Appl Biochem Biotechnol (2009) 159:78–84
DOI 10.1007/s12010-008-8413-y

Improved Production of Raw Starch Degrading Enzyme by *Aspergillus oryzae* F-30 Using Methyl Glucoside Sesqui-Stearate

Hai-Yan Sun · Lu Wang · Jian-Wen Liu · Ming Peng

Received: 30 July 2008 / Accepted: 24 October 2008 /
Published online: 11 November 2008

Abstract The effect of methyl glucoside sesqui-stearate (MGS) on the production of raw starch degrading enzyme (RSDE) by *Aspergillus oryzae* F-30 was studied in this paper. The activity of RSDE formed by *Aspergillus oryzae* F-30 was enhanced dramatically by the addition of MGS to the medium. As a result, with the addition of 1.5 g MGS in 1 L basal medium, RSDE activity and productivity were 107 U mL^{-1} and 1.49 U mL^{-1} h^{-1}, 4.3-fold and 7.1-fold greater than the values obtained in the basal medium, respectively. The effect of MGS on the synthesis of RSDE by *Aspergillus oryzae* F-30 was also studied on a molecular level. It was observed that the regulation of RSDE synthesis in *Aspergillus oryzae* F-30 occurs at both transcriptional and translational level and the enzyme synthesis was provoked by the addition of MGS at transcriptional level.

Keywords *Aspergillus oryzae* · Methyl glucoside sesqui-stearate · Raw starch degrading enzyme · Transcriptional level · Translational level

Introduction

Starch is the most abundant form of storage polysaccharides in plants and constitutes an inexpensive source for production of syrups containing glucose, fructose, or maltose, which are widely used in food industries [1]. In addition to that, the sugars produced from starch can be fermented to produce bioethanol, amino acids, organic acids, and others [2]. In the conventional starch processing industry, conversion of starch to glucose and other

H.-Y. Sun · M. Peng (✉)
State Key Laboratory of Tropical Corp Biotechnology, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Danzhou, Hainan 571101, China
e-mail: mmpeng_2000@yahoo.com

L. Wang
Wuxi Scientific Research and Designing institute of the State Administration of Grain Reserve, Wuxi, Jiangsu Province 214036, China

J.-W. Liu (✉)
Research Center of Enzymes, AsiaPac Bio-technology Co. Ltd., Dongguan, Guangdong Province 523808, China
e-mail: jliu1997@126.com

oligosaccharides requires a three-step process namely gelatinization, liquefaction, and saccharification. This process, although currently used by starch processing industries, is energy intensive thus increasing the production cost of starch-based products. With the view of reducing the energy cost, a worldwide interest has been focused on raw starch degrading enzymes (RSDE) in recent years [3–6]. Microorganisms reported to be good producers of RSDE include *Aspergillus* sp. [7], *Rhizopus* sp. [8], *Penicillium* sp. [9–11], *Bacillus* sp. [12], etc. Much work has been carried out to improve RSDE production such as screening potential strains, optimization of medium composition, and cultural conditions. We recently isolated a good RSDE producer, *Aspergillus oryzae* F-30, which had a high hydrolyzing activity toward raw starch. It was found that methyl glucoside sesqui-stearate (MGS) significantly improved RSDE production by *Aspergillus oryzae* F-30. Methyl glucoside sesqui-stearate, a kind of chemical material, is generally used in cosmetic industry. However, there is no description about its application in enhancing RSDE production until now. This paper describes the improvement in RSDE production by *Aspergillus oryzae* F-30 using MGS.

Materials and Methods

Microorganism

Aspergillus oryzae F-30, a RSDE producer, newly isolated from Chinese wheat koji, was used in the experiment.

Materials

The corn starch and soybean meal were obtained from the local market (commercial grade). MGS was obtained from Guangzhou Kemeida Company (China). All other chemicals were purchased from Sigma.

Basal Medium

The basal medium used for the enzyme production containing (g L^{-1}): corn starch 30, soybean meal 20, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, NaCl 0.5, and KCl 0.5, natural pH. Fifty milliliters of medium in a 250-mL flask was sterilized at 121 °C for 20 min. The medium in each flask was inoculated with 1 mL spore suspension (with cell count of 10^6 mL^{-1}) and incubated at 30 °C for 144 h on a rotary shaker (140 rpm).

Effect of MGS Concentration on RSDE Production by *Aspergillus oryzae* F-30

To investigate the effect of MGS concentration on RSDE production by *Aspergillus oryzae* F-30, MGS was added to the basal medium at different concentrations in the beginning of culture. The obtained RSDE activities were compared to search for the optimum concentration of MGS.

Effect of the Addition Time of MGS on RSDE Production by *Aspergillus oryzae* F-30

To investigate the effect of addition time of MGS on RSDE production by *Aspergillus oryzae* F-30, MGS (separately sterilized) was added to the basal medium at different stages

of the culture at the optimum concentration determined from the above experiments. The obtained RSDE activities were compared to determine the optimum addition time.

Regulation Mechanism of RSDE Synthesis by *Aspergillus oryzae* F-30

To determine whether the regulation of RSDE synthesis in *Aspergillus niger* F-30 occurs at transcriptional or translational level, actinomycin D (a repressor of transcription) or cycloheximide (a repressor of translation) was added in the basal medium after 36 h of culture with the concentration of 15 and 20 μg mL^{-1}, respectively. The reason for using these concentrations is that our earlier tests indicated that higher concentrations inhibited the growth of the organism while lower concentrations had no significant influence on the synthesis of the enzyme. To investigate the effect of MGS on RSDE synthesis, MGS (1.5 g L^{-1}) was added in the medium repressed by actinomycin D or cycloheximide at 48 h of culture. The samples were periodically withdrawn and used for the analysis of the enzyme activities.

Analytical Methods

The RSDE activity was determined using in a reaction mixture consisting of 0.5 mL of appropriately diluted enzyme solution and 4.5 mL of 1% (*w*/*v*) raw starch in 50 mM acetate buffer (pH 5.0). The reaction was carried out at 60 °C for 30 min and terminated by heating in boiling water bath for 5 min. One unit of RSDE activity was defined as the amount of enzyme that liberated 1 μmol glucose from raw starch per minute under the standard assay conditions. Glucose released was detected by using the dinitrosalicylic acid method [13]. Biomass was determined by harvesting the mycelia pellets and freeze drying them to a constant weight; the dry weight was expressed as grams per liter of culture. All the experiments were repeated three times. All values given are means of three determinations.

Results and Discussion

Effect of MGS Concentration on RSDE Production by *Aspergillus oryzae* F-30

As shown in Table 1, the addition of MGS into the medium at different concentrations could improve RSDE production by *Aspergillus oryzae* F-30 to different levels, while MGS had no effect on biomass. When 1.5 g L^{-1} MGS was added, RSDE activity reached the maximum (107 U mL^{-1}) after 72 h of culture. Compared to the basal medium (25 U mL^{-1}),

Table 1 Effect of MGS concentration on RSDE production by *Aspergillus oryzae* F-30.

MGS (g L^{-1})	Biomass (g L^{-1})	Peak hour (h)	RSDE activity (U mL^{-1})	RSDE productivity (U mL^{-1} h^{-1})
0	16.8	120	25	0.21
0.5	16.7	108	49	0.45
1.0	17.1	96	60	0.63
1.5	16.5	72	107	1.49
2.0	16.6	72	63	0.88
2.5	16.8	72	58	0.81

RSDE activity was enhanced 4.3-fold and the time needed to attain the maximum activity was shortened from 120 h to 72 h. Correspondingly, RSDE productivity increased by 7.1-fold. When higher concentration of MGS was used, RSDE activity began to decline. The possible reason is that higher concentration of MGS influenced the cell metabolism and RSDE synthesis by *Aspergillus oryzae* F-30.

Effect of the Addition Time of MGS on RSDE Production by *Aspergillus oryzae* F-30

The data in Table 2 revealed the effect of varying the time of MGS addition on RSDE production by *Aspergillus oryzae* F-30. The best result was (107 U mL^{-1}) obtained when an optimal concentration (1.5 g L^{-1}) of MGS was added in the beginning of the culture. Under optimum conditions, RSDE activity and productivity were 107 U mL^{-1} and 1.49 U mL^{-1} h^{-1}, respectively. When 1.5 g L^{-1} MGS was added after 24 h and 48 h of culture, maximum RSDE activity was 85 U mL^{-1} and 55 U mL^{-1}, respectively. MGS supplementation at 60 h increased RSDE activity to 37 U mL^{-1}, which was only an improvement of 12 U mL^{-1} compared to the basal medium. These observations indicated that the action of MGS on RSDE production was mostly complete within 60 h. The later MGS was added, the lower RSDE activity was obtained.

Comparison of RSDE Production by *Aspergillus oryzae* F-30 in the Basal and MGS-Containing Medium

MGS resulted in a 4.3-fold improvement in RSDE activity, while it had no effect on the cell growth (Fig. 1a,b). It was supposed that MGS stimulated RSDE production by *Aspergillus oryzae* F-30 via a special mechanism: MGS (methyl glycoside sesqui-stearate), composed of a methyl glycoside residue and a stearic acid residue through ester-type linkage. The ester molecules are amphipathic, containing both hydrophilic methyl glycoside and hydrophobic stearic acid residues. In aqueous solution, molecules of MGS form themselves into spherical micellar structures in which the stearic acid residues are hidden inside the micelle. The methyl glycoside residues interact with the surrounding water molecules. The surface of lipid bilayers of the microorganism cell membranes is hydrophilic; therefore, it is easy for the methyl glycoside residues to attach to the cell membrane. Because there are no covalent bonds between the lipids in the bilayer, the cell membrane is not static but fluid. Those movements make it possible for the stearic acid region of MSG to approach the hydrophobic hydrocarbon chains inside the lipid bilayer of the cell membrane. Thereby the highly ordered packaging of the fatty acid chains of lipid bilayer is disrupted, thus reducing the interactions between neighboring lipid molecules and enhancing the membrane permeability for the secretion of enzymes. On the other hand, methyl glycoside residues

Table 2 Effect of the addition time of MGS on RSDE production by *Aspergillus oryzae* F-30.

Addition time (h)	Biomass (g L^{-1})	Peak hour (h)	RSDE activity (U mL^{-1})	RSDE productivity (U mL^{-1} h^{-1})
0	16.5	72	107	1.49
12	16.9	72	96	1.33
24	16.8	72	85	1.18
36	16.4	72	73	1.01
48	17.1	96	55	0.57
60	17.0	120	37	0.31

might act as an effective inducer of RSDE because methyl glycoside was a structural analogue of maltose. It was reported that the productivity of RSDE from *Aspergillu* sp. K-27 was doubled by methyl glycoside [14]. Methyl xyloside also proved a good inducer of xylase from *Pullularia pullulans* [15]. Therefore, MGS molecules attach on the surface of fungual cells, acting as a persistent inducer to initiate RSDE production by *Aspergillus oryzae* F-30.

Regulation Mechanism of RSDE Synthesis by *Aspergillus oryzae* F-30

As shown in Fig. 2a, with the addition of actinomycin D (a repressor of transcription) or cycloheximide (a repressor of translation) in the basal medium after 36 h of culture, RSDE biosynthesis nearly ceased. These results suggested that the synthesis of RSDE by *Aspergillus oryzae* F-30 is regulated at both transcriptional and translational level. When

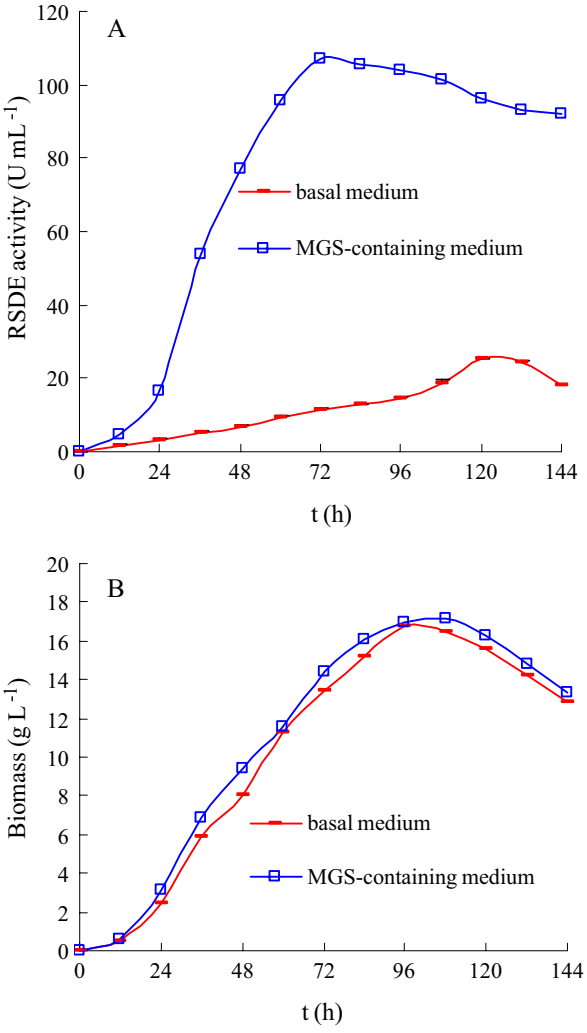

Fig. 1 Comparison of RSDE activity (**a**) and biomass (**b**) produced by *Aspergillus oryzae* F-30 in the basal and MSG-containing medium

MGS was added in the medium repressed by actinomycin D, RSDE biosynthesis started again. When MGS was added in the medium repressed by cycloheximide, no revival of RSDE synthesis was observed (Fig. 2b). These results suggested that the enzyme synthesis was provoked by MGS only at transcriptional level.

Conclusion

With the addition of 1.5 g MGS in 1 L medium, RSDE activity and productivity by *Aspergillus oryzae* F-30 were 107 U mL^{-1} and 1.49 U mL^{-1} h^{-1}, 4.3-fold and 7.1-fold greater than the values obtained in the basal medium, respectively. The regulation of RSDE

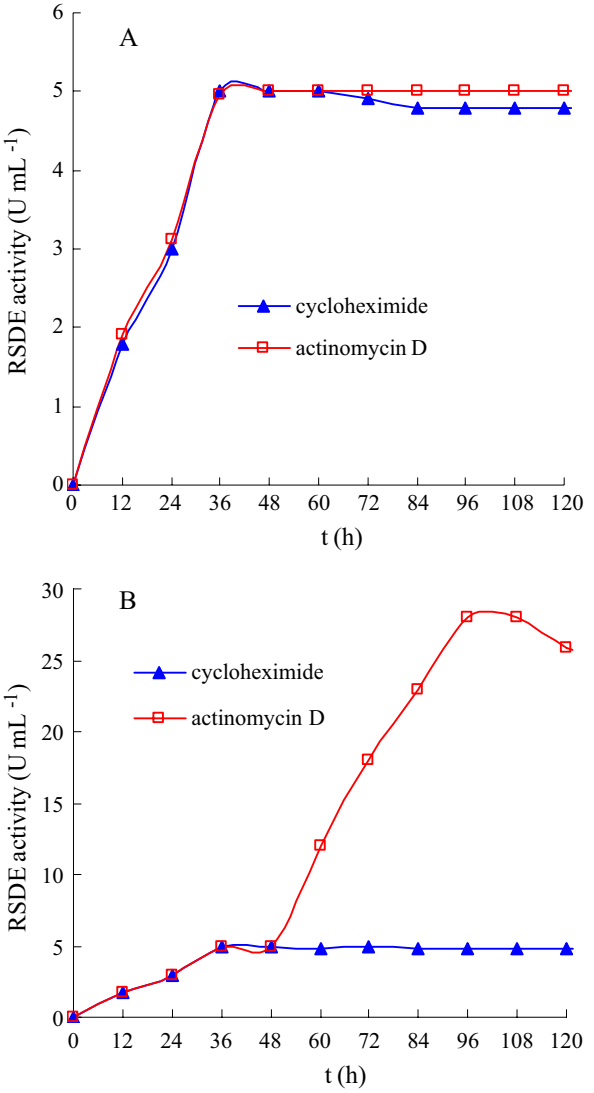

Fig. 2 RSDE synthesis regulated by actinomycin D and cycloheximide (**a**) and provoked by MGS (**b**) in *Aspergillus oryzae* F-30

synthesis in *Aspergillus oryzae* F-30 occurs at both transcriptional and translational level. RSDE synthesis is provoked by MGS only at transcriptional level.

Acknowledgements This research was supported by Chinese 863 Project (no. 2007AA021307).

References

1. Roy, I., & Gupta, M. N. (2004). *Enzyme and Microbial Technology*, *34*, 26–32. doi:10.1016/j.enzmictec.2003.07.001.
2. Polakovic, M., & Bryjak, J. (2004). *Biochemical Engineering Journal*, *18*, 57–64. doi:10.1016/S1369-703X(03)00164-5.
3. Matsubara, T., Ammar, Y., Anindyawati, T., Yamamoto, S., Ito, K., Iizuka, M., et al. (2004). *Journal of Biochemistry and Molecular Biology*, *37*, 429–438.
4. Rajoka, M. I., & Yasmeen, A. (2005). *World Journal of Microbiology & Biotechnology*, *21*, 179–187. doi:10.1007/s11274-004-1766-7.
5. Ezeji, T. C., & Bahl, H. (2007). *World Journal of Microbiology & Biotechnology*, *23*, 1311–1315. doi:10.1007/s11274-007-9353-3.
6. Xudong, L., & Yan, X. (2008). *Bioresource Technology*, *99*, 4315–4320. doi:10.1016/j.biortech.2007.08.040.
7. Pothiraj, C., Balaji, P., & Eyini, M. (2006). *Mycobiology*, *34*, 128–130.
8. Lisen, W., Xiangyang, G., & Weiguo, Z. (2007). *World Journal of Microbiology & Biotechnology*, *23*, 461–465. doi:10.1007/s11274-006-9247-9.
9. Haska, N., & Ohta, Y. (1994). *Starch*, *46*, 480–485. doi:10.1002/star.19940461207.
10. Haiyan, S., Xiangyang, G., & Weiguo, Z. (2006). *Biotechnology Letters*, *28*, 1719–1723. doi:10.1007/s10529-006-9151-2.
11. Haiyan, S., Xiangyang, G., & Weiguo, Z. (2007). *World Journal of Microbiology & Biotechnology*, *23*, 603–613. doi:10.1007/s11274-006-9269-3.
12. Goyal, N., Gupta, J. K., & Soni, S. K. (2005). *Enzyme and Microbial Technology*, *37*, 723–734. doi:10.1016/j.enzmictec.2005.04.017.
13. Miller, G. L. (1959). *Analytical Chemistry*, *31*, 426–427. doi:10.1021/ac60147a030.
14. Abe, J., Bergmann, F. W., Obata, K., & Hizukuri, S. (1988). *Applied Microbiology and Biotechnology*, *27*, 447–450.
15. Pou-Llinas, J., & Driguez, H. (1987). *Applied Microbiology and Biotechnology*, *27*, 134–138. doi:10.1007/BF00251935.