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Characterization and immunological activity of different forms of recombinant secreted Hc of botulinum neurotoxin serotype B products expressed in yeast

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The recombinant Hc proteins of botulinum neurotoxins and tetanus toxin are exclusively produced by intracellular heterologous expression in *Pichia pastoris* for use in subunit vaccines; the same Hc proteins produced by secreted heterologous expression are hyper-glycosylated and immunologically inert. Here, several different recombinant secreted Hc proteins of botulinum neurotoxin serotype B (BHc) were expressed in yeast and we characterized and assessed their immunological activity in detail. Recombinant low-glycosylated secreted BHc products (BSK) were also immunologically inert, similar to hyper-glycosylated BHc products (BSG), although deglycosylation restored their immunological activities. Unexpectedly, deglycosylated proBHc contained an unexpected pro-peptide of an α-factor signal and fortuitous N-linked glycosylation sites in the non-cleaved pro-peptide sequences, but not in the BHc sequences. Notably, a non-glycosylated secreted homogeneous BHc isoform (mBHc), which we successfully prepared after deleting the pro-peptide and removing its single potential glycosylation site, was immunologically active and could confer effective protective immunity, similarly to non-glycosylated rBHc. In summary, we conclude that a non-glycosylated secreted BHc isoform can be prepared in yeast by deleting the pro-peptide of the α-factor signal and mutating its single potential glycosylation site. This approach provides a rational and feasible strategy for the secretory expression of botulism or other toxin antigens.

Botulinum neurotoxins (BoNTs) produced by bacteria of the genus *Clostridium* are among the most toxic proteins for humans with estimated 50% lethal dose (LD_{50}) values of 1 ng/kg body weight. BoNTs can be classed into eight serotypes: A, B, C, D, E, F, and G, and the recently reported serotype H[1]. Among of BoNTs, serotypes A, B, E, and F can cause disease in humans, and serotypes C and D can cause disease in cattle and horses under normal circumstances. BoNTs are synthesized as ~150 kDa single-chain proteins that are composed of a N-terminal catalytic light chain (LC, 50 kDa) and heavy chain (HC, 100 kDa) linked by a disulfide bond. The heavy chain is composed of two domains, the N-terminal translocation domain (Hn domain, 50 kDa) and the C-terminal receptor-binding domain (Hc domain, 50 kDa), which mediates binding to target neurons[2,3]. Among these domains, the nontoxic Hc alone seems to confer protective immunity against the toxin[4–6]. Thus, the Hc domain of BoNTs contains most of the neutralizing epitopes[7,8] and is the leading candidate for inclusion in recombinant botulinum vaccine preparations[6,9].

Recombinant botulinum antigens can be produced in large quantities using expression systems, such as yeast or *Escherichia coli*[10,11]. For vaccine development, production in *Pichia pastoris* offers additional advantages over *E. coli* as it avoids inclusion body formation and eliminates pyrogens owing to the presence of bacterial endotoxins in *E. coli*. Additionally, secreted recombinant vaccine antigens from *P. pastoris* are easier to scale-up for expression and purification compared to intracellular production from yeast or *E. coli*. Some secreted recombinant bacterial toxins and their derivatives from *P. pastoris* have been shown to be biologically active and immunogenic[10], while the recombinant Hc of TeNT and BoNTs secreted into the culture medium are glycosylated as a consequence of the presence of fortuitous N-linked glycosylation sites and this hyper-glycosylation renders them immunologically inactive[12–14]. Notably, three intramuscular vaccinations with the hyper-glycosylated Hc of BoNT/B (BHc) failed to induce protective immunity in mice[13].

To prepare a good candidate subunit vaccine against BoNT/B, several different recombinant secreted BHc proteins were expressed in yeast and their immunological activities were assessed in detail. After laborious efforts,

a non-glycosylated secreted homogeneous BHc product, termed mBHc (BHcN957Q), was shown to be biologically and immunologically active and could confer effective protective immunity against challenge with high doses of active BoNT/B.

Results

Characterization and immunogenicity of recombinant BSG and BSK products. The purified BSG or mBSG expressed in *P. pastoris* GS115 proteins were visualized by SDS–PAGE as a major band of ~150 kDa and a smear with lesser electrophoretic mobility as a result of hyper-glycosylation (Figure 1A, Table 1). By contrast, the purified BSK protein expressed in *P. pastoris* GJK01[15] appeared by SDS–PAGE as a major band of ~60 kDa because of low-glycosylation (Figure 1B, Table 1).

To evaluate the immunity induced by the BSG or BSK subunit vaccines, mice were immunized i.m. with BSG or BSK antigen, and then were serologically monitored. Low anti-BSG or BSK antibody titers were observed in mice vaccinated with two or three doses of 1 or 10 μg BSG or BSK formulated with aluminum hydroxide adjuvant, respectively (Figure 2). ELISA analysis of anti-BSG sera showed no obvious reactivity with BSK, which indicated the absence of cross-reactivity between BSG and BSK antigen. Sera from mice immunized two or three times with 1 or 10 μg BSG or BSK subunit vaccine all showed no detectable neutralizing antibody titers against BoNT/B (< 0.16 IU/mL). Furthermore, these multiple immunizations did not produce protective responses against low doses (100–1000 50% lethal doses [LD_{50}]) of BoNT/B challenge in mice. Our data show that hyper-glycosylated BSG vaccination has low immunogenicity and does not evoke protective immunity in mice, as previously reported[12–14]. Low-glycosylated BSK vaccinations also failed to induce protective immunity in mice. In sum, both low- and hyper-glycosylations rendered the antigens non-protective and low immunologically inactive, indicating that these glycosylations might alter the correct conformation of BHc antigen and obscure the neutralizing epitopes.

Characterization and immunogenicity of the deglycosylated proBHc product. A deglycosylated BHc product (proBHc) composed of recombinant BSG/mBSG or BSK was generated by *in vitro* deglycosylation. Deglycosylated proBHc showed an unexpected size of ~57 kDa (>50 kDa BHc, Figure 1C, Table 1). The N-terminal sequence of the proBHc product was also unexpected, APVXT-(X=N, which is glycosylated), based on N-terminal amino acid sequencing (Supplementary Figure S1). This finding was consistent with the amino acid sequences that we predicted from the α-factor pro-peptide (66 amino acids, 7 kDa), which indicated that the α-factor pre-peptide was precisely cleaved in *P. pastoris*, but that the α-factor pro-peptide was not processed. Thus, our data show that the non-cleaved glycosylated pro-peptide yielded the unexpected glycosylated BHc products, and BHc or mBHc might be not glycosylated in *P. pastoris*.

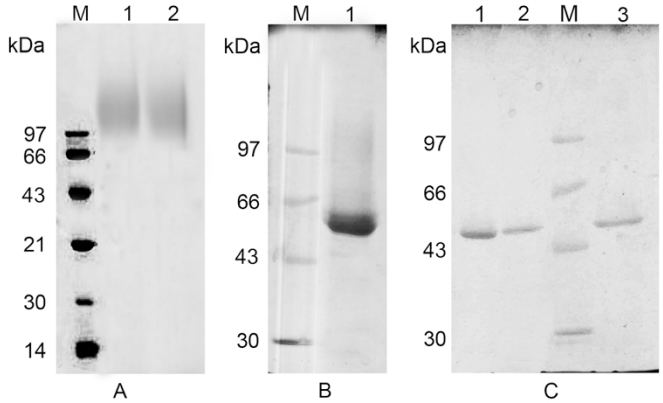

Figure 1 | Analysis of purified recombinant BHc products by SDS–PAGE. (A) Hyper-glycosylated BSG and mBSG products. Lane 1, BSG; lane 2, mBSG. (B) Low-glycosylated BHc products. Lane 1, BSK. (C) Deglycosylated proBHc. Lane 1, a deglycosylated BHc product derived from deglycosylated BSG; lane 2, a deglycosylated BHc product derived from deglycosylated mBSG; lane 3, a deglycosylated BHc product derived from deglycosylated BSK. M, protein standard.

The immunogenicity and protective capability of the 5 μg deglycosylated proBHc subunit vaccine was also evaluated and compared to the equivalent molar dosage (15 μg) of BSG antigen in mice. The mean antibody titers to proBHc in the proBHc-immunized mice were significantly higher than those in the BSG-immunized mice. The BSG vaccination still resulted in low anti-proBHc antibody titers (Table 2) as well low anti-BSG antibody titers as shown in above Figure 2A. ELISA analysis of the proBHc-immunized sera showed low reactivity to BSG comparable to the BSG-immunized sera (data not shown), which indicated that there was cross-reactivity of the deglycosylated proBHc and hyperglycosylated BSG antigens. Mice that received a single proBHc vaccination were completely protected (100% survival) against 10^3 LD_{50} active BoNT/B challenge, and two or three proBHc vaccinations provided complete protection against 10^4 or 10^5 LD_{50} BoNT/B challenge (Table 2). The neutralizing antibody titers against BoNT/B were positively correlated with both the frequency of proBHc immunizations and the proBHc-specific ELISA antibody titers. In a control group, multiple BSG vaccinations still failed to provide protective immunity or elicit strong BoNT/B neutralizing antibody titers in mice. Our results indicate that the glycosylated BHc products have low immunogenicity and fail to elicit protective immunity, while deglycosylation improves immunologically activity and confers the capacity to induce protective immunity.

Characterization and immunogenicity of recombinant non-glycosylated rBHc and mBHc products. Based on our results, we hypothesized that the undesirable glycosylation of secreted BHc products could be mitigated in *P. pastoris* by deleting the pro-region of the α-factor signal peptide fused to rBHc or mBHc, or by introducing one silent mutation to remove the potential glycosylation site that is present in the wild BHc amino acid sequence. We successfully expressed and purified these secreted BHc products (50 kDa, rBHc or mBHc, Figure 3A, Table 1). The rBHc products could be detected as two bands by SDS–PAGE (Figure 3B) and western blotting (Figure 3C), but only one band was detected for the mBHc product (Figures 3A and C). The main rBHc band was observed at the same position as mBHc (50 kDa), but the very weak band (low-glycosylated BHc, about 53 kDa, less than 5%) with fewer N-glycans was found at a slightly higher molecular weight position (>50 kDa). The low-glycosylated fraction of rBHc products could be bound by ConA Sepharose 4B to prepare non-glycosylated rBHc (Figure 3B). After treatment with Endo H, the low-glycosylated fraction showed a reduced molecular weight and a mobility similar to the non-glycosylated protein (Figure 3B). The five N-terminal amino acids of rBHc or mBHc were sequenced, and the sequences showed that complete cleavage had occurred (Supplementary Figure S2). The α-factor pre-signal peptide of rBHc or mBHc was precisely cleaved in *P. pastoris*, resulting in a homogeneous recombinant mBHc protein without any glycosylated product.

To assess the immunogenicity and protective capability of the non-glycosylated secreted rBHc or mBHc subunit vaccines, mice were immunized one, two, or three times with different doses of rBHc or mBHc formulated with aluminum hydroxide adjuvant. An antigen dose- and frequency-dependent immune response against BoNT/B was observed in rBHc or mBHc-immunized mice (Table 3). Mice that received one injection of ≥0.2 μg and two injections of ≥0.2 μg were completely protected (100% survival) follow-

Table 1 | A summary of the different forms of recombinant BHc proteins used for analysis and immunizations in this study

Product	Characterization	Description
BSG	A ~150 kDa, hyper-glycosylated BHc product. A His-tag was fused to the C-terminus of the recombinant BSG product.	BSG was expressed in the culture supernatant of pPICZαA-BHc-transformed GS115 through an α-factor pre-pro-peptide and purified by nickel affinity column chromatography to yield the 6×-His-tag fusion protein.
mBSG	A ~150 kDa, hyper-glycosylated mBHc product. A His-tag was fused to the C-terminus of the recombinant mBSG product. The point mutation N957Q of BHc did not change the glycosylation of mBHc.	The mBSG protein was expressed in the culture supernatant of pPICZαA-mBHc-transformed GS115 through an α-factor pre-pro-peptide and purified by nickel affinity column chromatography to yield the 6×-His-tag fusion protein.
BSK	A ~60 kDa mostly low-glycosylated BHc product. A His-tag was fused to the C-terminus of the recombinant BSK product.	The BSK was expressed in the culture supernatant of pPICZαA-BHc-transformed GJK01 through the α-factor pre-pro-peptide and purified by nickel affinity column chromatography to yield the 6×-His-tag fusion protein.
proBHc	A 57 kDa, deglycosylated BHc product containing an unexpected pro-peptide sequence (66 amino acids, 7 kDa). The pro-peptide sequence was determined by sequencing and the molecular weight of proBHc.	The glycosylated BHc product (BSG/mBSG or BSK) was treated with endoglycosidase H (endo H) to generate a similarly deglycosylated proBHc protein. The proBHc includes BHc and the non-cleaved pro-peptide. The glycosylated BHc product was immunologically inactive, while the deglycosylation restored immunological activity.
rBHc	The recombinant BHc product without a signal peptide contained the main non-glycosylated rBHc (50 kDa) and less than 5% of low-glycosylated BHc (~53 kDa). The low-glycosylated site was predicted to be Asn (N) residue 957.	The BHc product was expressed in the culture supernatant of pPICZαA-pre-BHc-transformed GS115 through the α-factor pre-peptide and purified using sequential cation-exchange chromatography and gel filtration chromatography. The low-glycosylated fraction of the BHc product could be bound by ConA Sepharose 4B or deglycosylation to prepare a non-glycosylated rBHc.
mBHc	A 50 kDa recombinant non-glycosylated mBHc (BHcN957Q) protein without a signal peptide. The non-His-tagged and homogeneous mBHc without any glycosylation represents a good potential candidate subunit vaccine for use in humans.	The mBHc protein was expressed in the culture supernatant of pPICZαA-pre-mBHc-transformed GS115 through an α-factor pre-peptide and purified using sequential cation-exchange chromatography and gel filtration chromatography. The mBHc protein was immunogenically active and could confer effective protective immunity against BoNT/B similarly to wild-type rBHc.

ing challenge with 1000 or 10,000 LD_{50} BoNT/B, respectively. The geometric mean titer (GMT) of mice that received a single vaccination was relatively low (2.72–3.61), and was dependent upon the injection dose. Low titers of neutralizing antibodies were measured (≤0.32 IU/mL) and the boost immunizations obviously augmented the group GMT (≥4.89) and induced high levels of neutralizing antibodies (≥1.28 IU/mL). Specially, the sera of mice that received three immunizations had strong neutralizing antibody titers against BoNT/B (≥20.48 IU/mL).

Furthermore, ELISA analysis of the immunized-rBHc sera showed identical reactivity to both rBHc and mBHc. The IgG isotype profiles of the rBHc and mBHc-immunized mice were very similar and showed a predominant IgG1 basis, which indicated a Th2-type humoral immune response. These findings suggest that the non-glycosylated secreted rBHc and mBHc containing one silent point mutation (N957Q) antigens elicited similar immune responses and provided equivalent protection in mice. In summary, a non-His-tagged and non-glycosylated secreted homogeneous mBHc antigen with biological and immunological activity can be prepared using a yeast expression system with low costs and biosafety, including an easier scale-up manufacturing process, which make it a good candidate subunit vaccine that might be suitable for human use.

Discussion

A safe and effective second generation botulism subunit candidate vaccine can be prepared by replacing the formalin-inactivated toxoid with recombinant Hc of BoNTs[4–6]. *E. coli*[10,16–18] and yeast[10,11,14,19,20] are the most widely-used expression systems for these recombinant Hc proteins, which can be produced in high quantities and purified to apparent homogeneity using conventional chromatography. *P. pastoris* allows for the secretion of recombinant proteins, resulting in an easy and efficient scale-up manufacturing process for vaccine development for human use. Proteins expressed in *P. pastoris* can be hyper-glycosylated when directed to secretion, which might affect the immunogenicity of these protein antigens if the native structures do not contain sugars. Previously, the recombinant Hc of TeNT and BoNTs secreted into the culture medium were found to be hyper-glycosylated because of the presence of fortuitous N-linked glycosylation sites in the prokaryotic primary amino acid sequences[12–14].

Here, we found that the hyper-glycosylated BSG/mBSG and low-glycosylated BSK were produced in the culture supernatant of pPICZαA-BHc/mBSG-transformed GS115 or GJK01, respectively. Multiple immunizations with these hyper-glycosylated BSG/mBSG and low-glycosylated BSK antigens also failed to induce neutralizing antibody or protective immunity in mice, as was previously reported for the glycosylated Hc of TeNT and BoNT/B[12,13]. Similar results were also obtained using the hyper- and low-glycosylated Hc of TeNT produced in our laboratory (data not shown). Although three immunizations with other glycosylated Hc antigens could induce protective immunity in mice[13], the protective potencies evoked by this approach were lower than for the same intracellular Hc antigens produced by yeast[14,19] or *E. coli*[17], indicating that this glycosylation still affected the immunological activity of Hc. Based on our study and previous reports[12,13], both low- and hyper-glycosylation renders these antigens low immunogenic and unable to induce protective immunity, which indicates that these glycosylations affect the correct conformation of BHc or other Hc antigens that do not disrupt the neutralizing epitopes.

Conceptually, a non-glycosylated Hc protein can be prepared by a deglycosylation step to remove the carbohydrate moieties of glycosylated products. After the BSG/mBSG or BSK was treated with endo H, a core deglycosylated product, proBHc, with the unexpected size of 57 kDa (>50 kDa of BHc) was obtained (Figure 1C, Table 1). The deglycosylated proBHc used in a subunit vaccine elicits a very strong BoNT/B neutralizing antibody response and provides protective immunity in mice, which indicates that the deglycosylation restores immunological activity and confers the capacity to protect animals, as previously reported[12–14]. However, a costly *in vitro* deglycosylation

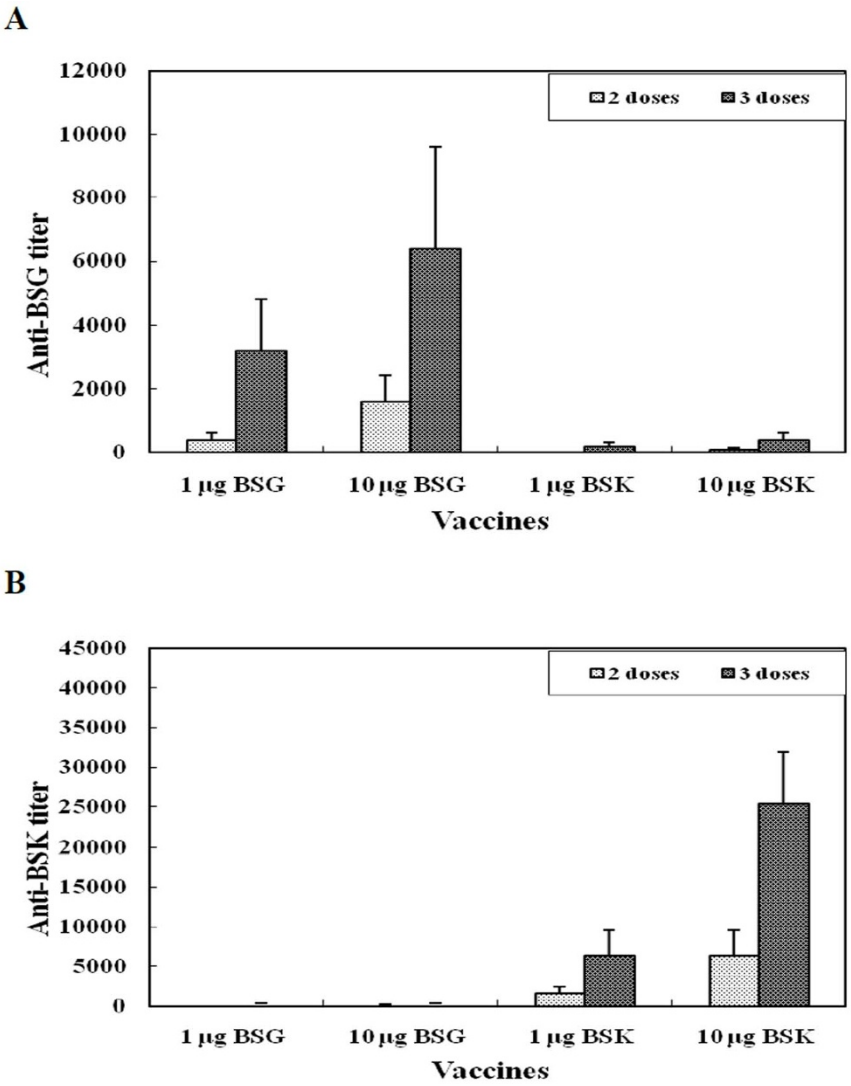

Figure 2 | Anti-BSG or BSK antibody titers in mice vaccinated with two or three doses of recombinant BSG or BSK antigen formulated with aluminum hydroxide adjuvant. Mice were immunized two or three times with 1 or 10 μg of BSG or BSK formulated with aluminium hydroxide adjuvant, respectively. Sera from individual mice from each group (n=8) 3 weeks after the final immunization were collected, and the specific anti-BSG (A) or BSK (B) antibody titers were analyzed by ELISA. Serum samples from individual mice were assayed and the geometric mean titer (GMT) was calculated for each group.

step is not suitable for vaccine development in an industrial scale manufacturing process. Additionally, our N-terminal amino acid sequencing indicated that the proBHc product contained an unexpected N-terminal sequence of a pro-peptide of an α-factor signal and the first of its three potential N-glycosylation sites was glycosylated (Supplementary Figure S1). These results indicate that the pro-peptide of 66 amino acids was not successfully cleaved in the secreted BHc products, which is abnormal[21] and yields unwanted hyper- and

Table 2 | The survival, serum antibody titers, and neutralizing antibody titers of mice following immunization with 5 μg recombinant proBHc or 15 μg BSG formulated with aluminum hydroxide adjuvant

	Number Alive[a]	Number Alive[b]		Log_{10} GMT (SD)[c]			Sera neutralizing titer (IU/mL)[d]		
Vaccine	1[e]	2[e]	3[e]	1[e]	2[e]	3[e]	1[e]	2[e]	3[e]
proBHc	8*	8*	8*	3.74 (0.25) #	5.06 (0.24) $	5.29 (0.31) $	0.16	5.12	81.92
BSG	0	0	0	2.83 (0.25)	3.75 (0.17)	3.86 (0.23)	<0.16	<0.16	0.16
Control	0	0	0	<2	<2	<2	<0.16	<0.16	<0.16

[a]Balb/c mice that survived (8 mice per group) after i.p. challenge with a dose 10^3 LD_{50} BoNT/B 3 weeks after the final injection. Statistical significance was determined by Fisher's exact test. * $p = 0.00016 < 0.001$, compared to BSG or the negative control group.
[b]Balb/c mice that survived (8 mice/group) after i.p. challenge with a dose 10^4 or 10^5 LD_{50} BoNT/B 3 weeks after the final injection. * $p = 0.00016 < 0.001$, compared to BSG or the negative control group.
[c]Serum antibody titers for individual mice after the final immunization were analyzed by ELISA and the geometric mean titer (GMT) for each group (n=8) was determined. Antibody titers to proBHc are shown in the table. Statistical significance was determined by Student's *t*-test. # $p < 0.05$; $ $p < 0.01$, compared to the BSG-immunized group.
[d]Serum neutralizing titers against BoNT/B are shown in the table.
[e]Number of vaccinations. Control mice were injected with PBS and Alhydrogel.

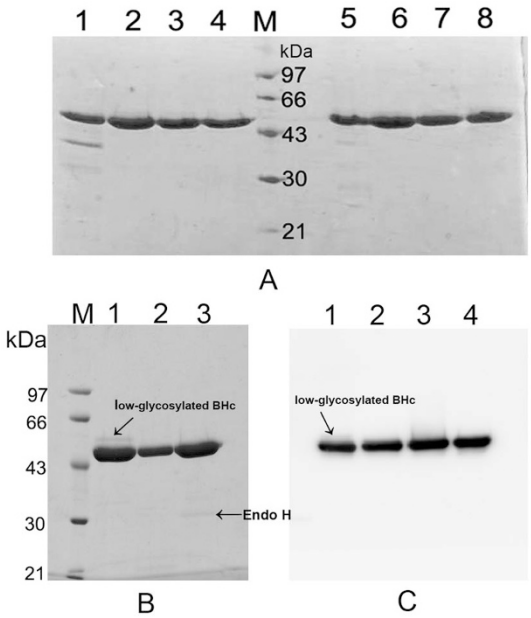

Figure 3 | Analysis of purified recombinant BHc products by SDS–PAGE (A and B) and western blotting (C). (A) SDS–PAGE for each process step for rBHc (lanes 1–4) and mBHc (lanes 5–8) purification. Lanes 1 and 5, capto MMC product; lanes 2 and 6, Source 30s product; lanes 3, 4, 7, and 8, Superde ×200 product. (B) SDS–PAGE of low-glycosylated BHc and non-glycosylated rBHc. Lane 1, rBHc and low-glycosylated BHc (less than 5%) products; lane 2, the rBHc product without low-glycosylated BHc. The rBHc and low-glycosylated BHc products were applied to a ConA Sepharose 4B column (a chromatography medium for the separation and purification of glycoproteins, polysaccharides, and glycolipids) according to the supplier's protocol (GE Healthcare); the low-glycosylated BHc was bound by ConA Sepharose 4B, whereas rBHc flowed through the column; lane 3, the rBHc product without low-glycosylated BHc. The rBHc and low-glycosylated BHc products were treated with Endo H. (C) Western blot of purified rBHc and mBHc products. Lane 1, rBHc and low-glycosylated BHc products; lane 2, rBHc and low-glycosylated BHc products treated with Endo H; lane 3, mBHc; lane 4, mBHc treated with Endo H. M, protein standard.

low-glycosylated BHc products. Based on these findings, we conclude that fortuitous N-linked glycosylation sites exist in the non-cleaved pro-peptide sequences, not in the BHc sequences, which is different from previously reported glycosylated Hc products[12–14]. Furthermore, the glycosylation of the pro-peptide fused to the BHc affects its cleavage and renders the BHc products immunologically inert.

These findings also indicated that the undesirable glycosylation of secreted BHc products could be addressed by deleting the pro-region of the α-factor signal. Although there were no hyper-glycosylated BHc products, it is regretful that the rBHc products contained a low-glycosylated fraction of less than 5% (~53 kDa), in which a single potential glycosylation site might have been present on the Asn (N) 957 residue of BHc[13,22], except for the main non-glycosylated fraction (50 kDa). The low-glycosylated fraction could be bound by ConA Sepharose 4B (Figure 3B) or eliminated by deglycosylation (Figure 3C) to prepare a non-glycosylated homogeneous rBHc. Subsequently, we introduced one point mutation (N957Q) in the BHc amino acid sequences to eliminate the potential glycosylation site (Table 4). We obtained a non-glycosylated secreted homogeneous mBHc (50 kDa) that we could observe at the same position as the main band of the rBHc products. N-terminal amino acid sequencing showed that the sequenced N-terminal amino acids were sequences of rBHc or mBHc. The α-factor pre-peptide was precisely cleaved and successfully directed the secretion of BHc or mBHc products from *P. pastoris*. Therefore, we inferred that the low-glycosylation site occurs at the Asn (N) residue 957 of BHc. The secreted rBHc or mBHc used in a subunit vaccine induced strong immunogenicity and protective immunity in mice, similarly to intracellular BHc antigen from yeast[13,14,20] or *E. coli*[18,23]. Our results also suggest that both rBHc and mBHc antigens can elicit similar immune responses and provide equivalent protection against BoNT/B. In summary, a non-glycosylated secreted homogeneous mBHc with immunological activity was prepared in yeast by mutating a single putative glycosylation site in BHc. Therefore, a substitution of a chemically similar Gln for Asn residue in BHc can entirely avoid the glycosylation of the expressed product in yeast and restore its full bioactivity.

In summary, non-glycosylated secreted BHc products are immunologically active and can confer very effective protective immunity against BoNT/B, whereas both low- and hyper-glycosylated BHc products cannot induce protective immunity. Therefore, this non-His-tagged homogeneous mBHc antigen developed by secretory expression by yeast shows biologically activity, can be produced at a low costs, and is therefore a good candidate subunit vaccine that might be suitable for human use. To the best of our knowledge, this is the first report of a non-glycosylated secreted BHc isoform produced in yeast by deleting the pro-peptide of an α-factor signal and by mutating its single potential glycosylation site. This strategy can

Table 3 | The survival, serum antibody titers, and neutralizing antibody titers of mice following immunization once, twice, or three times with different doses of rBHc or mBHc formulated with aluminum hydroxide adjuvant

	Number Alive[a]	Number Alive[b]		Log_{10} GMT (SD)[c]			Sera neutralizing titer (IU/mL)[d]		
Vaccination dose	1[e]	2[e]	3[e]	1[e]	2[e]	3[e]	1[e]	2[e]	3[e]
0.04 μg rBHc	4	4	8	2.72 (0.16)	4.89 (0.16)	5.25 (0.25)	<0.16	2.56	40.96
0.2 μg rBHc	8	8	8	2.95 (0.25)	4.89 (0.16)	5.30 (0.30)	<0.16	2.56	40.96
1 μg rBHc	8	8	8	3.44 (0.25)	4.95 (0.14)	5.43 (0.27)	0.16	5.12	81.92
5 μg rBHc	8	8	8	3.49 (0.30)	5.03 (0.13)	5.43 (0.27)	0.16	5.12	81.92
0.04 μg mBHc	5	4	8	2.72 (0.16)	4.89 (0.16)	5.25 (0.25)	<0.16	1.28	20.48
0.2 μg mBHc	8	8	8	3.20 (0.22)	4.89 (0.16)	5.37 (0.25)	<0.16	2.56	81.92
1 μg mBHc	8	8	8	3.43 (0.30)	5.01 (0.21)	5.43 (0.27)	0.16	5.12	81.92
5 μg mBHc	8	8	8	3.61 (0.26)	5.08 (0.13)	5.43 (0.27)	0.32	5.12	81.92

[a]Balb/c mice that survived (8 mice per group) after i.p. challenge with a dose of 10^3 LD_{50} BoNT/B 3 weeks after the final injection.
[b]Balb/c mice that survived (8 mice per group) after i.p. challenge with a dose of 10^4 LD_{50} BoNT/B 3 weeks after the final injection.
[c]Serum antibody titers for individual mice after the final immunization were analyzed by ELISA and the geometric mean titer (GMT) for each group (n=8) was determined. Antibody titers to rBHc or mBHc are shown in the table.
[d]Serum neutralizing titers against BoNT/B are shown in the table.
[e]Number of vaccinations. Mice were vaccinated once, twice, or three times at four different doses of BHc formulated with aluminum hydroxide adjuvant, ranging from 0.04 to 5 μg.

Table 4 | A summary of the different forms of recombinant BHc products expressed in the supernatant of *P. pastoris* cultures using different transformed host strains and signal peptides

BHc product	α-factor signal	Recombinant expression plasmid	Host strain (P. pastoris)	cryptic N-glycosylation site	
				pre-pro-peptide	BHc[d]
BSG	pre-pro-peptide[a]	pPICZαA-BHc	GS115	3	1
mBSG	pre-pro-peptide	pPICZαA-mBHc	GS115	3	0
BSK	pre-pro-peptide	pPICZαA-BHc	GJK01[c]	3	1
rBHc	pre-peptide[b]	pPICZαA-pre-BHc	GS115	0	1
mBHc	pre-peptide	pPICZαA-pre-mBHc	GS115	0	0

[a]The pre-pro-peptide sequence is MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKR. The red amino acids (N) indicate cryptic N-glycosylation sites. The pro-region peptide (7 kDa) sequence is the later 66 amino acids of the pre-pro-peptide sequence.
[b]The pre-peptide sequence is MRFPSIFTAVLFAASSALA (19 amino acids). The pro-peptide sequence of 66 amino acids is deleted in the pPICZαA-pre-BHc vector, which can express the rBHc in yeast.
[c]A previously reported *P. pastoris* mutant strain GJK01[15], which is an *OCH1* (α-1, 6-mannosyltransferase gene) knock-out based on the autotrophic strain JC308, was also used as a host strain to express a low-glycosylated secreted heterologous protein.
[d]BHc, the Hc domain of BoNT/B (amino acids 853–1291, ~50 kDa) contains a single potential N-glycosylation site on Asn (N) residue 957. The mBHc protein is a BHc mutant containing one point mutation (N957Q) and involving a substitution of the chemically similar Gln for the Asn residue.

tested for the secretory expression of botulism or other toxin antigens.

Methods

Construction of recombinant plasmids. For the inducible expression of BHc in *P. pastoris* (Table 4), four recombinant plasmids were constructed by cloning the *BHc* or *mBHc* genes into pPICZαA (Invitrogen, Carlsbad, CA, USA) or pPICZαA-pre (a pre-peptide instead of the pre-pro-peptide) at *Xho* I (5′) and *Not* I (3′), respectively. Briefly, the *BHc* gene was amplified by PCR from pGEM-BHc (containing a completely synthetic gene encoding the Hc domain of BoNT/B, amino acids 853–1291, ~50 kDa)[24] and cloned into pPICZαA or pPICZαA-pre to produce recombinant pPICZαA-BHc and pPICZαA-pre-BHc, respectively. To eliminate the potential N-linked glycosylation site of BHc, a *mBHc* gene encoding a BHc mutant, in which N957 of BHc was mutated to Q957, was cloned by mutagenesis. The *mBHc* gene was cloned into pPICZαA or pPICZαA-pre to construct pPICZαA-mBHc and pPICZαA-pre-mBHc, respectively. The pPICZαA is an expression vector, in which the expression of BHc is under the control of the *P. pastoris Alcohol oxidase 1* (*AOX1*) promoter. The α-factor pre-pro-peptide containing a KEX2 cleavage site was used to direct the secretion of BHc. The α-factor pre-peptide could also direct the secretion of BHc[21].

Screening and selection of transformed *P. pastoris* strains. The expression plasmid vectors were linearized by *Sac* I digestion and the cassettes were integrated into the chromosomal *AOX1* locus of *P. pastoris* (GS115 or GJK01; Table 4). Transformants were selected for zeocin (Invitrogen) resistance and the integration of DNA fragments was confirmed by PCR with the primers 5′-GACTGGTTCCAATTGACAAGC-3′ and 5′-GCAAATGGCATTCTGACATCC-3′, which were located in the *AOX1* promoter and *AOX1* transcriptional terminator, respectively. Secretion of BHc in these yeast transformant strains was screened and assessed by a dot blot immunoassay using hyper-immune horse BoNT/B antiserum. High expressing clones were used to seed subsequent cultures.

Fermentation and protein expression. The primary culture was prepared by inoculating a 1-L baffled flask containing 200 mL buffered methanol complex medium (BMMY) with 10 mL seed culture. The cells from the primary culture were transferred to a fermenter. Fermentations were conducted in 5-L (the initial volume was 3 L) glass bioreactors (Sartorius, Goettingen, Germany). The fermentation process was divided into three different stages: a glycerol batch phase, a glycerol fed-batch phase, and a methanol induction phase, as previously described[25]. After fermentation, the supernatant was cleared by centrifugation and used for further purification of the BHc product.

Purification and analysis of recombinant BHc protein. All chromatography steps were performed on an AKTA Explorer (GE Healthcare, Piscataway, NJ, USA). The supernatants containing secreted BHc products were purified to obtain the initial three recombinant BHc products listed in Table 1 by nickel affinity column chromatography (GE Healthcare) for the six His-tag fusion proteins, according to the manufacturer's instructions. A His-tag was fused to the C-terminus of the recombinant BHc product. The purified glycosylated BHc products (BSG/mBSG or BSK) were also treated with an endoglycosidase H (endo H) deglycosylation kit (NEB, Beverly, MA, USA) according to the manufacturer's instructions to generate deglycosylated proBHc (Table 1).

For the last two recombinant BHc products listed in Table 1, supernatants containing secreted non-His-tagged BHc products were purified using sequential cation-exchange chromatography and gel filtration chromatography. Briefly, a capto MMC (Φ: 1.6 × 10 cm, GE Healthcare) column was used for the first purification step. The column was equilibrated with buffer A (20 mM sodium phosphate, pH 6.0). Next, the column was equilibrated with 4 column volumes of 1 M NaCl in buffer A and then loaded with the supernatants. The protein products were eluted with 100 mM sodium phosphate (pH 7.5). A Source 30 s (Φ: 1.6 × 10 cm, GE Healthcare) was used for the second purification step. The column was equilibrated with buffer A and then loaded with previously eluted pooled solutions. The protein products were eluted with 100–200 mM NaCl in buffer A. Finally, the eluate containing BHc was passed through a gel filtration chromatography column (Superde ×200, Φ: 1.6 × 90 cm, GE Healthcare) equilibrated with 20 mM sodium phosphate, 100 mM NaCl, pH 7.0.

Overall, a total of six secreted products were obtained, as shown in Table 1. The purified BHc products were verified by 12% SDS–PAGE or immunoblotting using hyper-immune horse BoNT/B antiserum. The N-terminal amino acid sequences of recombinant BHc products were determined using automated Edman degradation that was performed on a model 470A amino acid sequencer (Applied Biosystems, Foster City, CA, USA).

Vaccination of mice and challenge with BoNT/B. Specific pathogen-free female Balb/c mice 6 weeks (purchased from Beijing Laboratory Animal Center, Beijing, China) were randomly assigned to different treatment groups (8 mice per group). For preparation of subunit protein vaccines, different doses of different forms of recombinant BHc products (Table 1) in PBS were formulated with 0.33% (w/w) alhydrogel (Aluminium hydroxide gel, 1.3%, Sigma–Aldrich, St. Louis, MO, USA). Mice were i.m. vaccinated one, two, or three times with the previously described subunit protein vaccines. Injections were administered at 2-week intervals (100 μL/injection). Mice from all groups were challenged i.p. with different doses of active BoNT/B (strain Okra, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) diluted in 20 mM sodium phosphate buffer (pH 6.5) 3 weeks after the final vaccination. Mice were observed for 1 week after challenge, and survival was determined for each vaccination group. All experimental protocols were approved by Beijing Institute of Biotechnology Institutional Animal Care and Use Committee. All animal procedures were carried out in accordance the approved guidelines.

Antibody titer measurement and BoNT/B neutralization assay. Sera from individual mice of each group collected 3 weeks after the final immunization were screened for antigen-specific antibodies by ELISA, as previously described[17]. Briefly, ELISA plates (Corning Inc., Corning, NY, USA) were coated overnight at 4°C with 100 μL of different forms of recombinant purified BHc products (10 μg/mL) for the different treatment groups. Serum samples were two-fold serially diluted beginning at a 1 : 100 dilution and 100 μL was added to each well for 1 h at 37°C. Total IgG titers were measured using HRP-conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilutions of 1 : 2000. Antibody titers were estimated as the reciprocal of the maximum dilution of serum that yielded an absorbance reading greater than 0.3 units following the subtraction of non-specific binding detected in control sera. Serum samples from individual mice were assayed and the geometric mean titer (GMT) for each group (n=8) was determined.

The neutralization potency of the sera was assayed using a BoNT/B neutralization assay, as described previously[26]. Briefly, mixtures of serial dilutions of pooled sera from eight mice per group along with a standard concentration of BoNT/B were incubated for 30 min at room temperature and the mixtures were injected i.p. into mice (18–22 g) using a volume of 500 μL/mouse (four mice per group). Mice were observed for 1 week, and survival was recorded. The relative concentration of neutralizing antibody in the sera was calculated by comparison to a World Health Organization BoNT/B antitoxin and neutralizing antibody titers in sera were reported as international units per milliliter (IU/mL).

Statistical analysis. Differences in antibody titers were analyzed statistically using Student's *t*-test for between group differences. Fisher's exact test was used to determine statistically significant differences in survival between the treatment

groups. For all tests, only data resulting in p- values < 0.05 were regarded to be statistically significant.

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Author contributions

Y.Z.Y., B.L., Z.W.S. and J.W. conceived and designed the experiments. B.L., D.Y.S., S.H.C., X.G. and Y.Z.Y. performed the experiments. D.Y.S., Y.Z.Y. and B.L. analyzed the data. S.H.C. and X.G. contributed reagents/materials/analysis tools. Y.Z.Y. and B.L. wrote the paper.

Additional information

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