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Research paper

Surfactin variants for intra-intestinal delivery of insulin

Li Zhang, Xiaoying Xing, Jia Ding, Xiuyun Zhao, Gaofu Qi *

College of Life Science and Technology, Biomedical Center, Huazhong Agricultural University, Wuhan 430070, China

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ABSTRACT

Surfactin is a *Bacillus*-produced natural lipopeptide, which can overcome the epithelial cell barriers for orally delivering insulin, but its ability to promote uptake of insulin by the intestine need to be further improved for a higher oral bioavailability. Here, we designed and synthesized several surfactin variants to improve its ability for oral delivery of insulin. Firstly, we replaced Glu with Gln in surfactin for decreasing its negative charges, but found this replacement weakened its ability to orally delivery insulin. We further chemically synthesized surfactin variant by replacing its fatty acid chain (C15) with a shortened one (C14), and found this replacement did not influence its ability to orally deliver insulin. Lastly, we replaced its amino acids (Leu) with more hydrophobic ones (Ile), and found the replacement could significantly improve its ability to deliver insulin, with a maximal blood glucose decrease to 27.33% of the initial level and an insulin bioavailability of 18.25%. We also replaced its amino acids of Leu with Val, and Val with Ile, and found this replacement could also significantly improve its ability to deliver insulin with a maximal blood glucose decrease to 18.36% of the initial level and a high insulin bioavailability of 26.32% in diabetic mice. Further analysis by CD, we found the surfactin variants with more hydrophobic amino acid residuals significantly induced insulin from rigid (α-helix) to flexible structure (β-sheet and random coil), that is favorable for insulin to permeate across the intestine epithelial membrane. Collectively, surfactin variants with more hydrophobicity are very potential for delivery of insulin in the everyday control of blood glucose.

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1. Introduction

Bacillus can produce many secondary metabolites, some of which are found to have use in industry and medicine. For example, *Bacillus*-produced surfactin is a natural lipopeptide, which can be used as an antibiotic, immune adjuvant and immunomodulator [1]. Notably, this lipopeptide can be used for oral delivery of insulin by its biosurfactant activity [1].

Insulin is generally used to treat diabetes for maintaining a controlled glycemia in blood by subcutaneous (*s.c.*) injection [2]. Compared to *s.c.* injection, oral insulin is the most attractive option regarding its convenience, patient compliance, fewer side effects, mimic of physiological fate of insulin and improvement of glucose homeostasis [3,4]. However, the high molecular weight of insulin, along with the lack of lipophilicity, limits this polypeptide to permeate across the intestinal epithelium, so the oral bioavailability of insulin is generally very low (<1%) [5]. To overcome these limitations, several strategies have been developed for oral delivery of insulin, including formulation with surfactin [1,6–8].

Surfactin, comprising of a heptapeptide (ELLVDLL) linked to a β-hydroxy fatty acid chain via a cyclic ester, is a natural lipopeptide gaining more and more attentions in recent years [9,10]. Due to cyclic structure, surfactin is resistant to acidic digestion and proteases attack [10]. We previously demonstrated that *Bacillus*-produced surfactin can bind to insulin protecting it from acidic digestion and proteases attack in the gastrointestinal tract after oral administration. Moreover, surfactin can effectively improve the transepithelial transport of insulin [1]. The hydrophobic property from hydrophobic amino acid residuals and fatty chain of surfactin plays a vital role for facilitating insulin to permeate through the intestinal epithelium. Otherwise, Glu and Asp are also very important for forming the special structure of "horse-saddle" topology of surfactin [11,12], which is favorable for forming electrostatic interactions between surfactin, insulin and cell membrane. By these two acidic amino acid residues, surfactin has a pI value about 2.0 and possesses negative charges at neutral pH, that is necessary for the "detergent-like" role of surfactin. By "detergent-like" role, surfactin firstly inserts into cell membrane by hydrophobic interactions, following acts as a "crowba" to disturb cell membrane to allow insulin into the cells by the

* Corresponding author.
E-mail address: qigaofu@mail.hzau.edu.cn (G. Qi).

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electrostatic interactions between surfactin and cell membrane with negative charges [10,13].

Although the natural surfactin is effective for overcoming the epithelial cell barriers, its ability to promote uptake of insulin by the intestine need to be further improved for a higher oral bioavailability. Based on this requirement, we deduced that the surfactin variants might be more effective for promoting uptake of insulin by the intestine. In this study, we designed and chemically synthesized several surfactin variants with more hydrophobicity, and hypothesized that these surfactin variants would be more efficient to promote insulin across the intestine epithelial cell membrane with high bioavailability.

2. Materials and methods

2.1. Materials and reagents

Surfactin (C15) was purified from *Bacillus amyloliquefaciens* WH1 culture as described previously [14]. Recombinant human insulin (25 IU/mg) was a gift from JS Bioway pharmaceutical Co., Ltd (Changzhou City, China). NHS-Rhodamine B and streptozotocin were purchased from Sigma-Aldrich (USA). Human insulin enzyme-linked immunosorbent assay (ELISA) Kits were purchased from RayBiotech (Norcross, USA). Ketamine and Xylazine was purchased from Daiichi-Sankyo (Tokyo, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively. All other chemicals are of analytical grade supplied by Sinopharm Chemical Reagent (China).

2.2. Chemically synthesizing surfactin variants

Lichenysin (Fig. 1A), purchased from Sigma-Aldrich (USA), is a natural surfactin variant, in which Glu is replaced with Gln, and this replacement decreases its negative charges at neutral pH. Other three chemically synthesized surfactin variants were designed as 876634, 879214 and 879216 in this study (Fig. 1A). Compared to surfactin, the fatty acid chain with 15 carbons is replaced with a chain with 14 carbons in 876634. The other two variants of 879214 and 879216 were also with a fatty acid chain of 14 carbons. Otherwise, all Leu are replaced with Ile in 879214, and Val is replaced with Ile and all Leu are replaced with Val in 879216. Among these surfactin variants, 879214 is with the strongest hydrophobicity, followed by 879216. Variants were chemically synthesized for a purify >90%.

The strategy for the solid-phase synthesis of cyclic lipopeptides involves side-chain attachment and on-resin head-to-tail cyclization. Since in surfactin or variants 3-hydroxy pentadecanoic acid (C15) is attached via ester bond to the C-terminal carboxy group of Leu (876634), Ile (879214) or Val (879216), we decided to couple the closest commercially available analogue to this acid instead, 3-hydroxy myristic acid (C14). The key step in the synthesis of cyclic lipopeptides is the ring closure.

The synthesis is started by attaching the C-terminal Fmoc-Glu-OAllyl via side chain to amide resins using HBTU/HOBt/DMAP procedure. Standard Fmoc-chemistry was used throughout [15,16]. After the solid-phase assembly of heptapeptide sequences of surfactin variant 876634, 879214 or 879216, 3-hydroxy myristic acid pentafluorophenyl ester (2 equiv) was reacted to give the desired linear lipidic peptide precursor. The last amino acid in the linear peptide sequence, Leu (876634), Ile (879214) or Val (879216), was coupled with the hydroxy of 3-hydroxy myristic acid tail via ester bond using previously mentioned DIC/DMAP method [16,17], followed with simultaneous Alloc and Allyl removal by treatment with Pd $(Ph_3P)_4$ [16]. Treatment of the crude product with $MeOH/H_2O$/ammonia mixture, that is, known to hydrolyze only ester bond, followed by RP-HPLC and MALDI-TOF MS analyses of the reaction mixture, confirmed that major product is the desired surfactin variants [16].

2.3. Induction of diabetic mice

Balb/C mice (5–6 weeks old, 18–20 g body weight) were purchased from the center for disease prevention and control of Hubei province (Wuhan, China) following the University Ethics Committee's guidelines. Streptozotocin was injected intraperitoneally at a dose of 70 mg/kg for induction of Type 1 diabetes mellitus in mice [1].

2.4. Determination of hypoglycemic effects of insulin delivered by lichenysin

Insulin (90 IU/kg) was mixed with 3.6 mg/kg surfactin or lichenysin in 200 μl phosphate buffer (PB), then used for oral administration of diabetic mice (25–33 mmol/L of blood glucose, 7 mice per group) by an oral feeding needle. Blood samples were collected from the tail for detecting blood glucose prior to oral administration as fasting glucose levels, and at different time intervals post oral administration. Results are presented as the% reduction from the initial value calculated by Eq. (1)

$$\% \text{ change} = [(F - P_t)/F] \times 100 \quad (1)$$

where F is the fasting blood glucose level, P_t is the blood glucose level at time (t) after administration [1,18–20].

2.5. Assessment of insulin bioactivity influenced by ethanol

The chemically synthesized surfactin variants cannot be dissolved in water. Thereby, 60% (v/v) ethanol was used for dissolving these variants and insulin. To know possible influence of 60% ethanol on insulin bioactivity, the insulin dissolved in 60% ethanol (1.2 mg/mL) was kept at 4 °C for 30, 60 and 90 days, then used for *s.c.* injection of diabetic mice (n = 7 per group) at a dose of 1 IU/kg body weight. Another group of diabetic mice were injected with the same dose of freshly prepared insulin dissolved in phosphate buffer (PB, 10 mmol/L, pH = 7.0) as control. After administration, the blood glucose was determined at different time points.

2.6. Determination of hypoglycemic effects of insulin delivered by surfactin variants

We used microsurgery to deliver insulin into the intestine for mimicking oral release of insulin in the intestine. In detail, the diabetic mice were anesthetized by intraperitoneal injection with Ketamine (150 mg/kg body weight) / Xylazine (10 mg/kg body weight) combination, then the diabetic mice were randomly divided into 10 groups (n = 7 per group), following with microsurgery to exposure the intestine. 200 μl insulin (45 IU/kg body weight) plus 1.8 mg/kg surfactin or variant (876634, 879214 or 879216) dissolved in 60% ethanol, 200 μl surfactin or variant (876634, 879214 or 879216, 1.8 mg/kg) dissolved in 60% ethanol, 200 μl insulin (45 IU/kg) dissolved in 60% ethanol, or just 200 μl 60% ethanol solution, were injected into the duodenum at the site of 1-cm downstream of pylorus by a syringe (28G needle). After administration, the wound was stitched up, and the mice were intramuscularly injected with penicillin (20,000 U/mouse) for prevention of possible infection. The blood glucose was determined at different time points and the% reduction from the initial value was calculated using Eq. (1).

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