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Functional expression of recombinant insulins in *Saccharomyces cerevisiae*

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Abstract

Background Since 1982, recombinant insulin has been used as a substitute for pancreatic insulin from animals. However, increasing demand in medical and food industries warrants the development of more efficient production methods. In this study, we aimed to develop a novel and efficient method for insulin production using a yeast secretion system.

Methods Here, insulin C-peptide was replaced with a hydrophilic fusion partner (HL18) containing an affinity tag for the hypersecretion and easy purification of proinsulin. The HL18 fusion partner was then removed by in vitro processing with the Kex2 endoprotease (Kex2p), and authentic insulin was recovered via affinity chromatography. To improve the insulin functions, molecular chaperones of the host strain were reinforced via the constitutive expression of *HAC1*.

Results The developed method was successfully applied for the expression of cow, pig, and chicken insulins in yeast. Moreover, biological activity of recombinant insulins was confirmed by growth stimulation of cell line.

Conclusions Therefore, replacement of the C-peptide of insulin with the HL18 fusion partner and use of Kex2p for in vitro processing of proinsulin guarantees the economic production of animal insulins in yeast.

Keywords Insulin, Secretion, Kex2p, Cultured meat, *Saccharomyces cerevisiae*

Background

More than 500 million people are estimated to have type 2 diabetes by 2030 owing to the changes in eating habits. Increasing incidence of diabetes has considerably increased the global demand for insulin, which regulates the blood glucose levels [1]. With the global population expanding rapidly, innovation of food systems is under way for sustainable supply. To meet the growing demand for meat, cultured meat is used as a promising alternative to livestock products owing to its high degree of similarity to existing meat; replacing conventional meat with cultured meat further reduces greenhouse gas emissions and use of land, water, and energy resources [2]. Cell culture media are responsible for most of the marginal cost of cultured meat, and fetal bovine serum (FBS) accounts for 99% of this cost [3, 4]. Insulin is one of the key constituents of FBS that enhances cell proliferation [5]. Therefore, insulins and growth factors from various animals are required to develop a serum-free culture medium for the economical production of cultured meat even without cross-species compatibility.

Insulin is produced by the β-cells of pancreas as proinsulin composed of A- and B-chains that are connected by the C-peptide. Mature insulin is formed by the proteolytic processing of the C-peptide in

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secretory granules. The A- and B-chains are linked by two intermolecular disulfide bonds, and an intramolecular disulfide is present within the A-chain [6]. Since its discovery, bovine or porcine insulin has been used to treat diabetes; however, owing to the advancements in molecular biology, recombinant insulins produced in various expression systems have now substituted pancreatic insulins from animals. Recombinant human insulin was the first drug licensed for therapeutic application in humans by the U.S. Food and Drug Administration [7]. Although mammalian cells and plant expression systems were developed for insulin production [8, 9], *Escherichia coli* and yeasts are mainly used as hosts for the large-scale production of recombinant human insulin [10]. Compared to the *E. coli* expression system, which requires solubilization and refolding steps for the production of functional insulin [11], a yeast-based expression system is preferred as it directly secretes the soluble insulin precursors into the culture supernatant [12–14].

As the secretory expression of proinsulin in yeast using the mating factor (MF)-α leader is inefficient [12], the secretion of proinsulin can be improved by the manipulation of insulin gene to encode proinsulin-like single-chain proteins by direct fusion of the A- and B-chains or replacement of the C-peptide with short synthetic peptides [15–17]. These proinsulin-like single-chain precursors are treated with trypsin to produce a two-chain mature insulin digested at the end of the B-chain [17]. However, trypsin digestion generates non-functional insulin as the B-chain contains two internal basic residues cleavable by trypsin, leading to a significant decrease in the yield of functional insulin. Sreenivas et al. solved this problem by overexpressing the endogenous Kex2 endoprotease (Kex2p) cleaving after dibasic residues (Arg–Arg and Lys–Arg) in the late Golgi network [18] instead of trypsin digestion, as the C-terminal amino acid sequence of the B-chain is Arg–Arg [14].

In this study, the C-peptide of insulin was replaced with a hydrophilic fusion partner (HL18) derived from the hydrophilic domain of yeast *VOA1* [19] and an affinity tag for the hypersecretion and easy purification of insulin precursors. Kex2p was used for in vivo processing of insulin precursors in *Saccharomyces cerevisiae* and in vitro processing for the production of authentic human (hINS), bovine (bINS), pig (pINS), and chicken (cINS) insulins. Moreover, molecular chaperones of the host strain were reinforced via the constitutive expression of *HAC1* to improve the functional expression of insulin.

Methods

Strains, chemicals, and media

Haploid yeast *S. cerevisiae* 2805 allgal (Mat α, *pep4::HIS3*, *prb1*, *can1*, *his3-200*, *ura3-52*, *gal1*, *gal2*, *gal7*, *gal10*, and *gal80*) was used for the constitutive expression of recombinant insulins under the control of *GAL10* promoter [20]. *E. coli* DH5α [F−*lacZΔ* M15 *hsd*R17(r-m-) *gyr*A36] was used for general DNA recombination procedures. Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), restriction endonucleases, ligase, and In-Fusion HD Cloning Kit were purchased from Takara Bio Company (Shiga, Japan). Bovine pancreatic insulin and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). DNA was purified using the Zymoclean Gel DNA Recovery Kit (ZYMO RESEARCH, USA). Yeast cells were cultured in the yeast peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose). Yeast transformants were selected on a synthetic defined medium lacking uracil (SD-ura; 0.67% yeast nitrogen base without amino acids, 0.077% ura-dropout supplement, 2% glucose, and 2% agar). Uracil auxotroph strains were selected on 5-fluoroorotic acid (5-FOA) medium (0.67% yeast nitrogen base without amino acid, 2% glucose, 0.05 mg/mL uracil, and 0.1% 5-FOA).

Plasmid construction and protein expression analysis

For the construction of YGαbINS, YGαbINSΔC, and YGαHL18bINS vectors, genes encoding bINS (FVNQHLCGSHLVEALYLVCGERGFFYTPKAKREVEGPQVGALELAG GPGAGGLEGPPQKRGIVEQCCASVCSLYQLENYC), bINSΔC (FVNQHLCGSHLVEAL YLVCGERGFFYTPKAKRGIVEQCCASVCSLYQLENYC), and HL18bINS (FVNQHLCG SHLVEALYLVCGERGFFYTPKAKRSVEDEDGDDEYATEETLSHHHHHHGDDDDKGAAKRGIVEQCCASVCSLYQLENYC) were synthesized by Bioneer (Daejeon, Republic of Korea) based on the codon usage of *S. cerevisiae* and then cloned between the MFα leader and *GAL7* terminator of the 2-μm plasmid-based episomal expression vector, YEGα-HL28-EGF [19], containing the *GAL10* promoter, MFα leader, *GAL7* terminator, and *URA3* selectable marker using the In-Fusion cloning kit. To construct the hINS, pINS, and cINS expression vectors (YGαHL18hINS, YGαHL18pINS, and YGαHL18cINS, respectively), bINS gene of the YGαHL18bINS vector was replaced with hINS, pINS, and cINS genes by site-directed mutagenesis.

Yeast was transformed using the lithium acetate method, as previously described [21]. The transformants were aerobically cultivated in 10-mL test tubes containing 2 mL of YPD broth medium for 40 h at 30 °C. Then,

0.6 mL of culture supernatants was precipitated with 0.4 mL of cold acetone and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 4–12% gradient polyacrylamide gels (Thermo Fisher Scientific) with and without β-mercaptoethanol, followed by Coomassie blue staining. For in vitro processing with Kex2p, protein precipitates were dissolved in the Kex2p working buffer (50 mM Tris–HCl [pH 8.0], 50 mM NaCl, and 2 mM $CaCl_2$), followed by the addition of 0.1 μg of Kex2p prepared in-house and incubation at 37 °C for 1 h.

Construction of an unfolded protein response (UPR)-enforced strain

For the constitutive expression of *HAC1*, the intron in *HAC1* was removed by polymerase chain reaction (PCR) and expressed under the control of *GAL10* promoter using the integration vector, pTU2-HAC1 (Fig. S1). This vector contains an expression cassette for *HAC1*, a fragment of YDRCTy1-1 (284 bp) for random integration, and *URA3* as a selection marker. *HAC1* expression cassette and YDRCTy1-1 fragment were flanked by a 100-bp vector sequence to excise the selection marker and vector backbone via homologous recombination. The linearized pTU2-HAC1 vector was prepared by inverse PCR using primers targeting the YDRCTy1-1 element for integration into the YDRCTy1-1 loci of *S. cerevisiae* genome. Transformants were selected on SD-ura medium and confirmed by colony PCR using primers targeting the *HAC1* and *GAL10* promoters. Finally, strains without *URA3* and vector backbone were selected by counter screening on the 5-FOA medium.

Fed-batch fermentation

Fed-batch fermentation was carried out in a 5-L fermenter (KoBiotech Co., Incheon, Republic of Korea). Seed culture was prepared by cultivation in 50 mL of SD-ura broth at 30 °C for 24 h. The seed culture was mixed with 200 mL of YPD broth, incubated at 30 °C for 24 h, and inoculated into a 5-L jar fermenter containing 1.75 L of the main culture medium (2% glucose, 3% yeast extract, and 1.5% peptone). Temperature was maintained at 30 °C, and the pH was adjusted to 5.5 using NH_4OH. During fermentation, the feeding medium (5% yeast extract and 30% glucose) was added to maintain the glucose concentration in the main culture. Agitation and aeration were maintained at 900 rpm and 1 vvm, respectively, and culture samples were collected every 4 h. Cell mass and secreted proteins were analyzed by measuring the optical density at 600 nm (OD_{600}) and performing SDS-PAGE, respectively. Quantitative analysis of the expressed proteins was performed using the Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions, with bovine serum albumin as the standard.

Direct purification of recombinant insulins

Fermentation broth was filtered through a 0.44-μm filter (Sartoclear S9-P Cap; Sartorius, Goettingen, Germany), concentrated tenfold by ultrafiltration using the 3000 NMWC ultra centrifugal filter device (GE Healthcare, Chicago, IL, USA), and adjusted using buffer A (50 mM Tris–HCl [pH 8.0] and 0.5 M NaCl). Insulin fusion proteins were purified by immobilized metal affinity chromatography (IMAC) on a nickel-NTA agarose column (Promega, Madison, WI, USA) using AKTA FPLC (GE Healthcare). After loading onto the column at a flow rate of 1 mL/min, the insulin fusion proteins were eluted in a gradient of buffer B (50 mM Tris–HCl [pH 8.0], 0.5 M NaCl, and 0.5 M imidazole). Fractions containing the insulin fusion proteins were concentrated and replaced with the Kex2p working buffer using the 3000 MWCO filter device. The HL18 fusion partner was separated by digestion with in-house prepared Kex2p [20] at 30 °C for 1 h. Notably, 1 μg of Kex2p was used per 1 mg of the fusion protein. Intact insulin was obtained by repeated IMAC. Finally, molecular weight of purified insulin was determined by Protein Works (Daejeon, Republic of Korea).

Analysis of insulin activity

Bioactivity of insulin was determined by proliferation assays using HaCaT cells purchased from Thermo Fisher Scientific. Cells were cultured in the growth medium (Dulbecco's modified Eagle's medium [DMEM; Thermo Fisher Scientific] + 10% FBS [Thermo Fisher Scientific] + 100 U/mL penicillin–streptomycin [Thermo Fisher Scientific]) in a humidified incubator containing 5% CO_2 at 37 °C. After the cells reached 80–90% confluency in the T75 culture flasks, the medium was replaced with DMEM + 100 U/mL penicillin–streptomycin containing different concentrations of insulin and cultured for 48 h before harvesting. After removing the growth media, the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (Promega) for 1 h at 37 °C. Absorbance was measured at 490 nm using a microplate reader (Molecular Devices, San Jose, CA, USA). Statistical comparison of growth was performed using a two-tailed Student's *t*-test (Microsoft Excel). Statistical significance was set at $p < 0.05$.

Results and discussion

Analysis of the amino acid sequences of animal insulins

Amino acid sequences of bINS, pINS, and cINS were compared with those of hINS using the ClustalW program [22]. In contrast to the highly conserved B- and

A-chains, amino acid sequences of the C-peptide were not conserved (Fig. 1). This indicates that the amino acid sequence of C-peptide is not strictly related to the proteolytic maturation of insulin. As the C-peptide is not a constituent of mature insulin, and the expression of proinsulin-like single-chain proteins without the C-peptide is more advantageous than that of proinsulin [15–17], replacement of the C-peptide with a functional peptide acting as a secretion enhancer and use of affinity tags can provide novel strategies for insulin production. Therefore, in this study, C-peptide was replaced with the HL18 peptide derived from the hydrophilic domain of yeast *VOA1* [19]. The secretion-enhancing effects of HL18 fusion partners have been demonstrated via the expression of various recombinant proteins [23].

Expression of recombinant bINS

Although the amino acid sequences of all mature insulins, except cINS, were almost the same, bINS was expressed in *S. cerevisiae* to develop a serum-free culture medium for the production of cultured beef. Three episomal expression vectors (YGαbINS, YGαbINSΔC, and YGαHL18bINS) expressing the three types of bINS, bovine proinsulin (bINS), bovine proinsulin without C-peptide (bINSΔC), and bovine proinsulin in which the C-peptide is replaced with HL18 peptide and 6 histidine (HL18bINS), were constructed (Fig. 2A). These vectors

Fig. 1 Alignment of the amino acid sequences of human (hINS), bovine (bINS), pig (pINS), and chicken (cINS) insulins. Signal peptide, B-chain, C-peptide, and A-chain are indicated by boxes. Disulfide bonds are indicated by red lines

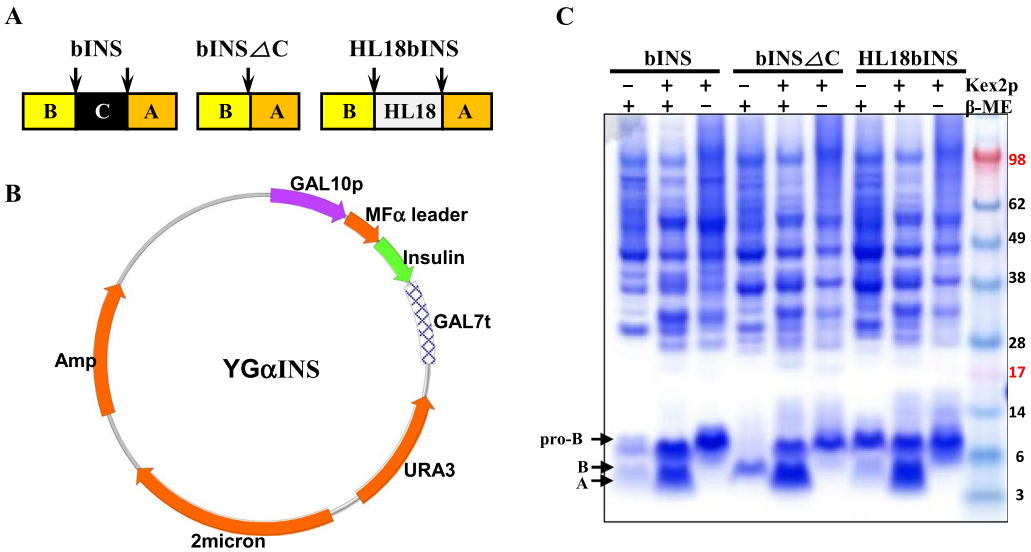

Fig. 2 Expression of bINS in *Saccharomyces cerevisiae*. **A** Schematic diagram of the three bINS constructs and insulin expression vectors (**B**). **C** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the concentrated culture supernatants expressing YGαbINS, YGαbINSΔC, and YGαHL18bINS. Kex2 endoprotease (Kex2p) cleavage sites and corresponding protein bands are indicated by arrows. M: protein molecular weight markers

contain the *URA3* selectable marker and recombinant insulin expression cassette, comprising the *GAL10* promoter, MFα leader, coding sequence of bINS, and *GAL7* terminator (Fig. 2B). To ensure the in vivo processing of proinsulin by Kex2p in the Golgi complex, each chain was connected by the Lys–Arg sequence. *S. cerevisiae* allgal strain was transformed with these expression vectors. After 40 h of cultivation at 30 °C, the same volume of culture supernatants was analyzed with and without β-mercaptoethanol. When the culture supernatants were analyzed under reducing conditions, peptides corresponding to the A-, B-, and unprocessed MFα pro-B chains were detected in all the tested constructs in different amounts. As is known [12], expression of bINSΔC was higher than that of bINS, and the amount of proinsulin was highest in the recombinant yeast expressing HL18bINS. These peptide bands thickened after incubation with Kex2p and converged to form larger smear bands under non-reducing conditions (Fig. 2C). This result imply that although the in vivo processing of MFα pro-peptide was incomplete, secretion of proinsulin was significantly enhanced by replacing the C-peptide with the HL18 fusion partner. Furthermore, the secreted proinsulin peptides were connected by intermolecular disulfide bonds.

Production of bINS via in vitro processing with Kex2p

When proinsulin folds in the endoplasmic reticulum of β-cells, the C-peptide is removed by proteolytic processing with prohormone convertase (PC1/3) in secretory vesicles [24]. Kex2p is a functional equivalents of the PC1/3 that cleaves after dibasic sequences of substrates in the late Golgi network of yeast. We hypothesized that Kex2p works similar to the PC1/3 in the proteolytic processing of proinsulin in yeast. At first, enterokinase was used for in vitro processing of proinsulin fused with HL18, but we could not get functional insulins due to the ectopic cleavage at the B-chain of insulin. Therefore, Kex2p was employed instead of enterokinase. We previously developed a mass production system for Kex2p from *Candida albicans* and used it for recombinant protein production [25]. To produce fusion proteins joined by the Kex2p cleavage sequence in yeast, a *kex2* mutant strain is needed to block the in vivo processing by Kex2p during secretion. However, the *kex2* mutant strain is not an appropriate host for recombinant protein expression as it is very sensitive to stress, leading to its slow growth in complex media [26]. Therefore, in this study, instead of using the *kex2* mutant strain, Kex2p cleavage was controlled by manipulating the cleavage sites because the substrate specificity of Kex2p is affected by the amino acids adjacent to the cleavage site [23, 27, 28]. Expression vector YGαHL18bINS contained three Kex2p cleavage sites: after MFα pro-peptide, the B-chain and HL18 fusion partner (Fig. 3A). The 1st and 2nd Kex2p cleavage sites should be processed in vivo for correct folding of insulin and further processing of C-terminus of the B-chain via intracellular protease. Processing of the 3rd Kex2p cleavage site should be controlled during secretion for direct purification of fusion proteins because the affinity tag is contained in the HL18 fusion partner. For the efficient in vivo processing of the MFα pro-peptide and B-chain during secretion, adjacent sequences of 1st and 2nd Kex2p cleavage sites were modified based on

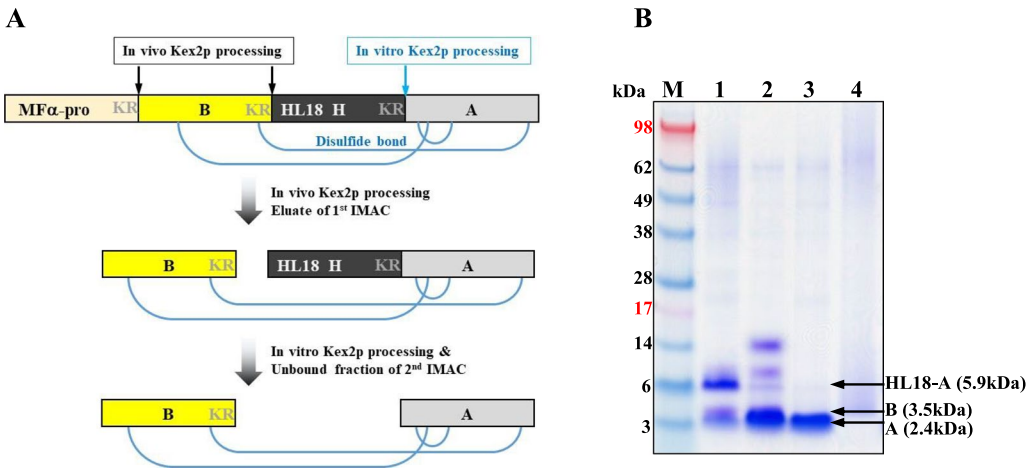

Fig. 3 Production of bINS using the HL18 fusion partner. **A** Schematic diagram of the in vivo and in vitro processing of the insulin precursor by Kex2p. **B** SDS-PAGE analysis of the purification process using immobilized metal affinity chromatography (IMAC). Eluate of 1st IMAC (lane 1), eluate of 1st IMAC digested with Kex2p (lane 2), unbound fraction of 2nd IMAC (lane 3), and unbound fraction of 2nd IMAC without β-mercaptoethanol (lane 4) are shown. M: protein molecular weight markers. The corresponding protein bands are indicated by arrows

the ProP 1.0 server (https://services.healthtech.dtu.dk/service.php?ProP-1.0), which predicts the pro-peptide cleavage sites in eukaryotic protein sequences [29]. As a high ProP score is more confident in the prediction of the pro-peptide cleavage site, ProP scores of 1st and 2nd Kex2p cleavage sites were adjusted to 0.412 and 0.535, respectively. To block the in vivo processing of the 3rd Kex2p cleavage site during secretion and ensure in vitro processing by Kex2p after purification of proinsulin, ProP score of the 3rd Kex2p cleavage site was reduced to 0.228 by modifying the P_3 and P_4 sites. This score was predicted to not indicate a cleavage site using the ProP 1.0 server.

If the 1st and 2nd Kex2p cleavage sites were completely processed and the 3rd Kex2p cleavage site was not processed during secretion, the fusion protein was expected to be expressed as one B chain and one HL18-A-chain connected by two disulfide bonds (Fig. 3A). HL18-proINS fusion protein produced by fed-batch fermentation was purified via IMAC and analyzed by SDS-PAGE (Fig. 3B). Two protein bands representing the HL18-A-chain (5.9 kDa) and the B-chain (3.5 kDa) were identified after 1st IMAC (Fig. 3B, lane 1). After digestion with Kex2p, the larger band (HL18-A-chain) disappeared and the intensity of smaller band increased, implying the cleavage of the HL18-A-chain into HL18 (3.5 kDa) and A-(2.4 kDa) chains (Fig. 3B, lane 2). The Kex2p-digested fusion protein was further purified by IMAC, and bINS was obtained by collecting the unbound fractions (Fig. 3B, lane 3). As the sizes of the B-chain, HL18 peptide, and A-chain were similar, these protein bands were indiscernible by SDS-PAGE under reducing conditions. To identify the link between B- and A-chains, the unbound fraction was analyzed without β-mercaptoethanol (Fig. 3B, lane 4). As the disulfide bonds connecting the B- and A-chains are maintained under these conditions, their bands converge to yield larger smear bands. These results suggest that the HL18 fusion partner is completely removed by in vitro processing with Kex2p, and intact insulin chains are connected by disulfide bonds.

UPR activation for functional expression of bINS

HAC1 is a key transcriptional regulator of UPR, a quality control mechanism for the proper folding of proteins in the secretion pathway. Overexpression of *HAC1* gene increases the expression of ER chaperones and components of the ER associated degradation machinery resulting in the enhanced expression of heterologous proteins [30, 31]. As bINS contains two intermolecular and one intramolecular disulfide bonds, a *HAC1*-overexpressing recombinant strain was constructed for the proper folding and enhanced secretion of bINS. The YGαHL18bINS vector was expressed in this strain, and bINS was purified after fermentation as described above. Then, molecular weights of the purified B- and A-chains were determined via LC–MS (data not shown) to confirm their intactness. As the C-terminal Arg–Arg residues of the B chain are removed by carboxypeptidase-E in the secretory vesicle of pancreatic islets, we expected the removal of the C-terminal Lys–Arg residues of the B-chain by the carboxypeptidase encoded by *KEX1* gene in the Golgi apparatus of yeast, as observed in the processing of MFα. However, the Lys–Arg residues were retained in the final purified insulin. To identify the effects of these artificial residues on insulin activity, the growth-stimulating activity of recombinant bINS prepared from the wild-type (WT) and *HAC1*-overexpressing strains was compared with that of bovine pancreatic insulin using HaCaT cells. Recombinant bINS expressed in the *HAC1*-overexpressing strain exhibited comparable growth to that of pancreatic insulin. Recombinant bINS expressed in the WT strain also demonstrated growth-stimulating activity, but it was inferior to that of bINS expressed in the *HAC1*-overexpressing strain (Fig. 4). These results suggest that the artificial residues remaining in B-chain do not affect the growth-stimulating activity of insulin and that *HAC1* overexpression improves the functional expression of recombinant insulin.

Production of hINS, pINS, and cINS in yeast

Expression and purification methods used for bINS were further applied to produce hINS, pINS, and cINS. As the amino acid sequences of the B- and A-chains are highly conserved, expression vectors for hINS, pINS, and cINS (YGαHL18hINS, YGαHL18pINS, and YGαHL18cINS, respectively) were directly constructed by site-directed mutagenesis of the YGαHL18bINS vector. These vectors were subsequently expressed in the *HAC1*-overexpressing strain and insulin was purified by sequential IMAC after fed-batch fermentation. No differences were observed in the growth of the four strains expressing bINS, hINS, pINS, and cINS, but a slight difference was observed in the amount of purified insulin after 1st and 2nd IMAC. Overall, 16–21 mg of insulin was produced after fermentation (Table 1). Purified insulin was analyzed using SDS-PAGE with and without β-mercaptoethanol (Fig. 5A). High molecular weight smeared protein bands were detected for hINS and pINS. These protein bands were considered to be glycosylated unprocessed fusion proteins (MFα pro-B-chain) as MFα pro-peptide contains three N-glycosylation sites and insulin does not contain any glycosylation sites. All insulin bands changed to larger smeared bands under non-reducing conditions, indicating the link between the A and B chains by disulfide bonds. All recombinant insulins

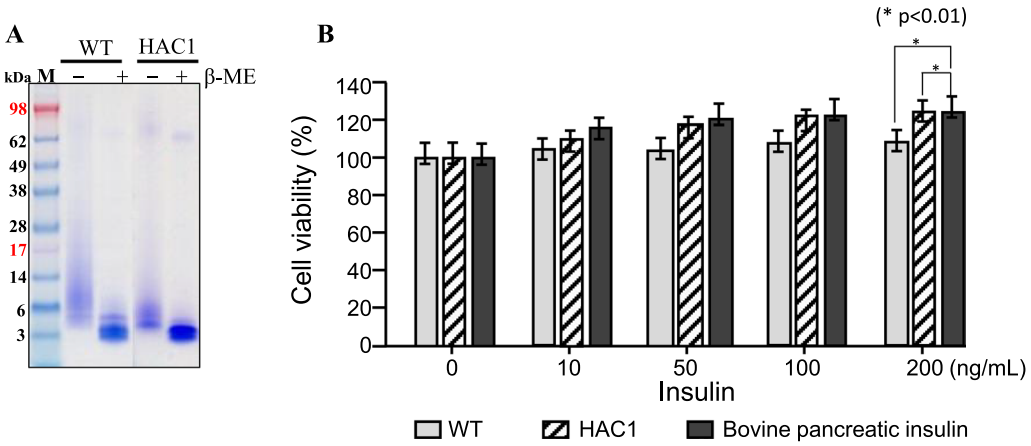

Fig. 4 Bioactivity of bINS. HaCaT cells were cultured in the presence of pancreatic and recombinant bINSs. Cell proliferation was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) assay kit

Table 1 Purification table of recombinant insulins

Insulin (mg)				
IMAC	**bINS**	**hINS**	**pINS**	**cINS**
1st IMAC	123	141	136	115
2nd IMAC	18	25	21	16

Insulins produced by 2L fed-batch fermentation were purified by IMAC

demonstrated growth-stimulating activity comparable to that of pancreatic insulin in HaCaT cells (Fig. 5B). These results highlight the efficiency of our method in producing insulin from various sources.

Conclusions

To the best of our knowledge, this study is the first to establish an efficient method for the production of hINS, bINS, pINS, and cINS using *S. cerevisiae*. Functional expression of insulin was enhanced by replacing the C-peptide with a novel hydrophilic fusion partner, HL18, and expressing it in a UPR-enforced strain. For the production of intact insulin, HL18 fusion partner was efficiently removed by controlled processing using Kex2p, which was mass-produced in our laboratory. Although the recombinant insulin produced in this study contained artificial residues at the C-terminus of the B-chain, its growth-stimulating activity was not affected. These findings highlight the potential of recombinant insulin for cultured meat production.

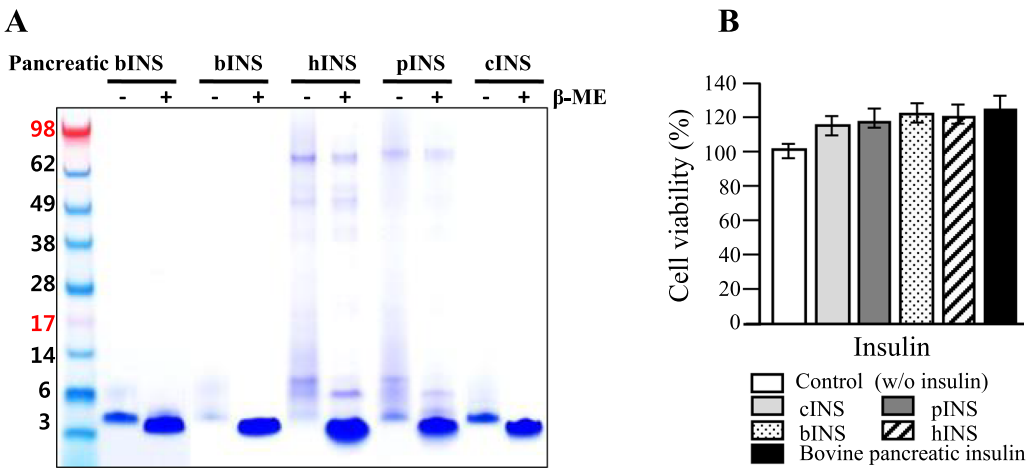

Fig. 5 Production and activities of hINS, pINS, and cINS. **A** SDS-PAGE analysis of bINS, hINS, pINS, and cINS. Insulins produced by fed-batch fermentation were purified using IMAC. Same amount insulins were compared with and without β-mercaptoethanol. **B** Cell proliferation was analyzed using the MTT assay kit (Promega, Madison, WI, USA)

The production method developed in this study can be applied to other expression systems such as *E. coli* and *Komagataella phaffii* (*Pichia pastoris*).

Abbreviations

bINS	Bovine insulin
cINS	Chicken insulin
FBS	Fetal bovine serum
hINS	Human insulin
IMAC	Immobilized metal affinity chromatography
Kex2p	Kex2 endoprotease
MF	Mating factor
pINS	Pig insulin
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
UPR	Unfolded protein response

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02571-2.

Supplementary Material 1.

Acknowledgements
This work was partly supported by the High Value-added Food Technology Development Program funded by the Ministry of Agriculture, Food, and Rural Affairs (MAFRA; 321026053SB010), Agriculture Science and Technology Development Program funded by the Rural Development Administration of Korea (PJ014938012023), Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MSIT; 2023M3A9l308236712), and the Research Initiative Program (KGM5402423) of KRIBB.

Author contributions
JHB and JHS designed this study. MJK, SLP, and HJK performed the experiments. BHS analyzed the data. JHB and SLP wrote the manuscript. All authors have read and approved the manuscript. We certify that the above information is true and correct. All authors contributed to the study and manuscript.

Funding
This work was partly supported by the High Value-added Food Technology Development Program funded by the Ministry of Agriculture, Food, and Rural Affairs (MAFRA; 321026053SB010), Agriculture Science and Technology Development Program funded by the Rural Development Administration of Korea (PJ014938012023), Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MSIT; 2023M3A9l308236712), and the Research Initiative Program (KGM5402423) of KRIBB.

Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 8 April 2024 Accepted: 28 October 2024
Published online: 11 November 2024

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