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Registry No. Ergotamine, 113-15-5; ergotaminine, 639-81-6; ergostine, 2854-38-8; ergocristine, 511-08-0.

Expression of Bovine β-Casein in *Saccharomyces cerevisiae* and Characterization of the Protein Produced in Vivo

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Recombinant DNA technology offers numerous opportunities for engineering food proteins and for studying their structure–function relationship. As part of the study of the structure–function of bovine caseins, it is necessary to produce mutant proteins in experimental amounts and correlate their new structure to their physicochemical characteristics. To this end, bovine β-casein was expressed in the yeast *Saccharomyces cerevisiae* by a fusion to the *HXK1* (hexokinase P1) gene. Casein was produced during late exponential/early stationary phase of growth on glucose as would be predicted for a gene under the control of the *HXK1* promoter. Bovine β-casein was posttranslationally modified by yeast. Internal phosphorylated forms were observed as well as a high molecular weight form that appeared to be O-glycosylated and largely localized to the periplasmic space.

Bovine caseins serve as important sources of nutrition in the human diet, and the proteins themselves are used as food additives to enhance the nutritional value and functional characteristics of a variety of products. Proteolytic digestion coupled with decreased solubility of the caseins at low pH is central to the production of cheese and other dairy products. Because of their availability and precipitation at low pH, bovine caseins are used as clarification agents in wine processing. One approach to further elucidate the structural features of caseins important for proper function in micelle formation and functionality in foods is to alter the protein sequence by altering the DNA sequence encoding the protein and to obtain sufficient quantities of the protein for analysis. All of the bovine caseins have been cloned (Bonsing and Mackinlay, 1987), and some have been expressed in *Escherichia coli* (Kang and Richardson, 1988). Bovine β-casein is modified posttranslationally with the addition of five phosphate groups to the protein. Such modification does not occur in *E. coli*. Therefore, we sought to obtain expression of β-casein in the eukaryotic microorganism *Saccharomyces cerevisiae*. Numerous heterologous proteins have been expressed in *Saccharomyces* species (Brake et al., 1984; Hitzeman et al., 1982; Kingsman et al., 1985). Since glycolytic enzymes are normally produced in large quantities in yeast cells (any given enzyme may represent from 1 to 5% of total cellular protein), glycolytic promoters have been exploited in constructs to obtain high yields of foreign proteins. However, the glycolytic enzymes are generally produced constitutively during growth, and such a strategy for expression of a heterologous protein may affect growth efficiency, thereby selecting for variants in the population producing less of the protein product. The hexokinase P1 enzyme encoded by the *HXK1* gene catalyzes the phosphorylation of fructose and glucose at the 6-position. However, the synthesis of hexokinase P1 is glucose repressible, and this isozyme becomes the predominant species only in the absence of glucose or following glucose exhaustion in the medium (Gancedo et al., 1977; Kopperschlager and Hofmann, 1969; Muratsubaki and Katsume, 1979). Thus, the hexokinase P1 gene is a glycolytic gene regulatable by the glucose concentration in the medium. We decided to investigate the utility of the *HXK1* promoter for expression of a heterologous protein, bovine β-casein, in yeast. In addition, we undertook a preliminary characterization of

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the protein produced in yeast to determine whether the casein was modified via phosphorylation.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions. *E. coli* RR1 and HB101 were used for bacterial transformations and routine plasmid preparations. *E. coli* strain JM105 was used for the selection of plasmids derived from pUC18 and pUC19. Standard conditions and media were used for the growth of *E. coli* (Maniatis et al., 1982). *S. cerevisiae* strain AB116 a cir^0 derivative of BJ2168 (*MAT a, leu 2, trp 1, ura 3-52, prb 1-1122, pep 4-3, prc 1-407*) was used for the expression of β-casein. This strain is deficient in proteinases A, B, and C, the major yeast proteinases (Jones, 1977). Strain AB116 was obtained from Dr. Anthony Brake, Chiron Corp., Emeryville, CA. Plasmids YEp24 and YEp13 were purchased from New England Biolabs, Beverly, MA. Plasmid pAB24 was also obtained from Dr. Brake; this plasmid contains the *URA3* gene and a defective copy of the *LEU2* gene, *LEU2-d*, which is capable of complementing a genomic *leu2* mutation only if present in high copy number. Thus, the presence of the *LEU2-d* gene therefore allows the selection of cells that contain a high plasmid copy number. Plasmid pAB24 contains a complete 2 μ region that both provides stability to the plasmid and ensures plasmid maintenance when transformed into (cir^0) strains of *Saccharomyces*.

The conditions for growth in yeast were optimized at 20 °C and 250 rpm in 1-L flasks. Minimal media (yeast nitrogen base, 6.7 g/L, 2% glucose) was used to prepare inocula. Growth and expression were carried out in yeast extract (10%) peptone (20%) medium with glucose (2%) (YPD) or galactose (1%) (YPG) as the carbon and energy source.

A genomic DNA fragment encoding for the complete gene of yeast hexokinase P1 plus flanking regions was obtained from Dr. D. G. Fraenkel as an insert in the yeast vector YEp13. The characterization of this plasmid has been previously described (Walsh et al., 1983), and the sequence of the *HXK1* gene has been determined (Kopetzki et al., 1984). The bovine β-casein gene was obtained from a cDNA library and has likewise been sequenced and characterized (Jimenez-Flores and Richardson, 1987).

General DNA and Protein Methods. Manipulations of subcloning, restriction enzyme digestion, and DNA recovery from gels and standard procedures in *E. coli* were as described in Maniatis et al. (1982) and Ausubel et al. (1987). Standard procedures for transformation and selection in *S. cerevisiae* were performed according to Sherman et al. (1986). Protein analysis by SDS-PAGE was performed according to Laemmli (1970), urea PAGE according to Medrano and Sharrow (1989), and immunoblot transfers according to Towbin et al. (1979). Rabbit antiserum for bovine caseins was purchased from Calbiochem (San Diego, CA), and all incubations for immunoblots were done in 0.25% gelatin, 1× PBS, 0.05% Tween-20, and 0.02% sodium azide. The commercial antibody preparation yielded an extremely high background, and it was thus necessary to further purify this antibody. Antibody was purified by affinity chromatography through protein A immobilized Sepharose (Bio-Rad, Richmond, CA), and the purified antibodies from this step were preincubated (preadsorbed) against a crude protein extract from yeast strain AB116 (Harlow and Lane, 1988). Expression of β-casein was initially demonstrated in Western blots with immunodetection using a commercial polyclonal antibody directed to bovine caseins.

Construction of a Yeast Expression Vector for Bovine β-Casein. The expression vector pJR221 for bovine β-casein was constructed from the promoter and transcription terminator regions of the hexokinase P1 (*HXK1*) structural gene. The β-casein fragment was obtained from plasmid pJR37 (Jimenez-Flores and Richardson, 1987), and it was necessary to construct an in-frame fusion to the coding sequence of hexokinase P1 with putative *Xba*I linkers. This resulted in the addition of seven amino acids (Figure 1a) to the 5′-end of the casein coding sequence. The casein cDNA contained a translational stop signal, but again, being derived from a cDNA clone, no transcriptional terminator. For this reason a DNA fragment containing a portion of the 3′-end of the hexokinase P1 gene extending through the terminator region was inserted, as a blunt end fragment, downstream of and adjacent to the β-casein gene. The β-casein gene with 5′ and 3′ *HXK1* flanking regions was then inserted into plasmid YEp24, yielding pJR221, with *URA3* as a selectable marker in yeast.

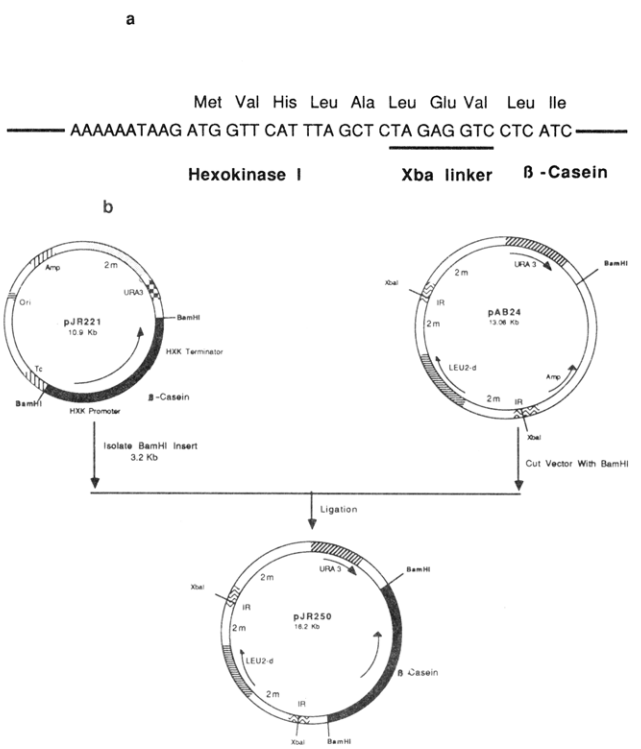

Figure 1. (a) Detailed nucleotide and amino acid description of the linkage between the *HXKI* promoter and β-casein. (b) Construction of expression vector pJR250.

Protein Extraction from *S. cerevisiae*. Total protein was extracted from yeast cells by disruption with glass beads. The cells were collected from the medium by centrifugation in 250-mL bottles at 5000*g*. The cell pellets were then resuspended in extraction buffer (6 M urea, 0.1 M Tris (pH 7.5), 1 mM DTT, 0.1% PMSF), mixed with glass beads (0.45–0.5-mm diameter) in equal volumes, and shaken in a B. Braun (Bronville Instruments, Inc., Rochester, NY) vibrator for five intervals of 1 min each. Final disruption of the cells was consistently over 95% when the disrupted cells were observed under the microscope. The extract was separated from the glass beads by filtration and then centrifuged at 10000*g* for 10 min, and the precipitate was discarded. The extract was then dialyzed against water for 20 h and the precipitate collected by centrifugation (10000*g* for 20 min) and resuspended in the appropriate buffer for chromatography.

Localization of the Protein. Lyticase was purchased from Sigma (St. Louis, MO), and enzymic treatment of the yeast cells was performed by the technique described by Scott and Scheckman (1980). Intact cells were collected from the media by centrifugation at 5000*g* for 3 min. The pellet was then resuspended in 1.2 M sorbitol to an A_{600} of 6.0. Lyticase was then added to a final concentration of 100 U/mL in 0.05 M phosphate buffer (pH 7.7) and incubated in the mixture for 30 min at 30 °C. Spheroplasts were then recovered by gentle centrifugation at 3000*g* for 5 min, and the supernatant fluids were separated from the pellet. The concentrated supernatant liquids constituted the periplasmic fraction, and the disrupted spheroplasts, the cytoplasmic fraction. Both fractions were then analyzed by SDS-PAGE and Western blot.

Purification of Bovine β-Casein from Yeast Extract. Several chromatographic procedures were used for the partial purification of the β-casein produced by yeast:

DE-52 chromatography of proteins from yeast was performed by a modification of the method of Davies and Law (1977). A Super-Flo (Sepragen, Emeryville, CA) column with a capacity of 100 mL was packed with DE-52 (Whatman Maidston, Kent, England) in the buffer described by Davies and Law

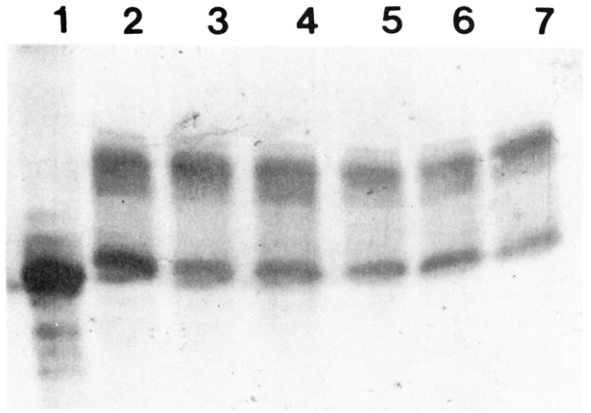

Figure 2. Western blot of an extract of different transformants containing the plasmid pJR250 immunostained with anti-casein antibodies. Lane 1 contains commercial standard β-casein. Lanes 2–7 represent different transformant strains grown under identical conditions.

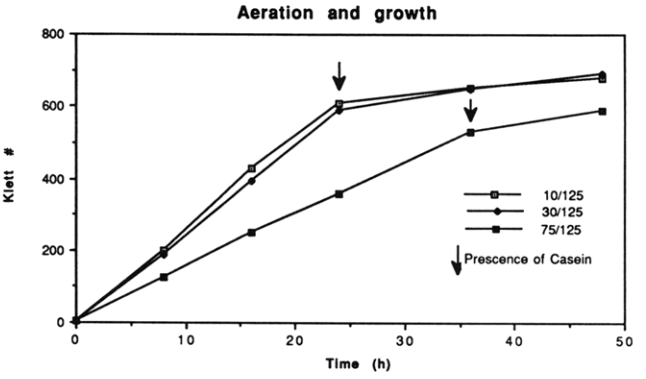

Figure 3. Growth curve at different aeration conditions of a selected strain that contains plasmid pJR250. Production of β-casein is first observed at the points indicated by arrows. Each line represents a different volume of culture in the same-size flask (as indicated in the legend, 10 mL of culture/125-mL flask, etc.). Temperature of growth was 30 °C, and all were shaken at 250 rpm.

(1977), and a NaCl gradient (0.05–0.5 M) was used for elution of the proteins.

Hydroxyapatite (HA) chromatography was performed on the fractions obtained from the DE-52 separation according to the method of Donnelly (1977). For dot blot analysis, 30 μL of each fraction (total volume of each fraction was 2.5 mL) was applied to a specific well in a dot blotter (Schleicher and Schuell). The nitrocellulose bound the protein, and the membrane was then treated as an immunoblot. The proteins were eluted with a linear NaCl gradient (0.05–0.5 M).

Test for Phosphorylation. Phosphatase treatment of β-casein was performed with use of calf intestine phosphatase (CIP) (Boehringer Mannheim, Indianapolis, IN) by solubilizing the protein in 20 mM Tris (pH 8.0), 1 mM $ZnCl_2$, and 1 mM $MgCl_2$. The addition of 0.1 U/mL of CIP was followed by incubation at 37 °C for 30 min. Results were analyzed by urea PAGE.

Glycosylation Tests. Several tests were performed on the expressed β-casein with an apparent molecular weight of 36K.

Endoglycosidase H was obtained from Miles Scientific (Naperville, IL). The sample protein was resuspended in 50 mM citrate (pH 5.5) and 0.1% SDS to a concentration of approximately 23 mg/mL. The sample was then boiled and after cooling the endoglycosidase H added at 30 mU/mL. Reaction was allowed to stand at room temperature for 8 h. The resulting mixture was analyzed directly by SDS-PAGE.

Concavalin A (Con-A) conjugated with horseradish peroxidase (HRP; Bio-Rad) was used for identification of glycoproteins according to the directions of the supplier (Clegg, 1982).

β-Elimination of the carbohydrate from the yeast protein was performed by resuspension of the protein in 0.1 M NaOH and 0.0 1 M $NaBH_4$. The reaction was kept at room temperature for 12 h, and the mixture was neutralized and analyzed by SDS-PAGE (Beeley, 1985).

RESULTS

Expression of β-Casein by Yeast. The *HXK1*–β-casein expression cassette was cloned into pAB24, generating plasmid pJR250 (Figure 1b). *S. cerevisiae* strain AB116 (*MATa, ura-3-52, leu2, trp1, prb1-1122, pep4-3, prc1-407 cir*^{0}) was transformed with pJR250. The transformants obtained varied in the amount of β-casein produced (Figure 2), and all displayed two molecular weight forms reacting with antibody that were not present in control transformants carrying the vector, pAB24, alone.

One of the transformants, ABY116-2 (Figure 2, lane 2), producing high levels of β-casein was chosen for further study. The effect of aeration was evaluated by varying the total culture volume in a fixed-size flask; the results of the growth curves are shown in Figure 3. The protein was expressed at the end of exponential growth/beginning of stationary phase as would be expected for a gene product under the control of the *HXK1* promoter. The highest yields of β-casein (approximately 10 mg/L of culture) were obtained from addition of inoculum in minimal media to complex media (YPD) at 15% of the total final volume. Cells were grown to stationary phase, harvested, and analyzed for β-casein production.

Preliminary Studies of Localization of β-Casein in Yeast. The β-casein construct contained the β-casein signal sequence for secretion, which is conserved among caseins in mammalian systems (Bonsing and Mackinlay, 1987). The yeast β-casein construct contained an additional seven amino acids preceding the β-casein leader and two amino acids of the leader sequence; the Met and the Lys had been deleted. In all previous experiments, the medium supernatant fluid had been assayed for the presence of β-casein and none had been found. The β-casein could therefore be localized in the periplasmic space or in the cytoplasm.

Strain ABY116-2 (carrying plasmid pJR221) was grown in minimal medium, and the cells were harvested and lysed with lyticase to produce spheroplasts. The spheroplasts were separated from the supernatant fluid by centrifugation, and proteins present in the supernatant solution were precipitated with trichloroacetic acid (TCA). Most of the recoverable β-casein was found in the supernatant fraction not associated with the spheroplasts and was the higher molecular weight form. These findings suggest, but by no means prove, that some of the β-casein may be localized in the periplasmic space. Additionally, the total amount of β-casein recoverable following lyticase treatment was very low as compared to extracts of whole cells, which may be a consequence of proteolytic activity occurring as an impurity in the lyticase preparation.

Partial Purification of β-Casein from Yeast. Yeast strain ABY116-2 appeared to produce two forms of β-casein, differing in molecular weight in denaturing gels. To characterize these proteins, the crude extracts were further fractionated.

Comparison of different extraction procedures revealed that simple stirring of cells for 2 h at 20 °C in reducing buffer, containing protease inhibitors, yielded the higher molecular weight band (Figure 4, lane 1). Breaking the cells open with glass beads yielded both putative casein bands in crude extracts (Figure 4, lanes 3 and 4). The ease of extraction of the upper casein band also suggests

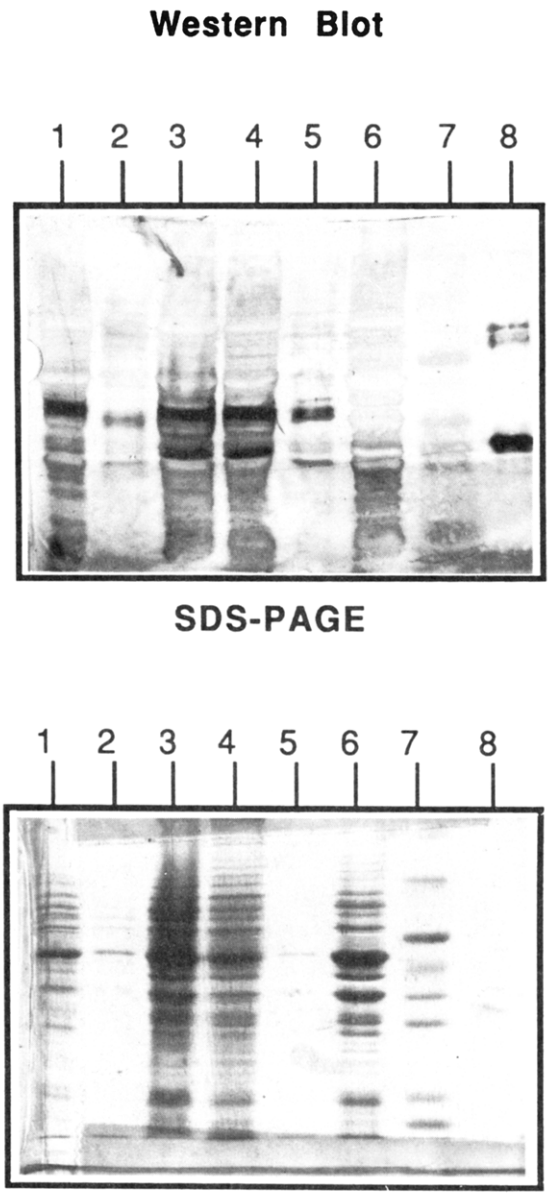

Figure 4. Analysis of the protein extraction procedure from yeast. Parallel SDS-PAGE (Coomassie-stained) and Western blot (immunostained) at different stages in the extraction. Lanes: 1, extract of nondisrupted cells (10 μg); 2, dialyzate precipitate of extract in lane 1 (1 μg); 3, total protein extract using glass beads (20 μg); 4, concentrated debris from cell disruption (15 μg); 5, total extract of cells grown in minimal media (1 μg); 6, control cells transformed with pAB24 (10 μg); 7, molecular weight standards (36 and 29K); 8, standard bovine β-casein (100 ng).

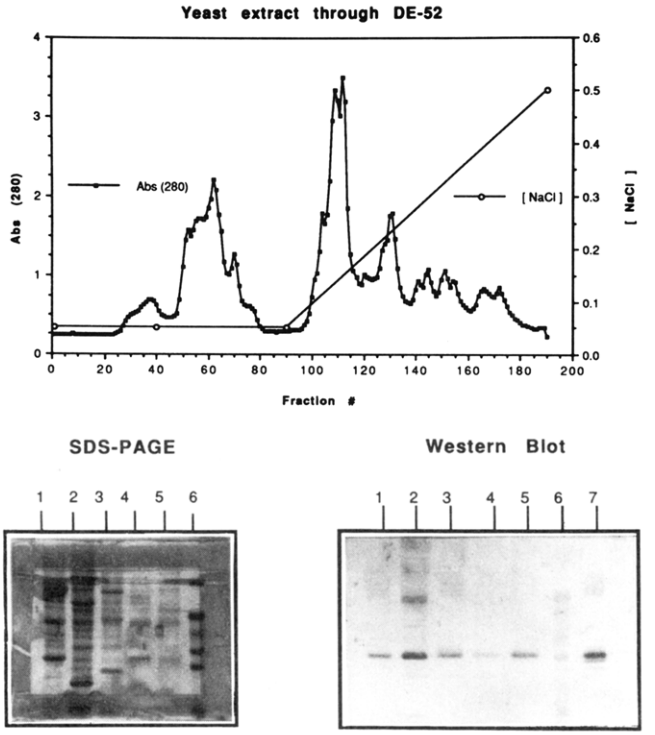

Figure 5. Chromatogram of the total cell extract through DE-52 and parallel SDS-PAGE and Western blot of some of the fractions obtained [lane, fraction]: 1, 100; 2, 110; 3, 130; 4, 145; 5, 155; 6, molecular weight standard (18, 29, 36, 43, 55, 95K); 7, standard β-casein (100 ng).

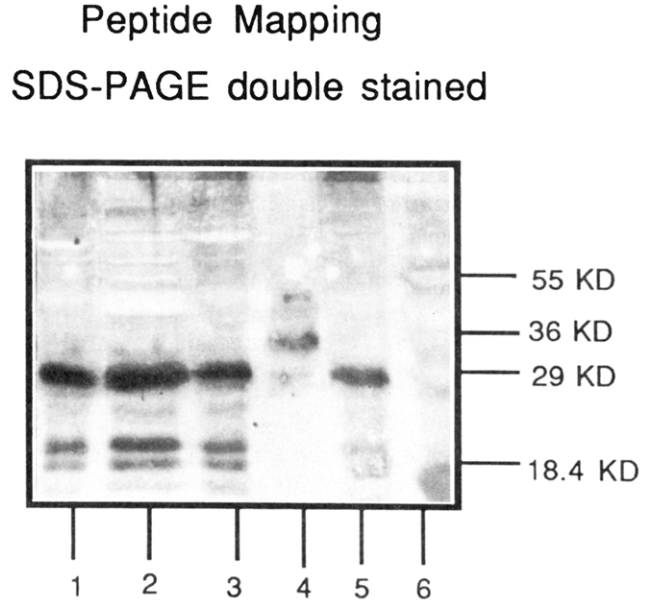

Figure 6. Proteolytic digests of different fractions obtained from the DE-52 column. A Western blot of the chymosin digest of the different fractions was double-stained, first with Coomassie and then by immunostaining using casein antibodies [lane, fraction]: 1, 110; 2, 140; 3, 150; 4, 40; 5, standard β-casein (100 ng) in yeast protein extract (10 μg); 6, molecular weight standard.

that this form of the protein may reside in the periplasmic space.

Purification procedures were carried out in total extracts obtained from glass bead-disrupted cells grown on YPD media. Initial fractionation was achieved by ion exchange chromatography through DE-52 cellulose (Figure 5). All fractions selected (100, 110, 130, 140, 150) for Western blot analysis revealed the presence of some β-casein. When these same fractions were analyzed on urea gels, somewhat different mobilities were observed. This result indicates that the β-casein produced by yeast was heterogeneous in nature, perhaps containing varying numbers of phosphate groups, for example (see below).

To verify the identity of the different casein fractions obtained from ion-exchange chromatography, different protein fractions collected from the ion-exchange column were subjected to partial hydrolysis by trypsin. As control, standard commercial bovine β-casein was added to a protein extract from the yeast strain AB116 to determine the peptide pattern of a digestion of β-casein by trypsin under similar conditions. The results of the proteolytic digests are shown in Figure 6. In fractions 110, 140, and 150 treated with trypsin, a pattern of three peptides identical with that of the control with standard β-casein was observed. Digestion of fraction 40 (lane 4, Figure 6) suggests that the high molecular weight band

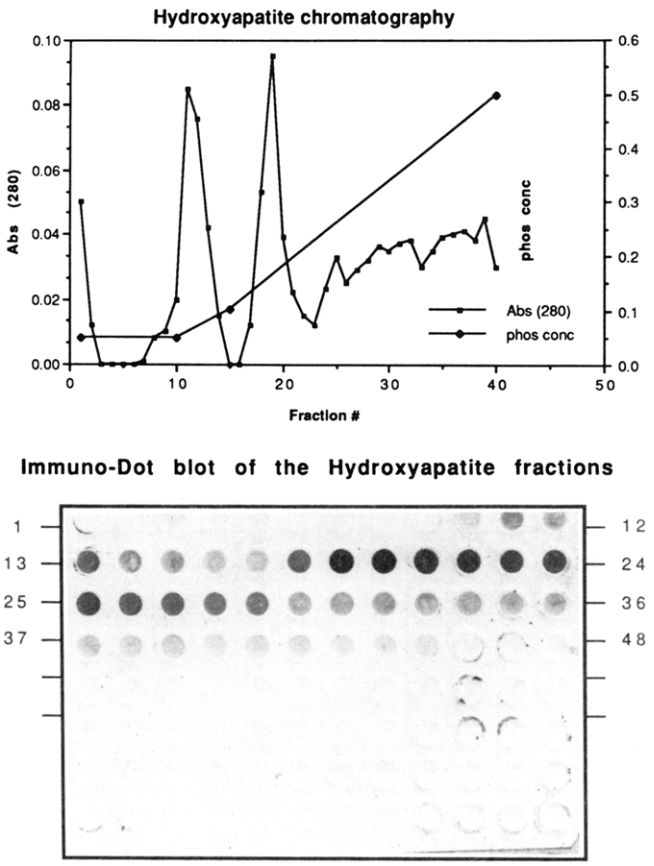

Figure 7. Top: Rechromatography on hydroxyapatite of fraction 155 collected from the DE-52 column. Bottom: Dot blot of HA column fractions, each dot corresponds to 30 μL of each fraction collected (total volume of each fraction 2.5 mL). The intensity of the dots indicates the concentration of casein in each fraction. Fractions 19–25 contain most of the casein.

is more resistant to proteolysis by trypsin.

Characterization of β-Caseins from Yeast. Bovine β-casein produced in cows is modified posttranslationally and contains five phosphate moieties attached to the protein via five closely linked serine groups in the N-terminal region of the mature protein (Eigel et al., 1984). Yeast extracts are known to possess casein kinases (Hathaway and Traugh, 1982), and it was of interest to determine whether β-casein produced in vivo could be phosphorylated in vivo. The fraction requiring the highest salt concentration for elution from DE-52 cellulose (fraction 150) was thought likely to contain protein with a higher negative charge or number of phosphate groups.

The proteins present in fraction 150 were further purified by hydroxyapatite chromatography. The different fractions collected from this column were tested directly for the presence of casein by dot blot analysis. This test was performed after verifying that the fraction loaded onto the HA column yielded a single band upon immunoblotting of an SDS-PAGE gel. A single peak of β-casein was observed upon HA chromatography (Figure 7).

To determine whether the β-casein in this fraction contained any phosphate moieties, the protein was subjected to treatment with calf intestine alkaline phosphatase following extensive dialysis and lyophilization. Standard bovine β-casein served as control. Two different polyacrylamide gels were run for each sample: an alkaline urea gel (which separates proteins mainly by charge) and a SDS gel (which separates proteins largely by molecular weight). The results are shown in Figures 8 and 9. Treatment of β-casein with alkaline phosphatase resulted in the generation of more slowly migrating bands in urea PAGE gels. Comparison of the standard and yeast-produced β-caseins revealed a series of comigrating bands. Approximately five bands were observed in the case of the β-casein obtained from yeast, implying that this protein is multply phosphorylated as it is in the cow. The SDS gels indicated the absence of proteases in the alkaline phosphatase preparation. This finding suggests that β-casein produced in yeast is phosphorylated in vivo, but, although unlikely, we could not rule out the possibility of a rapid phosphorylation in the crude extract prior to protein fractionation.

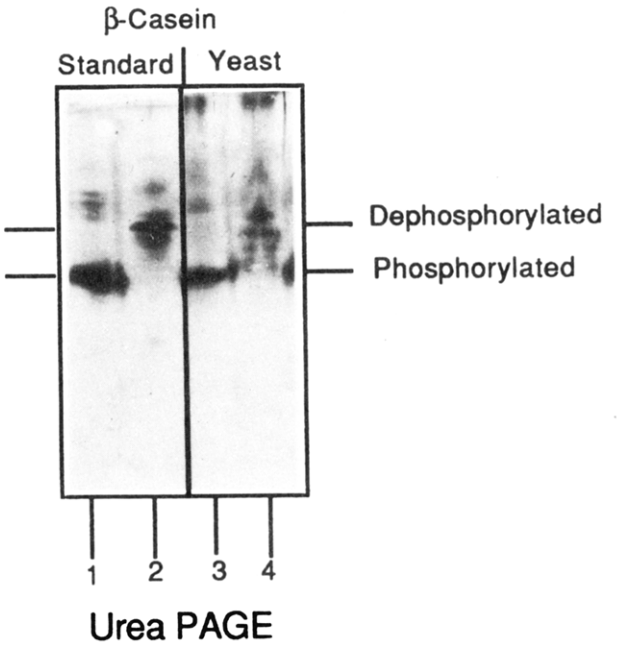

Figure 8. Western blot after urea PAGE of the dephosphorylation treatment of fraction 21 from HA chromatography. Standard β-casein and the proteins of fraction 21 were treated with calf intestinal alkaline phosphatase. Lanes: 1, standard β-casein; 2, standard β-casein dephosphorylated; 3, fraction HA21 of yeast protein; 4, fraction HA21 dephosphorylated (partial dephosphorylation).

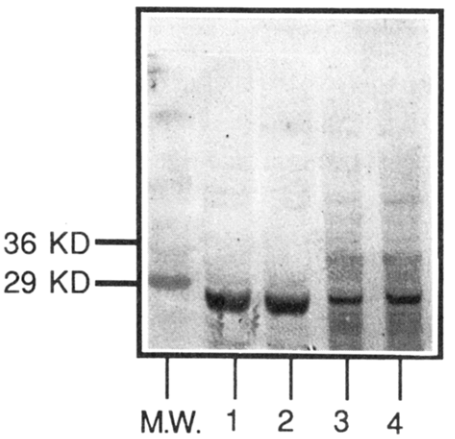

Figure 9. Western blot after SDS-PAGE on dephosphorylated samples using calf intestinal alkaline phosphatase. Lanes: MW, molecular weight standard (29 and 36K); 1, standard β-casein; 2, standard β-casein dephosphorylated; 3, fraction HA21 of yeast protein; 4, fraction HA21 of yeast protein dephosphorylated.

Characterization of the High Molecular Weight β-Casein. In extracts of AB116-2, a second higher molecular weight (36K) form of β-casein was observed. As previously discussed, this form seemed to be easily released from cells, suggesting a periplasmic location. This pro-

tein was found to be highly soluble in contrast to native β-casein. It did not precipitate at low pH or upon dialysis of the urea from extracts and was soluble at ammonium sulfate concentrations greatly exceeding that required to precipitate native β-casein as well as the lower molecular weight form also found in yeast extracts. These observations implied a dramatic modification of solubility resulting from the increase in molecular weight. Also, this form of casein was much more resistant to proteolysis.

β-Caseins that have been chemically glycoslyated are known to be more soluble than the unglycosylated proteins (Lee et al., 1979; Courthaudon et al., 1989). Therefore, we investigated the possibility that the higher molecular weight form was generated by glycosylation. The high molecular weight protein was treated with endoglycosidase H, which specifically cleaves N-linked sugars from proteins. This treatment did not affect the apparent migration of the protein (data not shown). This was not surprising, because the β-casein protein fusion does not contain any putative N-glycosylation sites (Asn-X-Ser/Thr). However, β-caein contains many potential O-glycosylation sites.

The ability of the 36K form of the protein to react with the lectin concanavalin A was investigated. This lectin has been linked to horseradish peroxidase, making it suitable for identification of glycoproteins in a similar way as immunostaining, described above. The high molecular weight protein was partially purified, taking advantage of its property of high solubility. This fraction was found to contain many higher molecular weight proteins that are glycosylated. However, a glycoprotein migrating at the same position as the high molecular weight casein was observed to be glycosylated (Figure 10). In control extracts not containing the expression vector for β-casein, no glycosylated protein was observable at this molecular weight.

As a third test of the potential glycosylation of β-casein, the high molecular weight form was subjected to mild alkaline hydrolysis, known to result in loss of O-glycosylated sugars through β-elimination. This treatment resulted in the formation of a lower molecular weight casein migrating at 29K from the high molecular weight form. These results suggest that the higher molecular weight form is glycosylated.

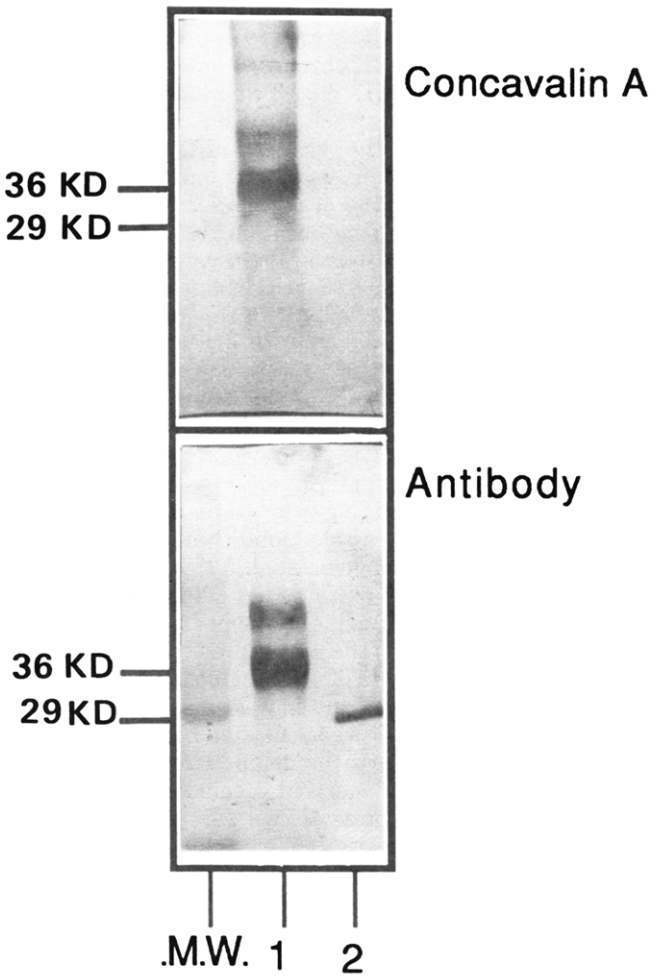

Figure 10. Test for glycosylation of yeast-produced β-casein. Parallel Western blots stained with either antibodies for casein (upper half) or Concavalin A (lower half). Both systems used HRP as active enzyme, and the substrate was 3,3′-diaminobenzidine (DAB). Lanes: MW, molecular weight standard (29 and 36 K); 1, fraction 40 from DE-52 (10 μg); 2, standard β-casein (100 ng).

DISCUSSION

Bovine β-casein was produced in *S. cerevisiae* by a novel yeast expression system relying on promoter and terminator sequences of the structural gene for hexokinase P1. We chose to develop the hexokinase P1 (*HXK1*) system rather than utilize an existing expression system for the following reasons. First, this gene encodes a glycolytic enzyme that is under glucose repression control meaning that high-level expression occurs in the absence of glucose as carbon and energy source or upon glucose exhaustion in the medium. This expression system, therefore, offers many of the same advantages as the inducible GAL promoters, without the strict requirement for galactose. The production of recombinant β-casein by yeast will enable us to study the relationship between the structure of bovine caseins and their functionality. Second, as a particular application, we are interested in exploiting the role of β-casein as a protein fining agent for wine production and ultimately want to study expression of this protein by yeast during vinification, the goal being to construct a yeast strain capable of production of its own protein fining agent. This fining agent would have to be produced at the end of the fermentation and without reliance on the introduction of any inducers (such as galactose or metal ions) that would impair the quality or stability of the wine itself. The *HXK1* gene seemed ideal for this purpose. Indeed, studies of expression of β-casein during growth revealed that it was not produced during growth on glucose until late exponential/early stationary phase of growth.

In the construction of the *HXK1*–β-casein fusion, we wanted to keep the native signal sequence for casein secretion intact to see whether this protein would be secreted by yeast. However, for ease of construction it was necessary to include an additional seven amino acids to the N-terminus of the protein. While signal sequences in yeast are not highly conserved (Kaiser et al., 1987), it is not clear whether this construction would have interfered with signal sequence recognition. Also, the signal sequence is removed during processing of β-casein to the mature form, and it is unclear whether such N-terminal processing will occur in yeast. The signal sequence of chicken lysozyme has been used successfully in its expression and secretion from yeast (Oberto and Davidson, 1985), albeit not very efficiently. Preliminary localization studies revealed that the protein was not being secreted into the medium, since the medium was devoid of β-casein. Casein expression was investigated in a yeast strain, AB116, mutated to reduce proteolytic activity. Transformants of AB116 were found to produce two forms of β-casein, one with a molecular weight 29K as previously seen, and a second

higher molecular weight form of 36K. Significantly less degradation of these polypeptides was observed in crude extracts. Again, the 29K form was only released from cells upon breakage of the cells. Preliminary studies suggest that at least some fraction, if not all, of the 36K form is associated with the yeast cell surface, being selectively extracted by spheroplast production procedures and by extended urea washing of intact cells. These observations imply a periplasmic location for the gene product, which awaits confirmation by in vivo immunolocalization. This would suggest that the bovine signal sequence may function in yeast for export to the periplasm.

Bovine β-casein is processed during secretion in the cow such that the mature protein contains five phosphate groups. The location of the five phosphate moieties to a run of serines at the mature N-terminal region has been well documented; however, this protein contains several other potential phosphorylation sites (Eigel et al., 1984). Proper folding prior to phosphorylation is believed to play a role in selection of serine residues by bovine casein kinase (Bingham et al., 1988; Holt and Sawyer, 1988). Yeast crude extracts possess kinases capable of phosphorylation of bovine β-casein in vitro (Meggio et al., 1986). Therefore, the casein produced in vivo was examined for the presence of organic phosphate. In order to address this question, β-casein was purified from crude extracts. Purification was complicated by the facts that the 29K casein seemed to be comprised of a group of heterogeneous proteins and that casein seemed to form stable associations or aggregates with other proteins during fractionation procedures. We suspected that heterogeneity upon ion exchange may be due to heterogeneity in the number of phosphate groups associated with the protein. We examined the protein form requiring the highest salt concentration for elution from DE-52. Treatment of this protein with calf intestine alkaline phosphatase and examination of protein products on urea PAGE gels revealed that this protein contained multiple phosphate moieties. Both yeast β-casein and the native bovine casein displayed the same mobilities in urea gels before dephosphorylation and changed in the same way following treatment. Additionally, in the lane corresponding to the yeast-produced protein, several bands are apparent, which indicates partial dephosphorylation (observed occasionally in bovine β-casein). The exact location of the phosphate residues on the protein is not known; however, the analysis and characterization of casein kinase II from yeast (Hathaway and Traugh, 1982; Kuenzel et al., 1987; Chen-Wu et al., 1988; Meggio et al., 1986; Padmanabha and Glover, 1987) seem to indicate that it may be very similar to the kind of phosphorylation found in bovine caseins (Bingham et al., 1988). Casein kinase II is structurally a very similar enzyme in many different species (i.e., yeast, fly, and rat). Its function is also conserved in many species: it phosphorylates Ser and Thr residues in protein substrates, where a cluster of acidic residues immediately C-terminal to the modified amino acid appears to be important for recognition. Therefore, β-casein produced in yeast seems to be phosphorylated in vivo.

The nature of the 36K form of casein was also investigated. We could not account for the increase in molecular weight (7K) from failure to process the 17 amino acid leader or from read through into the terminator regions of the *HXK1* construct. The β-casein translational terminator was unaltered in the construction. Other possible protein modifications accounting for dramatic increases in molecular weight include glycosylation. The β-casein construct does not include a single N-glycosylation consensus site (Asn-X-Ser/Thr) so it is unlikely that this protein is N-glycosylated. However, O-glycosylation is not precluded. Several lines of evidence suggest that the 36K protein is indeed glycosylated. First, there is a dramatic increase in solubility of the 36K protein as compared to the 29K form, and chemical glycosylation is known to increase solubility of the commonly insoluble caseins (Courthaudon et al., 1989). Second, the 36K protein during purification seemed to fractionate and copurify in a manner similar to that of other glycosylated proteins in the yeast extract. This form was more resistant to proteolysis, as might be examined by the presence of bulky carbohydrate groups. The 36K form, when partially purified, seemed to react with the lectin concanavalin A, suggesting glycosylation, and finally, mild alkaline hydrolysis yielded a 29K protein from the 36K form, again consistent with β elimination of O-glycosylated carbohydrate. Final confirmation of glycosylation will require purification of the 36K protein to homogeneity, which is in progress. It is interesting to note that we only observed the 36K form in a strain greatly overproducing β-casein and that this form seemed to be located in the periplasmic space. Overproduction of proteins in yeast generally leads to secretion, since secretion is the default pathway rather than transport to the vacuole [reviewed in Pfeffer and Rothman (1987)]. This may explain, at least partially, the observed localization of the protein. Many secreted proteins are glycosylated, and O-glycosylation has not been thoroughly studied in yeast (Kukuruzinska et al., 1987). If the 36K protein is indeed O-glycosylated, this may represent an opportunity for the further analysis of O-glycosylation in *Saccharomyces*.

In summary, the *HXK1* regulatory sequences were successfully exploited in the construction of an expression vector for bovine β-casein in *S. cerevisiae*. The pattern of expression was consistent with the known regulatory mechanisms for *HXK1* expression. The protein produced in yeast seems to be present in heterogeneous forms, differing in extent of phosphorylation, apparent cellular localization, and glycosylation. Further studies are under way to elucidate the nature of the protein modifications during in vivo expression of β-casein.

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