

## Secretion of Bovine $\beta$ -Casein by *Saccharomyces cerevisiae*

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**Yeast expression plasmids containing an appropriate leader sequence and bovine  $\beta$ -casein cDNA were constructed to produce  $\beta$ -casein for the study of its functional characteristics. Two kinds of expression systems for  $\beta$ -casein were constructed using pCGY1444 as a precursor plasmid. This plasmid is a yeast-*E. coli* shuttle vector which contains the chelatin promoter. The plasmid pISB202 contains the invertase leader sequence and  $\beta$ -casein gene. The plasmid pDEB303 contains the original bovine  $\beta$ -casein leader sequence gene. These two plasmids were introduced into *S. cerevisiae* AB116 which is a strain deficient in the major yeast proteinases. Each clone was grown in minimal media for 24 h before induction by CuSO<sub>4</sub>. The cells were thus grown under expression conditions. Both strains harbouring pISB202 and pDEB303 expressed bovine  $\beta$ -casein. The  $\beta$ -casein was detected using immunochemical staining after western blot. Secretion of  $\beta$ -casein was detected in the culture broth. The estimated amount of secreted  $\beta$ -casein was approximately 50  $\mu$ g/l.**

Bovine milk proteins are some of the most widely consumed human food proteins which makes them good candidates for use as models for genetic modification of their functionalities. The caseins are the major protein fraction in milk and are comprised of 4 major polypeptide families;  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -caseins (14). All of the bovine caseins have been cloned (3) and some have been expressed in *E. coli* (11).

Bovine  $\beta$ -casein is modified post-translationally with the addition of five phosphate groups to the protein (6). Such modification does not occur *E. coli*. Numerous heterologous proteins have been secreted in *S. cerevisiae* (4, 7). We have previously reported the expression of  $\beta$ -casein in *S. cerevisiae* ABY116-2 using a fusion to the HXK1 (hexokinase P1) gene (9). But this strain did not secrete the  $\beta$ -casein into the culture broth. Therefore, we sought to obtain expression and secretion of  $\beta$ -casein in the eukaryotic microorganism, *Saccharomyces cerevisiae*, because secretion of the protein into the culture broth will simplify protein recovery and purification.

In this paper, we describe the construction of bovine  $\beta$ -casein expression systems for *S. cerevisiae* harbouring the invertase leader sequence and the  $\beta$ -casein leader

sequence, respectively, using pCGY1444 vector containing the chelatin promoter. We show that these systems express and secrete the  $\beta$ -casein whose molecular weight and antigenicity are consistent with bovine  $\beta$ -casein.

### MATERIALS AND METHODS

#### Strains, Growth Conditions, Plasmids and DNA

*E. coli* DH5 $\alpha$  was used for bacteria transformation and plasmid preparations. Standard conditions and media were used for the growth of *E. coli* (13). *S. cerevisiae* AB116 (*MAT $\alpha$* , *leu2*, *trp1*, *ura3-52*, *prb1-1122*, *pep4-3*, *prc1-407*, *cir<sup>o</sup>*) (10) was used for the expression of  $\beta$ -casein. This strain is deficient in proteinase A, B and C. The conditions for the growth of yeast was at 30°C, 250 rpm using minimal media (yeast nitrogen base w/o amino acid and ammonium sulfate 1.7 g, ammonium sulfate 5 g per liter, 2% glucose). After growing of the yeast, the induction solution (30 mM CuSO<sub>4</sub>, 2% glucose) was added and growth was continued. The plasmid pCGY1444 and the invertase leader sequence gene were kindly supplied by Dr. Hitzeman of Genentech, Inc., South San Francisco, CA. The plasmid pJR1 was used as the source of cDNA for bovine  $\beta$ -casein (8). The oligonucleotides for synthetic linkers were purchased

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from Operon Technologies Inc (Alameda, CA).

### General DNA and Protein Methods

Manipulations of subcloning in *E. coli* were described in Maniatis *et al.* (13). Procedures for transformation and plasmid isolation in *S. cerevisiae* were performed according to Ausubel *et al.* (1). Protein analysis using SDS-PAGE was performed according to Laemmli (12), and immunoblot transfers according to Bers and Garfin (2). After electrophoresis, the proteins were blotted onto nitrocellulose membranes and tested for the presence of  $\beta$ -casein with antibodies against bovine caseins. Primary antibody (rabbit antiserum) for bovine caseins was purchased from Calbiochem (La Jolla, CA). The secondary antibody, goat anti rabbit IgG-horseradish peroxidase (GAR-HRP), was purchased from Bio-Rad Lab. (Richmond, CA). To prevent non-specific immunostaining, the primary antibody was pre-adsorbed with a cell free extract of *S. cerevisiae* AB116 harbouring pCGY1444. The intensities of the  $\beta$ -casein bands in western blot were compared with the various concentrations of standard bovine  $\beta$ -casein, and the amounts of expressed  $\beta$ -casein were estimated from a standard curve prepared after laser densitometry of the standard  $\beta$ -casein.

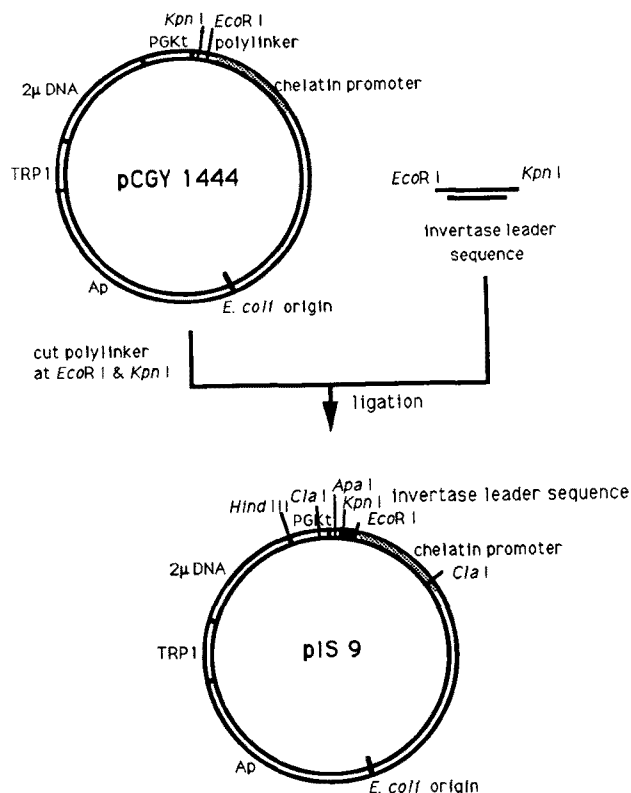
### DE52 Anion Exchange Chromatography

The anion exchange chromatographic procedure was used for the concentration and partial purification of the secreted  $\beta$ -casein in the culture broth. DE52 anion exchange (Whatman) column chromatography was performed by a modification of the method of Davies and Law (5). Fractions were eluted using a 0.03 to 0.3 M NaCl (in 3 M urea, 0.005 M tris, pH 8.2, buffer) linear gradient. Each fraction from the chromatography were analyzed using dot blot and immunochemical methods. Fractions giving positive signals were further analyzed using SDS-PAGE, western blotting and immunochemical staining.

## RESULTS AND DISCUSSION

### Construction of the Yeast Expression Systems for Bovine $\beta$ -Casein

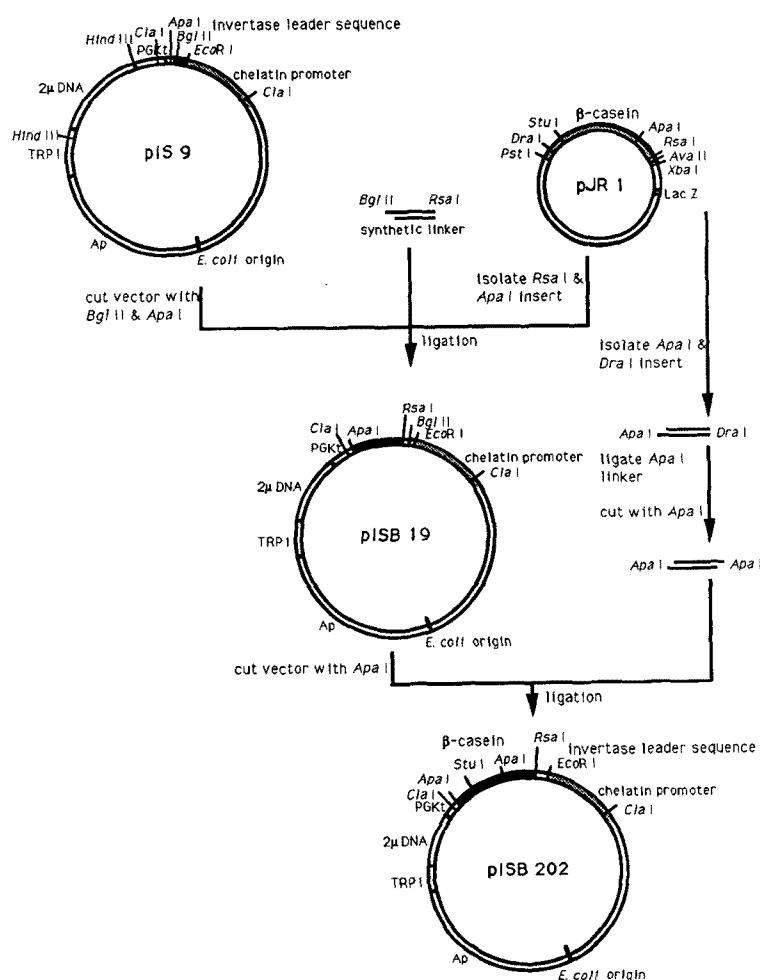
We constructed two kinds of expression systems; one is pISB202 harbouring the invertase leader sequence, and the other is pDEB303 harbouring the original bovine  $\beta$ -casein leader sequence coupled to the  $\beta$ -casein cDNA. The *E. coli* and yeast shuttle vector pCGY1444 was used to construct two kinds of expression systems for bovine  $\beta$ -casein. The plasmid pCGY1444 contains the chelatin promoter, the phosphoglycerate kinase terminator, the 2  $\mu$ m DNA origin of replication, *TrpI*, the ampicillin resistance marker and a polylinker region. The  $\beta$ -casein cDNA was obtained from pJR1. For the construction of the pISB202, the invertase leader sequence



**Fig. 1a. Schematic diagram representing the subcloning of the invertase leader sequence into a precursor plasmid, pCGY1444.**

The resulting plasmid pIS9 was used for the construction of the expression plasmid pISB202 containing the invertase leader sequence.

was inserted between the *KpnI* and *EcoRI* sites of pCGY 1444, yielding pIS9 (Fig. 1a). Two  $\beta$ -casein fragments, *RsaI/ApaI* (168 bp) and *ApaI/DraI* (539 bp) fragments were inserted into the pIS9 sequentially (Fig. 1b). These inserted DNA sequences of  $\beta$ -casein (707 bp) are only the coding sequence for the bovine  $\beta$ -casein protein. The restriction map of pISB202 is shown in Fig. 2. There are two fragments (540 bp, 180 bp) for  $\beta$ -casein when the plasmid pISB202 digested with *BglII* and *ApaI*. Also, the *StuI* and *ClaI* digestion generated two fragments (1.6 kb, 280 bp). From these restriction patterns, we can confirm that the pISB202 contains the bovine  $\beta$ -casein gene and the inserted orientations of these fragments are correct. The *StuI* site is situated in the *ApaI/DraI* (539 bp) fragment asymmetrically. On the other hand, the pDEB303 was constructed as shown in Fig. 3. Preferentially, the 5'-region of  $\beta$ -casein (*AvaII/ApaI* fragment, 229 bp) containing the original bovine  $\beta$ -casein leader sequence was inserted into pCGY 1444 using an *EcoRI/AvaII* synthetic linker. And then, the 3'-region of  $\beta$ -casein (*ApaI/DraI* fragment, 539 bp)



**Fig. 1b. Insertion of the bovine  $\beta$ -casein cDNA coding for bovine  $\beta$ -casein into pIS9.**

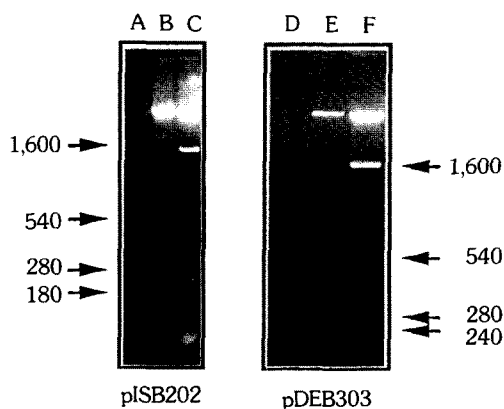
The *Rsa*I/*Apa*I fragment (168 bp) of the 5'-region of  $\beta$ -casein cDNA was inserted into pIS9 using *Bgl*III/*Rsa*I synthetic linker, yielding pISB19. Then the *Apa*I/*Dra*I fragment (539 bp) for the 3'-region of  $\beta$ -casein cDNA was isolated, ligated to the *Apa*I oligonucleotide linker, generating the *Apa*I/*Apa*I fragment cut with *Apa*I. This *Apa*I/*Apa*I fragment was inserted into pISB19 at the *Apa*I site, yielding the expression plasmid pISB202. The invertase leader sequence codes for 18 amino acids.

was inserted, generating plasmid pDEB303. The restriction map of plasmid pDEB303 yielded two fragments (540 bp, 240 bp) when digested with *Apa*I and *Eco*RI, and two fragments (1.6 kb, 280 bp) when digested with *Stu*I and *Cla*I (Fig. 2).

#### Expression of $\beta$ -Casein by Yeast

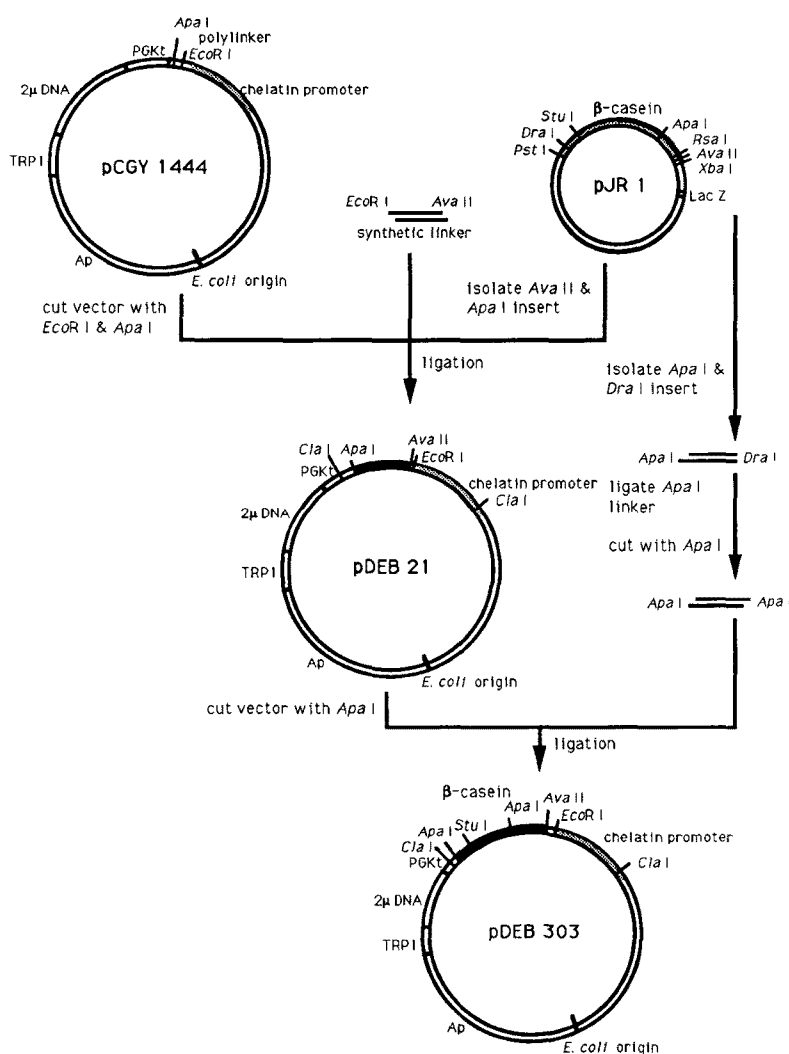
*S. cerevisiae* AB116 was transformed with pISB202 and pDEB303 respectively. The transformed yeast cells were plated in supplementary media lacking tryptophan. The colonies from the plates were restreaked onto the same plate again. Both transformants were screened for expression of  $\beta$ -casein. This was done by growth in minimal media, supplemented with uracil and leucine. The cells were grown in the media for 24 h, and then the induction solution (30 mM  $\text{CuSO}_4$ , 2% glucose) was ad-

ded and growth was allowed for an additional 24 to 36 h. After cultivation, the cells were sedimented and the cell pellets were extracted. *S. cerevisiae* AB116-234 (harbouring pISB202) and *S. cerevisiae* AB116-314 (harbouring pDEB303) both produced bovine  $\beta$ -casein. The presence of  $\beta$ -casein in the cell extracts was detected using SDS-PAGE, western blotting and immunochemical staining (Fig. 4). The expression level could not be measured accurately, however, western blots containing  $\beta$ -caseins from these transformants along with different concentrations of standard  $\beta$ -casein allowed the estimation of approximately 0.5~1 mg of  $\beta$ -casein produced from the cell mass of one liter culture. Also, these yeast transformants maintained both plasmids as authentic plasmids (Fig. 5).



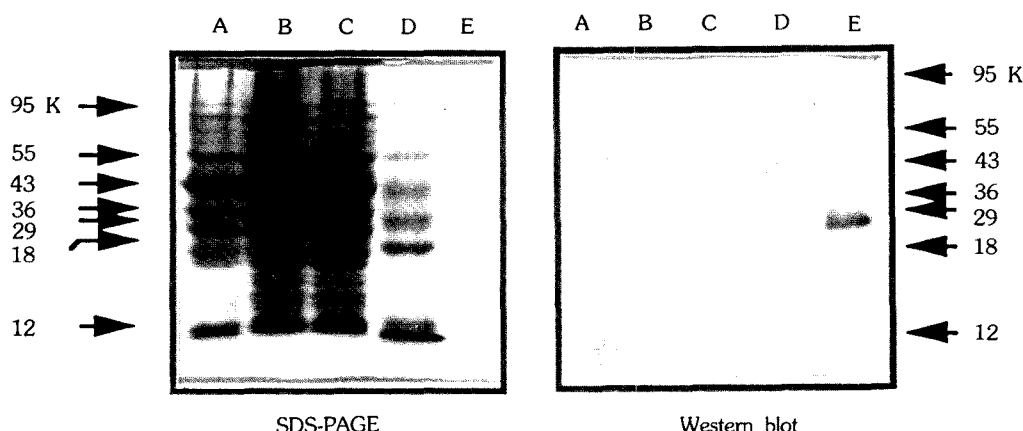
**Fig. 2. Agarose gels of the restriction fragments of the constructed plasmid pISB202 and pDEB303 containing the  $\beta$ -casein cDNA insert.**

Lane A and D, molecular weight markers, pBR322 digested with *Hae*III, Lane B, plasmid pISB202 digested with *Apa*I and *Bgl*II, Lane C, plasmid pISB202 digested with *Stu*I and *Cla*I, Lane E, plasmid pDEB303 digested with *Apa*I and *Eco*RI, Lane F, plasmid pDEB303 digested with *Stu*I and *Cla*I.



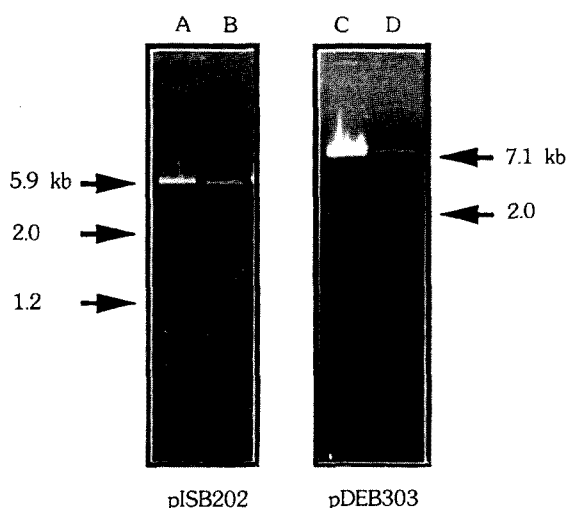
**Fig. 3. Schematic diagram representing the construction of bovine  $\beta$ -casein expression plasmid pDEB303 containing the  $\beta$ -casein leader sequence.**

The *Ava*II/*Apa*I fragment (229 bp) from the 5'-region of  $\beta$ -casein cDNA harbouring the  $\beta$ -casein leader sequence was inserted into pCGY1444 using an *Eco*RI/*Ava*II synthetic linker, yielding pDEB21. Then the *Apa*I/*Apa*I fragment generated from the 3'-region of  $\beta$ -casein cDNA (*Apa*I/*Dra*I fragment, 539 bp) was inserted into pDEB21 at the *Apa*I site. The resulting plasmid pDEB303 contained the leader sequence of  $\beta$ -casein coding for 15 amino acids.



**Fig. 4.** SDS-PAGE and western blot patterns of the extract of strains AB116-234 and AB116-314 cells transformed with the plasmid pISB202 and pDEB303, respectively.

Lane A, cell extract of the strain harbouring pCGY1444 as a control, Lane B, cell extract of AB116-234, Lane C, cell extract of the AB116-314, Lane D, prestained molecular weight standards (mid range kit, Diversified Biotech. Inc.), Lane E, standard bovine  $\beta$ -casein.

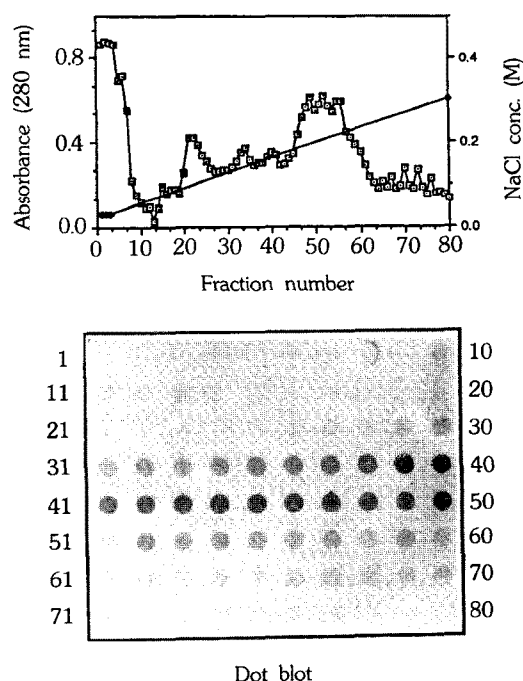


**Fig. 5.** Agarose gels of the digestion of plasmid pISB202 and pDEB303 which were harboured in yeast transformants.

Lane A, transformed pISB202 digested with *Bgl*II and *Hind*III, Lane B, authentic pISB202 digested with *Bgl*II and *Hind*III, Lane C, transformed pDEB303 digested with *Hind*III, Lane D, authentic pDEB303 digested with *Hind*III.

### Secretion of $\beta$ -Casein into Culture Broth

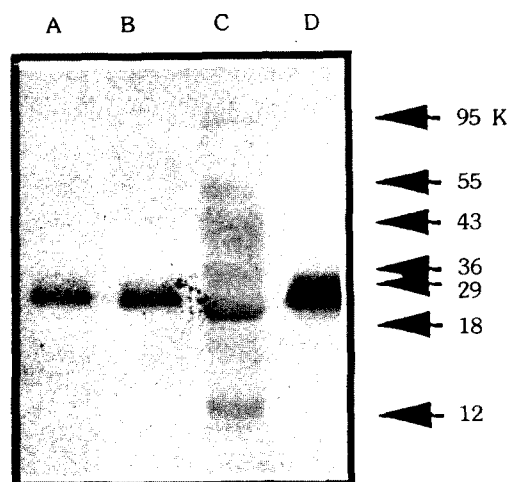
Study of the structure and function relationship for  $\beta$ -casein would require a purified protein from the microorganism. The constructed plasmid pISB202 contained the invertase leader sequence for secretion, and also, the constructed plasmid pDEB303 contained the bovine  $\beta$ -casein leader sequence which is conserved among caseins in mammalian systems (3). The invertase signal was more effective in the release of human inter-



**Fig. 6.** DE52 anion exchange chromatography of secreted  $\beta$ -casein and dot blot analysis of the fractions.

$\beta$ -Casein secreted into the culture broth of strain AB116-234 was applied to the column and eluted using 0.03~0.3 M NaCl linear gradient. Fractions were analyzed using dot blotting and immunochemical staining.

feron than a mammalian secretion signal in the yeast system (4). To concentrate secreted  $\beta$ -casein, the culture broth was applied to a DE52 anion exchange column. After the column chromatography, the  $\beta$ -casein fractions were detected using dot blotting and immunochemical



**Fig. 7. Western blot patterns of concentrates of the positive fractions obtained from DE52 chromatography.**

Lane A, the concentrate of the culture broth from strain AB116-234, Lane B, the concentrate of the culture broth from the strain AB116-314, Lane C, molecular weight standards, Lane D, standard bovine  $\beta$ -casein.

staining (Fig. 6). Both expression systems yielded very similar results in the chromatographic profile and dot blot pattern (the data for strain AB116-314 are not shown). The  $\beta$ -casein fractions were collected, dialyzed, and freeze-dried. In Fig. 7, it is evident that  $\beta$ -casein is secreted into the culture broth of both expression systems. These two secreted  $\beta$ -caseins had the same molecular weight as that of natural bovine  $\beta$ -casein, and the amounts of secreted  $\beta$ -caseins were almost the same for both expression systems. The difference in the two expression systems is in the secretion stage during the growth. The invertase leader sequence system (strain AB116-234) secreted  $\beta$ -casein at an earlier stage than the  $\beta$ -casein leader sequence system (strain AB116-314). The estimated amount of secreted  $\beta$ -casein was approximately 50  $\mu$ g per liter of culture broth. The percentage of secreted  $\beta$ -casein of the total expressed  $\beta$ -casein were only 5–10%. This fact means that the optimization of secretion conditions are needed. Previous results obtained using the HXK1 (hexokinase P1)- $\beta$ -casein expression system (9) indicated that the highest expression level of  $\beta$ -casein was 10 mg per liter of culture under optimized conditions, however, HXK1 system did not secrete the  $\beta$ -casein. Although the expression levels of the chelatin promoter systems were lower than for the HXK1 system, these expression systems secreted the  $\beta$ -casein into culture broth.

It is not clear whether these secreted  $\beta$ -caseins are exactly the same as the natural bovine  $\beta$ -casein. Proof would require N-terminal amino acid sequence analysis of these secreted  $\beta$ -caseins.

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