Process Biochemistry 58 (2017) 224–229

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

L-glutamine efficiently stimulates biosynthesis of bacillomycin D in *Bacillus subtilis* fmbJ

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ARTICLE INFO

Keywords:
Amino acids
Bacillomycin D production
Fed-batch fermentation
Gene expression

ABSTRACT

The present study investigated the stimulation effect of L-glutamine (L-gln) on bacillomycin D biosynthesis of *Bacillus subtilis* fmbJ and the transcription of the synthetase genes and the signal proteins that related to the biosynthesis of bacillomycin D. It was found that L-gln significantly increased the production of bacillomycin D, in comparison with other amino acids. L-gln significantly enhanced the expression level of *bmyA* (*BYA*), *bmyB* (*BYB*), *bmyC* (*BYC*), the thioesterase gene (*TE*) and up-regulated *DegU*, σ^H and *Spo0A*, but down-regulated *AbrB*, therefore the yield of bacillomycin D significantly increased. Furthermore, when conjointly feeding 5 g/L of L-gln and 30 g/L of inulin, the production of bacillomycin D was up to 1.93 g/L.

1. Introduction

Lipopeptide antibiotics were widespread among *Bacillus subtilis* and the skeletons of cyclic lipopeptides usually contained various amino acid residues that could cause the differential production of certain lipopeptides. Amino acids were the organic nitrogen sources which contributed to the biosynthesis of the cylic lipopeptides in *B. subtilis*. It was evidenced that L-valine (L-val) or L-isoleucine (L-ile) could cause an increase of the production of Val7-surfactin while L-leu could make very low Val7-surfactin production [1]. The structure of bacillomycin D (Fig. 1) contained a β-amino fatty acid and a cyclic heptapeptide that determinately characterized as: L-asn-D-tyr-D-asn-L-pro-L-glu-D-ser-L-thr [2]. However, how these amino acids affect bacillomycin D production is not clear.

Bacillomycin D was cyclic lipopeptide that exhibited strong antifungal effects for agriculturally pathogenic fungi and anticancer activity [3–6]. The biosynthesis of bacillomycin D were involved in the non-ribosomal peptide synthetases (NRPSs), which named the bacillomycin D synthetases that mainly encoded by the genes: *bmyA* (*BYA*), *bmyB* (*BYB*) and *bmyC* (*BYC*). The bacillomycin D synthetases contained amino acid-activating modules that catalyzed the formation of the cyclic structure of bacillomycin D [7]. Additionally, signal proteins like DegU, ComA, σ^H and Spo0A could directly or indirectly act on the expression of bacillomycin D synthetases and therefore affected bacillomycin D biosynthesis. It was evidenced that DegU, ComA and σ^H could play a positive role in the biosynthesis of bacillomycin D [7]. Our previous work also appeared that ComA, DegU, σ^H and Spo0A up-regulated the expression of bacillomycin D synthetases and therefore promoted the production of bacillomycin D when using inulin as a sole carbon source [8]. Amino acids played an important role in the regulation of biosynthesis of lipopeptide, which mainly relied on NRPSs [9,10]. For example, the biosynthesis of surfactin was involved in three interactive amino acid activating modules of surfactin synthetase SrfA, B and C alone. Particularly, L-glu-activating module functionally acted on SrfD that stimulated the initiation of surfactin biosynthesis [11]. However, up to now, there is no paper on systematically investigating how amino acids regulate the biosynthesis of bacillomycin D and its mechanism has not been clarified.

In this study, the effect of 20 amino acids on bacillomycin D production was analyzed and the expression profiles of the bacillomycin D synthetase genes: *BYA*, *BYB*, *BYC*, *TE* and the signal genes: *AbrB*, *ComA*, *DegU*, σ^H, *Spo0A* were investigated in order to make clear the role of amino acids in the biosynthesis of bacillomycin D. Also, different fermentation strategies were carried out to obtain a high production of bacillomycin D.

2. Materials and methods

2.1. Strains and culture media

The strain *Bacillus subtilis* fmbJ isolated in the previous work [4] was used in this study. Seed medium contained (g/L): beef extract 3,

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http://dx.doi.org/10.1016/j.procbio.2017.04.026
Received 28 September 2016; Received in revised form 11 April 2017; Accepted 12 April 2017
Available online 18 April 2017

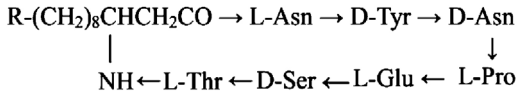

Fig. 1. Structure of bacillomycin D. R standed for the isomers of the alkane with three to five carbons.

peptone 10 and NaCl 15. Landy medium [12] modified (LM1) contained (g/L): yeast extract 1, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $MnSO_4$ 0.05, $CuSO_4{\cdot}5H_2O$ 0.0016, $FeSO_4{\cdot}7H_2O$ 0.0015, KH_2PO_4 1, inulin 30. Each of 20 amino acids (5 g/L) was separately added to LM1 to investigate the effect of every amino acid on bacillomycin D production. The batch fermentation medium was prepared by adding 5 g/L of L-gln to LM1. The pH value of the medium used in this work was controlled at 7.0 and the medium was sterilized at 115 °C for 20 min.

2.2. Fermentation conditions

B. subtilis fmbJ was cultured in 100 mL of seed medium in a 250 mL flask at 37 °C for 18 h. The fermentation of bacillomycin D was experimentalized in a 250 mL of flask contained 100 mL of fermentation medium at 33 °C. The batch fermentation was performed in a 5.0 L of fermenter (GUJZ, Zhenjiang, China) contained 1.0 L batch fermentation medium at 33 °C, pH 7.0, 200 rpm and 1.0 L^{-1} L^{-1} min. In feeding fermentation, 300 mL of 5 g/L L-gln, 300 mL of 30 g/L inulin and 150 mL of 5 g/L L-gln, 150 mL of 30 g/L inulin into 1.0 L was respectively added.

2.3. Analysis and determination of bacillomycin D

30 mL of fermentation broth was centrifuged at 5,000 g on a centrifuge (Thermo, USA) to collect the crude supernatant. The supernatant was adjusted to pH 2.0 and stayed overnight at 4 °C. Then, the precipitation was obtained by again centrifugation at 5,000 g, 100% of methanol was added to dissolve the precipitation and the mixture was adjusted to pH 7.0 by 4 M NaOH. The crude bacillomycin D were prepared with centrifuging at 10,000 g for 20 min. And then, reverse-phase high performance liquid chromatography (RP-HPLC) and high performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC–MS/MS) were used to characterize the structure of bacillomycin D and the production of bacillomycin D [8].

2.4. Measurement of biomass

40 mL of fermentation broth was centrifuged at 4 °C and 8,000 g for 10 min to collect wet cell. Then, the wet cell was washed with 5 mL of distilled water twice and re-centrifuged to remove any remaining supernatant. The wet cell was collected and dried at 80 °C to a constant weight and the biomass concentration was calculated based on the dry cell weight.

2.5. RT-qPCR

Total RNA was isolated according to the manufacturer's protocol of Trizol Reagent (Sangon, Shanghai, China).The RNA sample was electrophoresed on a 1.2% agarose gel and 1 μg RNA quantified by NanoDrop2000 (Thermo Scientific, USA) was used for cDNA synthesis, according to the procedure of HiScript™ 1 st Strand cDNA Synthesis Kit (Vazyme, USA).To analyze the expression level of *BYA*, *BYB*, *BYC*, *TE*, *AbrB*, *ComA*, *DegU*, σ^H and *Spo0A*, RT-qPCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) using SYBR Premix ExTaq™ (TaKaRa, Dalian, China). The reaction was performed in triplicate with a total volume of 20 μL containing 2 × SYBR Premix Ex Taq 10 μL, PCR Primer (10 μM) 1.0 μL and ddH_2O 6.0 μL. The primers used for amplification of target genes and the PCR program were described as the previous work [8].

Table 1
Effects of different amino acids on bacillomycin D production. Control: LM1. 5 g/L of each amino acids was added to 100 mL LM1 and the strain *B. subtilis* fmbJ was fermented at 33 °C for 96 h −: non detection of target products. All experiments were triplicated and values were the average ± standard deviations.* and ** were significantly different from controls at 0.05 and 0.01 levels.

Amino acids	Bacillomycin D production (g/L)	Biomass (g/L)
Control	0.06 ± 0.00	13.56 ± 0.41
L-ala	0.02 ± 0.00**	3.27 ± 0.1**
L-arg	0.06 ± 0.00	10.38 ± 0.24
L-asn	0.2 ± 0.00**	21.61 ± 0.42**
L-asp	–	–
L-cys	–	15.42 ± 0.47*
Gly	0.02 ± 0.00	9.27 ± 0.22
L-gln	0.46 ± 0.01**	29.56 ± 0.64**
L-glu	0.16 ± 0.00**	20.33 ± 0.42**
L-his	0.06 ± 0.00	11.53 ± 0.32
L-ile	0.01 ± 0.00**	2.87 ± 0.06**
L-leu	0.01 ± 0.00**	1.03 ± 0.02**
L-lys	0.01 ± 0.00**	1.07 ± 0.01**
L-met	0.1 ± 0.00**	14.27 ± 0.39*
L-phe	0.01 ± 0.00**	2.09 ± 0.05**
L-pro	0.14 ± 0.00**	18.76 ± 0.54**
L-ser	0.01 ± 0.00**	3.48 ± 0.07**
L-thr	0.15 ± 0.00**	20.51 ± 0.47**
L-trp	0.13 ± 0.00**	19.02 ± 0.56**
L-tyr	0.02 ± 0.00**	3.01 ± 0.05**
L-val	0.01 ± 0.00**	1.87 ± 0.06**

2.6. Statistical analysis

The experiments data were analyzed by the SPSS software (SPSS 20.0version, IBM, USA).All experiments were triplicated and the results were showed by means ± SD. Significant differences between mean values were evaluated by Duncan's test at 0.05 and 0.01 levels.

3. Results and discussion

3.1. Effects of amino acids on bacillomycin D production

The HPLC–MS/MS analysis showed that bacillomycin D contained a fat acid chains with fourteen to seventeen carbons and a cyclic peptide with seven amino acid residues (Asn-Tyr-Asn-Pro-Glu-Ser-Thr), which agreed with the structure of bacillomycin D [4]. Influences of 20 amino acids on bacillomycin D production were investigated by flask fermentations and the results were presented in Table 1. In the LM1, adding L-arg, L-met, L-thr, L-trp, L-pro, L-asn, L-glu and L-gln had positive effects on bacillomycin D biosynthesis ($P < 0.01$). When adding 5 g/L of L-gln into LM1, the production of bacillomycin D and the concentration of biomass were 0.46 g/L and 29.56 g/L, respectively, which were much higher than that of other amino acids. However, L-serine (L-ser), L-tyrosine (L-tyr), L-alanine (L-ala), L-phenylalanine (L-phe), L-isoleucine (L-ile), L-lysine (L-lys), L-leu and L-val decreased bacillomycin D production in *B. subtilis* fmbJ ($P < 0.01$). Interestingly, there was no bacillomycin D detected in fermentation broth when adding L-aspartic acid (L-asp) and L-cysteine (L-cys) in LM1, suggesting that L-asp and L-cys completely inhibited the biosynthesis of the bacillomycin D in *B. subtilis* fmbJ.

Bacillomycin D belonged to iturin and its cylic peptide chain was verified as: L-asn-D-tyr-D-asn-L-pro-L-glu-D-ser-L-thr [2]. From our evidences, most amino acids related to bacillomycin D facilitated bacillomycin D production, but L-tyr was disadvantageous for producing the bacillomycin D. Similarly, L-val, L-leu and L-ile were three amino acid residues consisted in the cylic peptide chains of surfactin and it was evidenced that L-val or L-ile could cause an increase of the production of Val7-surfactin while L-leu could make very low Val7-surfactin production [13]. It was revealed that not all the amino acid residues in the cylic peptide chains of bacillomycin D were beneficial for the