Appl Biochem Biotechnol (2011) 163:162–172
DOI 10.1007/s12010-010-9025-x

Effects of Low-Shear Modeled Microgravity on the Characterization of Recombinant β-D-Glucuronidase Expressed in *Pichia pastoris*

Feng Qi · DaZhang Dai · Yanli Liu · Imdad Kaleem · Chun Li

Received: 4 April 2010 / Accepted: 23 June 2010 /
Published online: 6 July 2010

Abstract In this study, we used a high-aspect-ratio vessel (HARV), which could model environment of microgravity on ground to investigate for the first time the effects of low-shear modeled microgravity (LSMMG) on the characterization of recombinant β-D-glucuronidase expressed in *Pichia pastoris*. The β-D-glucuronidase gene (GenBank accession no. EU095019) derived from *Penicillium purpurogenum* Li-3 encoding β-D-glucuronidase (PGUS) was expressed in *P. pastoris* GS115 in two different environments of LSMMG and normal gravity (NG). Results manifested that both LSMMG and NG conditions had insignificant effects on temperature and pH activity (optimal temperature and pH were 55 and 5.0 °C, respectively) and characteristic stability of recombinant PGUS. However, the catalytic activity of recombinant PGUS expressed under LSMMG was less affected by metal ions and EDTA as compared with that of NG. Furthermore, K_m value of the recombinant PGUS expressed under LSMMG was nearly one fifth of that under NG (1.72 vs. 7.72), whereas catalytic efficiency (k_{cat}/K_m) of PGUS expressed under LSMMG (13.55) was 3.7 times higher than that of NG (3.61). The results initially reveal the significant alterations in catalytic properties of recombinant enzyme in response to LSMMG environment and have potential application in bioprocessing and biocatalysis.

Keywords Low-shear modeled microgravity (LSMMG) · Normal gravity (NG) · *Pichia pastoris* · Recombinant PGUS

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Introduction

In recent years, studies concerning the influences of microgravity on microbial cells are drawing more and more attention, and such information will lead to the advancement of knowledge regarding microbes' adaptations under the microgravity conditions. Most of these studies remained focus on the safety and health of astronauts during a long spaceflight time. A few studies also demonstrated that several microbial cellular processes, such as cell growth, gene expression [1], resistance to radiations, phage induction [2], contraction of cell size [3], secondary-metabolites production [4–7], and efficient utilization of the available nutrients [8], were altered when cells were cultured in microgravity or LSMMG conditions. However, little is known about the mechanism through which microbial cells sense the reduced gravity conditions and also how they convert these mechanical signals into molecular and biochemical responses. Because of high cost value, long duration of spaceflight, and the need of sensitive instrumentations, it has become difficult to meet the standards of space experiments, so the studies of space biology are limited to a large degree. To overcome these problems, several forms of ground-based low-shear suspension culture that could model aspects of spaceflight studies of "weightlessness" were invented [9, 10]. One such bioreactor, named as high-aspect-ratio vessel (HARV, Synthecon) [11] has been used in this study (Fig. 1). The HARV bioreactor used for cell suspension culture and tissue growth permits cell growth in suspension and minimizes the fluid shear levels encountered by cells [12, 13]. The low-

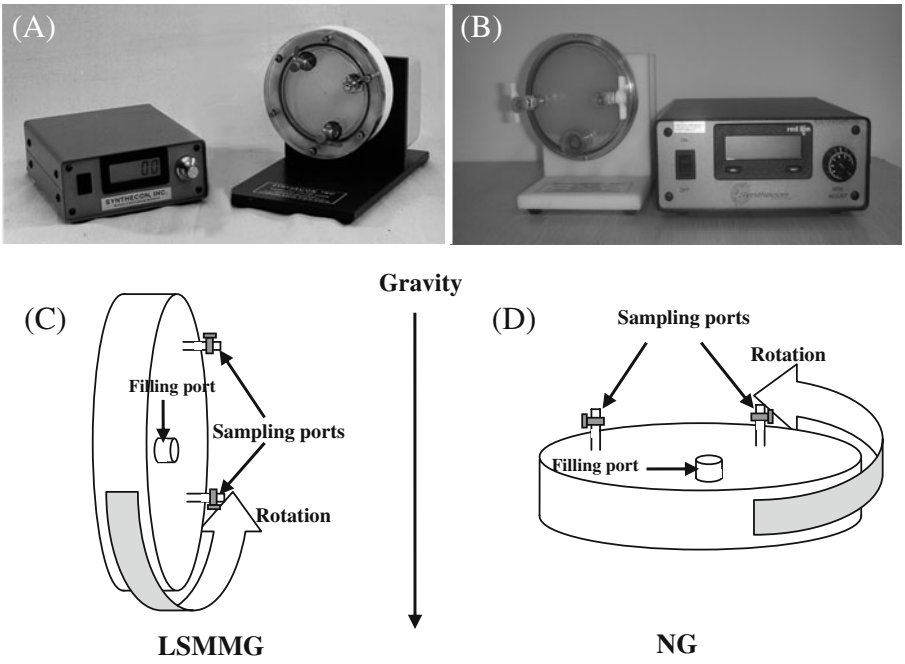

Fig. 1 The introduction of a HARV system used to generate LSMMG and NG conditions. **a**–**b** The HARV system used to generate LSMMG conditions. The back of the HARV is a gas-permeable membrane for the exchange of O_2 and CO_2. **c** LSMMG orientation: axis of rotation of HARV is perpendicular to the direction of the gravity vector. **d** NG orientation: axis of rotation is parallel to the gravity vector

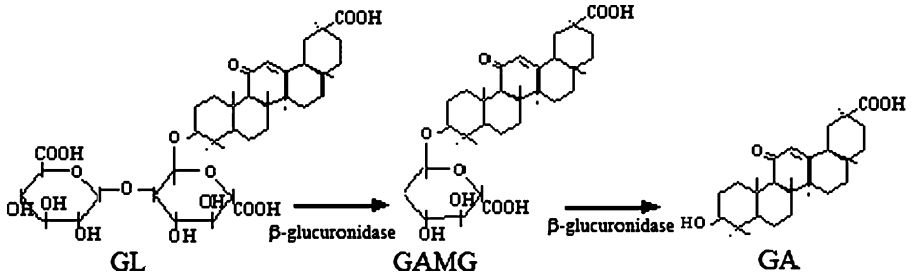

Fig. 2 Principle of biosynthesizing GAMG and GA by β-D-glucuronidase

shear environment associated with cell growth in HARV is the result of a combination of fluid forces principles and gravitational factors. When the cell culture vessel rotates, microbial cells do not settle down but revolve around a horizontal axis and continuously fall through the fluid at 1×g terminal velocity conditions, which lead to the different dispersion of nutrients and wastes within the vessel as compared with the environment of normal gravity (NG) [13, 14]. Exchange of dissolved gases has been achieved through a permeable membrane at the back of the vessel.

Our research especially focused on the effects of LSMMG on microbial processes and the recombinant enzymes expressed by microbial host cells. Although the studies of enzymatic reactions [15, 16] under microgravity are available, no reports have been published on the effects of LSMMG on the properties of enzymes. In this study, we aimed to determine the effects of LSMMG on the characterization of recombinant PGUS expressed by *Pichia pastoris*. PGUS from *Penicillium purpurogenum* Li-3 screened in our previous work [17] can directly biosynthesize glycyrrhetinic acid monoglucuronide (GAMG) from glycyrrhizin (GL) (Fig. 2) [17, 18]. GAMG is useful in clinical treatment of inflammatory diseases and is safer, more effective, and absorbable than GL [19]. We previously transformed *pgus* using the vector pPIC9K into *P. pastoris* GS115 expression system. *P. pastoris* is widely used as a host for the production of heterologous proteins because its expression system presents many advantages. Besides its high-level expression of recombinant proteins, *P. pastoris* is also known for its strong but tightly regulated alcohol oxidase-1 (AOX1) gene promoter [20, 21]. In the first growth phase, glycerol is used as a carbon source, whereas the second phase is an induction phase where methanol is added for biosynthesis of the recombinant protein under the control of alcohol oxidase-1 promoter. The characterization of recombinant enzyme PGUS expressed by *P. pastoris* GS115 cells under the environments of LSMMG and NG has been evaluated in this study.

Materials and Methods

Strain and Plasmid

The recombinant strain *P. pastoris* GS115 used in this study was constructed in our previous research. The constructed vector, pPIC9K–*pgus*, was integrated into the genome of the yeast cells. Standard procedure was used for the recombinant DNA manipulations [22]. The expression of *pgus* was under the control of alcohol oxidase-1 gene promoter for the production of recombinant protein.

Growth Conditions

A single colony of recombinant *P. pastoris* clone isolated from the YPD (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) plate was used to inoculate 200 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 1.61 μM biotin, 0.004% histidine, 1% glycerol) in a 500-ml shaker flask and incubated at 30 °C and 220 rpm for 18 hours. The cells were then harvested by centrifugation at 8,000 rpm for 10 minutes and resuspended in 100 ml BMMY medium (1% methanol instead of glycerol as the sole carbon source). Then aliquots of the BMMY cultures were loaded into two sterile 50-ml HARV vessels for LSMMG and NG cultures, respectively. Both of the two HARV vessels were incubated at the rotary speed of 30 rpm and 30 °C. The LSMMG of HARV rotated horizontally (with the axis of vessel perpendicular to the gravitational vector), whereas the NG control rotated vertically (with the axis of vessel parallel to the gravitational vector) (Fig. 1). All bubbles were removed to reduce shear. Methanol was added after every 24 hours to a final concentration of 1%. After 120 hours of induction, the culture medium was centrifuged at 8,000 rpm for 10 minutes at 4 °C, and the supernatant was analyzed for the characterization of recombinant PGUS within 1 hour.

PAGE Analysis of the Recombinant PGUS

Samples taken from the supernatant of cultured recombinant *P. pastoris* GS115 with methanol induction after 24, 48, 72, 96, and 120 hours under LSMMG and NG conditions were detected by polyacrylamide gel electrophoresis (PAGE), respectively. Equal amount (15 μl) of the samples was loaded on the gel. SDS-PAGE procedure was performed with 12% polyacrylamide gels using the Bis–Tris SDS-PAGE (Invitrogen) system [23]. The gels were then stained by Coomassie brilliant blue G250 (Amresco), and the recombinant PGUS was identified by densitometric analysis (ProExpress Imaging System, Perkin Elmer).

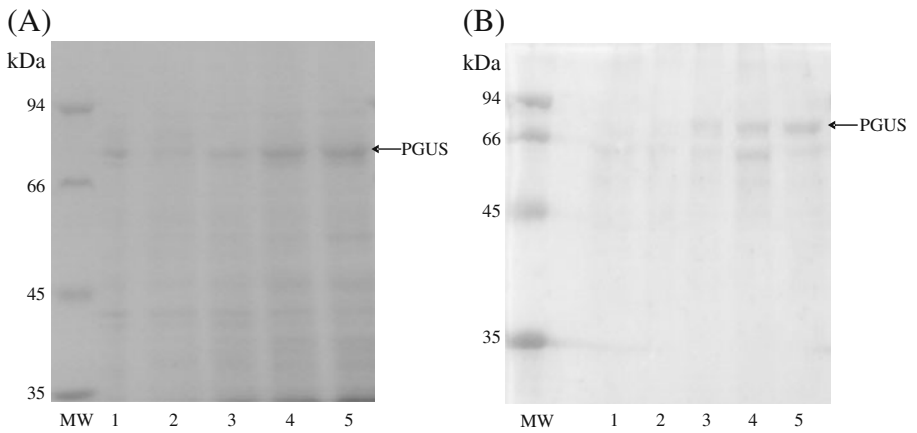

Fig. 3 Analysis of recombinant PGUS by Coomassie-stained SDS-PAGE (12%) expressed under NG (**a**) and LSMMG (**b**) conditions. Equal amounts of the supernatant from the two different conditions were collected after every 24-hour intervals, respectively. *Lane MW*: Marker weight; *Lane 1–5*, samples taken from the supernatant of cultured recombinant *P. pastoris* GS115 with methanol induction after 24, 48, 72, 96, and 120 hours, respectively

Enzyme Assay

PGUS activity was determined by using Glycyrrhizin (GL) as the substrate. GL (2 g/l) was prepared at 4 °C and pH 5.0. The assay mixture consisted of 200 μl enzyme solution and 800 μl 2 g/l GL. After incubation at 55 °C for 30 minutes, the reaction was stopped by heating in boiling water for 5 minutes and then centrifuged at 10,000 rpm for 5 minutes.

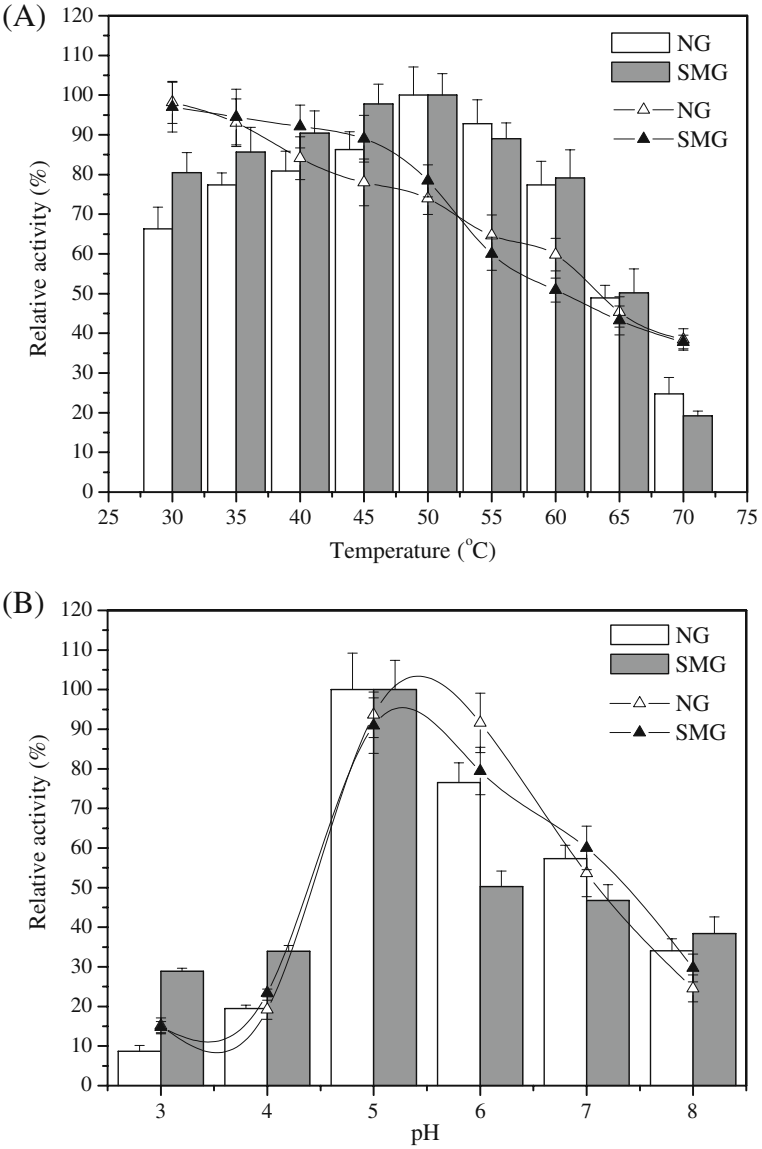

Fig. 4 Effects of LSMMG on temperature and pH activity and stability of the recombinant PGUS expressed by *P. pastoris* GS115. **a** Temperature; **b** pH. The enzymatic activity at the optimal temperature and pH value was defined as 100%. The *bars* and *lines* indicate the activity and stability, respectively. The relative activities were determined through triplicate independent experiments

The supernatant was used for enzyme analysis using high pressure liquid chromatograph (HPLC).

Chromatographic Conditions

The reaction mixture (10 μl) was subjected to reverse-phase HPLC on a C18 column (4.6×250 mm, 5 μm particle size, Kromasil) at 40 °C. Separation was achieved with mobile phase consisting of a mixture of methanol–0.6% acetic acid (81:19 v/v) in 25 minutes. Elution was monitored with UV detection at 284 nm. The amount of GAMG could be read from the standard curve between the peak area and concentration of GAMG. One unit (U) of activity was defined as the amount of enzyme that released 1 μmol of biosynthesized glycyrrhetinic acid monoglucuronide (GAMG) in the reaction mixture per minute.

Effects of LSMMG on Temperature and pH Activity and Stability of PGUS

The catalytic activities of recombinant PGUS expressed under LSMMG and NG conditions were examined at different temperatures ranging between 30 and 70 °C in 25 mM sodium-acetate buffer (pH 5.0) and at different pH values (3.6–8.0) using 100 mM glycine–HCl (pH 2.0–4.0), 100 mM sodium-acetate buffer (pH 4.0 – 6.0), and 100 mM potassium phosphate buffer (pH 6.0–8.0) at 55 °C.

We consequently examined temperature and pH stability of the recombinant PGUS. The enzyme was incubated at different temperatures within the range of 30–70 °C for 1 hour at pH 5.0 (optimum pH) without the substrate GL, and then residual activity was determined at 55 °C (optimum temperature). The pH stability of the enzyme was determined after incubation at different pH values (3.6–8.0) without the substrate for 1 hour, and then the residual activity was determined at 55 °C.

Effects of LSMMG on PGUS in Response to Metal Ions and EDTA

The effect of several metal ions on the activity of recombinant PGUS expressed under LSMMG and NG conditions was analyzed, respectively. PGUS activity was determined

Table 1 Effects of LSMMG on PGUS in response to metal ions and EDTA.

Reagent	Concentration (mM)	Relative activity (%)[a] NG	Relative activity (%) LSMMG
Control	0	100	100
K^+	2	168.68±6.7	125.34±7.0
Na^+	2	133.51±5.0	118.46±5.6
Ca^{2+}	2	135.64±8.2	121.12±4.5
Mg^{2+}	2	223.47±7.4	201.05±8.9
Fe^{2+}	2	150.21±6.1	109.56±3.2
Zn^{2+}	2	70.94±3.9	82.92±2.0
Cu^{2+}	2	87.28±5.0	94.38±3.2
Mn^{2+}	2	113.46±4.2	104.87±5.7
Ag^+	2	48.09±2.5	69.23±4.0
EDTA	2	66.72±1.8	82.56±3.5

[a] The activity assayed in the absence of metal ions or EDTA was taken as 100%. The data are reported as means ± SE derived from three replicate samples taken at three independent experiments.

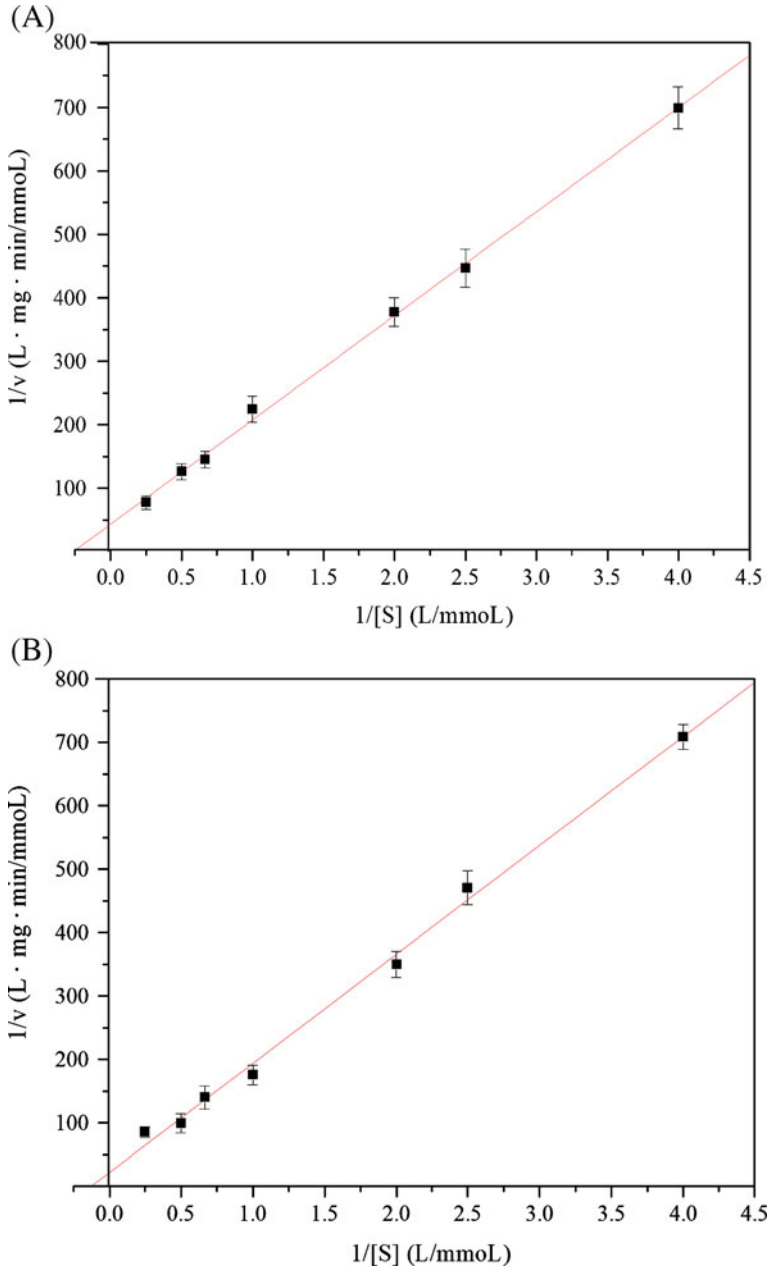

Fig. 5 The Lineweaver–Burk plots for the recombinant PGUS expressed by *P. pastoris* GS115 cells in NG (**a**) and LSMMG (**b**) using GL as the substrate. The relative activities were determined at different concentrations of the substrate GL through triplicate independent experiments

in reaction mixtures consisting of Mn^{2+} ($MnCl_2$), Na^{+} (NaCl), Ag^{2+} ($AgNO_3$), Ca^{2+} ($CaCl_2$), Cu^{2+} ($CuSO_4$), Mg^{2+} ($MgCl_2$), Fe^{2+} ($FeSO_4$), K^{+} (KCl), Zn^{2+} ($ZnSO_4$) metal ions, and EDTA at final concentration of 2 mM. Subsequently, the enzyme activity was examined at pH 5 and temperature 55 °C after incubation for 1 hour.

Effects of LSMMG on Kinetic Properties of PGUS

To determine the effects of LSMMG on the PGUS kinetic properties, the catalytic activities were examined with the concentrations of substrate GL ranging from 0.25 to 4 mM, and then the kinetics constants were determined. The catalysis reactions were followed continuously, and the initial velocities were fitted to the Michaelis–Menten equation using Origin 7.5 software (OriginLab). The values of the Michaelis–Menten constant (K_m), maximal velocity (V_{max}), catalytic turnover rate (k_{cat}), and catalytic efficiency (k_{cat}/K_m) were evaluated.

Results

Identification of PGUS

SDS-PAGE analysis of recombinant PGUS was collected after 24, 48, 72, 96, and 120 hours from *P. pastoris* GS115 cells cultured under LSMMG and NG conditions using HARV vessels (Fig. 1). The results showed the quantity of PGUS accumulated at different time intervals, which reached at its maximum after 120 hours (Fig. 3).

Effects of LSMMG on Temperature and pH Activity and Stability of PGUS

The temperature and pH stability profiles of PGUS from *P. pastoris* GS115 expressed under LSMMG and NG conditions have been shown in Fig. 4a and b. The optimal temperature and pH of PGUS expressed in LSMMG were same as in NG, i.e. 55 and 5.0 °C, respectively. Furthermore, there was little difference in temperature and pH stability of PGUS expressed in the two different environments. The activity of PGUS was least affected within the temperature range of 30–45 °C and pH 5.0–6.0. The relative activities were determined through triplicate independent experiments.

Effects of LSMMG on PGUS in Response to Metal Ions and EDTA

The influence of various metal ions and EDTA (at 2 mM) on the recombinant PGUS activity has been shown in Table 1. The activity assayed in the absence of metal ions or EDTA was taken as 100%. Cu^{2+}, Ag^{+}, Zn^{2+}, and EDTA had negative effects on PGUS, whereas the other ions could increase the PGUS activity in both NG and LSMMG, especially Mg^{2+} (NG 223.47±7.4%, LSMMG 201.05±8.9%), and K^{+} (NG 168.68±6.7%, LSMMG 125.34±7.0%). The results indicated that the effects of various metal ions and EDTA on the PGUS expressed in NG were more significant than in LSMMG, including both negative and positive effects. It showed that the recombinant PGUS expressed in LSMMG was less sensitive to metal ions and EDTA than in NG. The data were reported as means ± SE derived from three replicate samples taken at three independent experiments.

Table 2 Apparent kinetic constants of recombinant PGUS expressed in NG and LSMMG.

PGUS expression	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
NG	42.30	7.72	27.84	3.61
LSMMG	11.25	1.72	23.30	13.55

Effects of LSMMG on Kinetic Parameters of PGUS

The reaction kinetics of PGUS expressed in NG and LSMMG were determined (Fig. 5). By using Lineweaver–Burk plots, the apparent K_m and V_{max} values of PGUS towards GL were 7.72 mM and 42.3 μmol min^{-1} mg^{-1}, and 1.72 mM and 11.2 μmol min^{-1} mg^{-1} expressed in NG and LSMMG, respectively. The K_m value of the recombinant PGUS expressed in LSMMG (1.72) was nearly one fifth of that in NG (7.72) estimated from Lineweaver–Burk plots, whereas the catalytic efficiency (k_{cat}/K_m) of PGUS in LSMMG (13.55) was 3.7 times higher than that of NG (3.61). The relative activities were determined at different concentrations of the substrate GL through triplicate independent experiments.

Discussion

Low-shear fluid dynamics, a key aspect of the microgravity environment, can be well modeled in the specialized HARV bioreactor. The cells cultured in HARV were optimized to produce laminar flow and minimized the mechanical stresses that would encounter a low-shear suspension environment [10]. The previous researches showed that microgravity or LSMMG effects lead to a shortened lag phase, an increase in growth rate, and higher final cell counts as compared with the NG, which were coincident and reported more frequently [3, 24]. The altered physiology of the cells cultured under LSMMG condition would potentially influence expression and function of the recombinant proteins. However, there is no relevant study available. Here, we used HARV to study the effect of LSMMG on the expression and properties of the recombinant PGUS expressed in *P. pastoris*. This work is concerning the applications of LSMMG in the areas of biochemistry and fermentation biotechnology.

It was found that there were little difference in PGUS activity and stability in different temperatures and pH values under LSMMG and NG conditions. These results might be exclusively for glycoside hydrolases such as PGUS, but there is no relevant study about any other enzymes available until now. In addition, Cu^{2+}, Ag^{+}, Zn^{2+}, and EDTA had negative effects on PGUS expressed under the two different conditions. It was reported that most of the glycoside hydrolases could be strongly inhibited by Cu^{2+}, Ag^{2+}, and EDTA [25, 26]. On the contrary, the activity of PGUS could be mainly stimulated by Mg^{2+} and K^{+}. However, what made us interested was that LSMMG altered the degree of responses of the recombinant PGUS to metal ions and EDTA. We also found that the K_m value of the recombinant PGUS expressed under LSMMG was almost 4.5 times lower than that in NG (Table 2). Microgravity could reduce the apparent constant K_m of the soybean lipoxygenase-1 to one fourth with respect to the NG control [15], which indicated that microgravity or LSMMG might affect the process of catalysis because of density fluctuations and different molecule–molecule interactions. However, the effect of microgravity or LSMMG on the catalytic process of enzymes should also be considered to study.

Under the LSMMG environment, cell culture medium remains evenly distributed and fluids keep quiescent. In this convection lack environment, microbial cells could not sense the direction of gravity, whereas cells grown under NG, however, were not equally subjected to these fluid phenomena. These apparent discrepancies of LSMMG maybe contribute to some changes in conformation of the recombinant PGUS, e.g. more flexible of the active site groups. These changes make it easier for PGUS expressed under LSMMG to adapt itself to the substrate GL as compared with NG on equal energy consumption, which

resulted in reduced K_m value. Another explanation could be that the transcription and expression of the *pgus* gene or genes related to the recombinant protein folding changed due to the two different environments of LSMMG and NG, and ultimately, the conformation of recombinant enzyme was altered. A total of 1,372 genes of *Saccharomyces cerevisiae* cultured in LSMMG, many of which were involved in protein biosynthesis and protein activity regulation, differentially expressed themselves in response to the changing environment [27]. A significant number of genes of yeast were found to be up- or down-regulated by more than one to sevenfold as a result of growth in LSMMG analyzed by DNA microarray [27, 28]. This changing pattern of protein conformation probably plays a significant role in the altered properties of recombinant PGUS. Unfortunately, the key factors involved in the influences of LSMMG on the properties of PGUS remained undetermined. So it is necessary and worthwhile to continue these studies. In addition, the present study about enhanced catalytic efficiency (k_{cat}/K_m) of PGUS in LSMMG (13.55) had potential significance in the new areas of biocatalysis and fermentation biotechnology. Taken together, our results provided clues for future research perspective about the changes in the characterization of recombinant enzymes by LSMMG and might be a new guideline for further studies.

Acknowledgments This work is supported by the National "863" High-Tech Project (2008AA12A218-2) and Natural Science Foundation of China (20776017, 20976014) and Natural Science Foundation of Beijing (5072028).

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