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Biocatalytic properties of a novel crude glycyrrhizin hydrolase from the liver of the domestic duck

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Abstract

A novel crude glycyrrhizin (GL) hydrolase preparation from the liver of domestic duck was used to produce glycyrrhetic acid monoglucuronide. To characterize the biocatalytic profiles of the crude enzyme, some effect factors were investigated. It had an apparent optimal pH of 6.0 and an optimal temperature at 55 °C. Most of the metal ions tested and ethylene diamine tetra acetic acid showed little effect on the crude enzyme activity except Cu^{2+}. The enzyme was stable only at pH 6. It was more prone to inactivity at high pH conditions than at low pH conditions. It was stable at temperatures below 55 °C and it will lost 90% GL hydrolytic activity exposed at 70 °C. GL hydrolytic activity declined by 30% compared with the control in aqueous solution (buffer pH 6.0) when pre-equilibrated at 55 °C for 5 days. It indicated that the novel crude GL hydrolase preparation had good biocatalytic ability for selective hydrolysis of one glucuronic acid from GL.
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Keywords: β-Glucuronidase; Biocatalysis; Glycyrrhizin; Glycyrrhetic acid monoglucuronide

1. Introduction

Glycosides represent an important class of biologically active organic compounds. The sugar moieties of the glycosides are crucial for their activities [1,2] and pharmacokinetic parameters [3]. Recent developments in glycobiology implicated that glycosyl-conjugates played essential roles in biological recognition and cell signalling [4,5]. Glycosides were involved in pathology of human severe diseases, such as cancer. Based on these findings, it is possible to develop new, more active or more effective glycodrugs [6]. With the increased understanding of drug metabolic processes, it has led to a growing appreciation of the role and significance of 'phase II' metabolites, glucuronides in particular. Glucuronides of xenobotics not only perform an important detoxification role, but also have significant biological activities. Therefore, glucuronylation of natural or synthetic compounds has been an attractive method for developing new active drugs [7]. A very illustrative recent example is the story of morphine-6-glucuronide [8,9].

Glycyrrhizin (GL) is a triterpenoid saponin found in the root of licorice (*Glycyrrhiza glabra*) and is composed of one molecule of glycyrrhetic acid (GA) and two molecules of glucuronic acid. It has been shown that GL induces various biological activities, such as an anti-inflammatory effect, and antiviral effects [10], anti-severe acute respiratory syndrome (SARS)–coV activity [11]. GA is metabolite of GL by removal of two molecules of glucuronic acid. Glycyrrhetic acid monoglucuronide (GAMG) is the intermediate metabolite between GL and GA. For having two molecules of glucuronic acid, the strong molecular polarity of hydrophilic GL is a hindrance of entering into hydrophobic cell membrane and then taking its effects. The obvious evidence is that there is no detectable level of GL in the patient blood after oral administration. Only GAMG and GA were detected in the patient blood [12]. Without glucuronic acid, hydrophobic GA had a too weak solubility in the blood because of a weak molecular polarity. With one molecule of glucuronic acid, GAMG had a suitable molecular polarity, and had many benefit properties compared to GL and GA. So GAMG exhibited the similar (or stronger) effects to (or than) glycyrrhizin and GA.

GAMG was produced by selectively removal of one molecule of glucuronic acid from GL [13]. There were many purified β-glucuronidases could hydrolyze GL [14–16] to GAMG. But only *Cryptococcus magnus* MG-27^{+} [17] had high selectivity to producing GAMG in industrial scale.

In this study, a novel crude GL hydrolase preparation from the liver of domestic duck was used to produce GAMG. Effects on its biocatalytic properties were investigated particularly.

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2. Experimental

2.1. Reagents

Standard glycyrrhizinic acid and glycyrrhetic acid were purchased from Sigma Co. (USA). Authentic GAMG was kindly gift from Prof. Dong-hyun Kim (College of Pharmacy, KyungHee University, Korea). Glycyrrhizinic acid (monoammonium salt) was purchased from Gansu Lante Phytochemical Co. Ltd. Methanol was of chromatography grade. All other chemicals were obtained commercially. Freshly deionized water (18.2 MΩ) prepared by a Millipore Milli-Q® was used in HPLC.

2.2. Preparation of crude GL hydrolase

Crude GL hydrolase was prepared with modification of the method in literature [18]. Fifty grams fresh duck liver prefrozen at 0–4 °C was homogenized four times in a Waring blender for 30 s each. The homogenate was poured into 200 ml acetone at −20 °C with stirring. When the suspension had settled, the precipitate was collected by filtration using a Büchner funnel with No. 1 Xinhua filter paper under vacuum, washed with 100 ml cold acetone followed by 50 ml cold ether and dried in a vacuum desiccator. The dry powder was ground and stored in refrigerator at 0–4 °C. The preparation retained their biotransformation activities for 3 years.

2.3. HPLC and NMR analysis

HPLC was performed using LB-5 pump with a UV detector and N-2000 workstation. GL and GAMG were analyzed on Apollo C_{18} column using methanol–water–acetic acid (75:25:5, v/v/v) at a flow rate of 1.0 ml min^{-1}. Concentrations of GL and GAMG were calculated from peak areas using a calibration curve. ^{1}H NMR and ^{13}C NMR spectra were recorded in CD_3OD–D_2O (1:1, v/v) using a Bruker 400 Hz spectrometer.

2.4. The assay of enzyme activity

GL hydrolase activity was measured at 55 °C by mixing 3 mg crude GL hydrolase with 6 mM GL, buffered with 0.2 M citrate buffer, pH 6.0 (final volume 600 μl). The mixture was incubated for 2 h. The reaction was stopped by adding 400 μl methanol. GAMG and GL were analyzed by HPLC.

2.5. Optimal conditions of GL hydrolysis by the crude GL hydrolase

The reaction mixtures were incubated for 2 h at various pH, temperatures and concentrations of GL while fixing the other conditions. Percentage conversion of GL was determined.

2.6. Effect of various metal ions and chelator

The effect of different metal ions on the crude GL hydrolase activity was determined by the addition of the corresponding ion at a final concentration of 1.0 mM to the reaction mixture, and assayed under above conditions. The enzyme assay was carried out in the presence of $CuSO_4$, $MnSO_4$, $BaCl_2$, $CaCl_2$ and $FeSO_4$. Additionally, the effect of a chelator of divalent cations (ethylene diamine tetra acetic acid, EDTA) was also determined by preincubation with the enzyme solution for 30 min at 55 °C before the addition of substrate.

2.7. Effect of pH and temperature on enzyme stability

The crude enzyme was added in different relevant buffers (pH 4.0–9.0) and incubated at 55 °C for enzyme stability. Aliquots were withdrawn at different time for assay of GL hydrolytic activities.

To determine the enzyme stability with changes in temperature, crude enzyme was preincubated at different temperatures (25–70 °C) and relative GL hydrolytic activities were assayed.

2.8. Enzyme stability in aqueous solution

The crude enzyme in aqueous solution (buffer pH 6.0) was pre-equilibrated at 55 °C and pH 6.0. Aliquots were withdrawn at different time for assay of GL hydrolytic activities.

3. Results and discussion

3.1. pH optimum and pH stability

For the determination of the pH optimum, phosphate (pH 5.0–7.0), Tris–HCl (pH 8.0), and glycine–NaOH (pH 9.0–10.0) buffers were used. The highest GL hydrolase activity was found to be at pH 6.0 using phosphate buffer (Fig. 1). These findings are in accordance with several earlier reports showing pH optima of 5–7 for partial purified β-glucuronidase from *Bacteroides* J-37 [19], *Eubacterium* sp. [20], *Cryptococcus magnus*

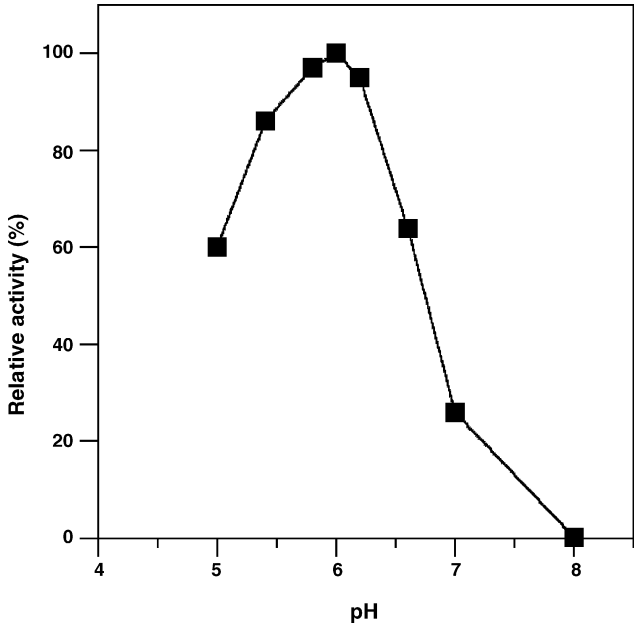

Fig. 1. Effect of pH on enzyme activity.

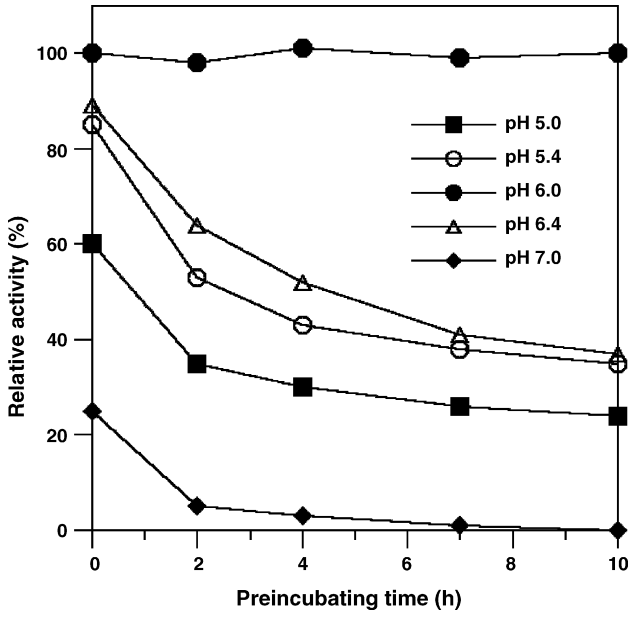

Fig. 2. Effect of pH on enzyme stability.

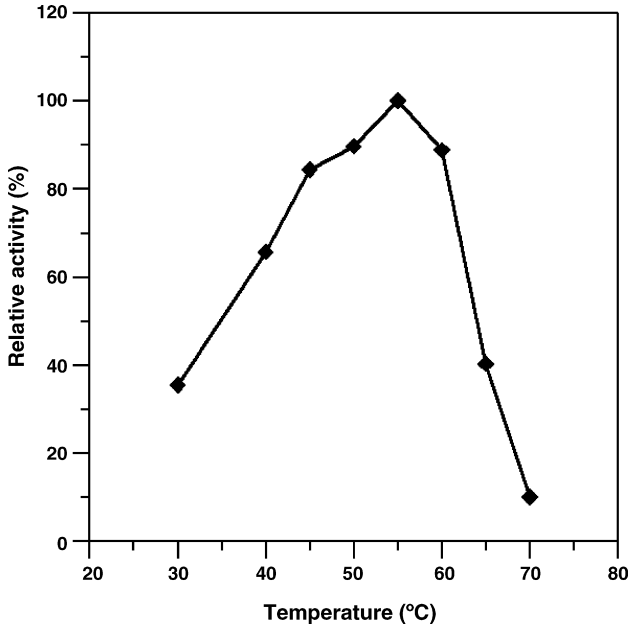

Fig. 3. Effect of temperature on enzyme activity.

MG-27$^+$ [17] and *Streptococcus* LJ-22 [16]. It also indicated that the GL hydrolase was very sensitive to pH and lost its activity entirely when pH is beyond 8.0. GL hydrolase had an optimum pH range of 5.8–6.2 at which their activity is maximal. This could be explained as follows. On one hand, amino acid side chains in the active site may act as weak acids and bases with critical functions that depend on their maintaining a certain state of ionization, and elsewhere in the protein, ionized side chains may play an essential role in the interactions that maintain protein structure. On the other hand, the substrate, GL is a weak tribasic acid and the variation of the degree of ionization of carboxyl group with pH affects it binding to enzyme molecule.

The stability of the GL hydrolase was also determined by the preincubation of the enzyme in various buffers of different pH values. In the case of 2 h preincubation, the enzyme was stable only at pH 6 (Fig. 2). It was more prone to inactivity at high pH conditions than at low pH conditions.

3.2. Temperature optimum and thermal stability

The activity of the crude enzyme was determined at different temperatures ranging from 30 to 70 °C. The optimum temperature was at 55 °C for GL hydrolytic activity. The enzyme activity gradually declined at temperatures beyond 55 °C (Fig. 3). This occurs because as the temperature changes this supplies enough energy to break some of the intramolecular attractions between polar groups as well as the hydrophobic forces between nonpolar groups within the protein structure. When these forces are disturbed and changed, this causes a change in the secondary and tertiary levels of protein structure, and the active site is altered in its conformation beyond its ability to accommodate the substrate molecules it was intended to catalyze. A optical temperature of 50 °C was recorded for a β-glucuronidase from *Cryptococcus magnus* MG-27$^+$ [17] and 45 °C for GL hydrolase derived from *Aspergillus niger* GRM3 [21], those were different in optical temperature with the crude enzyme reported in this work.

The thermal stability of the crude enzyme was tested at different temperatures ranging from 25 to 70 °C for 2 h. The crude enzyme was stable at temperatures below 55 °C (Fig. 4). When exposed at 70 °C, it lost 90% GL hydrolytic activity. It was indicated that the crude enzyme used in this study had better thermal tolerance while GL hydrolase derived from *A. niger* GRM3 was inactive entirely at 60 °C [21].

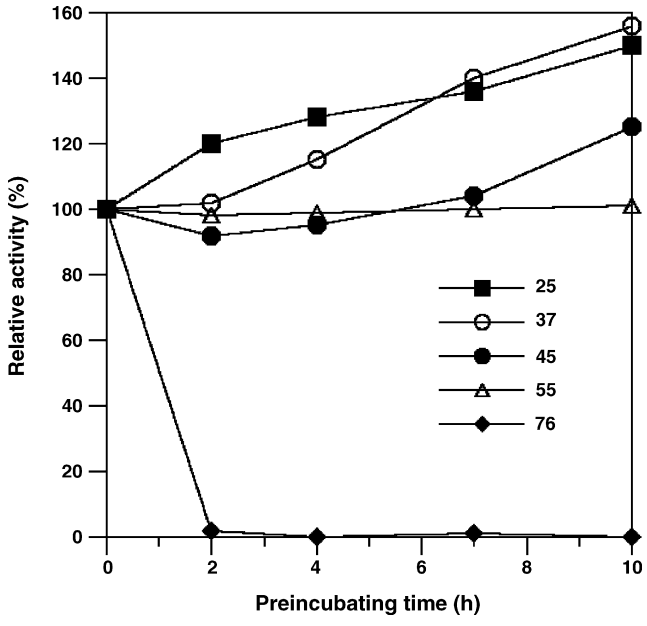

Fig. 4. Effect of temperature on stability of enzyme.

Table 1
Effect of various metal ions on GL hydrolase activity[a]

Metal ions (1 mM)	Residual GL hydrolase activity (%)
Control	100
$BaCl_2$	108
$CaCl_2$	99
$CuSO_4$	34
$FeSO_4$	99
$MnSO_4$	104
EDTA	103

[a] EDTA indicates ethylene diamine tetra acetic acid.

3.3. Effect of metal ions and chelator

Most of the metal ions tested had a slight stimulatory effect (Ba^{2+} and Mn^{2+}) or an inhibitory effect (other ions) on enzyme activity (Table 1). Some of the metal ions such as Ba^{2+} and Mn^{2+} increased GL hydrolytic activity of the crude enzyme; this is possible because of the activation by the metal ions. Cu^{2+} was an inhibitor to GL hydrolytic activity. EDTA also showed an appreciable effect on enzyme activity. The result may be due to EDTA bound to some inhibitory metal ions in the crude enzyme preparation. Other metal ions such as Ca^{2+} and Fe^{2+} did not show an appreciable effect on enzyme activity.

3.4. Stability in aqueous solution

Enzyme stability in aqueous solution (buffer pH 6.0) was studied at 55 °C for 5 days. As shown in Fig. 5, GL hydrolytic activity increased by 20% compared with the control during the first 2 days. This was probably a consequence of GL hydrolase was released gradually from the crude enzyme preparation. Interestingly, GL hydrolytic activity declined by 30% compared with the control during last 3 days. The results indicated that the crude enzyme was inactivated under the above conditions.

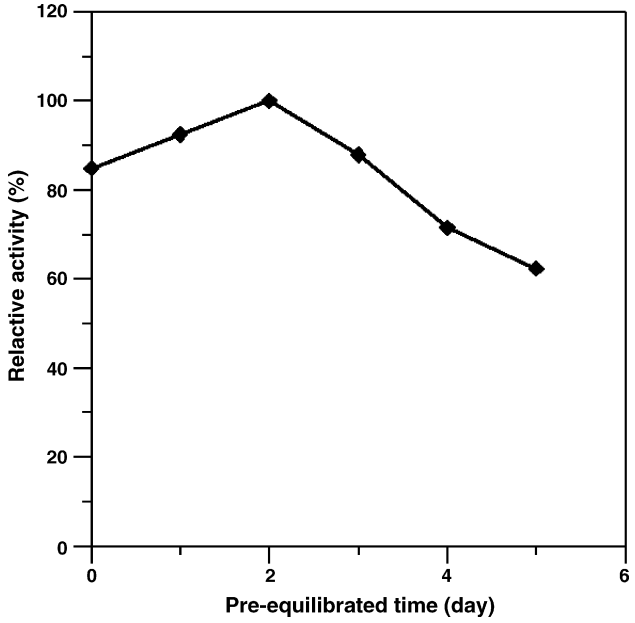

Fig. 5. Stability of the crude enzyme in aqueous solution.

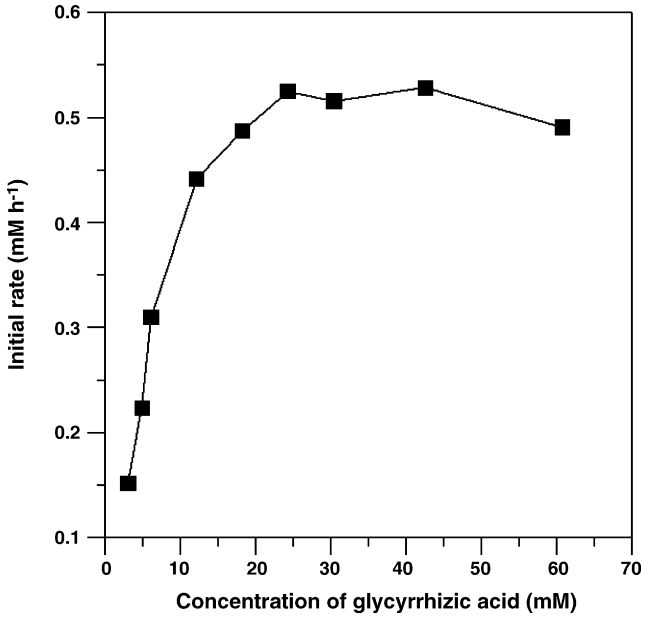

Fig. 6. Effect of concentration of glycyrrhizin on enzyme activity.

3.5. Effect of GL concentration

Effects of GL concentration on hydrolytic activity were studied under optimal pH and temperature. The data fitted well to Michaelis–Menten equation when GL concentrations lower than 42 mM. M–M parameters were calculated using Lineweaver–Burk plot. K_m and V_{max} were 10.98 mM and 0.74 mM/h respectively. It seems that GL inhibited the crude enzyme activity when concentration of GL up to 60 mM (Fig. 6).

3.6. Selectivity of the crude enzyme preparation

The crude enzyme preparation was measured for the selectivity of GL hydrolytic activities. It could effectively hydrolyze GL to produce GAMG without GA from the HPLC result under optimized conditions. When biotransformation reaction proceeded for 23 h, GL was completely converted to GAMG. Maintaining the reaction further, there was no GA produced (Fig. 7).

Various β-glucuronidases (EC 3.2.1.31) from bacteria to mammals are known. Although some β-glucuronidases were used for hydrolyzation of GL, only a few of them could selectively remove one molecular glucuronic acid from GL. In the present study, a crude enzyme preparation derived from the liver of the domestic duck, converted GL to GAMG, but did not hydrolyse GAMG further. It suggested that the crude enzyme preparation in this work differed from some reported ones and could be used for production of GAMG.

3.7. ^1H NMR and ^{13}C NMR spectra of GAMG

^1H NMR and ^{13}C NMR spectra were recorded in CD_3OD–D_2O (1:1, v/v) using a Bruker 400 Hz spectrometer.

For GAMG, ^1H NMR (400 MHz, CD_3OD/D_2O, 1:1, v/v) δ (ppm): 0.66 (s, CH_3), 0.71 (s, CH_3), 0.92 (s, CH_3), 0.98 (s, CH_3), 1.05 (s, CH_3), 1.09 (s, CH_3), 1.26 (s, CH_3), 4.32 (d,

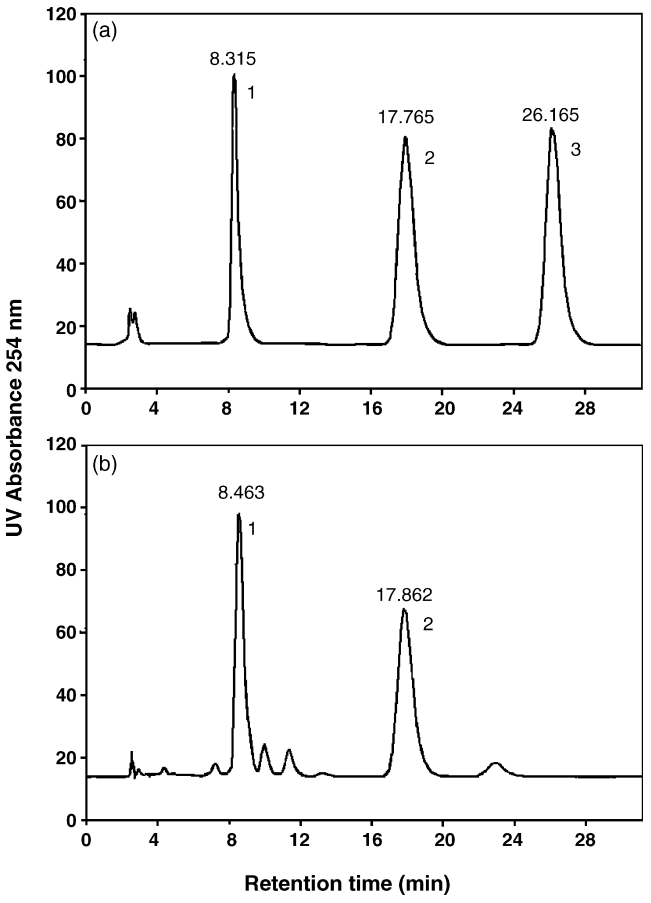

Fig. 7. HPLC chromatography of enzymatic reaction mixture: (a) chromatography of standard compounds and (b) chromatography of enzymatic reaction mixture. (1) glycyrrhizic acid; (2) glycyrrhetic acid monoglucuronide; and (3) glycyrrhetic acid.

J 7.4, anomeric proton), 5.45 (s, *CH*=). ^{13}C NMR (400 MHz, CD_3OD/D_2O, 1:1, v/v) δ (ppm): 39.1 (C-1), 26.5 (C-2), 90.1 (C-3), 39.4 (C-4), 55.1 (C-5), 17.2 (C-6), 32.6 (C-7), 43.6 (C-8), 62.0 (C-9), 36.8 (C-10), 203.1 (C-11), 127.5 (C-12), 175.5 (C-13), 45.8 (C-14), 25.7 (C-15), 26.2 (C-16), 31.9 (C-17), 49.9 (C-18), 41.2 (C-19), 44.1 (C-20), 30.9 (C-21), 37.8 (C-22), 27.4 (C-23), 15.9 (C-24), 16.1 (C-25), 18.3 (C-26), 22.8 (C-27), 27.9 (C-28), 28.2 (C-29), 181.1 (C-30), 105.3 (C-1′), 82.1 (C-2′), 76.3 (C-3′), 74.0 (C-4′), 72.3 (C-5′), 173.4 (C-6′).

From the data of ^{1}H NMR and ^{13}C NMR, it could be concluded that GAMG was effectively produced by selectively hydrolyzing one glucuronic acid from GL.

4. Conclusions

A crude GL hydrolase preparation from the liver of domestic duck was used to produce GAMG and the enzymatic reaction conditions were optimized. Most of the metal ions and EDTA had little effect on enzyme activity except Cu^{2+}. The enzyme was stable only at pH 6. It was more prone to inactivity at high pH conditions than at low pH conditions. It was stable at temperatures below 55 °C. GL hydrolytic activity declined by 30% compared with the control in aqueous solution (buffer pH 6.0) when pre-equilibrated at 55 °C for 5 days. It indicated that the novel crude GL hydrolase preparation had good biocatalytic ability for selective hydrolysis of one glucuronic acid from GL. The enzyme was expected to be used in selective hydrolysis oligosaccharide chain which had a terminal glucuronic acid in natural products.

It should be noted that this study has examined only the biocatalytic properties of the crude GL hydrolase preparation. Our results are lack of the data of the purified enzyme. Further study along this line will be reported in due course.

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