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Purification and characterization of wheat α-gliadin synthesized in the yeast, *Saccharomyces cerevisiae*

(Iso-1-cytochrome *c* promoter; heterologous expression; signal peptidase; seed storage protein)

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SUMMARY

The development of efficient methods for production and purification of plant seed storage proteins in heterologous microbial hosts would facilitate structure-function studies of these proteins. This report describes such methods applied to the production and isolation of wheat α-gliadin, a prolamine-type seed storage protein, from *Saccharomyces cerevisiae*. Beginning with the vector, growth conditions, and extraction methods of Neill et al. [Gene 55 (1987) 303–317], we implemented several improvements to increase the yields of α-gliadin per volume of yeast cell culture. The *CYC1*::*Gli-A2*-Y transcriptional fusion vector, pAY31 (Neill et al., 1987), was modified by replacing the *ARS1* region of replication with that of the 2 μ plasmid of yeast. We formulated a new medium, a derivative of synthetic defined (SD) medium supplemented with several nitrogen sources, that allows both selection for maintenance of plasmids and growth to high cell densities. Stationary phase cultures of cells bearing the modified expression vector, and grown in this medium with glycerol and lactate as carbon sources, contain significantly higher levels of α-gliadin than log-phase cultures grown in SD glucose. Sonication in 80% ethanol selectively and efficiently extracts the α-gliadin from cell pellets of small- or large-scale cultures, allowing the purification of several hundred μg of the wheat protein per liter in just a few high-yield steps. The α-gliadin isolated from yeast elutes at the same position in HPLC as the A-gliadin fraction purified from wheat flour. N-terminal amino acid (aa) sequencing reveals that the signal peptide is removed from the gliadin precursor in yeast cells. About two-thirds of the molecules have the same N terminus as A-gliadin made in wheat endosperm cells. The remaining third of the α-gliadin is apparently cleaved at a similar processing site three aa closer to the N terminus of the prepeptide.

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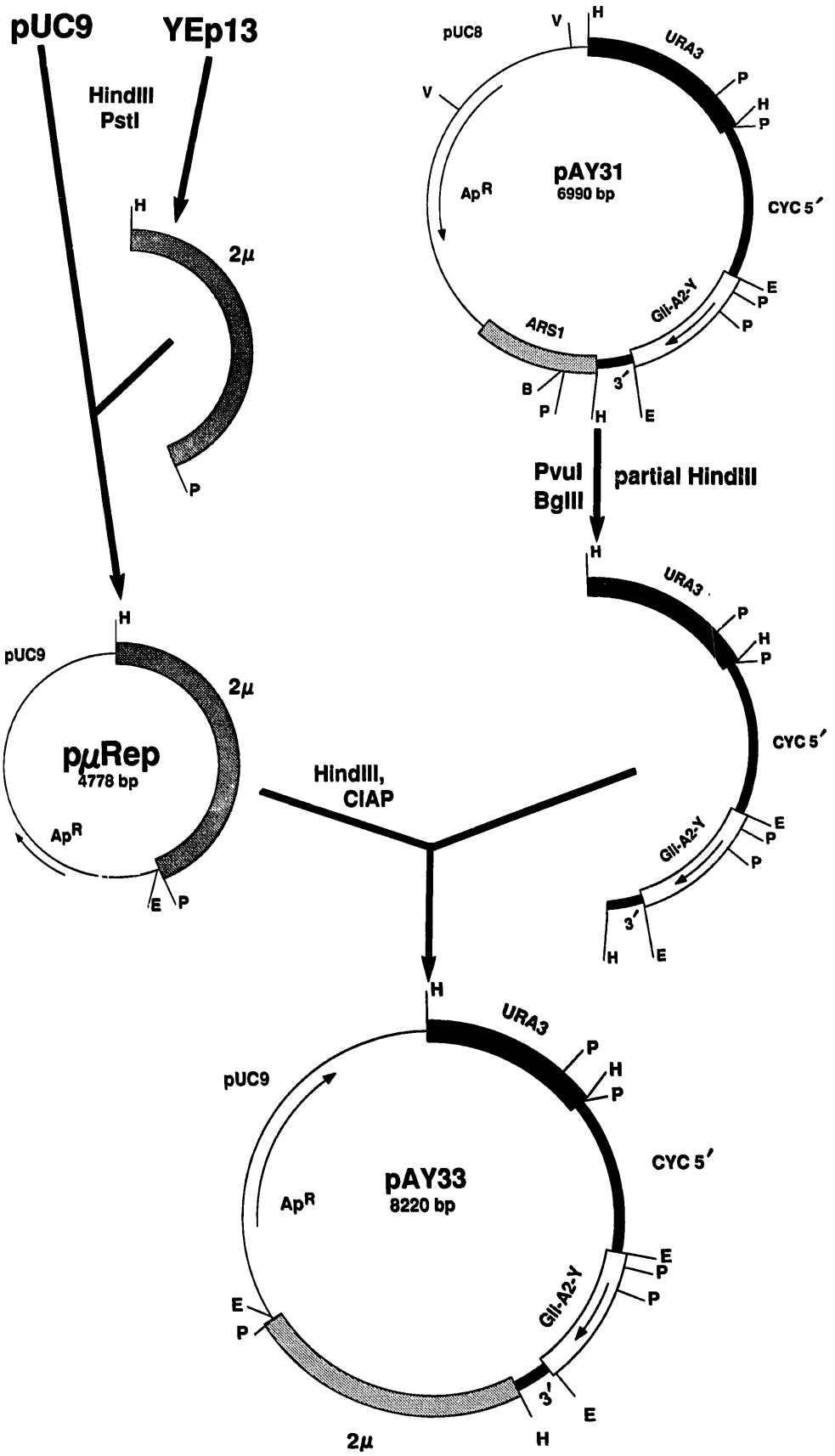
Abbreviations: A_{600}, absorbance (1 cm) at 600 nm; aa, amino acid(s); A-gliadin, fraction of wheat seed storage proteins with characteristic aggregation properties purified as described by Bernardin et al. (1967); α-gliadin, prolamine-type wheat seed storage protein; *ADC1*, yeast gene encoding alcohol dehydrogenase; Ap, ampicillin; *ARS1*, autonomously replicating sequence 1; BCIP, 5-bromo-4-chloro-3-indoyl-phosphate; bp, base pair(s); BSA, bovine serum albumin; CIAP, calf intestinal alkaline phosphatase; *CYC1*, yeast gene encoding iso-1-cytochrome *c*; ELISA, enzyme-linked immunosorbent assay; G/L, 3% (w/v) glycerol/2% (v/v) lactate; *Gli-A2*-Y (GenBank accession No. X01130) wheat gene encoding an α-gliadin cloned from chromosome 6A (cultivar Yamhill) and named YAM-2 by Anderson et al. (1984); HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; NBT, nitroblue tetrazolium; NSSD, nitrogen-supplemented SD medium (see Table I); p, plasmid; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; ^{R}, resistance/resistant; *S.*, *Saccharomyces*; SB, gel sample buffer: 35 mM Tris·HCl/1.1% (w/v) SDS/5% (v/v) β-mercaptoethanol/20% (w/v) sucrose/0.0567 mg per ml pyronin Y pH 6.8; SD (medium), synthetic defined medium (see Table I); SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline (50 mM Tris·HCl/75 mM NaCl pH 8.0); TFA, trifluoroacetic acid; *URA3*, yeast gene encoding orotidine-5′-phosphate decarboxylase; YP, rich broth for yeast: 1% (w/v) yeast extract/2% (w/v) Bacto peptone/0.001% (w/v) each adenine and uracil; [ ], denotes plasmid-carrier state; ′ (prime), truncated gene at the indicated side; ::, novel joint (fusion or insertion).


pUC9
YEp13
HindIII
PstI
2μ
pUC8
pAY31
6990 bp
ApR
URA3
CYC 5′
Gll-A2-Y
ARS1
3′
PvuI
BglII
partial HindIII
pμRep
4778 bp
HindIII,
CIAP
pAY33
8220 bp

INTRODUCTION

More than 50 individual components that serve as protein reserves for the germinating plant are made in the developing wheat endosperm. The aa compositions and structural characteristics of these storage proteins play an important role in determining the nutritional content, elasticity and extensibility of bread doughs made from wheat flour. These properties, in turn, determine the economic value of specific wheat cultivars in baking applications. Attempts to improve these characteristics by genetic engineering require an understanding of the relative contributions of the various storage protein classes to the functional properties of wheat gluten.

The most important proteins for dough quality are the glutenins and gliadins, members of the prolamine class of proteins distinguished by their solubility in aqueous alcohols and dilute acids. The large numbers of similar components and their virtual insolubility in salt solutions has hampered efforts to purify to homogeneity useful quantities of individual native subunits from wheat flour. The cloning of several wheat storage protein genes (Kreis et al., 1985) makes it feasible to express individual subunits in heterologous microbial systems (Neill et al., 1987; Scheets and Hedgcoth, 1989; Galili, 1989; Pratt et al., 1991) from which they can be more readily purified. Genetically modified versions of these genes can be constructed in vitro and expressed in these systems, in studies to define the structural motifs that underlie the unusual properties of these proteins.

With these goals in mind, we sought to improve the yield of a wheat α-gliadin protein from *S. cerevisiae* cells bearing a yeast *CYC1*::*Gli-A2*-Y expression vector (Neill et al., 1987). In this report, we describe improvements in growth conditions, including a new medium, that resulted in increased production of this protein. We exploit the unusual solubility characteristics of α-gliadin to selectively extract it from yeast cells. The aa sequence analysis of the purified protein reveals two N termini and provides insight into the recognition site specificity of the yeast signal peptidase.

RESULTS AND DISCUSSION

(a) Construction of a 2μ-based *Gli-A2*-Y expression plasmid

The *Gli-A2*-Y expression plasmid, pAY31, contains a wheat α-gliadin-encoding gene sequence flanked by the *S. cerevisiae CYC1* gene 5′ and 3′-untranslated regions (Neill et al., 1987). pAY31 is a multicopy, but unstable plasmid, relying on the *ARS1* region (Stinchcomb et al., 1979) for its replication in yeast cells. With the goal of increasing the plasmid average copy number per cell, we replaced the *ARS1* region with the replication region from the yeast 2μ plasmid. This region, which contains the origin of replication and *STB* locus for stability (Jayaram et al., 1983; Kikuchi, 1983), was isolated as a 2100-bp *Pst*I-*Hin*dIII fragment from YEp13 (Broach et al., 1979). Details of its insertion into pAY31 are described in Fig. 1. The resulting plasmid, pAY33, was transformed (Ito et al., 1983) into the yeast host strain D1113–10B [α *ura3*-52 *his3-Δ leu2*-3,112 *ilv3* (Neill et al., 1987)] and transformants were selected on SD medium lacking uracil.

(b) Extraction of wheat α-gliadin from yeast cells

Since stationary-phase cultures contain many more cells than log-phase cultures, it was expected that they would contain proportionately more wheat α-gliadin, assuming that its production were cumulative over time and that the protein was stable. However, stationary-phase cells are relatively resistant to the Zymolyase digestion procedure used by Neill et al. (1987) to extract total soluble proteins from log-phase D1113–10B[pAY31] cells (Kuo and Yamamoto, 1975, and our unpublished observations). In order to insure quantitative extraction of the wheat α-gliadin from stationary-phase cultures, a different method, based on sonication of cell pellets in 80% ethanol, was developed, as outlined in the legend to Fig. 2.

The Western blot shown in Fig. 2 is a comparison of the amounts of α-gliadin isolated from the same stationary phase culture, either by sonication in 80% ethanol (the S lanes) or by lysis of Zymolyase-digested cells (the Z lane). Extracts prepared by the sonication method contain at

Fig. 1. Construction of the *Gli-A2*-Y expression plasmid pAY33. The region of the 2μ plasmid contained in the 2100-bp *Hin*dIII-*Pst*I fragment of YEp13 (Broach et al., 1979) was isolated and subcloned into pUC9 (Vieira and Messing, 1982). The resulting plasmid, pμRep, was cut at its unique *Hin*dIII site, phosphatased, and ligated to the 3460-bp partial-*Hin*dIII fragment from pAY31 that contains the *URA3* selectable marker region and the *CYC1*::*Gli-A2*-Y expression cassette. (In order to isolate the partial *Hin*dIII digestion product by gel electrophoresis, pAY31 was simultaneously digested with *Bgl*II+*Pvu*I: these enzymes cut in the unwanted, but similarly sized pUC8-*ARS1* *Hin*dIII fragment.) The final construct, pAY33, has the same structure as pAY31 except that it contains the yeast 2μ replication region instead of *ARS1* and the prokaryotic vector sequences oriented in the opposite direction with respect to the eukaryotic sequences. The locations of restriction sites are indicated: B, *Bgl*II; E, *Eco*RI, H, *Hin*dIII; P, *Pst*I and V, *Pvu*I. The *Pvu*I and *Bgl*II sites are shown only in pAY31. Standard protocols (Sambrook et al., 1989) were used for molecular cloning and plasmid isolation. Restriction enzymes and DNA ligase were purchased from BRL (Gaithersburg, MD) and CIAP was from Boehringer Mannheim Biochemicals (Indianapolis, IN); all were used as specified by the manufacturers. The use of a brand name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

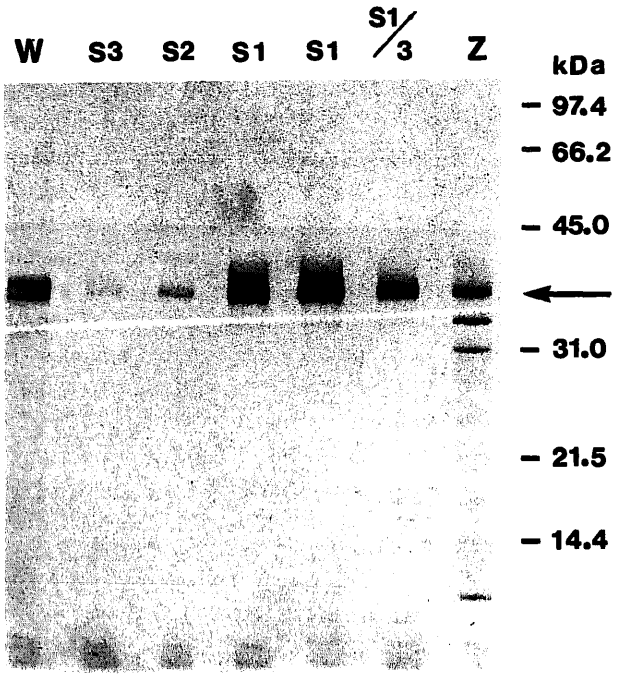


Fig. 2. Western-blot comparison of two α-gliadin extraction methods. Protein extracts were prepared from two 10-ml aliquots of the same stationary-phase culture of D1113–10B[pAY33] grown in SD G/L. The **S** lanes contain extract prepared by sonication in ethanol (see below) and lane **Z** contains extract prepared by lysis of Zymolyase-digested cells (Neill et al., 1987). Lanes **S1** and **Z** contain extract equivalent to 0.3 ml of the culture, while lane **S1/3** contains extract equivalent to 0.1 ml. Lanes **S1**, **S2**, and **S3** represent the first, second, and third, respectively, serial ethanol extractions of the same cell pellet. Lane **W** contains 40 ng of the A-gliadin fraction purified from wheat flour (Neill et al., 1987). To the right, the positions of protein standards (BioRad, Richmond, CA) run in an adjacent lane of the gel are indicated by their sizes in kDa. The arrow identifies the position of full-length α-gliadin. The double bands at the bottom of the transfer are the tracking dye, pyronin Y. **Methods.** To prepare protein extracts by the sonication method, the cell pellet from 10 ml of a D1113–10B[pAY33] culture grown to stationary phase (A_{600} 1.5) in SD G/L media was resuspended in 1 ml of 80% ethanol containing 0.2 mM PMSF/0.5 μg leupeptin per ml/0.7 μg pepstatin (Boehringer Mannheim Biochemicals, Indianapolis, IN) per ml/1 mM EDTA, and chilled in dry ice. Proteins were extracted from this suspension by sonication for 10–30 s using the tapered microtip of a Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT) set at output control 5 (about 40 W). The extracts were clarified by centrifugation, dried and resuspended directly in 50 μl SB. For the Zymolyase method of protein extraction, the procedure of Neill et al. (1987) for Zymolyase digestion was adapted to small cultures with the following modifications: the cell pellet was washed once in 5 ml of sterile water and resuspended in 3 ml of 0.125 mg Zymolyase (ICN Immunobiologicals, Lisle, IL) per ml of 1 M sorbitol pH 7.0. Cells were incubated at 37°C for 2 h, then washed in 100 μl 1 M sorbitol pH 7.0, and resuspended in 50 μl sterile water. The extract was combined with an equal volume of 2 × SB, boiled for 5 min and frozen at −35°C. For Western analysis (Towbin et al., 1979), proteins were fractionated by discontinuous 0.1% SDS–12%(0.2% bis-acrylamide)-PAGE (Laemmli, 1970). The gel was electro-blotted to nitrocellulose (Schleicher & Schuell, Keene, NH) for 1.5 h at 550 mA in a Hoefer (San Francisco, CA) Transphor electrophoresis chamber. The transfer buffer contained 20% ethanol and 0.02% SDS and was cooled by circulating water cycled through a −16°C 50% (v/v) ethylene glycol bath. The nitrocellulose filters were stained with amido black to verify that the transfer was uniform, and then incubated in 5% evaporated dry milk in TBS to block nonutilized protein-binding sites. The filter was incubated overnight with a polyclonal antibody to A-gliadins (Neill et al., 1987). Bands of gliadin complexed with antibody were detected by reaction for 2 h with goat anti-rabbit IgG conjugated to alkaline phosphatase. Both antibody incubations were at room temperature in TBS containing 3% fish gelatin. The complexes were visualized by reaction with 0.167 mg BCIP/ml in the presence of 0.333 mg NBT/ml (described and referenced in Sambrook et al., 1989). The staining reaction buffer consisted of 100 mM Tris·HCl/250 mM NaCl/10 mM $MgCl_2$ pH 9.5. All chemicals for visualizing immune complexes were from Sigma (St. Louis, MO). SDS was purchased from Pierce (Rockford, IL).

least threefold higher levels of gliadin: lane S1/3 contains more full-length gliadin (arrow) than lane Z, but in only one-third the amount of extract. The lower recovery of α-gliadin from Zymolyase-treated stationary-phase cells may be due to the insolubility of the wheat protein under the aqueous conditions used to prepare these extracts. In addition, a portion of the full-length α-gliadin is degraded during the 2-h incubation period required to digest the cell walls. These proteolytic digestion products are visible below the full-length α-gliadin band (arrow) in lane Z.

The efficiency of the sonication method in extracting α-gliadin was further tested by re-extracting the same yeast cell pellet three successive times. Nearly all of the wheat protein is obtained in the first extraction (Fig. 2, lane S1).

(c) Effects of carbon source and growth phase on gliadin synthesis in SD medium

Since the yeast *CYC1* promoter is regulated by the carbon source and subject to repression by glucose (Zitomer et al., 1979; Guarente and Ptashne, 1981), we tested a number of carbon sources for their effects on gliadin production. We found that cells grown on SD media with lactate (L) or glycerol+lactate (G/L) yielded higher amounts of α-gliadin than those grown with glucose or raffinose (Western blot data not shown). Yeast cells grow slowly on G/L as carbon sources, with doubling times in log phase of 4 h (vs. 2 h in SD glucose).

To assess the overall accumulation of α-gliadin in SD G/L cultures, protein extracts were prepared from D1113–10B[pAY33] cells in log phase (A_{600} 0.85) and stationary phase (A_{600} 1.5). Comparison of the relative levels of α-gliadin in these two extracts was achieved by determining the volume of each sample required to obtain similar staining intensities in Western analyses. Attempts to more precisely quantitate gliadin levels in the yeast extracts using ELISA methods were unsuccessful because the wheat protein did not bind quantitatively to polystyrene plastics under any of the conditions we tested. The Western blot in Fig. 3A demonstrates that about eightfold more α-gliadin is present per unit volume of stationary than log-phase SD G/L cultures. The sample representing fourfold greater volume of

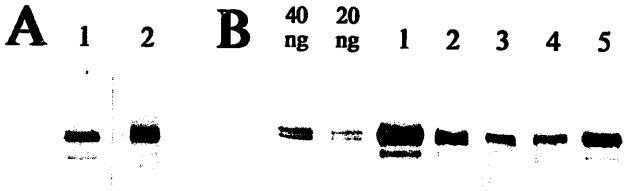


Fig. 3. Western blot comparison of α-gliadin quantities prepared from various cultures. (Panel A) The amounts from log (lane 1) and stationary (lane 2) phase cultures. Lane 1 contains extract equivalent to 1.0 ml of the log phase (A_{600} 0.8) culture, while lane 2 contains extract equivalent to 0.25 ml of the stationary-phase (A_{600} 1.5) culture. Protein was extracted for Western analysis by the sonication method described in Fig. 2 from 10-ml cultures of D1113–10B[pAY33] grown in SD G/L media. (Panel B) α-gliadin isolated from various SD-based media. Lanes 1, 3 and 5 contain extracts equivalent to 1, 0.05 and 0.5 ml, respectively, of culture grown in NSSD G/L media (A_{600} 2.2). Lane 2 contains extract equivalent to 1 ml of culture grown in NSSD with 2% glucose as the carbon source (A_{600} 2.15). Lane 4 contains extract equivalent to 0.4 ml of a culture grown in SD G/L media (A_{600} 1.8). For quantitation, 40 and 20 ng of the A-gliadin fraction purified from wheat flour (Neill et al., 1987) and run as standards on the same gel are shown in the leftmost two lanes. The compositions of SD G/L and NSSD G/L are listed in Table I. For each sample, 10-ml cultures of D1113–10B[pAY33] were grown to stationary phase for protein extraction by the sonication method and Western analysis as described in Fig. 2, except that the gel was 15% polyacrylamide. For determination of total soluble protein, a fifth portion of each original yeast cell culture was suspended in 0.1 N NaOH 1.0% SDS, and sonicated. This extract was used for determination of protein concentration by a modification of the Lowry procedure (Markwell et al., 1978), using BSA as the standard.

the log-phase culture (lane 1) yielded about twofold less staining than the sample from the stationary-phase culture (lane 2). Overall protein levels in these cells, as determined by Lowry analysis of the soluble proteins extractable by sonication in 0.1 *N* NaOH 1.0% SDS, actually decline from an average of 215 μg/ml in log phase to 100 μg/ml in stationary phase.

(d) NSSD: a nitrogen-supplemented selective medium for gliadin synthesis

In order to further increase gliadin production, we sought an alternative to the commonly used yeast growth medium: a defined medium that would support high final culture densities and/or total protein per unit of culture volume (like YP media), while selecting for maintenance of expression plasmids (like SD media). Several nitrogen-containing supplements to SD G/L were tested for their ability to increase stationary-phase culture densities and gliadin production. The nitrogen components of the medium derived as a result of these studies, called NSSD, are listed in Table I. In brief, NSSD medium contains twice as much yeast nitrogen base as SD medium, three times the aa, ten times the adenine and 0.3% Gln. A_{600} values attained by stationary-phase cultures in NSSD G/L are in the same range as those in YP G/L, usually exceeding 2.0. However,

TABLE I

Comparison of the nitrogen compositions of two synthetic defined media

Nitrogen component	Media composition[a]	
	SD G/L[b]	NSSD G/L[b]
YNB[c] without aa	0.67	1.33
Adenine	0.002	0.02
Arginine	0.002	0.006
Histidine	0.001	0.003
Isoleucine	0.006	0.018
Leucine	0.006	0.018
Lysine	0.004	0.012
Methionine	0.001	0.003
Phenylalanine	0.006	0.018
Threonine	0.005	0.015
Tryptophan	0.004	0.012
Tyrosine	0.004	0.016
Valine	0.015	0.045
Glutamine	None	0.3

[a] Compositions are expressed in % w/v.

[b] SD G/L and NSSD G/L media are buffered variants of the standard synthetic defined media used to test for aa and nucleotide auxotrophy (Sherman et al., 1986). Both media contained 20 mM $Na{\cdot}PO_4$ buffer pH 6.6, 2% Na·lactate and 3% glycerol as carbon sources, and lacked uracil so that only cells containing the expression plasmids pAY31 or pAY33 could grow.

[c] Yeast Nitrogen Base (Difco Laboratories, Detroit, MI).

the doubling time of cultures in NSSD G/L during exponential growth is 5–6 h, compared to 4 h in YP G/L. It is not the production of wheat α-gliadin that slows the growth rate: doubling times in NSSD G/L of D1113–10B cells are the same whether or not they contain pAY33. The generation time of cells in NSSD with glucose as the carbon source is the same as in YP glucose, 2 h.

Fig. 3B shows a Western-blot comparison of extracts from D1113–10B[pAY33] cells grown to stationary phase in NSSD G/L (A_{600} 2.2) and standard SD G/L media (A_{600} 1.8). Nearly equal amounts of extract from the SD (equivalent to 0.4 ml) and NSSD (equivalent to 0.5 ml) cultures are loaded in lanes 4 and 5, respectively, demonstrating that cultures grown in NSSD medium contain much more α-gliadin. Extract equivalent to 0.05 ml of the NSSD culture (lane 3) achieves the same staining intensity as extract equivalent to 0.4 ml from the SD culture (lane 4). Thus, the nitrogen supplements result in about an eightfold increase in gliadin per volume of culture.

Several investigators have previously reported increases in production of heterologous proteins in yeast by addition of between 0.5% and 4% Casamino acids to SD medium (Etcheverry et al., 1986; Ernst, 1986; Zsebo et al., 1986; Gabrielsen et al., 1990). Gabrielson and co-workers documented two effects of supplementing defined media: an increase in total cell protein and a decrease in certain proteolytic activities in the secretory pathway. Our version of

supplemented SD medium results in stationary-phase cell densities comparable to those of cells grown in YP medium. In addition, NSSD medium is superior to both YP and Casamino acids-supplemented SD media in that it retains the ability to select for maintenance of any of the plasmids commonly used for yeast transformation, simply by omission of either an aa or adenine or uracil, as appropriate.

Because of the high densities attained by cultures grown in NSSD medium, we reconsidered the use of glucose as a carbon source in hopes that its depletion late in the growth cycle would result in induction of the *CYC1* promoter. The Western-blot analysis in Fig. 3B shows that D1113-10B[pAY33] cultures grown to stationary phase in NSSD G/L (lane 1) contain much more α-gliadin than those grown in NSSD with 2% glucose as a carbon source (lane 2). Indeed, extract equivalent to 20 times the volume of the G/L-grown culture (lane 3) still has about one-half the staining intensity as the sample from the glucose-grown culture (lane 2). Clearly, the decrease in glucose concentrations during growth is not rapid or large enough to relieve the repression.

The amount of wheat α-gliadin in these protein extracts was estimated from Western blots by comparing the intensities of the stained bands with those of known amounts of purified wheat A-gliadin (leftmost two lanes), as demonstrated in Fig. 3B. Approximately 40 ng of the yeast product is apparent in the extract equivalent to 50 μl of cell culture grown in NSSD G/L (lane 3) or 400 μl of culture grown in SD G/L (lane 4). Total protein concentrations in these cultures were 570 and 155 ng/μl respectively. Thus, the wheat α-gliadin constitutes about 0.14% and 0.06% of the soluble protein in NSSD-grown and SD-grown cells, respectively. The yields of α-gliadin from larger scale NSSD G/L cultures (500 ml in 2.8-liter flasks) ranged from 0.5 to 4 μg/ml. These yields are more than tenfold higher than the 0.04–0.08 μg/ml estimated by Scheets and Hedgcoth (1989) for γ-gliadin produced in yeast from the *ADC1* promoter, but are still low compared to those expected by expression of the *CYC1* promoter from a high-copy-number plasmid. The endogenous single-copy *CYC1* gene produces 0.1% of the total cellular protein under derepressing growth conditions (Sherman et al., 1965). Although the wheat α-gliadin is apparently stable and nontoxic, it may not be as efficiently translated as the *CYC1* native gene product because of its yeast/wheat hybrid 5′ leader region or unfavorable codon bias (relative to highly expressed yeast proteins) (Neill et al., 1987). Recently, Pratt et al. (1991) obtained yields for wheat γ-gliadin of up to 10 mg per liter from stationary-phase yeast cells grown in SD galactose medium. Their higher yields are probably due to the use of a strong galactose/phosphoglycerate kinase hybrid promoter.

(e) Purification of wheat α-gliadin from yeast

As the first step in biochemical purification, the sonication method was used to prepare cell extracts from liter cultures of D1113[pAY33] grown in NSSD G/L medium. Very few of the yeast cell proteins are solubilized in ethanol as judged by Coomassie blue-stained SDS–PAGE of the extracts (not shown), but sugars and some lipids are efficiently extracted. Conventional procedures to remove these low-M_r contaminants, such as dialysis, were unsatisfactory because the wheat protein precipitated in aqueous solutions and bound irreversibly to the membranes. Gliadin solubility in the crude extracts could be maintained in 70–80% ethanol. In order to achieve size separations in this solution, we employed Sephadex LH-20, a chromatographic medium used to fractionate small molecules on the basis of their relative hydrophobicity in organic solvents. Two passages of the yeast extract over Sephadex LH-20 columns separated the proteins from nonproteinaceous components well enough to allow final purification on a reverse-phase HPLC column. Fig. 4A shows the absorption trace from one such HPLC fractionation. The α-gliadin from yeast elutes in a peak at the same position as the monomeric A-gliadin fraction purified from wheat flour. The broadness of the main peak in the wheat sample reflects the heterogeneity of this mixture, which contains six to eight closely related α-gliadin proteins (Kasarda, 1980). Fig. 4B shows a nitrocellulose filter transfer from a heavily loaded gel of the yeast extract before (lane 1) and after (lane 2) HPLC purification. The smaller proteins present after Sephadex LH-20 fractionation (bracket) are removed by HPLC, resulting in α-gliadin (arrow) that is estimated to be more than 95% pure. Yields for the preparation shown in this figure were estimated from Western-blot analyses (not shown) to be 2.7 mg of purified α-gliadin from 4 liters of D1113[pAY33] culture.

(f) Characterization of α-gliadin synthesized in yeast

The expression cassettes in pAY31 and pAY33 contain the entire coding region of the *Gli-A2*-Y gene, including its signal peptide sequence. In order to determine whether the prepeptide was properly processed during its synthesis in yeast cells, the N terminus of the HPLC-purified protein was microsequenced. Two sequences were obtained in a molar ratio of approx. 2:1. They are shown in Fig. 5 below the aa predicted by the wheat gene sequence (Anderson et al., 1984). The predominant sequence (capital letters) corresponds to one commonly obtained by sequencing the N termini of A-gliadins isolated from the seeds of several wheat cultivars (Kasarda et al., 1974; Kasarda, 1980). This result verifies the hypothesis of Neill et al. (1987), based on mobility in SDS–PAGE, that yeast recognizes and removes the wheat signal peptide. The minor sequence (includes the lower-case letters) is that of the α-gliadin precursor poly-

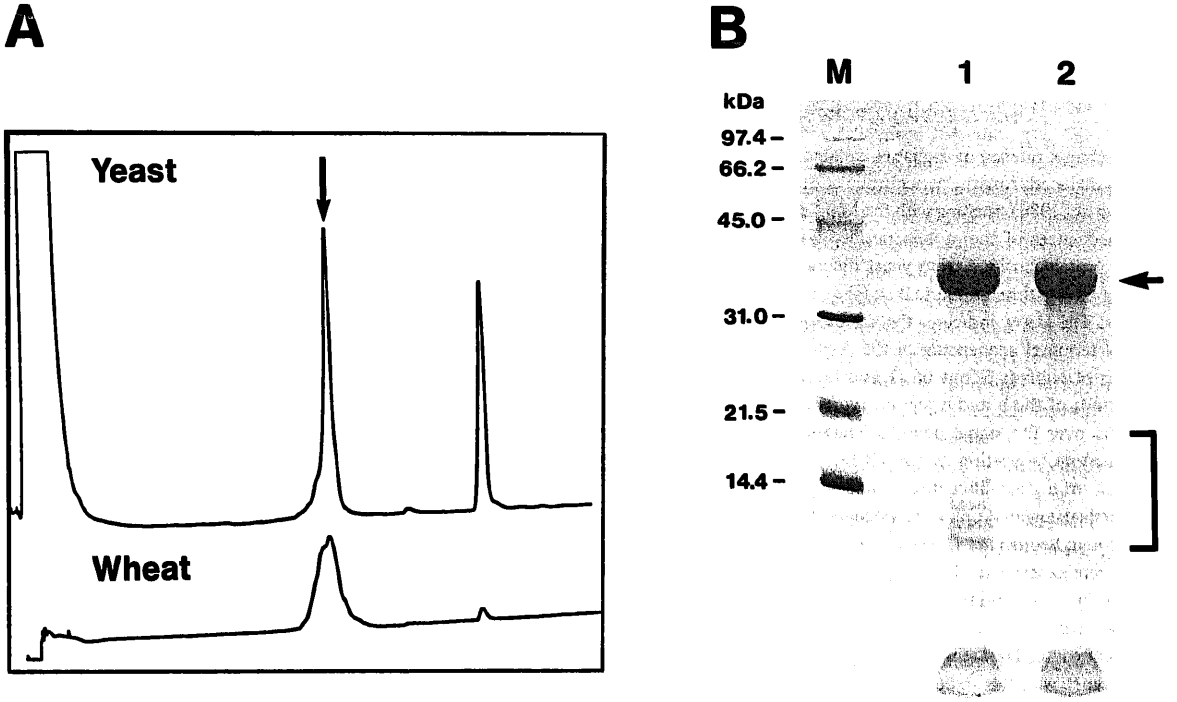


Fig. 4. Purity of α-gliadin isolated from yeast. Panel **A** shows absorbances (origin to the left) at 215 nm recorded during two HPLC fractionations run under identical conditions (described below). For the upper trace, extract from yeast cells producing wheat α-gliadin was loaded after partial purification by two rounds of chromatography on Sephadex LH-20 columns. The lower trace is from a sample of the A-gliadin fraction isolated from wheat flour. The peak fraction (arrow) from the yeast sample exhibiting the same migration as the major peak of wheat flour A-gliadin was collected. The late-eluting peak showed no visible protein bands when analyzed by Coomassie blue-stained 0.1% SDS-12% PAGE (data not shown). Panel **B** shows an SDS–PAGE analysis of the α-gliadin isolated from yeast cells before (lane **1**) and after (lane **2**) the HPLC purification step. The protein fractions were subjected to 0.1% SDS-12% PAGE, transferred to nitrocellulose and stained with amido black. Lane M contains molecular size standards in kDa on the left (see Fig. 2 legend). Lane **1** contains the proteins remaining after two rounds of separation on the Sephadex LH-20 column. The position of full-length α-gliadin is shown by the arrow to the right. Smaller contaminating peptides evident below the main band in lane **1**, but missing in lane **2**, are demarcated by the bracket. The gel was deliberately overloaded to visualize minor forms. **Methods.** To purify α-gliadin from yeast, D1113–10B[pAY33] cell pellets from eight half-liter cultures grown to stationary-phase (A_{600} 1.8–2.3) in NSSD G/L medium were suspended in 200 ml 80% ethanol containing protease inhibitors and extracted as described in Fig. 2 except that sonication was for 1 min using the standard tip of the sonifier set at output control 9 or 10. The supernate after centrifugation was concentrated to about 50 ml in a rotary evaporator. The resulting cloudy, yellow suspension was loaded directly onto a Sephadex LH-20 (Pharmacia, Piscataway, NJ) column and eluted in 70% ethanol at a flow rate of 0.5 ml/min. The protein (early) fractions were pooled and rechromatographed. The protein fractions were again pooled, reduced in volume, diluted with HPLC A solvent (0.1% TFA in water) and injected onto a Vydac (Hesperia, CA) C-4 reverse-phase column (0.46 i.d. × 25 cm) maintained at 25°C and equilibrated with the starting solvent (65% of the aqueous solvent A and 35% of the organic solvent B). Solvent B consisted of acetonitrile/2-propanol in a ratio of 3:1 and 0.1% TFA. After injection, the column was eluted at a flow rate of 1 ml/min with the starting solvent followed at 5 min by a linear gradient extending to 35% of solvent A and 65% solvent B in a period of 85 min.

peptide cleaved three aa upstream from the main processing site just after the preceding Thr-Thr-Ala triplet. This N-terminal sequence has not been detected in gliadin fractions from wheat flour (D. D. Kasarda, personal communication).

The wheat α-gliadin signal sequence has a typical structure: a short (2 aa) N-terminal region containing the charged aa Lys, followed by an uninterrupted hydrophobic core of 12 aa, and ending with the more polar C-region sequence, Thr-Thr-Ala-Thr-Thr-Ala (Von Heijne, 1984, and reviewed by Briggs and Gierasch, 1986). The major site of cleavage is consistent with the hierarchies proposed by von Heijne (1984) and demonstrated experimentally for canine signal peptidase by Folz et al. (1988). However, the yeast signal peptidase has a more relaxed spacing requirement than the canine enzyme, readily cleaving one-third of the molecules after the Ala that is only 3 aa from the hydrophobic core region. A similar case of multiple N termini for a single heterologous protein synthesized in yeast was described by Hitzeman et al. (1983). Human α2 interferon secreted from yeast was processed with equal efficiency at two major sites. One cleavage site was after the Cys, 4 aa past the hydrophobic core. The second cleavage site was 7 aa past the hydrophobic core following a Gly residue.

The ability to purify wheat α-gliadin from yeast will enable us to characterize, for the first time, the physicochemical properties of a single member of the A-gliadin seed storage protein class. Further structural studies, such as

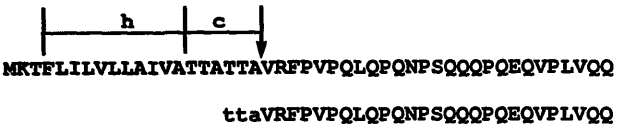


Fig. 5. N-terminal aa sequence of α-gliadin isolated from yeast. The top line shows the aa deduced from the nt sequence of this α-gliadin-encoding gene (Anderson et al., 1984), starting with the Met[1] codon. The lower line shows the two sequences of the protein purified from yeast. The minor sequence of the protein purified from yeast differs from the major one (capital letters) in having three additional aa (shown in lower-case letters) at its N terminus. The arrow indicates the site of signal peptide cleavage deduced from N-terminal sequencing of the A-gliadin fraction isolated from wheat flour of cultivar Scout 66 (Kasarda et al., 1974; Kasarda, 1980). The locations of the h and c regions (von Heijne, 1984) are indicated by brackets over the signal peptide. **Methods.** To obtain the N-terminal aa sequences, a portion of the HPLC-purified α-gliadin from yeast was spotted on a glass filter disc coated with polybrene and subjected to aa analysis using an Applied Biosystems (Foster City, CA) 477A Pulsed Liquid Protein Sequencer. Upon detection of two major sequences, a second sequencing experiment was designed to confirm and extend the results of the first. In the second run, the sample mixture was coupled with *o*-phthalaldehyde (Brauer et al., 1984) at the fourth aa, prior to coupling with phenylisothiocynate. Thus, the Val from the fourth cycle of the Thr-Thr-Ala-Val sequence was blocked and Edman sequencing was then carried out for another 16 aa residues of the main sequence. Cleavage times at Pro were 20 min, and 5 min for other aa. The combined repetitive yield for the background-subtracted, lag-corrected data was 94%.

circular dichroism, spectral determination, and solubility behavior are needed to reveal the extent of similarity between the higher order structures of α-gliadin synthesized in yeast and that synthesized in developing wheat endosperm tissue. The purification methods described in this report can be applied to any alcohol-soluble protein, including other wheat prolamines. The availablity of milligram quantities of these proteins would enable us to test their individual contributions to the properties of bread doughs in small-scale model mixing experiments.

(g) Conclusions

(*1*) Production of 0.5–4.0 μg of wheat α-gliadin per ml of *S. cerevisiae* culture, utilizing a *CYC1* expression cassette, has been achieved by two media improvements: the use of G/L instead of glucose as carbon sources and supplementation of SD G/L medium with nitrogen sources to allow growth under selective conditions to high cell densities.

(*2*) Wheat α-gliadin has been efficiently extracted from yeast cells by sonication in 80% ethanol and purified with an estimated yield of 675 μg per liter of stationary-phase NSSD G/L culture.

(*3*) In yeast cells, the wheat preprotein was cleaved after two different Ala that are three aa apart; yeast exhibit only a 2:1 preference for the site utilized by wheat endosperm.

(*4*) The protein purified from yeast cells elutes at the same position in HPLC as the monomeric A-gliadin fraction purified from wheat flour.

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