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BACILLUS AMYLOLIQUEFACIENS α -AMYLASE SIGNAL SEQUENCE FUSED IN FRAME WITH HUMAN PROINSULIN IS PROPERLY PROCESSED BY BACILLUS SUBTILIS CELLS

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SUMMARY. The plasmid pBINS1, containing the promoter, SD and leader peptide sequences of Bacillus amyloliquefaciens α-amylase gene and 267 bp long sequence coding for human proinsulin directs the efficient synthesis of hybrid preproinsulin, as well as quantitative secretion of proinsulin outside of protease-deficient Bacillus subtilis AJ73 cells. The recombinant proinsulin has been isolated from the culture medium and its N-terminal sequence shown to be identical with that of natural human prohormone.

During several last years various genetic regulatory regions of Bacilli genes have been widely used for constructing secretion vectors able to direct the synthesis, as well as secretion of the foreign proteins outside of the cells (1, 2, 3). Bacilli α -amylase gene fragments have been explored frequently for these purposes (2, 3). Nevertheless, it is always difficult to predict unambiguously whether the hybrid proteins, consisting of Bacilli signal sequence linked with the sequence of mature part of foreign protein, will be properly processed during the secretion. In any case at the present state of knowledge the processing should be experimentally tested by the analysis of the N-terminal sequence of the secreted foreign protein. The improper processing or the multiplicity of the cleavage sites, arising "ragged-ends" proteins is especially undesirable for the proteins potentially applicable as medical drugs (eg insulin, human growth hormone, interferons).

In the present communication we report that B. amyloliquefaciens α -amylase signal peptide sequence fused in frame with human proinsulin is properly processed in the course of hybrid preproinsulin secretion outside of B. subtilis cells. That arises the recombinant proinsulin with the N-terminal sequence indistinguishable from that of the natural human prohormone.

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MATERIALS AND METHODS

The plasmid pBINS1 (8,5 Kb, ori pSM19035, Em^r) (4) carrying 267 bp long human proinsulin gene sequence (5) and the promoter, SD and signal peptide sequence regions of B. amyloliquefaciens α-amylase gene (6) was constructed using the conventional techniques (7). Protease-deficient strain B. subtilis AJ73 (amy4 npr512 apr73) was used as a host. The cells were grown at 37° in L-broth, supplemented with erythromicin (8).

For the isolation of proinsulin the cells were removed by centrifugation, the supernatant treated with 5% trichloroacetic acid for 30 min at 4° and the pellet collected by centrifugation. Then the pellet was dissolved in minimal volume of 1 M Tris-buffer, pH 8,0, and applied onto a BrCN-activated Sepharose 4B column, containing covalently bound murine monoclonal antibodies E6E5 (2,3 mg of monoclonal antibodies per ml of wet gel). After washing out the impurities proinsulin was eluted with 0,1 M citrate buffer, pH 2,5, containing 0,3 M NaCl. The pooled active fractions were further purified with reverse-phase HPLC on a Ultrasphere C-8 column 4,6x250 mm (Beckman) developed in Buffer A (0,1% trifluoroacetic acid-0,1% triethylamine-10% acetonitrile). Proinsulin was eluted by a linear gradient of acetonitrile by increasing Buffer B (0,1% trifluoroacetic acid-0,1% triethylamine -90% acetonitrile).

Proinsulin concentration was measured by solid-phase sandwich ELISA, using murine monoclonal antibodies D3E7 as the first antibodies and murine monoclonal antibodies D6C4 conjugated with horse radish peroxidase as the second antibodies.

The N-terminal sequence of the isolated proinsulin was determined by automated Edman degradation with Beckman 890C sequenator, followed by identification of PTH-derivatives with HPLC on a Zorbax-PTH column 4,6x250 mm (DuPont).

E. coli-derived human recombinant proinsulin splitted off from the protein A-proinsulin fusion was used as a reference for ELISA and HPLC.

RESULTS

The plasmid pBINS1 directs not only the efficient synthesis of human proinsulin, but also the quantitative secretion of prohormone outside of B. subtilis AJ73 cells. Not more than 3% and 2% of the total detected amount of proinsulin were found within the cytoplasmic fraction and cell membrane fraction respectively, whereas 95% of proinsulin were measured in the culture fluid.

Table 1 summerizes the purification of proinsulin from the culture medium. The most powerful step in proinsulin isolation was immuno-affinity chromatography through BrCN-activated Sepharose 4B, containing covalently bound murine monoclonal antibodies E6E5. The final step of the purification procedure was HPLC on a Ultrasphere C-8 column, equilibrated in 0,1%

Table 1. The purification of proinsulin from B. subtilis AJ73 (pBINS1)

Purification step	Volume, ml	Protein, mg	Yield, %	Purification, -fold
Culture medium	250	25,5	100	1
TCA precipitation	15	25	100	1
Immuno-affinity chromatography	3	0,88	67,5	12
HPLC	2,5	0,58	57,4	16

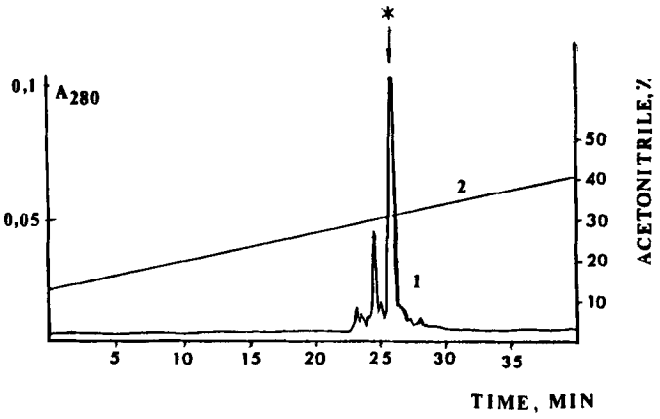

Fig. 1. Reverse-phase HPLC pattern of proinsulin purified by immuno-affinity chromatography. Active proinsulin shown by asterisk elutes as a single peak with 35% acetonitrile. The elution position of E. coli-derived reference proinsulin is indicated by arrow. 1 - $A_{280\ nm}$, 2 - acetonitrile concentration.

trifluoroacetic acid-0,1% triethylamine-10% acetonitrile. Active proinsulin was eluted with a linear gradient of acetonitrile (Fig. 1). The retention time of proinsulin isolated from B. subtilis AJ73 (pBINS1) culture medium exactly coinsided with that of recombinant human proinsulin, isolated from E. coli and splitted off from the protein A-proinsulin fusion. As revealed by SDS-PAGE under non-reducing conditions, there is no detectable amount of proinsulin oligomers in the isolated prohormone preparations (data not shown).

The N-terminal sequence analysis of proinsulin from B. subtilis AJ73 (pBINS1) unambiguously revealed one polypeptide chain with the N-terminal sequence Phe_1-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His_{10} indistinguishable from that of natural human prohormone.

DISCUSSION

Fig. 2 shows the sequence of signal peptides and mature proteins junction regions for B. amyloliquefaciens pre-α-amylase, human preproinsulin and hybrid preprotein (α-amylase-proinsulin). It is not evident that the proper processing and cleavage might be awaited for the hybrid preprotein in B. subtilis cells. Recently Inouye et al. (9) and Gierasch et al. (10) reported that the probability of the proper cleavage by signal peptidase correlates rather well with the β-turn of polypeptide chain in the region of processing site. The probabilities of β-turns, calculated according to Chou-Fasman (11) and Garnier (12) for the natural pre-α-amylase and preproinsulin, as well as for hybrid preprotein are represented in Fig. 2. The probability of β-turn formation within the sequence region enclosing the possible cleavage site of the hybrid preprotein is comparable with those for the natural preproteins. The experimental data obtained in the present study support this "β-turn" hypothesis, as the hybrid construction consisting of signal peptide sequence of B. amyloliquefaciens α-amylase and human proinsulin is

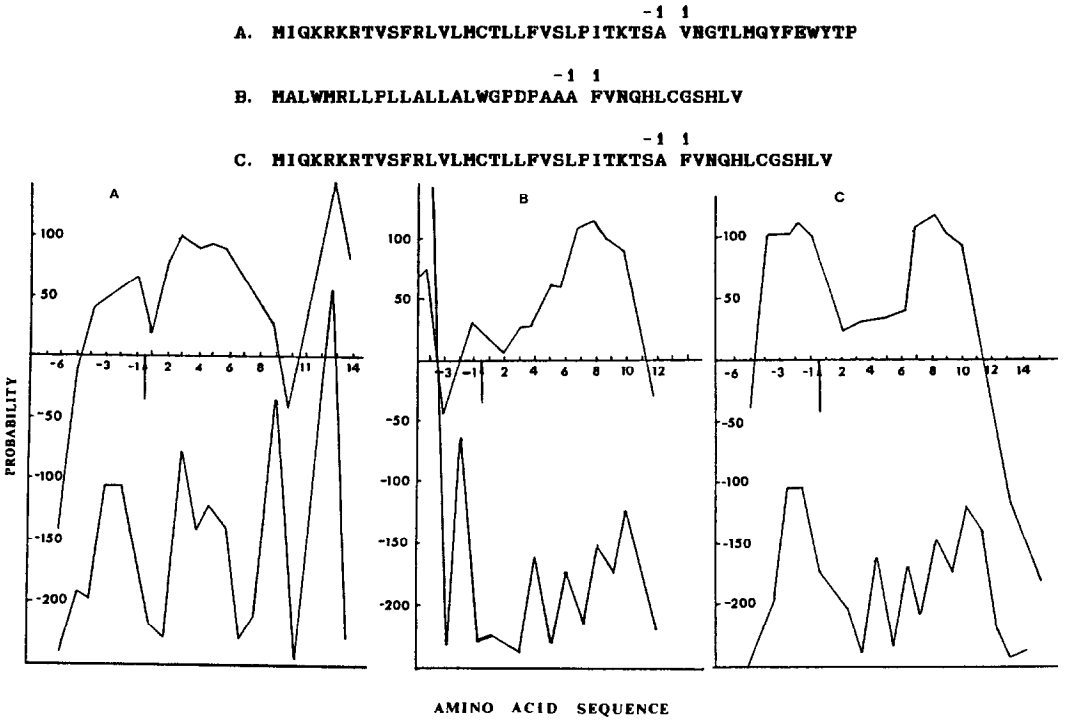

Fig. 2. Sequences and probabilities of β-turn formation, calculated according to Chou-Fasman (upper curve) and Garnier (lower curve) of the natural B. amyloliquefaciens pre-α-amylase (A), natural human preproinsulin (B) and hybrid preproinsulin (C). Arrow indicates the cleavage site.

properly processed in B. subtilis cells, and proinsulin with the N-terminal sequence identical to the natural human prohormone is quantitatively secreted into the culture medium.

Thus, in the nearest future the constructed hybrid sequence (α-amylase prepart fused in frame with proinsulin sequence) and host-vector system might be successfully applied in the production of human recombinant proinsulin for the preparative purposes, especially if the level of proinsulin gene expression is increased by the optimization of fermentation conditions and/or reducing the activity of proteases, secreted by B. subtilis host strains (8).

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