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Purification and Properties of a Novel β-Glucosidase, Hydrolyzing Ginsenoside Rb1 to CK, from *Paecilomyces Bainier*

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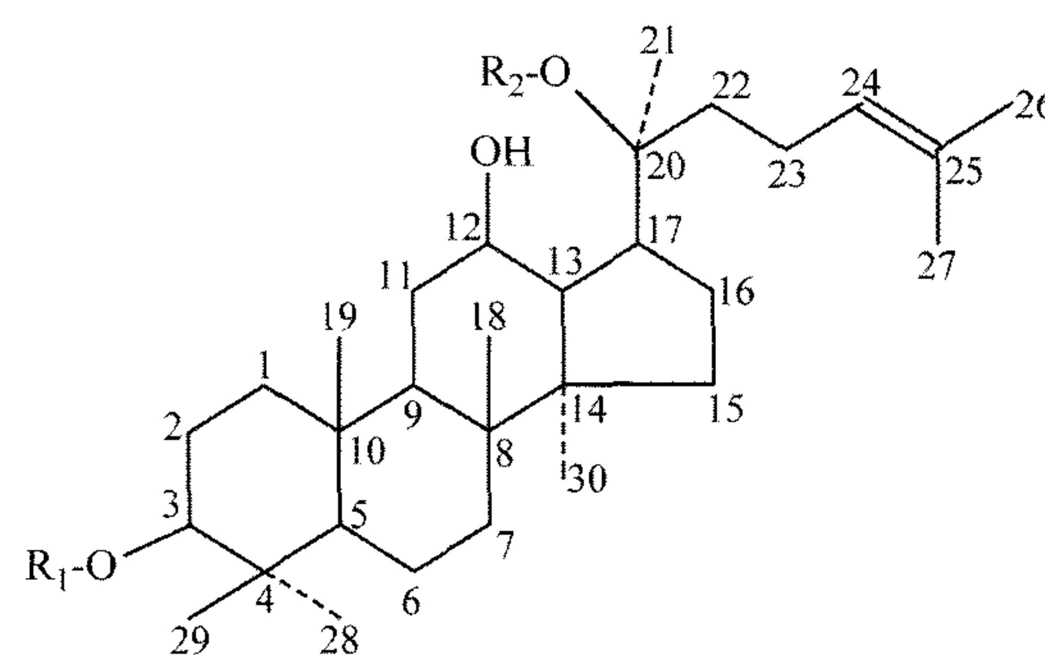
A novel ginsenoside-hydrolyzing β-glucosidase was purified from *Paecilomyces Bainier* sp. 229 by a combination of Q-Sepharose FF, phenyl-Sepharose CL-4B, and CHT ceramic hydroxyapatite column chromatography. The purified enzyme was a monomeric protein with a molecular mass estimated to be 115 kDa. The optimal enzyme activity was observed at pH 3.5 and 60°C. It was highly stable within pH 3–9 and at temperatures lower than 55°C. The enzyme was specific to β-glucoside. The order of enzyme activities against different types of β-glucosidic linkages was β-(1-6)>β-(1-2)>β-(1-4). The enzyme converted ginsenoside Rb1 to CK specifically and efficiently. An 84.3% amount of ginsenoside Rb1, with an initial concentration of 2 mM, was converted into CK in 24 h by the enzyme at 45°C and pH 3.5. The hydrolysis pathway of ginsenoside Rb1 by the enzyme was Rb1→Rd→F2→CK. Five tryptic peptide fragments of the enzyme were identified by a newly developed *de novo* sequencing method of post-source decay (PSD) matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. By comparing the five identified peptide sequences with the NCBI database, this purified β-glucosidase proves to be a new protein that has not been reported before.

Keywords: Ginsenoside CK, ginsenoside-hydrolyzing β-glucosidase, purification, *Paecilomyces Bainier*

Ginseng (the root of *Panax ginseng* C.A. Meyer Araliaceae) is a traditional herbal medicine that has been used for thousands of years in Asian countries for its various medicinal functions such as tonic, adaptogenic, immunomodulatory, and anti-aging effects [4, 12, 16, 29, 33]. The major active ingredients of ginseng are triterpene glycosides known as ginsenosides (Fig. 1) [3, 31]. However, these naturally occurring ginsenosides are observed to be poorly absorbed along the human intestinal tracts [20]. Their pharmacological actions are actually attributed to the deglycosylated aglycone metabolites formed in the intestine [7, 26]. Earlier reports demonstrated that protopanaxadiol-type ginsenosides such as Rb1, Rb2, and Rc are metabolized by intestinal bacteria after oral administration to a final derivative, 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol, also known as



Protopanaxadiol type ginsenoside	R$_1$	R$_2$
Rb1	-Glc^{2}-^{1}Glc	-Glc^{6}-^{1}Glc
Rd	-Glc^{2}-^{1}Glc	-Glc
F2	-Glc	-Glc
Rg3	-Glc^{2}-^{1}Glc	-H
CK	-H	-Glc
Rh2	-Glc	-H
Rb2	-Glc^{2}-^{1}Glc	-Glc^{6}-^{1}Arap
CO	-Glc	-Glc^{6}-^{1}Arap
CY	-H	-Glc^{6}-^{1}Arap
Rb3	-Glc^{2}-^{1}Glc	-Glc^{6}-^{1}Xyl
Mx	-H	-Glc^{6}-^{1}Xyl

Fig. 1. The chemical structures of relevant ginsenosides.

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ginsenoside CK [1, 11]. It has been suggested that ginsenoside CK can be easily absorbed by the human body and is actually the genuine active form of protopanaxadiol-type saponins [22].

Recently, ginsenoside CK has attracted more and more interest because of its intriguing biological actions. It has been widely reported that ginsenoside CK induces tumor cell apoptosis, inhibits tumor metastasis, and restrains tumor invasion [8, 9, 21, 27, 28]. In addition, ginsenoside CK also showed hepatoprotective and anti-inflammatory activities that appeared to be stronger than those of currently used drugs such as dimethyl diphenyl bicarboxylate (DDB) and indomethacin [15, 23]. Since ginsenoside CK does not exist in natural products and the natural transforming ability of human intestinal bacteria is rather limited, much attention has been paid to the preparation of CK. Several chemical approaches have been tried to deglycosylate ginsenosides (Yang *et al.*, China Patent CN03134090.3) [13]. However, none of them was suitable for preparation of ginsenoside CK, because both glycone moieties at C-20 could be cleaved nonspecifically under chemical hydrolysis conditions. Hence, enzymatic transformation, which is considered to be highly region-specific, might be a promising method.

Ginsenoside Rb1 is the main component in ginsenosides. It is a protopanaxadiol-type ginsenoside that has a structure similar to that of ginsenoside CK. By enzymatically hydrolyzing the two glucose molecules at C-3 and one glucose molecule at C-20, ginsenoside CK can easily be transformed from Rb1. However, owing to the special structure of the dammarane skeleton, only special β-glucosidases can hydrolyze ginsenoside-β-glucosidic linkages. So far, only one β-glucosidase has been reported to be able to slightly hydrolyze ginsenoside Rb1 to CK [10]. This reported enzyme, which was purified from China white jade snail, actually converted ginsenoside Rb1 mainly to Rd and F2. The content of ginsenoside CK was so low in the hydrolyzed products that it was unsuitable for practical application.

In this paper, a novel ginsenoside-hydrolyzing β-glucosidase that transforms ginsenoside Rb1 to CK specifically and efficiently was purified from *Paecilomyces Bainier* sp. 229, which was isolated from the soils of ginseng plantation localities. Some biological properties and the amino acid sequence of the purified enzyme were further studied.

Materials and Methods

Materials

p-Nitrophenyl-β-D-glucoside (*p*NPβG), *p*-nitrophenyl-α-D-glucoside (*p*NPαG), *p*-nitrophenyl-β-L-arabinopyranoside (*p*NPβA), and *p*-nitrophenyl-β-D-xyloside (*p*NPβX) were purchased from Sigma (St. Louis, MO, U.S.A.). Q-Sepharose FF, phenyl-Sepharose CL-4B, and Sephacryl S-300 HR were from Amersham Pharmacia (Uppsala, Sweden). CHT ceramic hydroxyapatite was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Ginsenosides Rb1, Rb2, Rb3, Rd, Rg3, F2, and Rh2 were from the China National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ginsenosides CY and Mx were isolated from the ginsenoside metabolites formed by *Paecilomyces Bainier* sp. 229 in our lab (unpublished paper). Ginsenoside CK was prepared in our previous study [34]. α-Cyano-4-hydroxycinnamic acid (CHCA), 4-sulfophenyl isothiocyanate (SPITC), and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, U.S.A.). Sequencing-grade modified trypsin was from Promega (Madison, WI, U.S.A.). The ZipTip reversed-phase C_{18} desalting tips were from Millipore (Bedford, MA, U.S.A.). Other chemicals were of analytical reagent grade.

Isolation of Ginsenoside-transforming Fungal Strain

The dried soil samples, which were collected from several ginseng plantation localities, were spread onto potato dextrose agar (PDA) plates containing 1 g/l of ginseng general ginsenosides. After incubation at 28°C for 2 d, more than 200 colonies were isolated by streak cultivation. Colony 229, which was identified as a *Paecilomyces Bainier* sp. by the Department of Microbiology and Microbial Engineering of Fudan University, was selected for its high ginsenoside transforming activity. The strain was maintained on PDA slants by periodical transfer, and stored at 4°C.

Assay of Enzyme Activity

β-Glucosidase activity was determined by a colorimetric method using *p*NPβG as a substrate. The reaction mixture, containing 40 μl of 6 mM *p*NPβG, 40 μl of enzyme solution, and 80 μl of 0.1 M formate buffer (pH 3.5), was incubated at 45°C for 20 min. The reaction was stopped by adding 840 μl of 0.5 M sodium hydroxide. Its absorbance at 405 nm was measured with a UNICO UV-2000 spectrophotometer (UNICO, Dayton, NJ, U.S.A.). When ginsenoside (or glucobiose) was taken as a substrate, the reaction was stopped by heating in boiling water for 3 min. The liberated glucose was measured by the glucose oxidase-peroxidase method (GOD-POD) using a glucose test kit (Shanghai Rongcheng Biotech, Shanghai, China). One unit of β-glucosidase activity (U) was defined as the amount of enzyme liberating 1 μmole of *p*-nitrophenol (or glucose) per minute under standard conditions. Specific activity was defined in terms of units per mg of protein.

Protein Measurement

Protein was measured by the Bradford method, using bovine serum albumin as a standard protein [5].

Preparation of Crude Enzyme

Paecilomyces Bainier sp. 229 was cultured in an aerobic fermentor (BIOFLO 110; New Brunswick Scientific, NJ, U.S.A.) at 28°C with stirring at 600 rpm/min and with ventilation of 4 l/min for 48 h in 8 l of medium containing 3% soybean powder, 3% sucrose, 0.2% calcium chloride, 0.1% magnesium sulfate, 0.1% ammonium sulfate, and 0.1% ginseng general ginsenosides. The cells were harvested by centrifugation at 3,000 ×*g* for 60 min and washed twice with cold 50 mM acetate buffer (pH 5.0). The pellets were then freeze-dried (Christ, Osterode a.H., Germany), and then pulverized by a jet mill (GJM-H100, Shanghai Hualy Superfines, Shanghai, China). The disrupted cells were suspended in 2 l of 50 mM acetate buffer (pH 5.0). After being stirred for 1 h, the cell pellets were removed by centrifugation at 3,000 ×*g* for 40 min. The supernatant was used as the crude enzyme solution in purification procedures.

Purification of β-Glucosidase

All the β-glucosidase purification steps were carried out at 4–6°C. The following buffers were used: buffer A, 10 mM sodium phosphate buffer (pH 7.0); buffer B, 10 mM sodium phosphate buffer containing 0.4 M ammonium sulfate (pH 7.0); buffer C, 10 mM potassium phosphate buffer (pH 6.8).

The crude enzyme was precipitated overnight at 4°C with 90% saturated ammonium sulfate and centrifuged at 12,000 ×g for 30 min. The pellets were collected, dissolved with 100 ml of buffer A, and dialyzed against 800 ml of the same buffer four times. After removing the nondissolved fraction by centrifugation, the dialysate was loaded onto a Q-Sepharose FF column (2.6×6 cm) previously equilibrated with buffer A. After washing with 4 column volumes of the same buffer, a 600-ml linear gradient elution of 0 to 0.3 M sodium chloride was done. The active fractions hydrolyzing ginsenoside Rb1 to CK were pooled and the same volume of buffer A containing 0.8 M ammonium sulfate (pH 7.0) was added. The mixed solution was then put on a phenyl-Sepharose CL-4B column (2.6×6 cm) previously equilibrated with buffer B. The column was washed with 4 column volumes of the same buffer and a 600-ml linear gradient elution of 0.4 to 0 M ammonium sulfate was done. The active fractions hydrolyzing ginsenoside Rb1 to CK were collected and dialyzed 4 times against buffer C. The dialysate was further applied to a CHT ceramic hydroxyapatite column (1.6×6 cm) previously equilibrated with buffer C. The column was washed with 4 column volumes of the same buffer and then eluted with a 400-ml linear 10 to 300 mM potassium phosphate buffer gradient. The active fractions were pooled and subjected to native and denatured PAGE.

Electrophoresis and Molecular Mass Determination

SDS-PAGE was performed on a 6% polyacrylamide gel by the procedure described by Laemmli [14]. The gel was stained by Coomassie Brilliant Blue R250. The molecular mass of β-glucosidase was estimated by both SDS-PAGE and gel filtration on a Sephacryl S-300 HR column. The standard protein markers for gel filtration were ferritin (440 kDa), catalase (250 kDa), bovine serum albumin (66 kDa), and ovalbumin (44 kDa).

Electrophoresis was also carried out on a 5% polyacrylamide gel under native conditions according to Reisfeld *et al.* [25]. The same enzyme sample was loaded into two tanks of the same gel. After the procedure of native electrophoresis, the two electrophoresed lanes were cut apart. One was stained by Coomassie Brilliant Blue R250, whereas the other was used for the staining of enzyme activity by the following procedure: the gel was cut at intervals of 0.4 cm, and each of the cut pieces, instead of enzyme solution, was immersed in the enzyme reaction mixture and incubated at 45°C for 2 h to assay enzyme activity.

Effect of pH on Enzyme Activity and Stability

The *p*NPβG hydrolyzing activity of the purified enzyme was studied at 45°C in the following buffers: 50 mM phosphate buffer (pH 1.5–3.0 and pH 6.0–8.0), 50 mM formate buffer (pH 3.0–4.5), 50 mM acetate buffer (pH 4.0–6.0), and 50 mM Tris-HCl buffer (pH 8.0–10.0). The pH stability of the enzyme was measured according to the residual activity after pre-incubating the enzyme in the above buffers (pH 1.5–10.0) at 45°C for 4 h.

Effect of Temperature on Enzyme Activity and Stability

β-Glucosidase activity was investigated at different temperatures ranging from 30 to 75°C in 50 mM formate buffer (pH 3.5). The thermal stability was examined according to the residual activity after pre-incubating the enzyme in 50 mM formate buffer (pH 3.5) at 25–70°C for 4 h.

Determinations of Kinetic Parameters

The enzyme activity was measured at 45°C and pH 3.5 with a final substrate concentration from 0.075 to 1.5 mM. The K_m and V_{max} values against *p*NPβG were determined using the Lineweaver-Burk plot [18].

Catalytic Properties on Ginsenosides

One hundred µl of 0.05 M formate buffer (pH 3.5) containing ginsenoside (2 mM) and β-glucosidase (total activity 0.02 U against *p*NPβG) was incubated at 45°C for 0.5–24 h. The reaction was stopped by adding 400 µl of methanol. Quantitative analysis of the hydrolyzed products was performed on a high-performance liquid chromatography (HPLC) system (Waters 2487 detector, 1525 binary pump; Milford, MA, U.S.A.) equipped with a C_{18} column (Waters Symmetry, 150 mm×4.6 mm, 5 µm) at 35°C. Water and acetonitrile were used as the binary gradient with the following profile: 0–20 min, 70–60% water, 30–40% acetonitrile; 20–30 min, 60–10% water, 40–90% acetonitrile. The flow rate was 1.0 ml/min. Eluted products were detected at a wavelength of 203 nm.

Analysis of Amino Acid Sequence

After SDS-PAGE of the purified enzyme, the target protein spot was excised from the gel. The in-gel trypsin digestion, sulfonation with SPITC, and sample desalting were carried out according to the description of Leon *et al.* [17]. The tryptic peptides, either sulfonated or intact, were applied on a 4700-Proteomics Analyzer with version 3.0 software (Applied Biosystems, Foster City, CA, U.S.A.) to

Table 1. Purification of β-glucosidase.

Purification step	Total activity[a] (U)	Total protein (mg)	Specific activity[b] (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	1,055.06	885.39	1.19	1.0	100
Ammonium sulfate precipitation	906.22	580.91	1.56	1.3	85.9
Q-Sepharose FF	188.26	19.99	9.42	7.9	17.8
Phenyl-Sepharose CL-4B	27.48	1.104	24.89	20.9	2.60
CHT ceramic hydroxyapatite	21.32	0.156	136.67	115	2.02

[a]One unit of β-glucosidase activity (U) was defined as the amount of enzyme liberating 1 µmol of *p*-nitrophenol per minute under standard conditions (pH 3.5, 45°C).

[b]Specific activity was defined as the β-glucosidase activity per mg of protein.

perform the analysis of post-source decay (PSD) matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) [6, 17]. The mass spectrometer used a 200 Hz frequency tripled Nd:YAG laser operating at a wavelength of 355 nm. For MS/MS, ions generated by the MALDI process were accelerated at 8 kV through a grid at 6.7 kV into a short, linear, and field-free drift region. MS/MS data were analyzed manually in order to obtain the *de novo* sequences, which were further compared with the NCBI database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

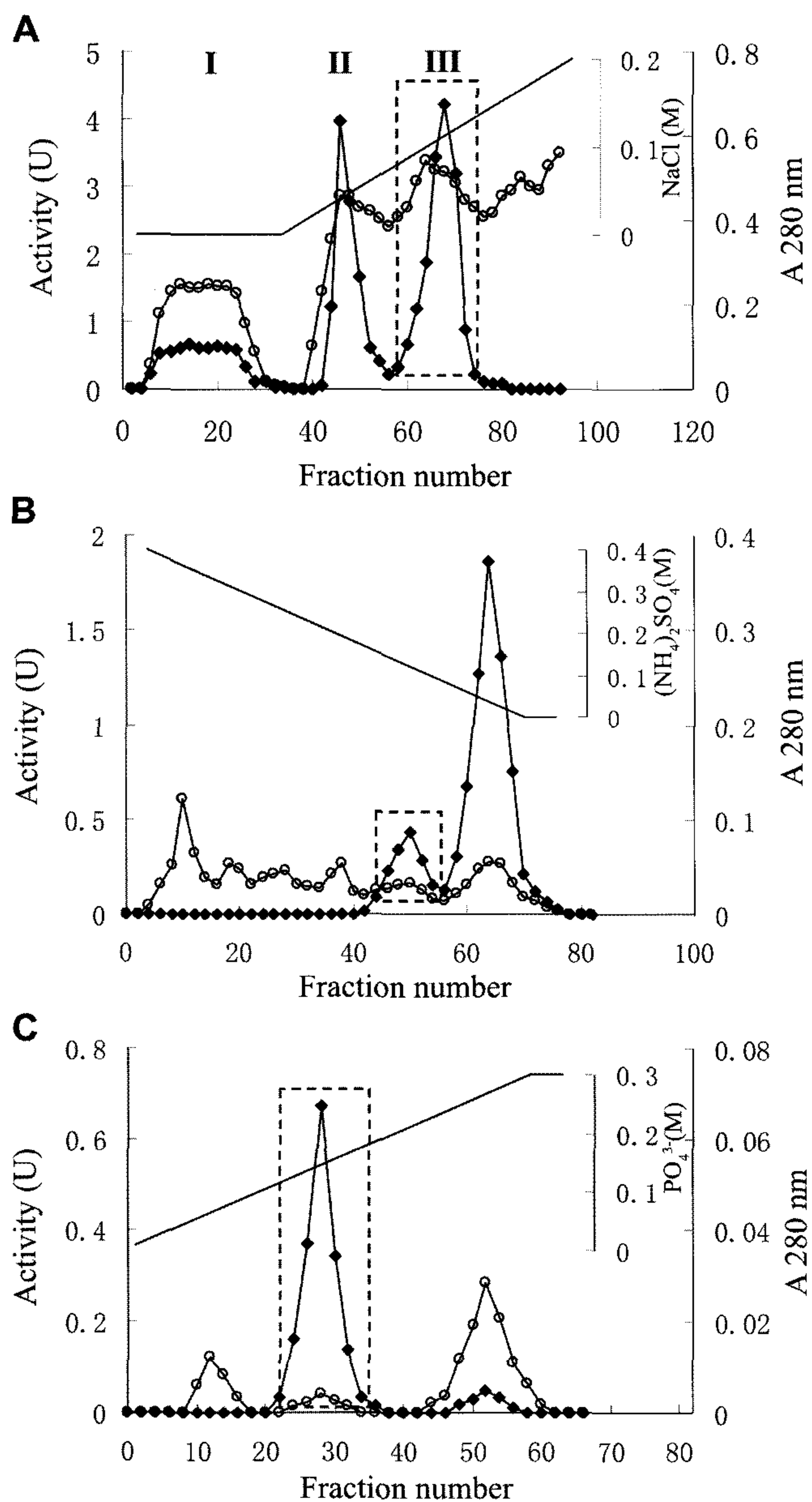

Fig. 2. Purification of β-glucosidase.
A. Ion-exchange chromatography on Q-Sepharose FF; **B**. Hydrophilic chromatography on phenyl-Sepharose CL-4B; **C**. CHT ceramic hydroxyapatite chromatography. ◆, *p*NPβG hydrolyzing activity; ○, absorbance at 280 nm. Fractions contained in broken-lined rectangles showed the activity of converting ginsenoside Rb1 to CK. Therefore, they were pooled for further purification or as final purified enzyme.

Results

β-Glucosidase Purification and Molecular Mass

β-Glucosidase was purified 115-fold from the crude enzyme with a final yield of 2.02% by the procedures shown in Table 1. The specific activity of the purified enzyme against *p*NPβG was 136.67 U/mg at pH 3.5 and 45°C. After ammonium sulfate precipitation (90% saturation), the crude enzyme was loaded on a Q-Sepharose FF column. The elution profile is shown in Fig. 2A. Three peaks containing β-glucosidase activity were eluted out. Only peak III was observed to have the activity converting ginsenoside Rb1 to CK. Hence, this peak was pooled and further purified to homogeneity by a combination of phenyl-Sepharose CL-4B and CHT ceramic hydroxyapatite column chromatography (Figs. 2A and 2B). The purified enzyme migrated as a single band in native and denatured PAGE (Fig. 3). In the native PAGE, the single band stained by Coomassie Brilliant Blue R250 was at the identical location as the enzyme activity peak seen on the gel. The molecular mass of the purified enzyme was approximately 115 kDa either by gel filtration on a Sephacryl S-300 column or by SDS-PAGE, which indicated that this enzyme was a monomeric protein.

Effect of pH on Enzyme Activity and Stability

The enzyme activity was assayed under standard conditions at different pHs ranging from 1.5 to 10.0. The optimal activity was observed at pH 3.5 (Fig. 4A). The enzyme

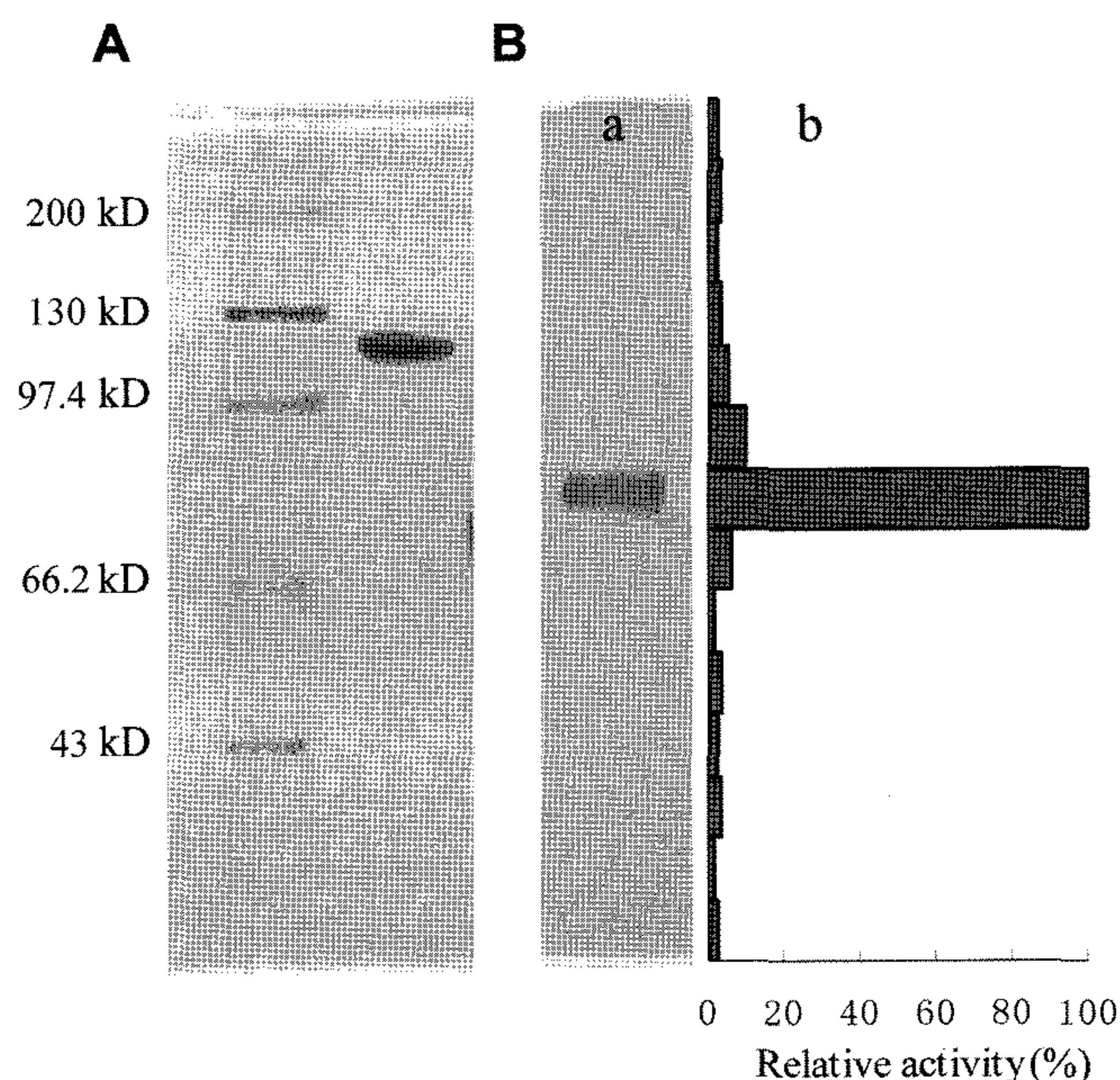

Fig. 3. SDS-PAGE (**A**) and native PAGE (**B**) of the purified β-glucosidase.
A. Coomassie Brilliant Blue R250 staining. **B**. Activity straining. The maximum activity was taken as 100%.

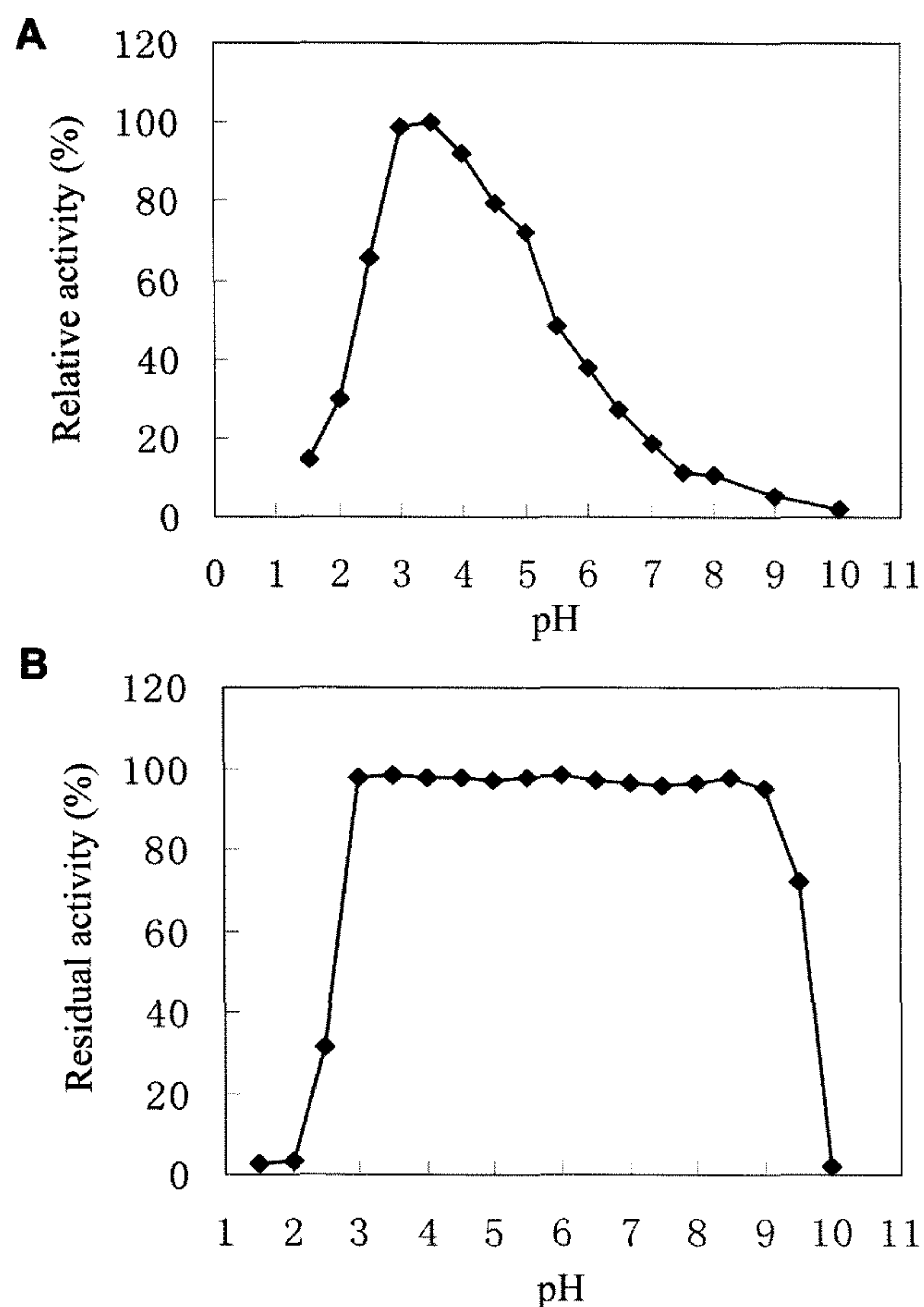

Fig. 4. Effects of pH on enzyme activity and stability.
A. Effect of pH on enzyme activity. The maximum activity observed at pH 3.5 was taken as 100%. **B.** Effect of pH on enzyme stability. The enzyme activity was assayed under standard conditions (pH 3.5, 45°C) after incubation at 45°C and different pHs for 4 h. The original activity before incubation was taken as 100%.

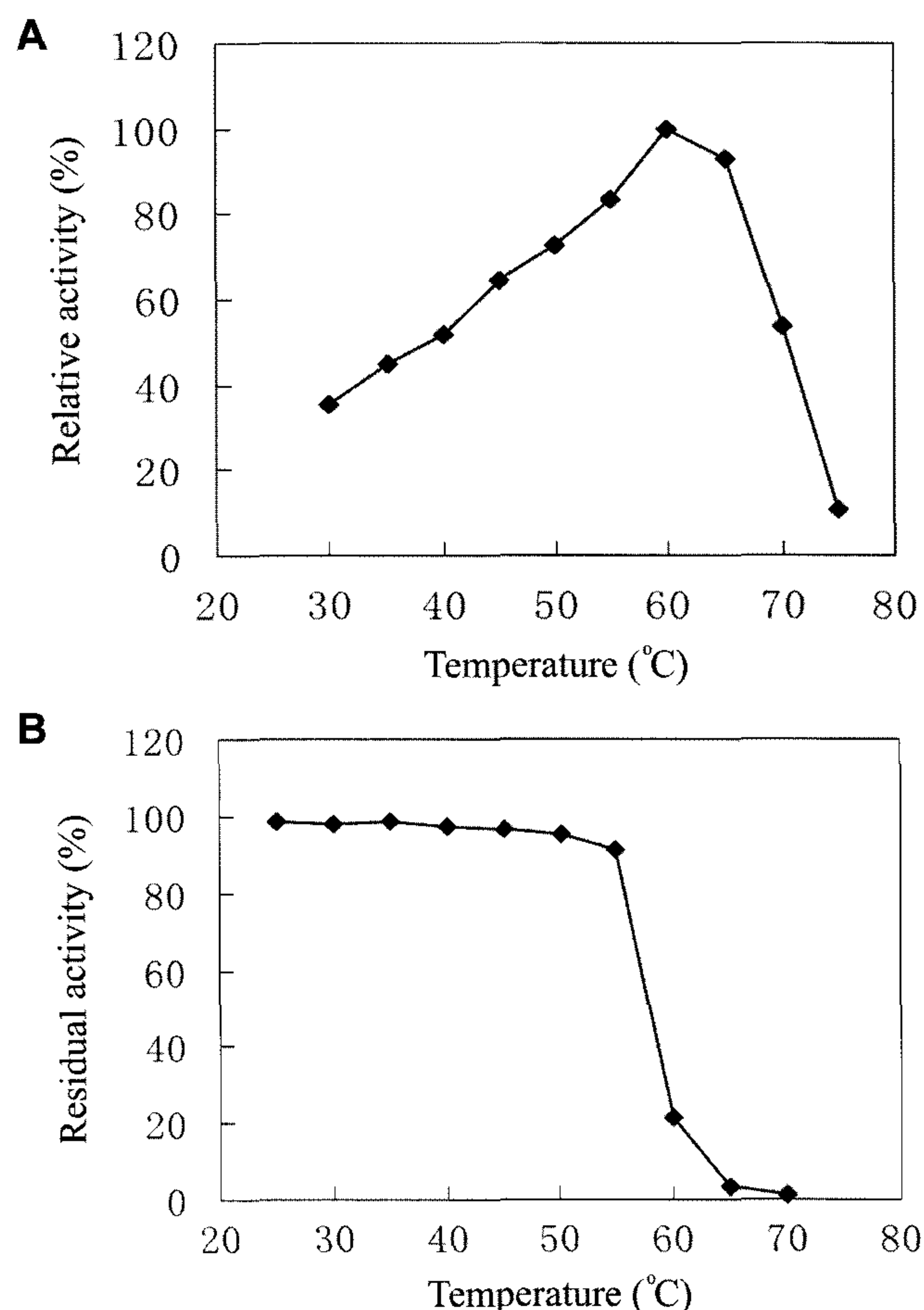

Fig. 5. Effects of temperature on enzyme activity and stability.
A. Effect of temperature on enzyme activity. The maximum activity observed at 60°C was taken as 100%. **B.** Effect of temperature on enzyme stability. The enzyme activity was assayed under standard conditions (pH 3.5, 45°C) after incubation at pH 3.5 and different temperatures for 4 h. The original activity before incubation was taken as 100%.

activity did not vary much within pH 3.0–3.5, whereas outside this range, the activity decreased rapidly. In order to study the effect of pH on the enzyme stability, residual activity was measured under standard conditions (pH 3.5, 45°C) after incubating the enzyme at 45°C for 4 h at a pH ranging from 1.5 to 10.0. The results showed that β-glucosidase was stable over the wide range of pH 3.0–9.0, within which the enzyme still retained more than 95% of original activity after the incubation (Fig. 4B).

Effect of Temperature on Enzyme Activity and Stability
When assayed at pH 3.5, β-glucosidase showed its optimal activity at 60°C (Fig. 5A). Within the temperature range of 55–65°C, the enzyme exhibited more than 80% of optimum activity, whereas at temperatures higher than 65°C, the activity declined sharply. The thermal stability tests showed that the enzyme was quite stable at temperatures lower than 55°C (Fig. 5B). After incubation at pH 3.5 and 55°C for 4 h, 91.4% of original activity still remained. When the incubation temperature was increased to 60°C, there was a steep decrease in residual activity and only 21.5% of original activity the retained under the same conditions.

Kinetic Parameters
The kinetic parameters of the enzyme were determined by typical Lineweaver-Burk double reciprocal plot. The K_m and V_{max} values against *p*NPβG were 0.102 mM and 0.158 mmol/min/mg, respectively, at pH 3.5 and 45°C (Fig. 6).

Catalytic Properties
Various substrates were incubated with the purified enzyme under standard conditions (Table 2). The optimal substrate was *p*NPβG, followed by ginsenoside Rb1, gentiobiose, and ginsenoside Rd. The enzyme activities on *p*NPαG, *p*NPβA, *p*NPβX, and maltose were not detectable, which indicated that this enzyme was specific to β-glucoside. Among the disaccharides with β-glucosidic linkages, the

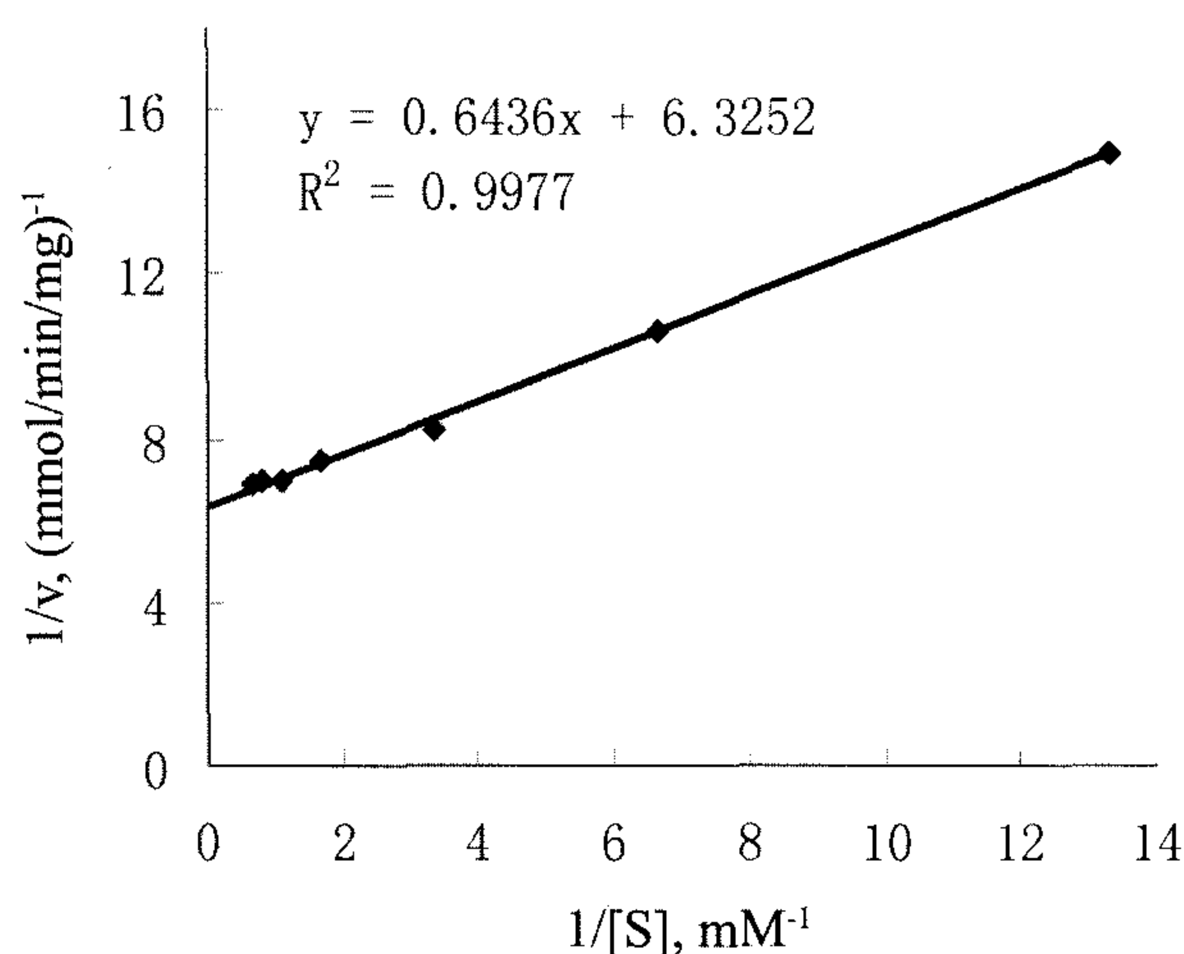

Fig. 6. Lineweaver-Burk plot for the purified β-glucosidase.
The values were assayed against *p*NPβG in 0.05 M formate buffer (pH 3.5) at 45°C.

Table 2. β-Glucosidase activity on various substrates.

Substrate[a]	Enzyme activity (%)[b]
*p*NPβG	100
*p*NPβG	0
*p*NPβA	0
*p*NPβX	0
Gentiobiose	39.2
Cellobiose	19.5
Sophorose	25.8
Maltose	0
Ginsenoside Rb1	67.3
Ginsenoside Rb2	17.6
Ginsenoside Rb3	26.8
Ginsenoside Rd	34.0
Ginsenoside F2	18.8
Ginsenoside CK	0

[a]Final concentration, 1.5 mM.
[b]The activity against *p*NPβG was taken as 100%.

order of enzyme activities was gentiobiose>sophorose>cellobiose.

In order to study the ginsenoside-hydrolyzing properties, the enzyme was incubated with ginsenoside Rb1 under standard conditions. The hydrolyzed products were periodically analyzed by HPLC (Fig. 7). The result showed that ginsenoside Rb1 was initially transformed to Rd, then to F2, and finally to CK. After 4 h, ginsenoside CK began to be detectable. After 24 h, 84.3% of ginsenoside Rb1 was transformed to CK. Neither Rg3 nor Rh2 was detected in the hydrolyzed products. We therefore concluded that the hydrolysis pathway of ginsenoside Rb1 by β-glucosidase was Rb1→Rd→F2→CK.

We also performed the hydrolysis experiment taking ginsenosides Rb2 and Rb3, respectively, as substrate. The results showed that, since this enzyme was specific to β-glucosidic linkage, it only hydrolyzed the two glucose molecules at C-3 of ginsenosides Rb2 and Rb3 and transformed them to CY and Mx, respectively (Fig. 8).

Analysis of Amino Acid Sequence

N-Terminal sequence analysis of β-glucosidase was done. However, this enzyme seemed to be *N*-terminal blocked and its *N*-terminal sequence was not available by the Edman method. Therefore, we turned to a newly developed SPITC-labeled PSD-MALDI technology for *de novo* sequencing of the enzyme protein. The PSD-MALDI spectra of a peptide from the tryptic digest are shown in Fig. 9. The spectrum obtained from the intact peptide demonstrated a complex fragmentation pattern consisting of incomplete series of *N*-terminal a- and b-type ions, *C*-terminal y- and z-ions, and even internal cleavage products. The spectrum was difficult to accurately interpret, whereas in the spectrum of the peptide sulfonated by SPITC, the

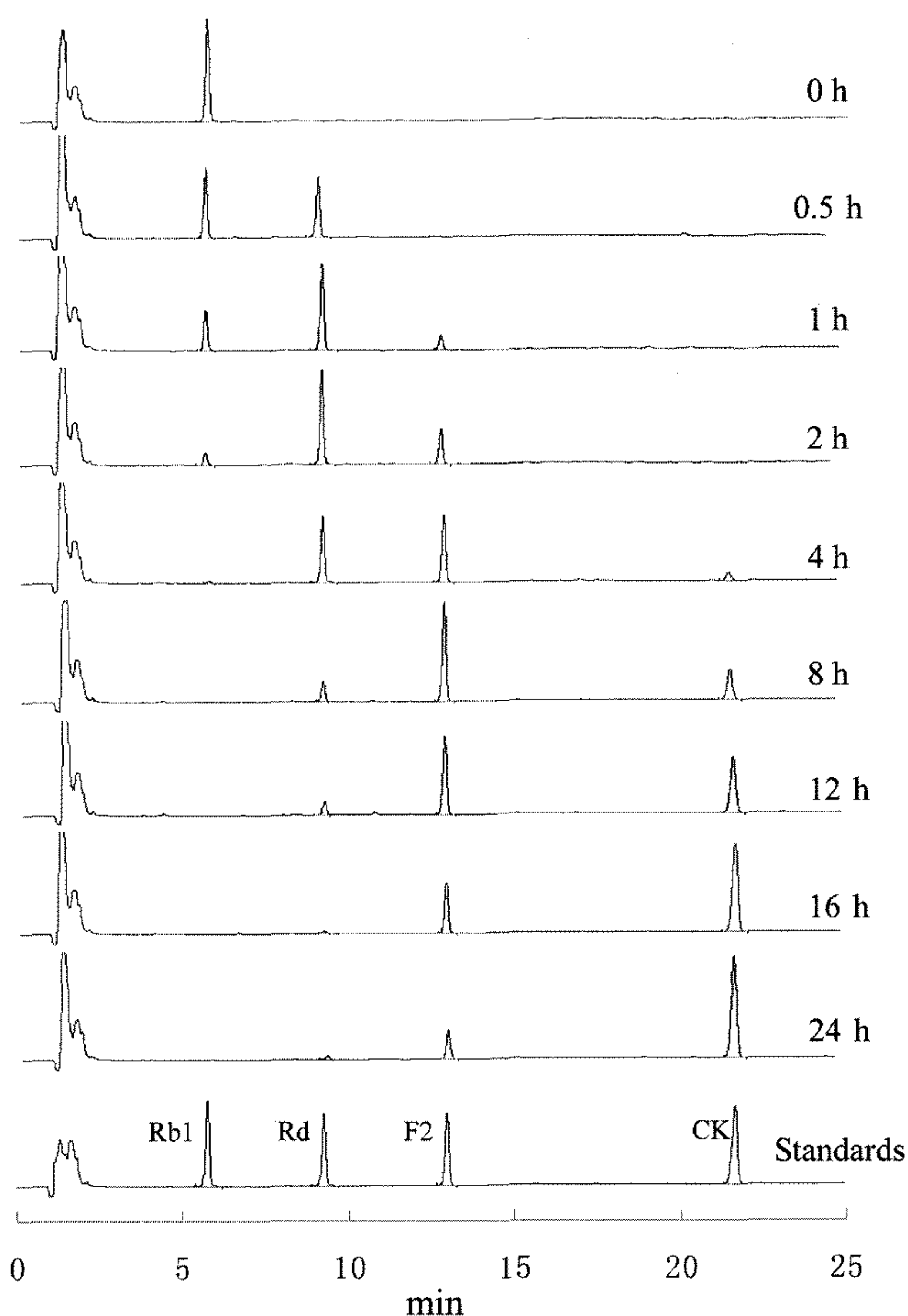

Fig. 7. HPLC profiles of hydrolyzed products of ginsenoside Rb1 by β-glucosidase.
One hundred μl of reaction mixture containing ginsenoside Rb1 (2 mM) and purified β-glucosidase (0.02 U against *p*NPβG) was incubated at 45°C and pH 3.5 for 0.5–24 h.

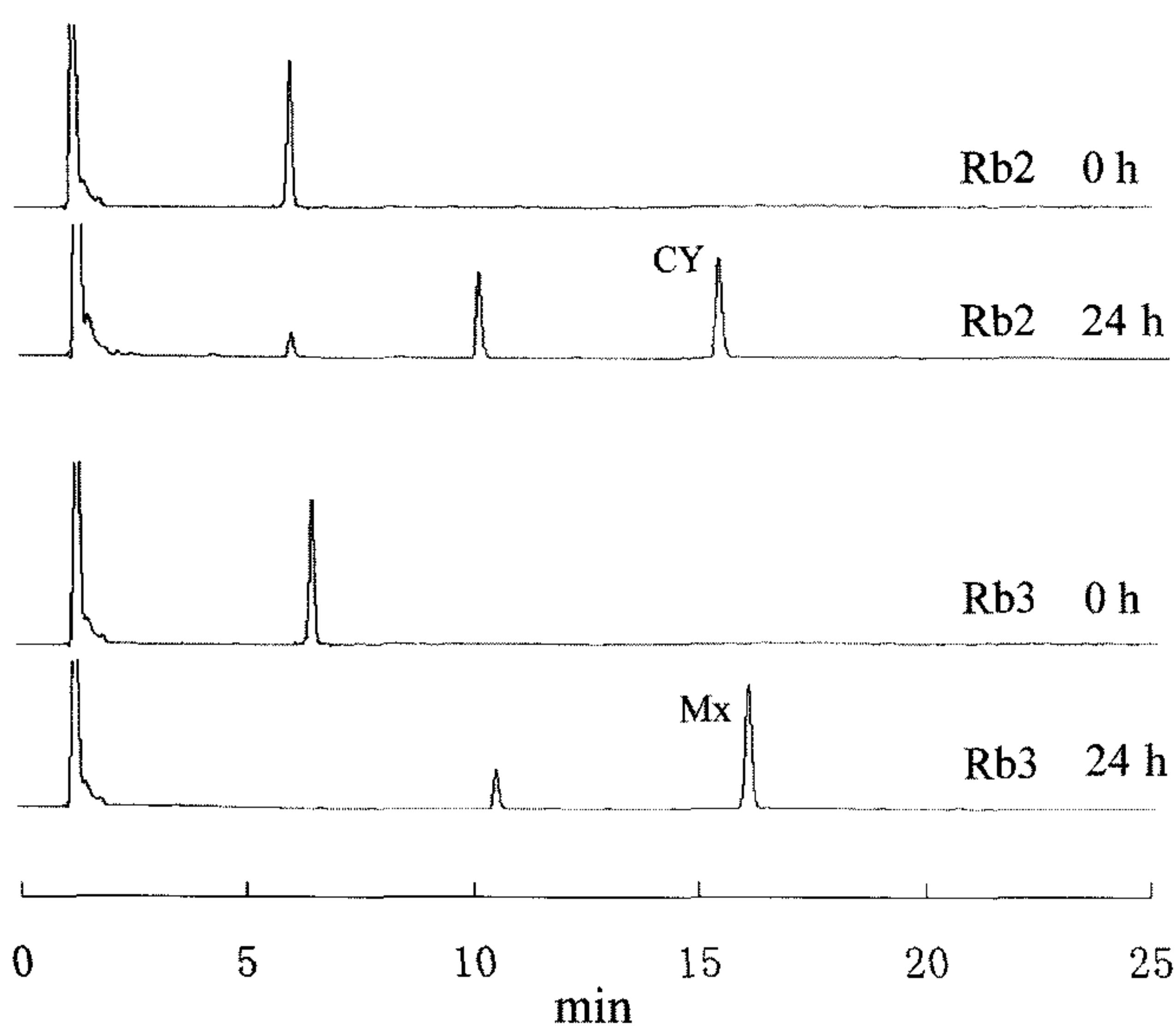

Fig. 8. HPLC profile of hydrolyzed products of ginsenosides Rb2 and Rb3, respectively, by β-glucosidase.

One hundred μl of reaction mixture containing ginsenoside Rb2 or Rb3 (2 mM) and purified β-glucosidase (0.02 U against *p*NPβG) was incubated at 45°C and pH 3.5 for 24 h.

fragment ion peaks represented the complete y-ion series plus the labeled precursor ion (+215 Da), making the amino acid sequence (S[I/L]VDV[I/L]YGR) easily interpretable by manual calculation of the differences between adjacent y-ion fragments. The total five peptides identified from the tryptic digest of β-glucosidase are shown in Table 3. Since

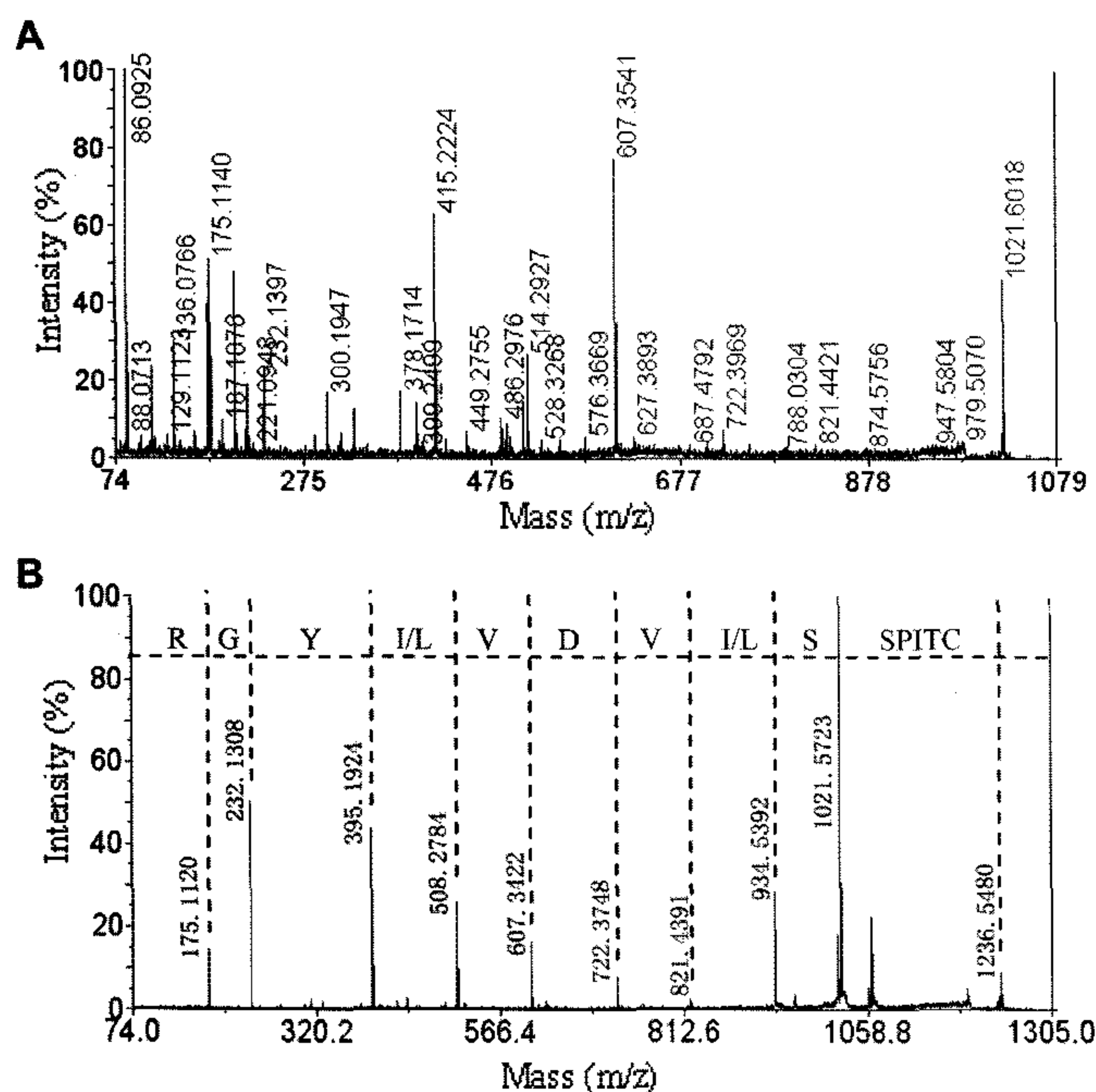

Fig. 9. PSD-MALDI spectra of a peptide from the tryptic digest of β-glucosidase.

A. Intact peptide; **B.** Peptide sulfonated by SPITC.

Table 3. Peptide fragments identified from tryptic digest of β-glucosidase.

Parent icon (*m/z*)	Peptide sequence
811.4366	DHAS[I/L][I/L]R
1021.5723	S[I/L]VDV[I/L]YGR
1107.5620	GG[I/L]P[I/L]THQER
1429.6443	HY[I/L]GNEQEHFR
1883.9839	VT[I/L]APGQQ[I/L]QWTAT[I/L]TR

the molecular weights of isoleucine and leucine are exactly the same, they could not be differentiated by this method.

We consequently searched the NCBI database for homologous proteins of the purified β-glucosidase. No protein has yet been found to contain more than two of the five identified peptide fragments, which suggested that the enzyme was a new unreported protein. By comparing the peptide sequences with known β-glucosidases in the database, it could be concluded that the two identified peptides, S[I/L]VDV[I/L]YGR and HY[I/L]GNEQEHFR, might actually be SLVDVLYGR and HYIGNEQEHFR, respectively (Table 4). They should be relatively conserved in the amino acid sequences of β-glucosidases. No reported β-glucosidase, however, was found to contain both of these two peptide fragments.

DISCUSSION

Ginsenoside CK is a promising drug in the treatments of numerous human pathologies including malignant tumors. Unfortunately, the difficulty in its preparation has severely limited the application and development of this compound. To date, a few β-glucosidases have been reported to show ginsenoside-hydrolyzing activity [10, 19, 24, 30]. However, none of them was suitable for preparation of ginsenoside CK. In this study, a novel β-glucosidase, which transforms ginsenoside Rb1 to CK very efficiently, was purified and characterized.

The purified enzyme is specific to β-glucoside. The order of enzyme activities against different β-glucose dimers indicates that the specificity on different types of β-glucosidic linkages is β-(1-6)>β-(1-2)>β-(1-4). The molecular mass of the enzyme, a monomeric protein estimated to be of 115 kDa, is similar to the subunit of a β-glucosidase isolated from China white jade snail [19]. However, it is higher than those of other reported β-glucosidases [10, 24, 30, 32]. The optimal pH of the enzyme was found to be 3.5, which is lower than previously reported β-glucosidases whose optimal pH ranges from 4 to 7 [10, 19, 24, 30, 32]. We suggest that, since the *Paecilomyces Bainier* sp. 229 grows best at pH 3.0–4.0, the enzyme in this microorganism should also show its optimal activity under this condition. The purified β-glucosidase was

Table 4. Comparison of two peptide sequences of purified enzyme with known β-glucosidases.

GenBank Accession No.	Source	Sequence
	Paecilomyces Bainier	S[I/L]VDV[I/L]YGR
AAZ95587	*Thermoascus aurantiacus*	572 AGLPGQESGN SLVDVLYGR VSPGGKTPFT
XP_750327	*Aspergillus fumigatus*	582 AGLPGQESGN SLVDVLYGR VNPSAKTPFT
AAL69548	*Talaromyces emersonii*	566 AGLPGQESGN SLVDVLYGR VNPGKTPFTW
XP_569544	*Cryptococcus neoformans*	588 AGLPGQESGN SLVDVLYGA YNPSARLPYT
XP_001398816	*Aspergillus niger*	571 GGLPGQESGN SLADVLYGR VNPGAKSPFT
	Paecilomyces Bainier	HY[I/L]GNEQEHFR
ABS71124	*Penicillium occitanis*	27 QDTGVIACAK HYIGNEQEHFR QGSQENFTVA
ABP88968	*Penicillium brasilianum*	198 QDTGVIACAK HYIGNEQEHFR QVGEAAGHGY
AAT95381	*Phaeosphaeria nodorum*	196 QDAGVIACTK HYIMNEQEHFR QPGNFEDQGF
CAB82861	*Phaeosphaeria avenaria*	193 QDAGVIACTK HYIMNEQEHFR QPGNFEDQGF

The identical amino acids are highlighted in grey.

highly stable at temperatures lower than 55°C within a wide pH range from 3 to 9, suggesting it is completely suitable for industrial application.

The study of ginsenoside Rb1 hydrolysis by the purified enzyme showed that it converted ginsenoside Rb1 to CK quite efficiently. A total 84.3% of ginsenoside Rb1, with an initial concentration of 2 mM, was converted into CK in 24 h by the enzyme at 45°C and pH 3.5. The pathway was observed to be Rb1→Rd→F2→CK, which indicates that the enzyme hydrolyzes the two glucose molecules at C-3 and one glucose at C-20 of ginsenoside Rb1. In an earlier report, a ginsenoside-hydrolyzing β-glucosidase from China white jade snail was also found to be able to transform Rb1 to CK by the same hydrolysis pathway [10]. However, in that report, the major hydrolyzed products actually were Rd and F2. The content of ginsenoside CK was extremely low and showed no obvious increase after 16 h. Given the intriguing pharmacological effects and the difficult availability of ginsenoside CK, this new finding is of great significance: It clearly provides a new and efficient approach for preparation of this useful compound.

We then sequenced five peptide fragments of the purified enzyme by the latest PSD-MALDI MS method according to previous reports [6, 17]. In this new analyzing method, the *N*-terminals of peptides from the tryptic digest are sulfonated by SPITC before the MS analysis. The presence of this newly formed acidic group greatly enhances the fragmentation efficiency of tryptic peptides. In addition, its negative charge neutralizes the positive charge of the captured proton in the b-type ions, making them undetectable by mass spectrometry. Hence, the resulting spectra are mainly composed of a well-characterized series of y-ions, which makes the interpretation much easier and more efficient for *de novo* sequencing. By comparing the five identified peptide fragments with the NCBI database using BLAST, the purified β-glucosidase proves to be a new protein that has not yet been reported. The two identified sequences, SLVDVLYGR or HYIGNEQEHFR, commonly exist in many β-glucosidases, indicating that they are relatively conserved in the amino acid sequences of β-glucosidases. On the other hand, no reported β-glucosidase contains both of these two peptide sequences. We therefore suggest that the enzyme gene might be able to be isolated from the microorganism using the primers designed according to these two peptide fragments. This study on amino acid sequencing is a remarkable contribution to further research on its gene cloning, which we are going to carry out.

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