For measurement of antioxidative activity, 1 mL of each fraction was taken and dried. The residue was dissolved in 0.1 mL of 50 mM ammonium bicarbonate and dried again before measuring. The active fractions were pooled and lyophilized to give 230 mg of antioxidative fraction III from 1 g of β-conglycinin.

Purification of Antioxidative Peptide. The antioxidative fraction was dissolved in distilled water and separated by reversed-phase HPLC on a TSK ODS 120T (5 μm, 7.5 × 250 mm, Toso, Tokyo, Japan) using a linear gradient of acetonitrile (0–40% in 60 min) in 0.1% trifluoroacetic acid (TFA). About 20 mg (in 0.7 mL) was separated at a run. Separations were accomplished at 35 °C and a flow rate of 1.5 mL/min. The elution peaks were monitored at 230 nm, and their antioxidative activities were measured using $^1/_{30}$ of each sample. The active peaks were concentrated to 1 mL using a centrifugal evaporator.

The antioxidative peptides were rechromatographed on a TSK ODS 120T column (5 μm, 4.6 × 250 mm) using a linear gradient of acetonitrile in 10 mM ammonium acetate at a flow rate of 1.0 mL/min.

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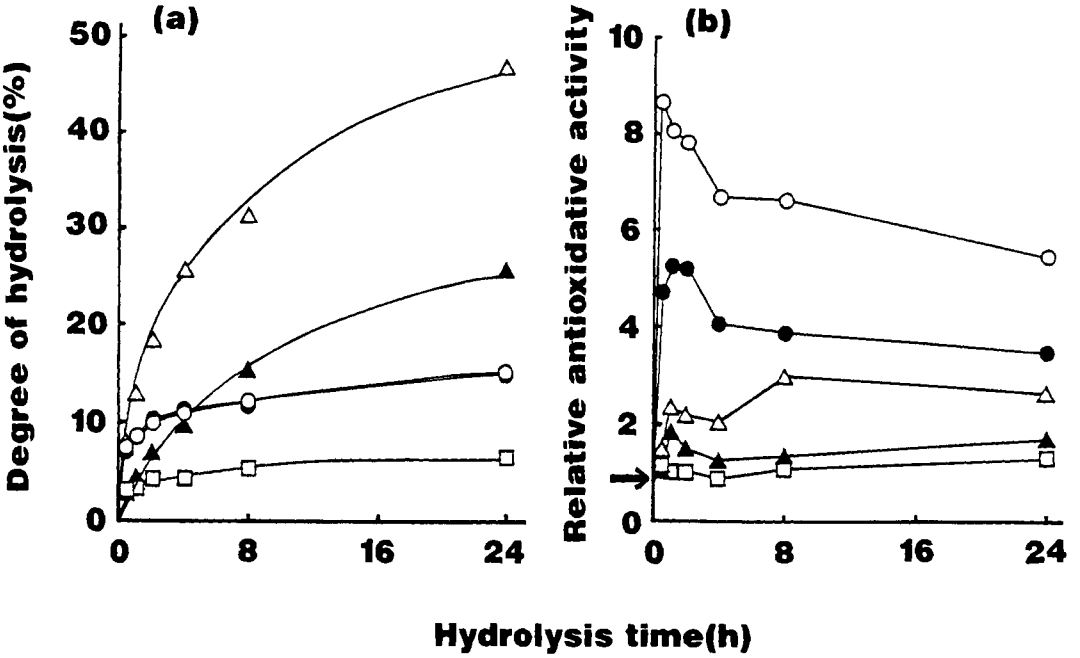

Figure 1. Time courses of hydrolysis and antioxidative activity of β-conglycinin treated with five different proteases at 3:100 (w/w) enzyme/substrate. Protein concentrations were 3%. (a) DH of β-conglycinin hydrolysates: pepsin, pH 2, 37 °C (□); protease M, pH 3, 50 °C (△); protease N, pH 7, 55 °C (●); protease P, pH 8, 45 °C (▲); protease S, pH 8, 70 °C (○). (b) Antioxidative activities of β-conglycinin hydrolysates. The numbers of the relative antioxidative activity correspond to the days of induction period. The arrow indicates the activity of unhydrolyzed β-conglycinin. Five milligrams of sample was used for the assay.

Amino Acid Analysis. The peptides were hydrolyzed with 6 N HCl at 110 °C for 24 h. The hydrolysate was derivatized with 4-(dimethylamino)azobenzene-4′-sulfonyl chloride and then analyzed by reversed-phase HPLC on a Capcell Pack ODS AG120 column (5 μm, 4.6 × 150 mm, Shiseido, Tokyo, Japan) (Knecht and Chang, 1986).

Mass Spectrometry. The molecular masses of purified antioxidative peptides were determined using a Finnigan MAT (San Jose, CA) TSQ-700 triple-quadrupole mass spectrometer equipped with a 15-keV conversion dynode for enhanced sensitivity (Fenn et al., 1989). The ion signals were recorded using a Finnigan ICIS data system operated on a DEC station 5000/25.

Sequence Analysis. Amino acid sequences of peptides were analyzed using a gas-phase sequencer (Model PSQ-1, Shimadzu, Kyoto, Japan) with fluorescein isothiocyanate as an Edman reagent (Muramoto et al., 1994). The peptide samples were covalently attached to arylamine-derivatized poly(vinylidene difluoride) membranes (Sequelon AA) (Milli-Gen, Burlington, MA) (Coull et al., 1991).

RESULTS AND DISCUSSION

Five different proteases—proteases M, N, P, and S and pepsin—prepared from different sources and with distinct specificities, were screened for the hydrolysis of soybean β-conglycinin. DH and the antioxidative activity of the hydrolysates were measured for a 24-h period for each protease. In each experiment, the antioxidative effects of β-conglycinin increased upon hydrolysis, while the induction period of the protein was less than 1 day without hydrolysis (Figure 1). The antioxidative activity did not increase with increasing DH. Protease M showed the highest DH values but only moderate activity. The hydrolysis proceeded with time to a DH of 45% at 24 h, while no increase of activity was observed after 8 h. Proteases S and N showed a similar hydrolysis rate but distinct activities. Protease S yielded the most active hydrolysate. The activity reached a maximum within 0.5 h and then decreased gradually. The activity changed similarly with other proteases, regardless of different patterns of hydrolysis. This result indicates that the antioxidative activity of the hydrolysates is inherent to their characteristic amino acid sequences of peptides depending on the protease specificities.

For measurement of antioxidative activity, it was important to dry peptide samples after they were dissolved in 50 mM ammonium bicarbonate to remove any residual acid, because the ferric thiocyanate method is very sensitive to acidic pH change. The dried samples were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) before mixing with linoleic acid ethanol solution. The presence of phosphate in the assay system was reported to enhance the antioxidative effect and lower the prooxidative effect with relatively high amino acid concentrations ($>10^{-4}$ M) (Marcuse, 1962).

To characterize the antioxidative peptides derived from soybean β-conglycinin, the protein was hydrolyzed with protease S for 1 h. The hydrolysate was then separated by size exclusion chromatography on Sephadex G-25 and fractionated to three fractions (Figure 2). Fractions I and II were subjected to reversed-phase HPLC to isolate antioxidative peptides; however, the antioxidative activity scattered all peaks, and no marked active peak was observed to allow for further purification. Fraction III was estimated to be about 1400 Da by the molecular weight standard.

Fraction III was separated by reversed-phase HPLC using a 0.1% TFA–acetonitrile system and fractionated to F1, F2, F3, F4, and F5 (Figure 3). These fractions were further separated by reversed-phase HPLC using a 10 mM ammonium acetate–acetonitrile system (Figure 4). From the protease S hydrolysate, six antioxidative peptides were finally obtained. The yields estimated by amino acid analysis of P1, P2, P3, P4, P5, and P6 were 2, 5, 3, 345, 245, and 1943 μg, respectively. The antioxidative activity was measured with 0.8 μg of P1, 1.6 μg of P2, 0.9 μg of P3, 69 μg of P4, 49 μg of P5, and 194 μg of P6. The peptide concentrations ranged from 3.3×10^{-7} to 4.5×10^{-5} M, in which range the

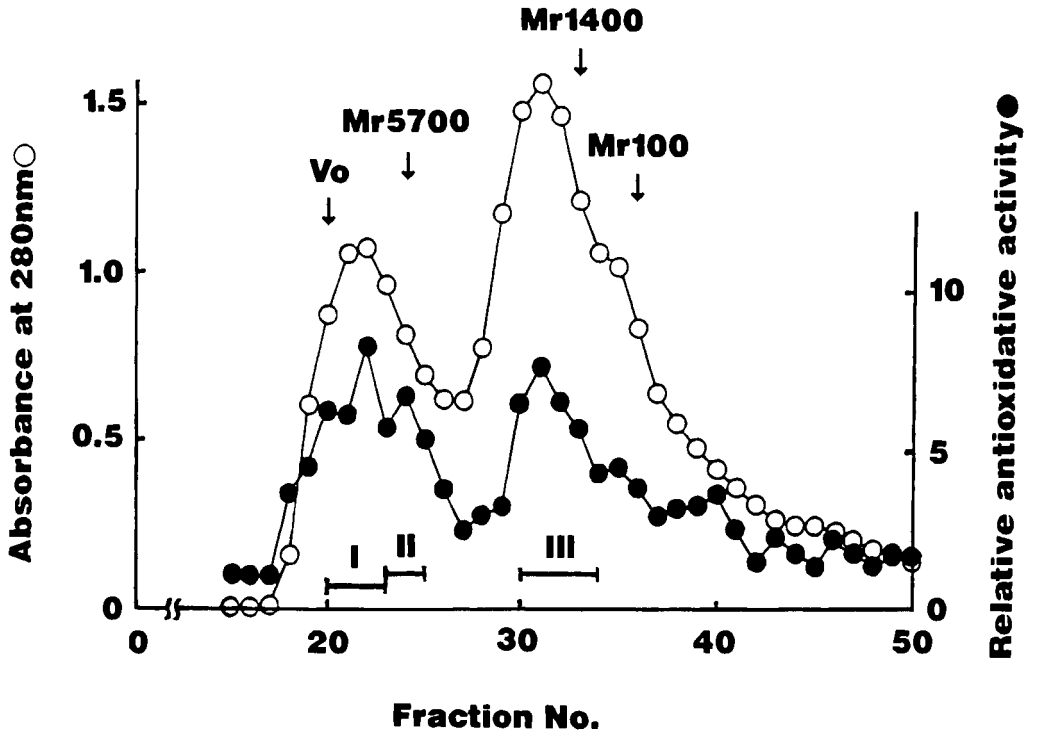

Figure 2. Elution profile of protease S hydrolysate separated by size exclusion chromatography on Sephadex G-25. The column (1.5 × 85 cm) was equilibrated and eluted with 50 mM acetic acid, at a flow rate of 20 mL/h. One milliliter of each fraction (4 mL) was used to determine the antioxidative activity. (○) Absorbance at 280 nm; (●) relative antioxidative activity.

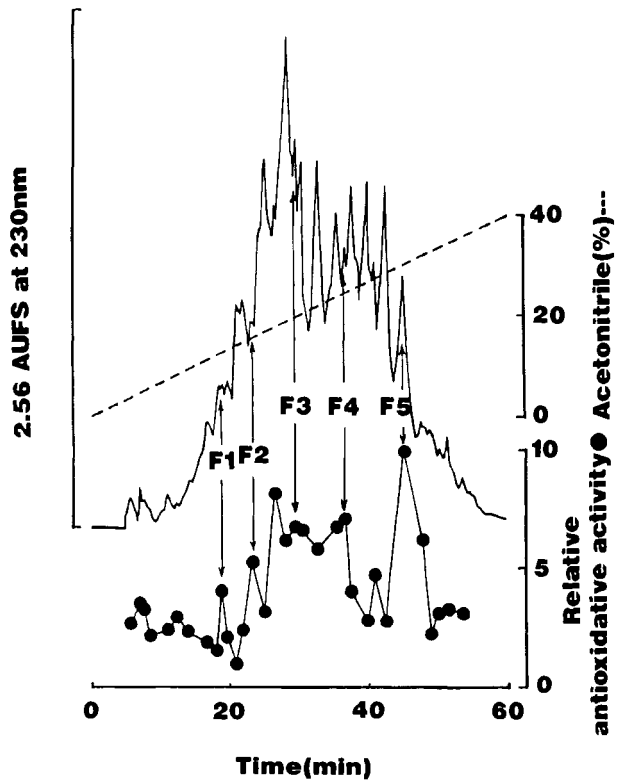

Figure 3. Elution profile and antioxidative activity of fraction III separated by reversed-phase HPLC. One-thirtieth of each fraction was used to determine the antioxidative activity (●).

antioxidative activity was dose dependent and no prooxidative activity was observed.

The amino acid sequences of these peptides are shown in Table 1. P1 had the same sequence as P2 except for the lack of the N-terminal leucine. P3 had a sequence common to the N-terminal sequence of P4. All of these peptides contained two histidine residues and one proline residue within the sequence. P5 and P6 had no histidine residue but contained a tyrosine residue in their sequences. The sequences of the peptides are in good agreement with their amino acid compositions and molecular masses analyzed by mass spectrometry (Table 2). Furthermore, the sequences were confirmed to be present in the amino acid sequence of β-conglycinin deduced from the nucleotide sequence (Doyle et al., 1986).

We compared the antioxidative effects of the isolated peptides with those of each constituent amino acid, since the antioxidative effects of some amino acids have been shown (Marcuse, 1960, 1962; Karel et al., 1966). When the constituent amino acids were mixed at the same concentration as the peptides, no antioxidative activity was observed. Thus, the characteristic amino acid sequences of peptides are required to express the antioxidative effects. The antioxidative activity of P3 was compared with that of butylated hydroxyanisole (BHA). Similar activities were observed in the range 10^{-6} –10^{-4} M.

All of the peptides isolated in this study had hydrophobic amino acids, valine or leucine, at the N terminus. The amino acid residues existing at the N termini of dipeptides were demonstrated to be antioxidative in an oil system (Kawashima et al., 1979). It is probable that the amino acid residues play a role in increasing the interaction between peptides and fatty acids.

The antioxidative activity of histidine-containing peptides has been reported (Uchida and Kawakishi, 1992; Murase et al., 1993). This activity may be attributed to the chelating ability and the lipid radical-trapping ability of the imidazole ring. The antioxidative activities of histidine-containing peptides were higher than that of histidine itself. This was partly explained by the increase of hydrophobicity of the peptides, which led to a higher interaction between the peptides and fatty acids. As shown in this study, four antioxidative

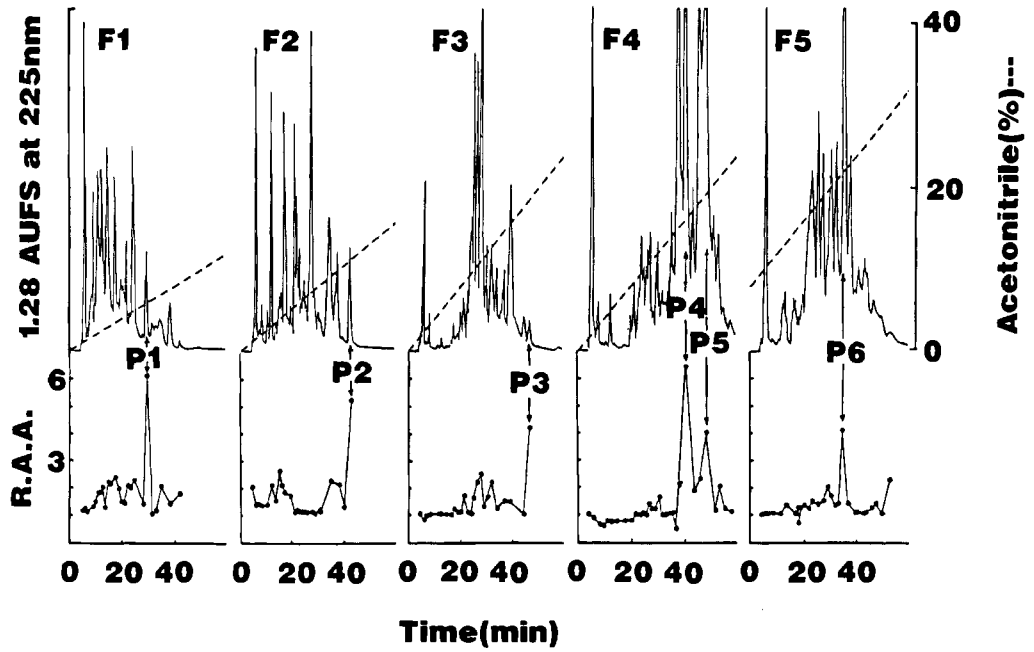

Figure 4. Elution profiles and antioxidative activities of F1, F2, F3, F4, and F5 separated by reversed-phase HPLC. One-fifth of each fraction was used to determine the antioxidative activity (●). RAA: relative antioxidative activity.

Table 1. Amino Acid Sequences of Isolated Antioxidative Peptides Derived from the Hydrolysis of β-Conglycinin with Protease S

P1	Val-Asn-Pro-His-Asp-His-Gln-Asn
P2	Leu-Val-Asn-Pro-His-Asp-His-Gln-Asn
P3	Leu-Leu-Pro-His-His
P4	Leu-Leu-Pro-His-His-Ala-Asp-Ala-Asp-Tyr
P5	Val-Ile-Pro-Ala-Gly-Tyr-Pro
P6	Leu-Gln-Ser-Gly-Asp-Ala-Leu-Arg-Val-Pro-Ser-Gly-Thr-Thr-Tyr-Tyr

Table 2. Amino Acid Compositions and Molecular Masses of Antioxidative Peptides Derived from the Hydrolysis of β-Conglycinin

	P1	P2	P3	P4	P5	P6
Asx	3.2 (3)[a]	2.9 (3)		1.5 (2)		1.0 (1)
Glx	1.2 (1)	1.4 (1)				1.2 (1)
Ser						1.1 (2)
Thr						1.2 (2)
Gly					1.4 (1)	1.4 (2)
Ala				1.6 (2)	1.0 (1)	1.1 (1)
Arg						0.8 (1)
Pro	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	2.0 (2)	1.0 (1)
Val	0.9 (1)	0.8 (1)			0.6 (1)	0.6 (1)
Ile					0.4 (1)	
Leu		0.9 (1)	2.0 (2)	1.9 (2)		1.5 (2)
His	2.2 (2)	2.1 (2)	1.9 (2)	1.7 (2)		
Tyr				0.5 (1)	1.2 (1)	1.3 (2)
total	8	9	5	10	7	16
M + H	960.0 (959.4)	1073.1 (1072.5)	616.3 (615.3)	1152.9 (1150.5)	716.4 (715.4)	1727.8 (1726.8)

[a] Numbers in parentheses are deduced from the sequences.

peptides of six peptides contained histidine residues in the sequence. The rest of the peptides contained tyrosine residues, which are potent hydrogen donors. To examine the structural change of antioxidative peptides, P3 was subjected to reversed-phase HPLC after incubating the peptide under the same condition as for the antioxidative activity measurement. The sample incubated beyond the induction period showed only a small decrease of P3 and several minor peaks (data not shown). This result suggests that the peptide was antioxidative with a minor change of its structure. It should be noted that three antioxidative peptides isolated from egg white albumin contained histidine residues at the second residue in the sequence; these were Ala-His, Val-His-His, and Val-His-His-Ala-Asn-Glu-Asn (Tsuge et al., 1991). Furthermore, all of the antioxidative peptides isolated from β-conglycinin contained a proline residue in the sequences. It is interesting that prolyl polypeptides are sensitive to oxidation (Uchida et al., 1992).

The varied yields of the antioxidative peptides from β-conglycinin depended on the purification procedure used and were not due to their contents in the hydrolysate, because all of them originated from a single polypeptide chain of a β-conglycinin subunit. Therefore, the peptides P1, P2, and P3 can be expected to be highly antioxidative in the unpurified hydrolysate in spite of their low yields in this study.

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