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Secretion of *Bacillus* Endoglucanase in *Saccharomyces cerevisiae* by Its Own Signal Sequence

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To examine whether the signal sequence of *Bacillus* endo-1,4-glucanase can act functionally in a yeast, a lower eucaryote, two recombinant plasmids were constructed and introduced into *Saccharomyces cerevisiae*: recombinant plasmid pGCMC10 containing the complete signal sequence of *Bacillus* endoglucanase, and pGCMC11 without the signal sequence. Secretion of endoglucanase into culture medium was obtained with the yeast transformant containing plasmid pGCMC10. The secreted endoglucanase was glycosylated and was apparently processed to be about 36 kilodaltons (KDa) and 43KDa proteins. The glycosylated endoglucanase from yeast transformant was more thermostable than the nonglycosylated endoglucanase from *Escherichia coli* transformant.

The ability of *Saccharomyces cerevisiae* to secrete proteins into extracellular region makes it an attractive host for the production of useful proteins. Glycosylation of the secreted proteins is another potential advantage of yeast over bacteria (13, 20). The proteins sercreted in eucaryotes usually have a *N*-terminal signal sequence which is around 20 amino acids long and composed of a positively charged *N*-terminal region, a long hydrophobic core region and a negatively charged *C*-terminal region (8). On the other hand, for many of the proteins secreted by *Bacillus*, the signal sequences are usually long (about 30 amino acids) compared to those of eucaryotic proteins (12), and the secreted proteins are not glycosylated. In many cases, the natural signal sequences of the heterologous proteins of animals, plants and fungi have been used in attempts to achieve secretion in *S. cerevisiae*: human α-interferon (9), *Bacillus* α-amylase (21), human serum albumin (16) wheat α-amylase (19), and *Aspergillus* glucoamylase (10). Therefore, it should be interesting to study the effect of procaryotic signal sequence on the secretion and biological activity of heterologous protein in yeast. Ruohonen *et al.* reported the successful secretion of *Bacillus* α-amylase in *S. cerevisiae* directed by its own signal sequence (20).

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In this paper, we describe the secretion of *Bacillus* endo-1,4-β-glucanase in *S. cerevisiae* by its own signal sequence, and the characterization of secreted enzyme, especially glycosylation and thermostability.

MATERIALS AND METHODS

Strains and Plasmids

The strains and plasmids used in this work are summarized in Table 1.

Media and Culture Conditions

Saccharomyces cerevisiae M1-2b was grown at 30°C in YEPD (1% yeast extract, 2% peptone, and 2% dextrose) medium. Yeast strains transformed with plasmids were grown at 30°C in a synthetic complete minimal medium containg 0.67% yeast nitrogen base and 2% dextrose supplemented with appropriate amino acids lacking uracil (SC-ura) (23). *Escherichia coli* MC1061 was grown at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) which was supplemented with 100 μg/ml of ampicillin when appropriate.

Plasmid Constructions and DNA Preparations

Standard recombinant DNA methods were used, and the plasmid DNA from *E. coli* was isolated by the alkaline extraction procedure described by Sambrook (22).

Transformations

S. cerevisiae strains were transformed by using the

Table 1. List of strains and plasmids used in this study

Strains or plasmids	Genotype or Characteristics	Source
Strain		
S. cerevisiae M1-2b	MAT α *ura3 trp gal2 sta*^{o}	Stinchcomb (25)
E. coli MC 1061	F^{-} *araD*139Δ(*ara-leu*)7696 Δ(*lac*)×74 *galU galK hsdR*2 (r_K-$m_K^{\times}$) *mcrB*1 *rpsL* (str^{r})	Wertman (26)
Plasmid		
pUBS101	*E. coli* vector of *Bacillus* endo-1,4-glucanase	S.H. Park (17)
pHY 101	Yeast *GAP* promoter	Y.I. Hwang
pBC 11	Structural gene of endo-1,4-glucanase	This work
pAA 7	Yeast *GAL* 7 gene	J.H. Kho
pGCMC 10	*Bacillus* endo-1,4-glucanase gene containing complete signal sequence	This work
pGCMC 11	*Bacillus* endo-1,4-glucanase gene without signal sequence	This work

lithium acetate method (13). Yeast transformants were selected by their growth on a synthetic complete minimal medium without uracil (SC-ura). Yeast colonies producing endo-1,4-β-glucanase were detected on a SC-ura medium containing 2% carboxymethyl cellulose (CMC) by the Congo-red staining method (3). *E. coli* transformation was performed as described by Chung *et al.*(2).

Cell Fractionation

Cultures of the yeast transformant (pGCMC 10) grown to 1×10^7 to 2×10^7 cells per ml in SC-ura media were centrifuged at 1,300xg for 10 minutes to separate the cells from the media containing secreted proteins (extracellular fraction). The cells were converted to spheroplasts by incubation at 37°C for 20 minutes in 100 mM sodium citrate (pH 8.0), 1 M sorbitol, 10 mM EDTA, 4 mg/ml of Zymolyase 200T. Spheroplasts were separated from the periplasmic fluids, resuspended in 50 mM Tris-HCl (pH 7.0), and vortexed for 2 to 5 minutes. The lysed spheroplasts were centrifuged at 3,000 xg for 10 minutes, and then the supernatants were transferred to a fresh tube (cytoplasmic intracellular fraction). All fractions were then dialysed against the buffer containing 50 mM Tris-HCl (pH 7.0), 5 mM EDTA, and 1 mM phenylmethyl sulfonyl fluoride (3).

Enzyme Assay

The sample containing endo-1,4-β-glucanase was mixed with 1 ml of 2% carboxymethyl cellulose in 200 mM acetate buffer (pH 5.0) and incubated at 50°C for 30 minutes. The released reducing sugar was determined by the method of dinitrosalicylic acid (DNS) (15). One unit was defined as the amount of enzyme liberating 1 μmole of glucose equivalent from substrate per minute.

Protein Analysis

Protein concentrations were determined by the Bradford method (1). The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was carried out by using the discontinuous buffer system of Laemmlis (6). To display the endo-1,4-β-glucanase secreted and to confirm its processing pattern, the proteins were immunoblotted as described by Sambrook (Protoblot Immunoassay system: Promega Corp. Medison, WA) (22). The primary antibody was a rabbit anti-endo-1,4-β-glucanase, which was raised in rabbit against the endo-1,4-β-glucanase of *Bacillus subtilis*.

Enzymes and Chemicals

Congo-red and carboxymethyl cellulose were purchased from Sigma (Sigma Chemical Company, St. Louis, MO). Restriction endonucleases, T4 DNA ligase, DNA polymerase (the Klenow fragment) and calf intestinal alkaline phosphatase were purchased from NEB(New England Biolabs Inc. Beverly, MA), KOSCO (Sungnam, Kyunggi, Korea) and IBI (International Biotechnologies Inc. New haven, CT). *Nco*I linker and *BamH*I linker were purchased from NEB.

RESULTS AND DISCUSSION

Construction of Plasmids and Secretion of Endoglucanase

For the expression and the secretion of *Bacillus* endo-1,4-β-glucanase in yeast, plasmids pGCMC10 and pGCMC11 were constructed as described in Figure 1 and Figure 2. Plasmid pGCMC10 has the structural gene of *Bacillus* endo-1,4-β-glucanase containing the signal sequence from plasmid pBC11 (1.4 Kb *Sal*I-*Eco*RI fragment). But, plasmid pGCMC11 has the structural gene of *Bacillus* endo-1,4-β-glucanase lacking in the signal sequence. The *Nco*I linker was attached to the 5′-terminus of endo-1,4-β-glucanase structural gene to make the initiation codon. Therefore, plasmid pGCMC11 generated two artificial amino acid residues, Met-Gly, at the *N*-terminal of the polypeptides (Fig. 3). As yeast-*E. coli* shuttle vectors, the plasmid pGCMC10 and pGCMC 11 were composed of: a yeast selectable marker, *URA*3 gene; the replication origin of yeast 2 μm plasmid; and a part of pBR 322 containing ampicillin resistance gene (β-lactamase) and the *E. coli* origin of replication. If the

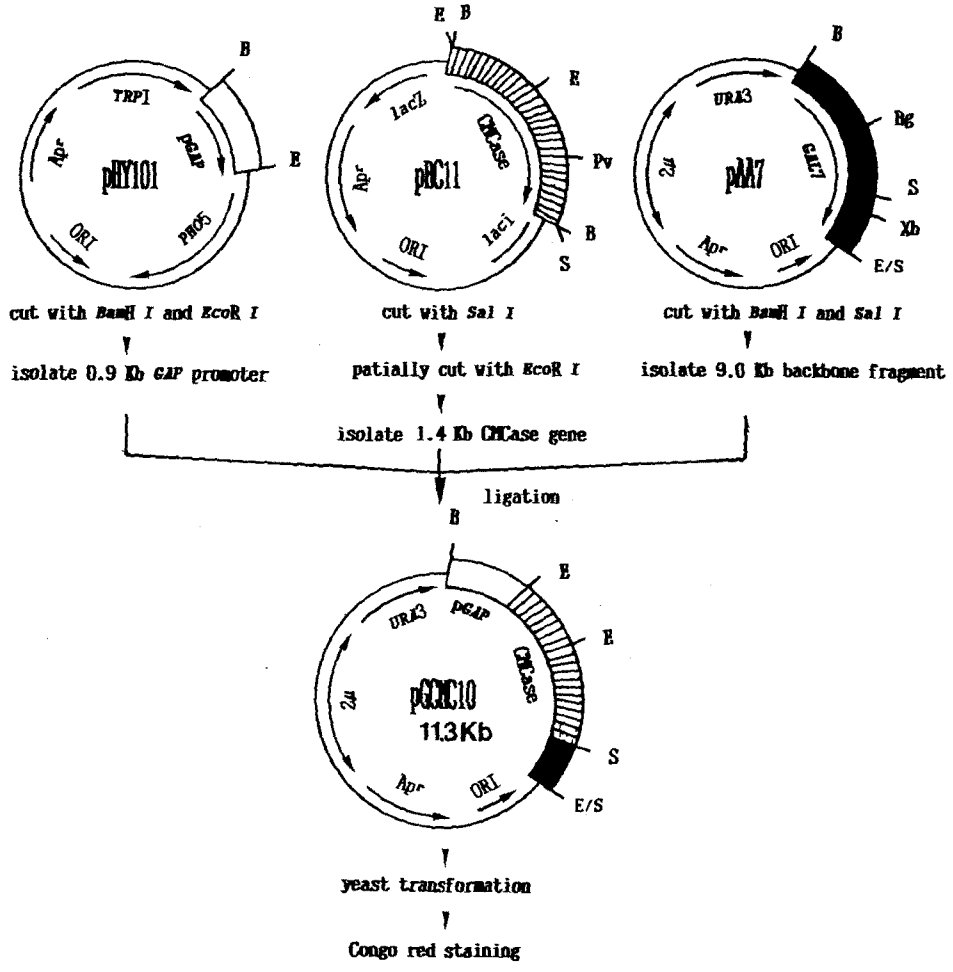

Fig. 1. Overall scheme for the construction of *Bacillus* endo-1,4-β-glucanase secretion vector pGCMC10.
Open box (pGAP): *GAP* promoter, closed box (tGAL7): *GAL* transcriptional terminator, hatched box:endo-1,4-β-glucanase (CMCase) with signal sequence, B: *Bam*HI, Bg: *Bgl*II, E: *Eco*RI, H: *Hind*III, Pst: *Pst*I Pv: *Pvu*II, S: *Sal*I, Sau: *Sau*3AI, Xb: *Xba*I.

translation initiation should occurs at the first ATG codon of each construction (Fig. 3), plasmid pGCMC10 will produce endo-1,4-β-glucanase with a complete signal peptide of 29 amino acid residues, and plasmid pGCMC11 will produce endo-1,4-β-glucanase lacking in the signal peptide and the N-terminal 11 amino acid residues of mature enzyme.

The recombinant plasmids pGCMC10 and pGCMC11 were introduced into *S. cerevisiae* M1-2b. After the yeast transformants had been grown on plates containing 2% CMC, and the plates were stained with 0.1% Congo-red. Yellowish haloes indicating the degradation of CMC by endo-1,4-β-glucanase were seen only around the colonies harboring recombinant plasmid pGCMC10. This suggested that *Bacillus* endo-1,4-β-glucanase had been secreted into the culture medium by its own signal sequence in yeast. Similar result was reported for *Bacillus amyloliquefaciens* α-amylase in yeast (20).

Enzyme Localization in Yeast Transformants

The efficiency of endo-1,4-β-glucanase secretion in transformants containing either plasmid pGCMC10 or pGCMC11 was studied by measuring the enzymatic activity in culture medium and periplasm (extracellular), and the spheroplast lysates (cytoplasmic). In parallel, as a negative control, transformants of plasmid YEp24 were fractionated and assayed by the same method. Secretion of enzyme in the transformant with plasmid pGCMC10 was very efficient, since the activity of secreted endo-1,4-β-glucanase during 2 days represented about 70% of the total activity. However, in the transformants with pGCMC11 containing the endo-1,4-β-glucanase gene without signal sequence, about 90% of the enzymatic activity was detected in the cytoplasmic fraction (Table 2).

Presumably, the *Bacillus* signal sequence can act functionally in yeast. The translation of protein is probably

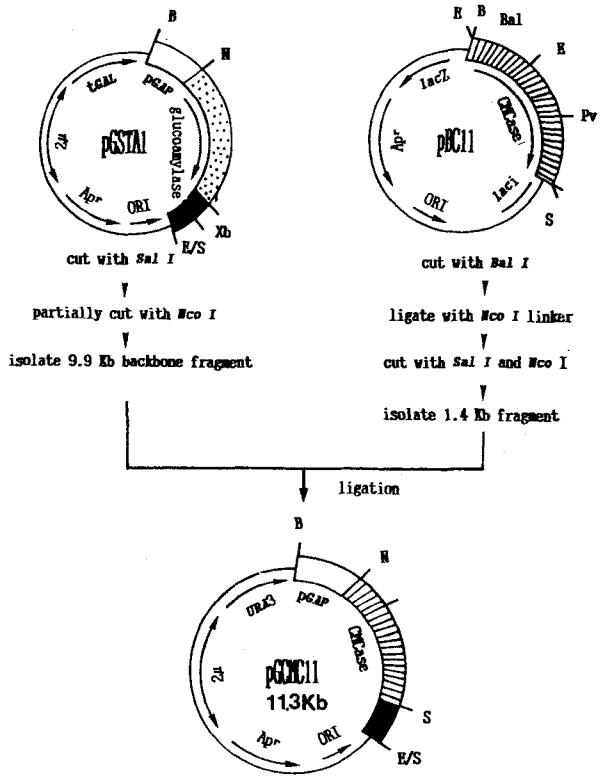

Fig. 2. Overall scheme for the construction of *Bacillus* endo-1,4-β-glucanase expression vector pGCMC11.
Open box (pGAP): *GAP* promoter, closed box (tGAL7): *GAL7* transcriptional terminator, hatched box: endo-1,4-β-glucanase (CMCase) without signal sequence, B: *Bam*HI, Bal: *Bal*I, E: *Eco*RI, H: *Hind*III, N: *Nco*I, Py: *Pvu*II, S: *Sal*I, Sma: *Sma*I, Xb: *Xba*I.

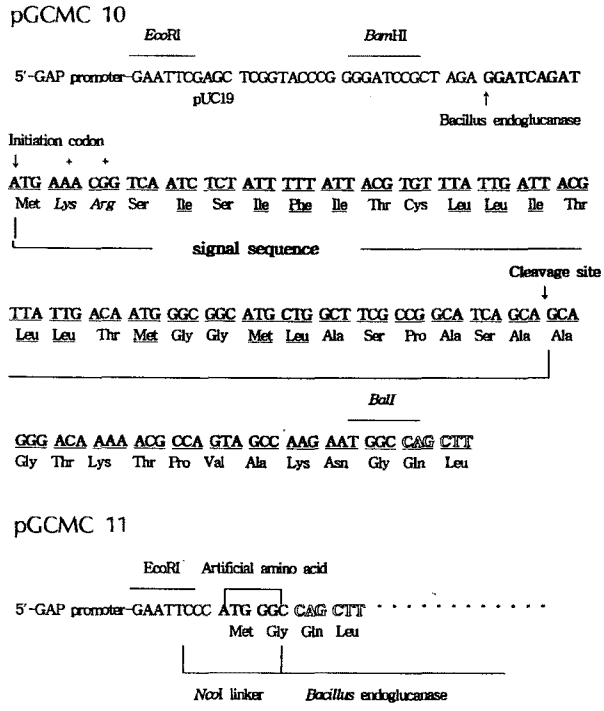

Fig. 3. Nucleotide sequences around junction sites between GAP promoter and endoglucanase.
Hydrophobic amino acids are underlined and positively charged amino acids are shown in italics. pGCMC10 aand pGCMC11 are yeast episomal plasmids containing *Bacillus* endoglucanase with complete or missing signal sequence.

coupled to the translocation, into the endoplasmic reticulum membrane, and then the protein passes through the correct secretory pathway. The lower level of total activity in *S. cerevisiae M*1-2b (pGCMC11) was believed to be due to the difference between the translation initiation region of the plasmid pGCMC10 and that of the plasmid pGCMC11 (21).

Characterization of the Secreted Endo-1,4-β-glucanase in Yeast

The endoglucanase gene of *Bacillus subtilis* encodes a protein of 499 amino acid residues (Mr=54,890) and possesses a typical *Bacillus* signal sequence. The native *Bacillus subtilis* endoglucanase was secreted into the medium and was then processed to give an enzymatically active form of about 35.8 KDa in *B. subtilis* and *E. coli* transformant. About 163 amino acids from C-terminus of immature endoglucanase were removed from the mature enzyme(6, 7, 11, 13, 14).

By using the anti-endoglucanase antibody in a Western blot analysis of the culture supernatant of *S. cerevisiae* transformant (pGCMC 10), the 35.8 KDa and 43.0 KDa proteins were detected (Fig. 4). Because *Bacillus*

Table 2. Endo-1,4-β-glucanase activity of cell fraction of transformants

Strain	Endoglucanase activity (Unit/ml)[a]			Total Activity (Unit/ml)
	Culture fluid	Peri-plasm	Cyto-plasm	
S. cerevisiae M1-2b[b]				
pGCMC 10	1.58	0.67	1.054	3.304
pGCMC 11	0.053	0.154	1.62	1.827
E. coli MC 1061[c]				
pUBS 101	0.28	1.65	1.33	3.31

[a] One unit was defined as the amount of enzyme liberating 1 μmole of glucose equivalent from substrate per minute.
[b] Yeast transformant was cultured in YPD broth at 30°C for 48 hours.
[c] *E. coli* transformant was cultured in LB broth containing ampicillin (100 μg/ml) at 37°C for 24 hours.

endoglucanase contains five putative *N*-glycosylation sites (Asn-X-Ser/Thr), we used tunicamycin to prevent *N*-glycosylation. When the yeast transformant was cultured in the medium containing tunicamycin, the 43 KDa band was reduced to about 35 KDa in size (Fig. 5). These results showed that *Bacillus* endoglucanase secreted by yeast had been glycosylated.

To compare enzymatic properties of the endogluca-

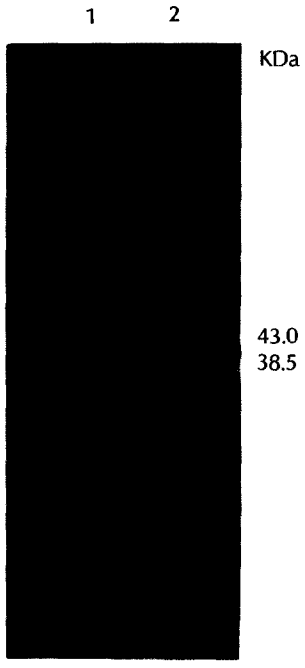

Fig. 4. Western blot analysis for the endoglucanase secreted in yeast.
Samples (lane 1 : 0.01 units, lane 2 : 0.02 units) were electrophoresed (15% SDS PAGE), blotted, and analysed for endoglucanase.

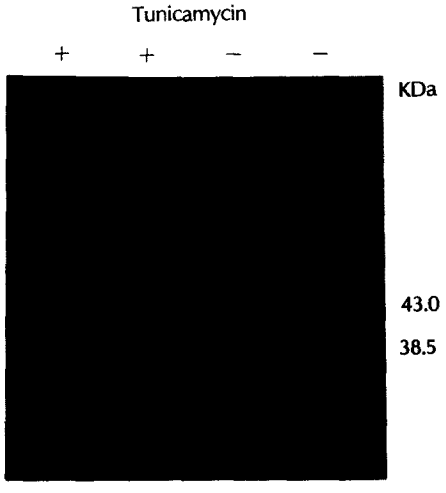

Fig. 5. Analysis of secretion and processing of endoglucanase in *S. cerevisiae*.
Yeast transformants (pGCMC10) were cultured at 30°C in YPD medium containing tunicamycin (10 μg/ml) or not. The growth was continued for 1 day. The culture broth was concentrated with PEG 4000 and separated by 15% SDS PAGE and immunoblotted.

nases produced by *S. cerevisiae* and *E. coli* transformants, the endoglucanases were partially purified from the culture supernatants of each strain. The two enzymes showed no difference in pH optimum (data not shown). In Fig. 6, the relationship between temperature and enzymatic activity is shown. Under the assay conditions described, the optimal temperature for enzyme activity was 50°C for both enzymes, but the enzyme of *E. coli* had a sharper optimum temperature profile. From this result, we could presume that the enzyme secreted from yeast is more thermostable than that of *E. coli* because of glycosylation. Enzyme thermostability was examined by measuring the loss of enzymatic activity as a function of incubation time at 60°C (Fig. 7). The

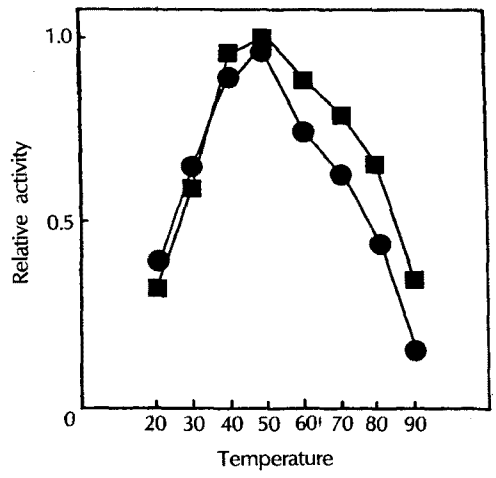

Fig. 6. Determination of temperature optimum.
Endoglucanase was incubated at the indicated temperature in 100 mM acetate buffer (pH 5.0), 1% CMC for 30 minutes and then endoglucanase activity was determined by DNS method. ■: yeast transformant (pGCMC10), ●: *E. coli* transformant (pUBS101).

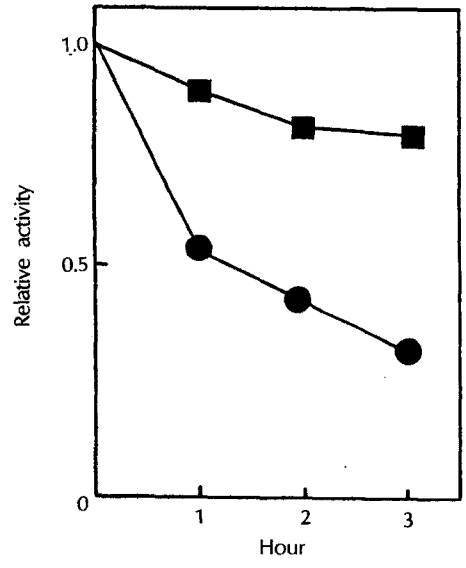

Fig. 7. Determination of thermal stability.
Endoglucanase (1 unit/ml) was incubated at 60°C in 100 mM phosphate buffer (pH 5.0) and then enzymatic activity was determined at the indicated times. ■: yeast transformant (pGCMC10), ●: *E. coli* transformant (pUBS101).

endoglucanase secreted from *S. cerevisiae* retained 87% of its initial enzyme activity after 3 hours of incubation at 60°C, whereas that from *E. coli* retained only 44%. This increase in thermal stability was thought to be due to glycosylation. It is well known that the glycan moieties somewhat enhance of protein stability, especially thermal stability(4, 16).

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