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The large subunit determines catalytic specificity of barley sucrose:fructan 6-fructosyltransferase and fescue sucrose:sucrose 1-fructosyltransferase

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Abstract Plant fructosyltransferases are highly homologous in primary sequence and typically consist of two subunits but catalyze widely different reactions. Using functional expression in the yeast *Pichia pastoris*, we show that the substrate specificity of festuca sucrose:sucrose 1-D-fructosyltransferase (1-SST) and barley sucrose:fructan 6-D-fructosyltransferase (6-SFT) is entirely determined by the large subunit. Chimeric enzymes with the large subunit of festuca 1-SST (LSuB) and the small subunit of barley 6-SFT have the same catalytic specificity as the native festuca 1-SST and vice versa. If the LSuB is expressed alone, it does not yield a functionally active enzyme, indicating that the small subunit is nevertheless essential.

Keywords: Cereal; Enzyme specificity; Fructosyltransferase; Sucrose:sucrose 1-β-D-fructosyltransferase; Sucrose:fructan 6-β-D-fructosyltransferase; *Pichia pastoris*

1. Introduction

Fructans are an important class of carbohydrates in plants [1–3]. The enzymes characteristic of plant fructan metabolism, fructosyltransferases and fructan hydrolases, have been found to be highly homologous to the plants' soluble acid β-fructosidases (invertases) belonging to glycoside hydrolase family 32 [2,4]. Indeed, the first plant fructosyltransferase to be cloned, the sucrose:fructan 6-β-D-fructosyltransferase (6-SFT) from barley (*Hordeum vulgare*), displayed both sucrose:sucrose 6-β-D-fructosyltransferase (6-SST)/6-SFT and β-fructosidase activity [5]. In contrast, the first 1-SST of grasses to be cloned, the one of tall fescue (*Festuca arundinacea*), produced almost exclusively 1-kestose and glucose when supplied with sucrose (suc) and had very little β-fructosidase activity [6]. In view of their striking homologies, fructosyltransferases may have evolved from β-fructosidases by relatively few mutational changes [4,5,7]. However, it is unknown which changes are essential for changes of catalytic specificity.

Typically, plant acid β-fructosidases and fructosyltransferases are synthesized as a primary translation product of ~85 kDa but then they are often cleaved into a large N-terminal subunit of ~60 kDa and a small C-terminal subunit of ~25 kDa [5,8–11]. The large subunit contains putative catalytic domains for suc binding and hydrolysis, namely the β-fructosidase motif, the RDP motif and the EC-domain [3,10]. The importance of these motifs was experimentally proven for the β-fructosidase motif and the EC-domain in the case of yeast invertase [12] and for the RDP motif in the case of a bacterial fructosyltransferase [13].

We have previously established a convenient heterologous expression system in *Pichia pastoris* to study fructosyltransferases of plants [6,14]. In the present work, we describe how this system can be optimized, and we use it to investigate the catalytic activity of chimeric enzymes generated by exchanging the large and small subunit of 6-SFT and 1-SST, respectively. Our results show that it is the large subunit of the enzyme which determines its catalytic properties.

2. Materials and methods

2.1. Microbial strains and vectors used for cloning and heterologous expression

Escherichia coli strain DH5α was used for amplification of the recombinant plasmids pK18, pBluescript KS$^+$ (Stratagene, Amsterdam, The Netherlands), pPICZαC. *P. pastoris* strain X-33 (wild-type) and the pPICZαC shuttle vector were obtained from Invitrogen BY (Leek, The Netherlands).

2.2. Cloning and mutagenesis

Constructs P1 and F1 in pPICZαC, representing the native coding sequences of barley 6-SFT (EMBL X83233) and festuca 1-SST (EMBL AJ297369), respectively (Fig. 1A and B), were described earlier [6,14]. To obtain myc- and 6× his-tagged versions of the two enzymes, their coding regions were excised with *Eco*RI and *Xba*I and subcloned into pK18. The stop codons were then altered to *Xba*I restriction sites by PCR, using primers P3f and P3r for 6-SFT and SST001 and SST002 for 1-SST (Table 1). The resulting PCR products were purified and digested with *Age*I and *Xba*I for 6-SFT and with *Nru*I and *Xba*I for 1-SST, respectively, ligated into the correspondingly digested parent plasmid, and excised from the plasmid by *Eco*RI and *Xba*I. These fragments were cloned in frame with the myc-epitope and his-tag into the *Pichia* shuttle vector, leading to pPICZαC-P3 and pPICZαC-F2 (Fig. 1C and D). Note that we used the tags here to simply verify that the recombinant proteins are expressed, but we would like to use them, in the future, to purify high levels of recombinant proteins to produce antibodies against them; there are still no specific antibodies against invertases or plant fructosyltransferases available.

Mutant P3[DTN], in which the first three amino acids (EAD) of the small subunit of barley 6-SFT are changed into the motif DTN typical of fructan:fructan fructosyltransferases (FFTs), was generated by site-directed mutagenesis using standard procedures (QuikChange™

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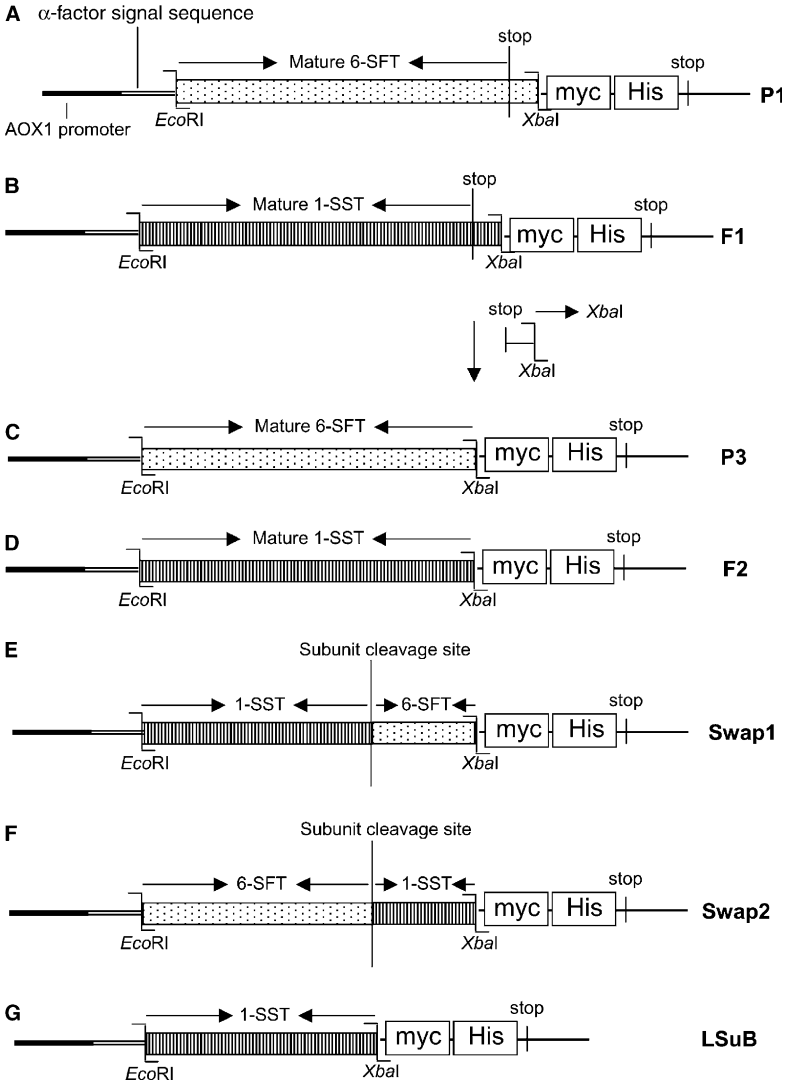

Fig. 1. Constructs introduced into plasmid pPICZαC for expression in *P. pastoris*. (A,B) Original constructs with the natural stop codon. (C,D) Constructs where the stop codon was changed to an *Xba*I site, yielding recombinant proteins with C-terminal tags. (E) Swapl: LSuB with the small subunit of barley 6-SFT fused to it. (F) Swap2: large subunit of barley 6-SFT with the small subunit of festuca 1-SST fused to it. (G) Large subunit of festuca 1-SST expressed alone.

Table 1
Oligonucleotides used for cloning

Primer	Sequence
P3f	5′-GTTCAACAACGCCACCGGTGCCAGC-3′
P3r	5′-CTTATTAATGACGAGTCTAGAGAACTTGATTGAAGATAC-3′
SST001	5′-CATTGTGCAGAGCTTCGCGATGGGTGGGAGGATTAC-3′
SST002	5′-CTCGACTTGGTTTCATCTCTAGAGCGTCGTTCGTGAAGATATG-3′
ADDA008	5′-GTGGCTGCCCTCAACGACACCAACGTTGGCTACAACTGCAG-3′
ADDA009	5′-CTGCAGTTGTAGCCAACGTTGGTGTCGTTGAGGGCAGCCAC-3′
SST004	5′-CCGACGTGGATGCTCTAGACGAGGCCGATGTCAGC-3′
SST005	5′-GCTGACATCGGCCTCGTCTAGAGCATCCACGTCGG-3′
SFT001	5′-TCCATTCAGTCAGTTCCTAGGACGGTGGCTCTG-3′
P4001	5′-GCTGACATCGGCCTCGTTGAGGGCAGCCACGGCGGAAGCATC-3′
P4002	5′-CGCCGTGGCTGCCCTCAACGAGGCCGATGTCAGCTACAAC-3′
SST006	5′-GAGTTTTTGTTCTAGAGCGTCG-3′

Site-Directed Mutagenesis Kit, Strategene), based on the pK18-clone containing the tagged version of barley 6-SFT. Primers ADDA008 and ADDA009 (Table 1) were designed to introduce the desired mutation and an additional restriction site (*Acl*I).

2.3. Construction of recombinant-tagged enzymes with exchanged large and small subunits

To obtain Swap1 (Fig. 1E), a construct with the large subunit of 1-SST at the N-terminus followed by the small subunit of 6-SFT, we made use

of a *Pst*I restriction site present close to the beginning of the small subunit in both festuca 1-SST and barley 6-SFT. In 1-SST there is another *Pst*I site; therefore the 1-SST-containing plasmid pPlCZαC-F1 was digested using *Kpn*I and *Xba*I, and the resulting SST-fragment was ligated into pBluescript leading to pBluescriptl. This plasmid was digested with *Pst*I and *Xba*I to cut off the small subunit of the festuca 1-SST. Digesting of pK18-P3 with *Pst*I and *Xba*I yielded the DNA-sequence corresponding to the small subunit of barley 6-SFT. This was ligated into pBluescriptl containing the *Kpn*I–*Pst*I-fragment of festuca 1-SST, leading to the plasmid pBlueSwap1. To include the entire mature sequence of the large subunit of festuca 1-SST (LSuB) in the swap-construct, the plasmids pBlueSwap1 and pPlCZαC-F1 were digested with *Kpn*I and *Xba*I, and the fragments ligated. This resulted in plasmid pPICZαC-Swap1.

For Swap2 (Fig. 1F), the large subunit of barley 6-SFT was coupled to the small subunit of festuca 1-SST by overlapping PCR [15], using the primer pairs SFT001/P4001 and SST006/P4002, respectively, to amplify the coding regions of the C-terminus of the large subunit of barley 6-SFT (from a conveniently located *Xma*JI site to the subunit cleavage site) and the small subunit of festuca 1-SST. The PCR-amplified fragments were mixed in equimolar amounts, denatured, and reannealed for a second PCR using SFT001 and SST006 as primers. The full-length product created in this way was digested with *Xma*JI and *Xba*I and ligated into the *Xma*JI/*Xba*I-digested pPICZaC-P3, resulting in pPICZαC-Swap2.

2.4. Cloning procedure to obtain the LSuB expressed alone

A *Xba*I site was introduced in the plasmid pK18-F2 5bp upstream of the beginning of the small subunit by site-directed mutagenesis (QuikChange™ Site-Directed Mutagenesis Kit, Stratagene), using primers SST004 and SST005 (Table 1). This allowed construction of a version of the large subunit (Fig. 1G).

All inserts in pPICZαC were sequenced after cloning and found to correspond exactly to the desired constructs.

2.5. Expression of fructosyltransferases in P. pastoris

The sequences were all cloned in frame behind the α-factor signal sequence of the expression vector pPICZαC, to allow entry into the secretory pathway. Competent *P. pastoris* cells were transformed according to the EasyComp transformation protocol (EasySelect™ *Pichia* Expression Kit, Invitrogen BV).

Expression in *P. pastoris* was performed as described [14], with minor modifications. The *P. pastoris* strain X-33 was transformed with 4 μg of *Pme*I-linearized constructs and plated on selective YPDS/Zeocin plates. To screen for activity, some of the newly grown colonies were inoculated in liquid culture. The best-growing colony was selected and used for further experiments. This strain was then grown in liquid culture, and the transgene was induced with 1% methanol, added at 15, 24, 36 and 42 h of induction. Generally, the cultures were used for experiments after 48 h of induction. The culture medium was harvested by centrifugation, concentrated 50-fold by dialysis against solid PEG 35000, and then desalted using desalting columns equilibrated with 50 mM MES (NaOH) buffer (pH 5.75).

2.6. Characterization of recombinant fructosyltransferases

Enzyme assays were performed for 1–5 h at 27 °C, with 100 mM suc or with 50 mM of all other substrates and the products formed were analyzed by anion exchange chromatography as described [16]. Catalytic specificity was tested with the following substrates: suc, 1-kestose, nystose, and a combination of suc with 1-kestose. A maximum of 7% of the substrates was used up after 5 h of incubation. In order to keep the substrate concentration constant during the experiments (maximal consumption of substrate = 7%), the amount of protein to be used per enzyme assay was ca 0.5 μg in the case of *Festuca* 1-SST and 5–10 μg for all other constructs.

3. Results and discussion

3.1. Expression of recombinant plant fructosyltransferases in P. pastoris

Previously, we introduced *P. pastoris* as a heterologous expression system for plant fructosyltransferases such as barley 6-SFT [14] and festuca 1-SST [6]. The fact that *Pichia* does not produce any invertases or fructosyltransferases makes it especially suitable for this purpose, since there are no background activities that might interfere with our activities of interest. In this early work, we used pPICZαC without making use of the potential of this plasmid to express a myc-epitope and a 6× histidine tag at the C-terminus of the recombinant protein. We now constructed barley 6-SFT and festuca 1-SST containing these tags (Fig. 1C and D) and compared their expression levels and enzymatic specificities with the parent untagged versions (Fig. 1A and B). In both cases, the tagged versions were expressed to a similar degree as the corresponding untagged parent enzymes, and the tagged versions had the same catalytic specificity as their native parent enzymes (data not shown). Western blots with antibodies against the tags revealed a major band of approximately 85 kDa (data not shown), indicating that the recombinant enzymes are well expressed but not cleaved into two subunits, as demonstrated previously for recombinant untagged 1-SST in *P. pastoris* [6].

Since the yield of recombinant proteins in our original protocol [6,14] was relatively low, we attempted to improve it. Previously, we had used 0.5% (v/v) methanol, applied every 24 h, to induce the transgenes (which are under control of the AOX1 promoter, see Fig. 1). With both 1-SST and 6-SFT, we achieved an approximately 50 times higher yield of enzyme activity when we added methanol at 1% (v/v) final concentration at 0, 15, 24, 36, and 42 h of induction (data not shown).

3.2. Study of the possible role of a conserved motif at the start of the small subunit

The putative catalytic domains (β-fructosidase motif, RDP motif, and EC-domain) of fructosyltransferases are located on the large subunit. In order to search for domains possibly important for enzyme specificity on the small subunit, we compared invertases and fructosyltransferases, and found the first three amino acids at the (putative) N-terminus of the small subunit to be conserved. In fact, for barley 6-SFT, the N-terminus of the small subunit was experimentally determined [5] and shown to start with the EAD motif. Remarkably, most of the related plant β-fructosidases, 1-SSTs, 6-SFTs, and also ^{6}G-FFTs, have the triplet EAD, while none of the currently known FFTs have this motif. The FFTs of *Cynara scolymus* (AJ000481) and *Cichorium intybus* (U84398) show the DTN motif at the corresponding position in sequence alignments. To investigate the importance of these differences on enzymatic specificity, we replaced the EAD motif in our construct P3 by DTN, using site-directed mutagenesis.

The enzymatic activities of the tagged recombinant barley 6-SFT (P3, with the native EAD sequence) and its derivative, the mutated 6-SFT (P3[DTN]), were compared using either suc alone or a mixture of suc and 1-kestose as substrate. When suc was offered as the sole substrate, fructose, 1-kestose and 6-kestose-levels linearly increased with incubation time in exactly the same way in both enzyme preparations (Fig. 2A and B). Similarly, with suc and 1-kestose as substrates, the products fructose, 6-kestose, and bifurcose were formed by both enzymes in an almost identical manner (Fig. 2C and D). Thus, the mutational change of the EAD motif to DTN does not affect enzymatic activity of recombinant 6-SFT.

3.3. Chimeric enzymes with exchanged large and small subunits

To learn more about the function of the two subunits of plant fructosyltransferases, we studied the enzymatic proper-

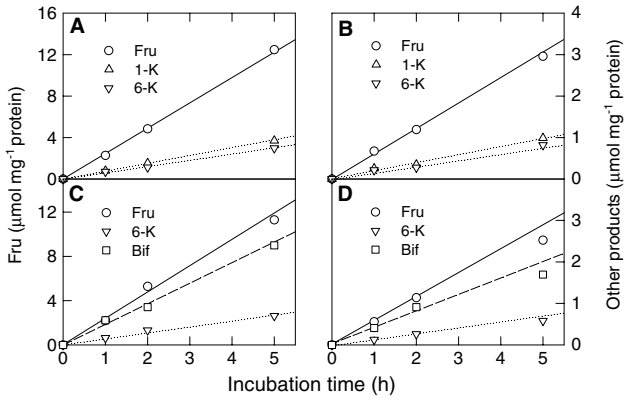

Fig. 2. Catalytic activities of recombinant-tagged barley 6-SFT (P3) with the native EAD motif (A,C) and comparison to the mutated version (P3[DTN]) (B,D). Time course of product formation over a period of 0–5 h with 100 mM suc (A,B) or 100 mM suc and 50 mM 1-kestose (C,D) as substrates.

ties of chimeric enzymes which contained the LSuB coupled to the small subunit of barley 6-SFT (Swap1) and vice versa (Swap2). Furthermore, we also expressed the LSuB alone.

The activity of the hybrid enzymes was markedly reduced compared to the "wild-type"-enzymes. Thus, the small subunit might play a role in the overall rate of catalysis, apparently in a cooperative way with its "own" large subunit. However, the recombinant proteins with exchanged (swapped) subunits were functional fructosyltransferases, and in qualitative terms, their catalytic functions (ratio of the different products formed) were almost identical to the parent enzyme contributing the large subunit.

Thus, when incubated with 100 mM suc, Swap1 produced mainly 1-kestose, a small amount of fructose and traces of nystose, just like the parent enzyme contributing the large subunit, 1-SST (Fig. 3A). There was no measurable production of 6-kestose with Swap1, as might have been expected if the small subunit derived from 6-SFT contributed to catalytic

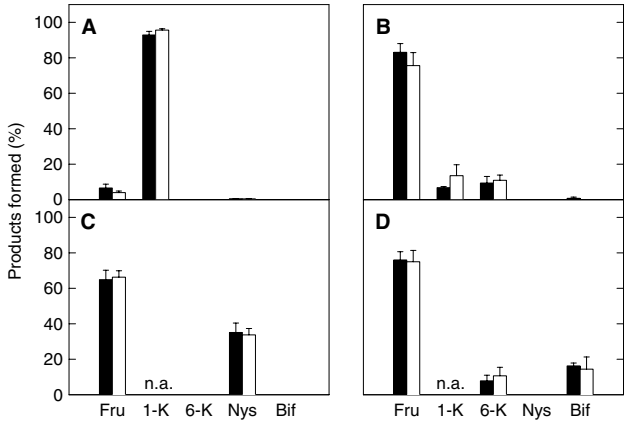

Fig. 3. Relative enzyme activities of 1-SST (F2)/Swap1 (A,C) and of 6-SFT (P3)/Swap2 (B,D), using 100 mM suc (A,B) or 100 mM suc and 50 mM 1-kestose (C,D) as substrates. In incubations with 100 mM suc, the total activity of the protein was approximately 125 nkat/mg protein for F2, 0.75 nkat/mg protein for P3, 0.7 nkat/mg protein for Swapl, and 0.05 nkat/mg protein for Swap2. Black bars: recombinant-tagged 1-SST and 6-SFT, respectively. Adjacent white bars: chimeric-tagged proteins with the same large subunit, but an exchanged small subunit. Data represent means and S.D. ($n = 6$, corresponding to six different enzyme preparations from six separate induction experiments). The sum of products formed is set to 100%.

specificity. Similarly, when Swap2 was incubated with 100 mM suc, it exhibited the same enzymatic activity as the parent enzyme contributing the large subunit, 6-SFT. Swap2 primarily acted as a β-fructosidase, yielding fructose, and produced 1-kestose and 6-kestose in the same proportions as the parent enzyme, 6-SFT (Fig. 3B).

The same picture emerged when enzymatic activities were compared in the presence of suc and 1-kestose as substrates. Swap1 yielded nystose and fructose in the same proportions as the parent enzyme delivering the large subunit, 1-SST, but no bifurcose as would have been expected if the enzyme contributing the small subunit, 6-SFT, had a role in enzymatic specificity (Fig. 3C). Vice versa, Swap2 yielded bifurcose in the same proportion as the parent enzyme delivering the large subunit, 6-SFT, but no nystose as would have been expected if the enzyme contributing the small subunit, 1-SST, had a role in enzymatic specificity (Fig. 3D).

1-SST acts as a fructan exohydrolase when incubated with 1-kestose or nystose alone [6]. This is also true for Swap1, comprising the large subunit of 1-SST: Both the parent enzyme 1-SST and Swap1 yielded considerable amounts of fructose when incubated with 1-kestose or nystose (Fig. 4A and B). Interestingly, both the parent enzyme and Swap1 additionally act as a FFT when incubated with 1-kestose, as indicated by the fact that about 15% of the products represent nystose (Fig. 4A).

In contrast, both 6-SFT and recombinant Swap2, comprising the large subunit of 6-SFT, displayed no measurable product formation in incubations with either 50 mM 1-kestose alone or nystose alone (data not shown).

To obtain further insight into the importance of the small subunit, we attempted to express a tagged version of the LSuB. However, we could not observe any enzyme activity in the corresponding preparations from the *P. pastoris* culture fluid (data not shown). In addition, immune blots with antibodies directed against the tags did not reveal any antigenic proteins in the preparations from the culture fluid; however, an immune-responsive protein of the correct size (approximately 50 kDa)

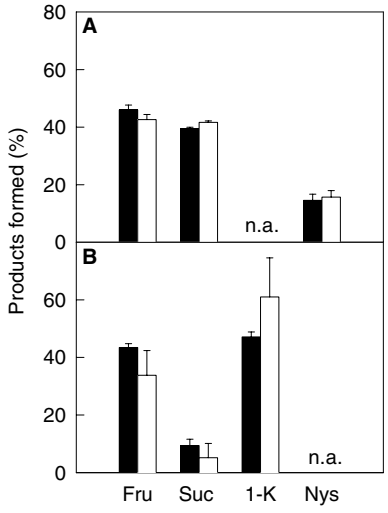

Fig. 4. Relative enzyme activities of 1-SST (F2, black columns) and Swap1 (white columns), using 50 mM 1-kestose (A) or 50 mM nystose (B) as substrates. Data represent means and SD ($n = 6$, corresponding to six different enzyme preparations from six separate induction experiments). The sum of products formed is set to 100%.

was present in preparations obtained by boiling induced cells in the presence of 0.5% SDS and 500 mM NaCl, followed by desalting (data not shown). These data indicate that the large subunit alone is translated correctly in *Pichia*, but not correctly folded and thus not secreted. The retained protein showed no activity in enzymatic assays using lysed cells (data not shown).

In conclusion, our data show that the small subunit of fructosyltransferases is essential for functional expression of the protein, but has no influence on the precise catalytic specificity of the enzyme. It is the large subunit alone which determines catalytic specificity and the nature of the products formed.

We are currently performing more detailed domain-exchange experiments within the large subunit, in conjunction with site-directed mutagenesis of some of its highly conserved domains, in order to narrow down the regions important for catalytic specificity.

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