The Journal of Microbiology (2011) Vol. 49, No. 3, pp. 376-380
DOI 10.1007/s12275-011-1165-3

Biochemical Analysis of a Fibrinolytic Enzyme Purified from *Bacillus subtilis* Strain A1

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(Received March 31, 2011 / Accepted June 8, 2011)

A fibrinolytic enzyme from *Bacillus subtilis* strain A1 was purified by chromatographic methods, including DEAE Sephadex A-50 column chromatography and Sephadex G-50 column gel filtration. The purified enzyme consisted of a monomeric subunit and was estimated to be approximately 28 kDa in size by SDS-PAGE. The specific activity of the fibrinolytic enzyme was 1632-fold higher than that of the crude enzyme extract. The fibrinolytic activity of the purified enzyme was approximately 0.62 and 1.33 U/ml in plasminogen-free and plasminogen-rich fibrin plates, respectively. Protease inhibitors PMSF, DIFP, chymostatin, and TPCK reduced the fibrinolytic activity of the enzyme to 13.7, 35.7, 15.7, and 23.3%, respectively. This result suggests that the enzyme purified from *B. subtilis* strain A1 was a chymotrypsin-like serine protease. In addition, the optimum temperature and pH range of the fibrinolytic enzyme were 50°C and 6.0-10.0, respectively. The N-terminal amino acid sequence of the purified enzyme was identified as Q-T-G-G-S-I-I-D-P-I-N-G-Y-N, which was highly distinguished from other known fibrinolytic enzymes. Thus, these results suggest a fibrinolytic enzyme as a novel thrombolytic agent from *B. subtilis* strain A1.

***Keywords*:** amidolytic activity, *Bacillus subtilis* strain A1, fibrinolytic enzyme, plasminogen activator, N-terminal amino acid sequence

Recently, cardiovascular diseases such as acute myocardial infarction have caused significant problems in society. These diseases are usually caused by the formation of fibrin clots adhering to the unbroken walls of blood vessels (Dobrovolsky and Titaeva, 2002). It is known that the main protein component of blood clots, fibrin, is generally formed from fibrinogen by the action of thrombin (EC 3.4.21.5), and fibrin deposition in vessels normally increases thrombosis, resulting in myocardial infarction and other cardiovascular diseases (Bode *et al*., 1996).

Typical fibrinolytic enzymes such as tissue plasminogen activator (*t*-PA) and urokinase (*u*-PA, EC 3.4.21.31) are used as thrombolytic agents (Blann *et al*., 2002). Fibrinolytic enzymes produced by food sources (Fujita *et al*., 1993; Sumi *et al*., 1995; Jeong *et al*., 2004b) and microbes (Arai *et al*., 1995; Kim *et al*., 1997; Lee *et al*. 1999; Hsu *et al*., 2009; Park *et al.*, 2010) have been extensively investigated, because of their high cost and unstable enzymatic properties. Additionally, fibrinolytic enzymes from microbial strains, including streptokinase produced by *Streptococcus haemolyticus* (Medved *et al*., 1966) and staphylokinase produced by *Staphylococcus aureus* (Lijnen *et al*., 1991), have been extensively studied due to their side effects such as gastrointestinal bleeding, resistance to repercussion, and allergic reactions, all of which significantly limit their extensive medical use (Blann *et al*., 2002). Fibrinolytic enzymes from *Bacillus* sp. have also been studied as thrombolytic agents since they offer high efficiency in fibrinolytic reactions such as plasmin activation (Nakamura *et al*., 1992). Although known fibrinolytic enzymes have been studied for a thrombolytic agent, more practical candidate for the agent needs to be developed.

A variety of extracellular and intracellular proteases, including subtilisins Carlsberg and BPN' (Smith *et al*., 1968), subtilisin nattokinase (NK) (Nakamura *et al*., 1992), and CK (Kim *et al*., 1996), have been found in *Bacillus* sp. We previously reported a fibrinolytic enzyme (Bacillokinase II, BK II) from *B. subtilis* strain A1, which is a metalloprotease (Jeong *et al*., 2004a). In this paper, the fibrinolytic enzyme, which is distinguished from BK II and other known fibrionlytic enzymes with regard to biochemical characteristics, was found and characterized by biochemical and molecular analyses.

Materials and Methods

Bacterial strain and culture conditions

B. subtilis strain A1 producing a fibrinolytic enzyme was isolated from soil sediments in Korea (Jeong *et al*., 2004a). The strain was aerobically cultured at 37°C overnight using Luria Broth (LB) medium consisting of 1% bacto-tryptone (Sigma, USA), 1% sodium chloride, and 0.5% bacto-yeast extract (Sigma).

Enzyme purification and electrophoresis

The extracellular enzyme of *B. subtilis* strain A1 was prepared by centrifuging the cultured cells at 12,000 rpm for 15 min and then adding butanol to the supernatant at a ratio of 2:1. To harvest the

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extracellular proteins, a mixture of the supernatant and butanol was subjected to centrifugation at 12,000 rpm for 15 min, followed by dialysis three times using 5 L of 20 mM Tris-HCl (pH 7.5). All purification steps were performed at 4°C, after which the protein concentration was measured according to the method of Bradford (Bradford, 1976). The crude enzyme solution was purified and lyophilized through a series of steps. Using DEAE Sephadex A-50 column chromatography, the enzyme was purified by gel filtration using a Tris-HCl buffer solution as an elution buffer. After 70% ammonium sulfate precipitation, gel filtration on Sephadex G-200 and G-50 columns using Tris-HCl buffer as an elution buffer was successively performed. The enzyme solution obtained by chromatography was then lyophilized. SDS-PAGE of the protein was carried out on a 10-15% gradient polyacrylamide gel and 4% stacking gel at 4°C, according to the method described by Laemmli (1970).

Biochemical analysis of the purified fibrinolytic enzyme

Fibrinolytic activity was determined by both plasminogen-free and plasminogen-rich fibrin plate methods (Astrup and Müllertz, 1952). The plasminogen-free fibrin plate consisted of a fibrinogen solution [2.5 ml of 1.2% (w/v) human fibrinogen (Sigma) in 0.1 M sodium phosphate buffer, pH 7.4], 10 U of thrombin solution (Sigma), and 1% agarose. To eliminate other fibrinolytic factors, fibrin plates were heated at 80°C for 30 min. A plasminogen-rich fibrin plate was made up of 2 ml of 1.5% fibrinogen, 5 U of plasminogen, and 1% agarose.

A hole (5 mm in diameter) was punched in the fibrin plate for sample application, after which the sterilized paper disc was placed on the plate. To observe the fibrinolytic activity of the enzyme, 100 µl of the purified protein solution was carefully dropped onto the disc and incubated at 37°C for 18 h. The activity of the fibrinolytic enzyme was determined by measuring the dimension of the clear zone on the fibrin plate and then plotting the standard curve, which was drawn by estimating the lysis area on plasminogen-rich fibrin plate to the amount of a standard protein, plasmin.

Effect of protease inhibitors and metal ions

The effect of protease inhibitors was observed by using phenylmethanesulfonyl fluoride (PMSF), diisopropylfluorophosphate (DIFP), chymostatin, tosyl phenylalanyl chloromethyl ketone (TPCK), antipain, leupeptin, soybean trypsin inhibitor (SBTI), mercuric chloride, tosyl-lysinechloromethylketone (TLCK), and EDTA. The effect of metal ions on fibrinolytic activity was also investigated by using $HgCl_2$, $MnSO_4$, $ZnCl_2$, $FeCl_3$, $CoCl_2$, $CuSO_4$, and $MgCl_2$. The concentration of all of the protease inhibitors and metal ions in the enzyme reaction was 1 mM. Amidolytic activity was measured spectrophotometrically by using the chromogenic substrates. The reaction mixture (1 ml) contained 20 µl of enzyme solution, 5×10^{-4} M chromogenic substrate, and 0.1 M sodium phosphate buffer (pH 7.4). After incubation for 5 min at 37°C, the amount of *p*-nitroaniline liberated was determined from the spectrophotometric absorption at 405 nm. One unit of amidolytic activity was expressed as µmol of the substrate hydrolyzed per min per mg of the protein at 25°C.

Effect of temperature on enzyme activity and stability

The optimum temperature for enzyme activity was determined by keeping the purified enzyme in 20 mM phosphate buffer (pH 7.4) for 30 min at various temperatures (20, 30, 40, 50, 60, 70, and 80°C). Stability of BK I was also investigated by measuring the residual activity after incubating the enzyme solution at 20-80°C for 30 min in 20 mM phosphate buffer (pH 7.4). All experiments were conducted three times.

Effect of pH on enzyme activity and stability

The optimal pH of the enzyme was determined between pH 3.0-12.0 using the following buffer systems: 10 mM citric acid buffer (pH 3.0-5.0), 0.05 M sodium phosphate buffer (pH 6.0-7.0), 0.05 M Tris-HCl (pH 8.0), 0.05 M glycine-NaOH (pH 9.0-10.0), 0.1 M sodium phosphate buffer (pH 11.0), and 0.05 M hydroxyl-chlorite buffer (pH 12.0), respectively. The pH stability in the range of 3.0-12.0 was examined by incubating the enzyme solution for 30 min at 25°C with the different buffers, and then the residual activity at pH 7.0 was determined. All experiments were conducted three times.

N-terminal amino acid sequencing

The fibrinolytic enzyme from *B. subtilis* strain A1 on SDS-PAGE gel was electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, USA), followed by staining with Coomassie Brilliant Blue R-250 (Sigma). The stained protein band was neatly excised and the amino acids of the N-terminal sequence were determined by the automated Edman method using a gas-phase protein sequencer (Applied Biosystems, USA).

Results and Discussion

Purification of the fibrinolytic enzyme and estimation of amidolytic activity

The fibrinolytic enzyme was purified by the steps listed in Table 1. DEAE Sephadex A-50 column chromatography, 70% ammonium sulfate precipitation, and gel filtration on Sephadex G-200 and G-50 columns were used in the purification of the enzyme. Sephadex G-50 column chromatography yielded a single polypeptide showing high fibrinolytic activity (Fig. 1 and Table 1). The purified fibrinolytic enzyme was an approximately 28 kDa, which was slightly different from the 31 kDa metalloprotease BK II identified in the same strain (Jeong *et al*., 2004a).

The fibrinolytic activities of the 28 kDa fibrinolytic enzyme, designated as Bacillokinase I (BK I), in the plasminogen-free

Table 1. Purification of the 28 kDa fibrinolytic enzyme, BK I from *B. subtilis* strain A1

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Fold	Yield (%)
Culture supernatant	9,385.0	15,652	1.7	1	100
DEAE Sephadex A-50	538.4	12,964	24.1	14.2	82.8
70% ammonium sulfate precipitation	62.7	9,725	155.1	91.2	62.1
Sephadex G-200	16.2	5,329	329.0	193.5	34.0
Sephadex G-50	1.8	2,938	1632.2	960.1	18.8

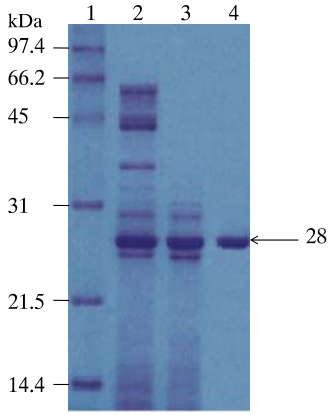

Fig. 1. Polyacrylamide gel electrophoresis of the fibrinolytic enzyme (BK I) purified from *B. subtilis* strain A1. Lanes: 1, protein molecular mass markers; 2, culture supernatant of *B. subtilis* A1; 3, proteins eluted by DEAE Sephadex A-50 ion exchange column chromatography; 4, purified enzyme eluted by Sephadex G-75 gel filtration. The arrow indicates the fibrinolytic enzyme purified from *B. subtils* strain A1.

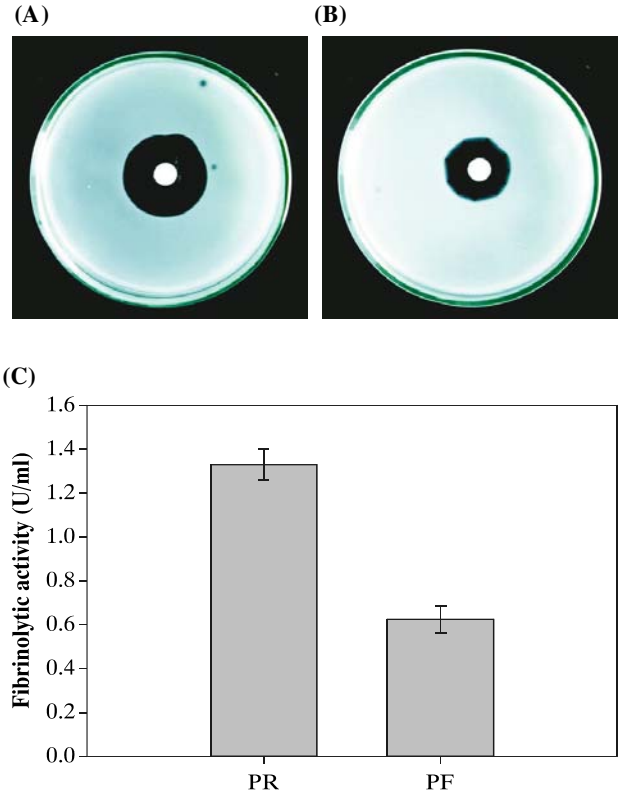

Fig. 2. Comparison of BK-I fibrinolytic activities of plasminogen-rich fibrin plate (A) with plasminogen-free fibrin plate (B), and estimation of fibrinolytic activity (C). The results in (C) represent the Mean±SD of three independent experiments performed in duplicate. PR, plasminogen-rich fibrin plate: PF, plasminogen-free fibrin plate. One unit was expressed as μmole of plasmin produced per minute by 1 mg of the protein.

and plasminogen-rich fibrin plates were 0.62 and 1.33 U/ml, respectively (Fig. 2). Fibrin of blood clots is formed from fibrinogen by thrombin and is lysed by plasmin, which is formed by activation of the proenzyme, plasminogen (*plasminogen activator type*). However, the fibrinolytic pattern of the newly identified 28 kDa BK I protein showed that the enzyme degraded fibrins acting as a plasminogen activator. In addition, the enzyme digested fibrins directly without acting as a plasminogen activator (direct type).

To observe substrate specificity of the BK I protein, we measured its amidolytic activity on commercial substrates and compared to that of known fibrinolytic enzymes (Table 2). In the presence of the synthetic substrate for plasmin, the amidolytic activities of the protein were found to be 46.3-, 26.5-, 6.9-, and 7.5-fold higher than those of NK (Nakamura *et al*., 1992), subtilisin BNP' (Smith *et al*., 1968), subtilisin Carlsberg (Smith *et al*., 1968), and CK (Kim *et al*., 1996), respectively. In the presence of synthetic substrate for thrombin, the amidolytic activities of the BK I protein were 18.1-, 39.7-, 5.4-, and 12.4-fold higher compared to those of NK, BNP', Carsberg, and CK, respectively. On a synthetic substrate for kallikrein, the amidolytic activities of the protein were 11.1-, 44.1-, 5.8-, and 8.9-fold higher, compared to those of NK, BNP', Carsberg, and CK, respectively. These results suggest that BK I is a serine protease and its fibrinolytic pattern is similar to that of plasmin.

Table 2. Amidolytic activity of the 28 kDa fibrinolytic enzyme (BK I), nattokinase (NK), subtilisin BNP', subtilisin Carlsberg, and CK for the synthetic substrates

Substrate	Enzyme activity[a]				
	BK I (nmol/min/mg protein)	NK (μmol/min/mg protein)	BNP' (μmol/min/mg protein)	Carlsberg (nmol/min/mg protein)	CK (nmol/min/mg protein)
H-D-Val-Leu-Lys-pNA[b]	3170 (100)	68.5 (100)	119.7 (100)	462.5 (100)	424.3 (100)
H-D-Phe-Pip-Arg-pNA[c]	270 (8.5)	14.9 (20.4)	6.8 (5.7)	50.3 (10.09)	21.7 (5.1)
H-D-Val-Leu-Arg-pNA[d]	150 (4.7)	13.5 (19.6)	3.4 (2.6)	25.9 (5.6)	16.9 (4.0)
H-D-Iso-Pro-Arg-pNA[e]	2390 (75.4)	ND	ND	ND	ND

The values in parentheses are percentages calculated on the basis of enzyme activity to H-D-Val-Leu-Lys-pNA. Each value is the mean of three determinations. ND indicates 'not determined'.

[a] Enzyme activity was expressed as nmol or μmol of substrate hydrolyzed per minute by 1 mg of the protein

[b] H-D-Val-Leu-Lys-pNA (Chromogenix, Italy): synthetic substrate for plasmin

[c] H-D-Phe-Pip-Arg-pNA (Chromogenix, Italy): synthetic substrate for thrombin

[d] H-D-Val-Leu-Arg-pNA (Chromogenix, Italy): synthetic substrate for kallikrein

[e] H-D-Iso-Pro-Arg-pNA (Chromogenix, Italy): synthetic substrate for serine protease

Table 3. Effect of protease inhibitors and metal ions on the activity of BK I protein purified from *B. subtilis* strain A1

Protease inhibitors or metal ions	Relative activity (%)[a]
None	100
PMSF	13±0.7
DIFP	33±2.7
Chymostatin	15±0.7
TPCK	22±1.3
Antipain	75±2.3
Leupeptin	77±2.2
SBTI	72±2.6
Mercuric chloride	71± 2.5
TLCK	76±3.1
EDTA	77±2.9
Hg^{2+}	43±1.5
Mn^{2+}	88±2.8
Zn^{2+}	91±2.1
Fe^{2+}	94±2.4
Co^{2+}	105±4.1
Cu^{2+}	101±3.2
Mg^{2+}	109±3.9

[a] Activity relative to the fibrinolytic enzyme, BK 1 from *B. subtilis* strain A1, which was taken as 100%. The values shown are the averages of at least three independent experiments. The concentration of all the protease inhibitors and metal ions was 1 mM.

Effects of protease inhibitors and metal ions

The effect of protease inhibitors on the fibrinolytic activity was estimated (Table 3). Serine protease inhibitors PMSF and DIFP reduced the fibrinolytic activity of the enzyme to 13.7 and 35.7%, respectively. The amidolytic activity of the protein upon the addition of chymotrypsin-like serine protease inhibitors chymostatin and TPCK was reduced to 15.7 and 23.3%, respectively, suggesting that BK I protein is a chymotrypsin-like serine protease. In addition, the effect of metal ions on the fibrinolytic activity of BK I protein was estimated (Table 3). The fibrinolytic activity of BK I protein was decreased

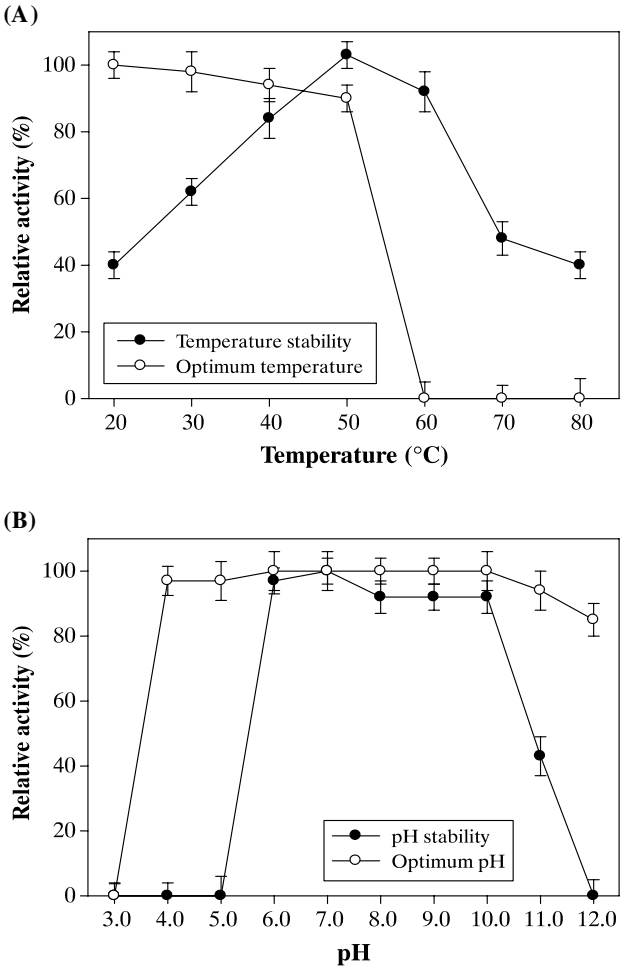

Fig. 3. Optimum temperature (A) and pH (B) of the fibrinolytic enzyme (BK I) purified from *B. subtilis* strain A1. The results represent the Mean±SD of three independent experiments performed in duplicate.

Protease	N-terminal amino acid sequence													
	+1				+5					+10				
BK I	Q	T	G	G	S	I	I	D	P	I	N	G	Y	N
BK II	A	R	A	G	E	A	L	R	D	I	Y	D	A	Q
Carlsberg	A	Q	T	V	P	Y	G	I	P	L	I	K	A	D
BPN'	A	Q	S	V	P	Y	G	V	S	G	I	V	A	P
NK	A	Q	S	V	P	Y	G	I	S	Q	I	K	A	P
CK	A	Q	T	V	P	Y	G	I	P	L	I	K	A	D

Fig. 4. Comparison of the N-terminal amino acid sequences of the fibrinolytic enzyme (BK I) purified from *B. subtilis* A1 with other proteases from the *Bacillus* genus. BK I, the 28 kDa fibrinolytic enzyme from *B. subtilis* A1; BK II (GenBank number, JF826532), the 31 kDa fibrinolytic enzyme from *B. subtilis* A1; Carlsberg (GenBank number, ZP08000924), subtilisin Carsberg from *B. licheniformis*; BPN' (GenBank number, AEB62660), subtilisin BPN' from *B. amyloliquefaciens*; NK (GenBank number, gi 14422313), subtilisin NK from *B. subtilis* (natto); CK (Kim *et al*., 1996), extracellular protease from *Bacillus* sp. strain CK 11-4. The consensus sequences of BK I, BK II, Carlsberg, and CK from *Bacillus* sp. are indicated by the box. The numbers represent the position from N-terminal sequences.

over 55% by the addition of Hg^{2+}. However, the addition of Mn^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, and Mg^{2+} in the reaction mixture did not significantly affect the activity of the fibrinolytic enzyme.

Effects of temperature and pH on fibrinolytic activity

The temperature activity profile showed that the optimum temperature for BK I was with maximum value at 50°C (Fig. 3A). As to the heat stability, BK I maintained over 85% of enzyme activity at a temperature range of 20 and 50°C. The pH activity profile of BK I showed that the enzyme was significantly active at pH values ranging from 6.0 to 10.0 (Fig. 3B), but sharply decreased at pH 3.0. Although the purified enzyme maintained a considerable degree of fibrinolytic activity at pH 5.0 and 12.0, but the pH stability abruptly decreased at the pH values, these results indicate that the BK I protein is relatively stable to a broad range of temperature and pH value.

N-terminal amino acid sequencing

The first 14 amino acid residues in the N-terminal sequence of BK I protein were Q-T-G-G-S-I-I-D-P-I-N-G-Y-N (Fig. 4). The N-terminal sequence of BK I protein shared two amino acid residues, glycine and isoleucin, with the BK II fibrinolytic enzyme and only one residue, proline, with the fibrinolytic enzymes Carlsberg (Smith *et al*., 1968) and CK (Kim *et al*., 1996), respectively. No homology in the N-terminal amino acid sequence was shown between the BK I protein and other fibrinolytic enzymes, such as BK II (Jeong *et al*., 2004a), BPN' (Smith *et al*., 1968), and NK (Nakamura *et al*., 1992). Of note, BlastP analysis revealed that the N-terminal sequence of BK-I is highly similar to the endo-beta-1,3-1,4 glucanase from *Bacillus subtilis* subsp. natto BEST195 (29QTGGSFFDPFNGYN42). This finding suggests that both enzymes may be functionally related because the endoglucanase has similarity to deblocking aminopeptidase by amino acid sequence (Ando *et al*., 1999). This study may suggest that at least a couple of fibrinolytic enzymes exist in *B. subtilis* strain A1, thus designated as BK I and BK II proteins. Therefore, these studies could support the fibrinolytic enzyme from *B. subtilis* strain as a potential thrombolytic agent.

Acknowledgements

This research was supported by the Technology Development Program for Agriculture and Forestry (610003-03-1-SB110), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea. This work was also supported by the Dong-A University Research Fund, Korea.

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