Antigenicity of the 139−149 α_{s1}-Casein Region in Different Species Revealed by ELISA and Immunoblotting Using Antipeptide Antibodies

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The antigenicity of the 139−149 α_{s1}-casein region has been studied by using polyclonal antibodies raised against four different peptides. Two antisera, directed against the bovine 141−148 and 139−149 α_{s1}-casein sequences, were found to be cow-specific in competitive ELISA tests. By immunoblotting, the recognized components corresponded to a set of minor bands distinct from the bovine α_{s1}-casein, also detected by polyclonal antibodies against purified bovine α_{s1}-casein fraction. The anti-(141−147) sequence stretch, common to bovine, water buffalo, caprine, and ovine α_{s1}-casein, recognized the main α_{s1}-casein components from the four milks, whereas the anti-(141−148) sequence stretch, present in water buffalo, caprine, and ovine α_{s1}-casein, detected only minor α_{s1}-casein components. Applications of these polyclonal antipeptide antibodies are suggested for the detection of bovine milk in mixture with milk from different species and for the evaluation of specific minor casein components.

Keywords: *Peptide antigens; polyclonal antibodies; α_{s1}-casein; ELISA; immunoblotting*

INTRODUCTION

Caseins are strictly conserved proteins (Swaisgood, 1992) so that polyclonal antibodies against each of the four bovine α_{s1}-, α_{s2}-, β-, and κ-casein fractions recognized both ovine and caprine counterparts (Chianese et al., 1992a,b) as well as large peptides arisen from the limited proteolysis of bovine casein (Addeo et al., 1995). Ovine α_{s1}-casein has been found to be constituted by two protein forms containing 191 and 199 amino acid residues differing for the deleted peptide 141−148 (Ferranti et al., 1995), whereas bovine α_{s1}-casein is only one peptide chain 199 residues long. Both the short and the long forms of five genetic ovine α_{s1}-casein variants at various phosphorylation levels were simultaneously recognized by polyclonal antibodies against bovine α_{s1}-casein (Chianese et al., 1996). The degree of identity between ovine (Ferranti et al., 1995) and caprine (Brignon et al., 1989) α_{s1}-casein is 97.9%, whereas the homology of the ovine with bovine counterpart is lower, 89%, owing to the presence of the long form. Therefore, tools more specific than polyclonal antibodies are needed to discriminate among homologous caseins from different species. To detect bovine milk in mixture with either ovine or caprine milk, one may specifically detect α_{s1}-casein long protein chain by taking advantage of the charged amino acid residue substitution differentiating ovine (Ferranti et al., 1995) and caprine (Brignon et al., 1989) from the bovine one, e.g. Gln^{148} instead of Glu^{148} in cow.

Closely related proteins have been distinguished in a number of cases, by using antisera as a probe for a specific substructure within the protein by using antipeptide antibodies technology (Groome, 1994). Obviously, the recognization of the whole protein by antipeptide antibodies can occur provided that the selected peptide contains at least one of the protein's epitopes and the synthetic peptide used as immunogen is able to mimic it. Polyclonal antibodies against the bovine α_{s1}-casein 140−149 fragment were found to be bovine species-specific (Rolland et al., 1993), and those against the whole protein were able to recognize its tryptic fragment 133−151 (Ametani et al., 1987).

In this work, by using competitive ELISA tests with antipeptide sera toward closely related synthetic peptides belonging to the 139−149 α_{s1}-casein region, the specificity of four antisera was studied to recognize parent α_{s1}-casein. The results of the competitive ELISA tests were compared with the immunoblotting experiments on whole casein from different species. An unexpected complexity was discovered: the bovine α_{s1}-casein consisted of the main α_{s1}-casein component, already sequenced by Mercier et al. (1971), and other minor components being probably nonallelic forms of mature protein. Three of our antipeptide antibodies did not recognize the long form of α_{s1}-casein from the bovine, caprine, ovine, and water buffalo species, even if the polyclonal antibodies were directed against actual sequences of the parent proteins, but only some minor components, the structural features of which remain to be identified.

MATERIALS AND METHODS

Synthetic Peptides, Conjugation to the Carrier, and Production of the Antisera. Peptides carrying segments of the amino acid sequences of α_{s1}-casein from different species were chemically synthetized by Neosystem S.A. (Strasbourg, France). They corresponded to the fragment 141−147, ELAYFYP (E7P), common to the four species of α_{s1}-casein; to the fragments 139−149, NQELAYFYPEL (N11L), and 141−148, ELAYFYPE (E8E), both specific for bovine α_{s1}-casein (Mercier et al., 1971); and to the fragment 141−148, ELAYFYPQ (E8Q), common to ovine (Ferranti et al., 1995), water buffalo (P. Ferranti, Servizio di Spettrometria di Massa, CNR, Naples, Italy, 1996, unpublished results), and caprine (Brignon et al., 1989) α_{s1}-casein, differing from the bovine counterpart for the E^{148}(bovine) → Q^{148}(other species) amino acid substitution.

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Crude preparation was fractionated by reversed-phase HPLC using a C_{18} Nucleosil column (150 × 4.6 mm) at a flow rate of 1.2 mL/min. Solvent A was 0.1% triethylammonium phosphate buffer (TEAP) at pH 3; solvent B was acetonitrile/0.1% TEAP. Elution was performed by a linear gradient from 5 to 40% solvent B over 20 min. The column effluent was monitored at 210 nm. Peptides eluted from the column were manually collected, dried down, and stored at −20 °C. The identity of the synthetic peptides was checked by fast atom bombardment mass spectrometry (FAB-MS) according to the procedure previously reported (Ferranti et al., 1995). Found quasi-molecular mass values (MH^+) were in full agreement with those theoretically deduced from the amino acid sequence: E7P, $m/z = 902$; N11L, $m/z = 1386$; E8E, $m/z = 1031$; E8Q, $m/z = 1030$.

To increase their immunogenicity, the peptides were conjugated through glutaraldehyde cross-linking to keyhole limpet hemocyanin (KLH) (1 μmol of peptide/1 mg of carrier) in phosphate-buffered saline (PBS: 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4), essentially according to the procedure reported by Reichlin (1980) followed by an extensive dialysis against PBS to eliminate the soluble contaminants. Each conjugated was used to immunize two rabbits; from six bleedings about 80 mL of antiserum per rabbit was obtained. They were labeled as anti-N11L, -E8E, -E8Q, and -E7P to indicate the target antigen.

Milk Collection and Casein Preparation. Milk from cow, ewe, goat, and water buffalo was drawn from private herds. Fat was removed by centrifugation at 3500 rpm for 10 min at 4 °C.

Whole casein was obtained from defatted milk by isoelectric precipitation at pH 4.6 according to the procedure of Aschaffenburg and Drewry (1959). The pellets were dissolved in water, the pH was adjusted to neutrality with 2 N NaOH, and the solution was lyophilized. The sodium caseinate was weighed and dissolved in PBS at the desidered concentration.

Enzyme-Linked Immunosorbent Assay (ELISA). All of the immunoassays belong basically to the "antibody capture assay" type; that is, the solid support is first coated with the antigen, and then the antibody is allowed to bind.

Indirect ELISA Assay. Stock solutions of the synthetic peptides (500 μg/mL) in PBS were prepared. Three aliquots of 100 μL of serial dilutions in PBS of stock solutions ($1:5^n$, $n = 1 \div 6$) were added to the wells of the microtiter plate (Falcon, cod. 3911) and incubated overnight at 4 °C for absorption. The wells were washed twice with PBS and then filled with blocking buffer (10% v/v heat inactivated horse serum in PBS) to saturate the remaining sites for protein binding.

After 1 h at 37 °C, the wells were emptied and 100 μL amounts of antiserum diluted in the blocking buffer were incubated in every well for 1 h at 37 °C in a humid atmosphere. The suitable dilution of each antiserum was determined in preliminary experiments as that which gave the highest binding to its respective antigen, keeping the background value below 10% of the maximal signal. According to this principle, it sufficed to use 1:2000 (v/v) dilutions of each antiserum with the exception of anti-E8Q, of which a 1:1000 (v/v) dilution was required.

At the end of the incubation in antibody excess, the unbound material was removed by washing five times with PBS. Then 100 μL aliquots of a 1:2000 (v/v) diluted goat anti-rabbit IgG antibody labeled with horseradish peroxidase (Bio-Rad, Hercules, CA) were added to reveal rabbit antibodies attached to the well. Following incubation for 1 h at 37 °C in a humid atmosphere and five washings with PBS, 100 μL amounts of a solution of tetramethylbenzidine (Sigma, St. Louis, MO), 0.1 mg/mL, in 50 mM citrate−phosphate buffer and 10% (v/v) DMSO, pH 5.0, containing 0.01% (v/v) hydrogen peroxide were put in each microtiter well. After 10−15 min at room temperature, the reaction was stopped by adding 100 μL of 1 M H_2SO_4. The resulting absorbance was measured automatically at 450 nm with a Model 450 microplate reader (Bio-Rad).

Nonspecific binding of the antibodies to the plates was determined in at least three wells treated with 100 μL of PBS instead of antigen solution, and the mean value was subtracted from the signals obtained.

Competitive Assay. All of the competitive assays were performed in microtiter plates coated with peptide antigens. To set up the experiments, first the amount of each standard antigen solution needed to coat the plate was titrated versus a fixed, high concentration of the respective primary antibodies. Then the values were plotted, and the lowest levels that yielded a strong signal were selected. N11L peptide at 1 μg/mL, E8Q and E7P peptides at 20 μg/mL, and E8E at 10 μg/mL were chosen as standard coating solutions. Next, using plates coated with these amounts of standard antigen solutions, primary antibodies were titrated and the linear portions of each curve were located. Analysis of peptide competitions was performed in maximum range by using the antisera dilutions that had yielded between 90 and 95% of the saturation signals in titration curves (anti-N11L, 1:8000; anti-E8E and anti-E7P, 1:4000; anti-E8Q, 1:2000 v/v). Casein competitions were more effective using antisera at dilutions giving linear response in titration curves (anti-N11L, 1:16000, anti-E8E and anti-E7P, 1:8000; anti-E8Q, 1:4000 v/v).

After peptide coating and saturation steps, performed as described above for indirect ELISA assay, 50 μL of serial dilutions ($1:5^n$, $n = 1 \div 6$) of the competitive antigens was introduced in the wells before 50 μL of antisera diluted in the blocking buffer at final values specified above was added.

The subsequent steps of washing, anti-rabbit enzyme-linked antibody reaction, and revelation were identical to those performed in the indirect ELISA assay.

Electrophoretic Separation Methods. Isoelectric focusing on thin-layer polyacrylamide gel (PAGIF) was carried out as described by Trieu-Cuot and Gripon (1981). The pH gradient in the range 2.5−6.5 was obtained by mixing Ampholine (Pharmacia, Uppsala, Sweden) 2.5−5, 4.5−5.4, and 4−6.5 in the ratio 1.6:1.4:1 (v/v/v). The gel was stained with Coomassie Brilliant Blue G-250 as described by Blakesley and Boezi (1977). Vertical polyacrylamide gel electrophoresis (disc-PAGE) in presence of 6.1 M urea was carried out at pH 8.6 as described by Chianese et al. (1992).

Immunoblotting. After gel electrophoresis, the proteins were carefully transferred by capillary diffusion from the gel onto nitrocellulose paper. Immunodetection was carried out using rabbit polyclonal antipeptide antisera as primary antibodies and horseradish peroxidase-labeled rabbit immunoglobulins as secondary antibodies. The procedure was essentially that already described by Chianese et al. (1992), but the blocking solution was replaced with PBS containing heat-inactivated horse serum at 10% in volume.

RESULTS

Antisera Performances in ELISA Tests. The specificity of each antiserum toward the different immunogens was first investigated through an indirect ELISA assay (Figure 1). In spite of the similarity among the antigen peptides, only E8Q in addition to the E8E peptide was recognized by anti-E8E (Figure 1C). Preimmune serum showed no activity against any of the synthetic peptides (results not shown).

To obtain a more reliable response for each antigen, inhibition ELISA tests were carried out by using plates coated with the respective original peptide antigen (Figure 2). As shown in Figure 2A, only anti-N11L retained its striking specificity for its N11L antigen. Anti-E8E recognized both the N11L and E8Q peptides, in addition to its antigen E8E (Figure 2C). Moreover, anti-E8Q also recognized both E8E and E8Q, which confirms that the two closely related peptides share similar immunogenicity (Figure 2D). As expected, anti-E7P recognized the four synthetic peptides containing the common stretch 141−147 (Figure 2B), whereas none of the antisera was able to interact with the E7P peptide, except anti-E7P.

Application of Antisera to Casein Detection in ELISA Tests. To assess the reactivity of the four antipeptide antisera toward the parent proteins, ELISA

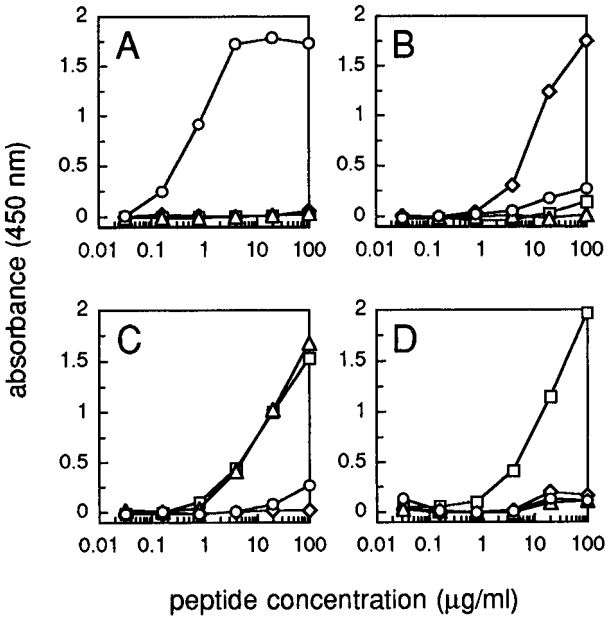

Figure 1. Reactivity of anti-N11L (A), anti-E7P (B), anti-E8E (C), and anti-E8Q (D) antisera toward N11L (○), E7P (◇), E8E (△), and E8Q (□) peptides in indirect ELISA tests. Antisera anti-N11L, anti-E7P, and anti-E8E were used at dilutions of 1:2000 (v/v), and anti-E8Q was used at a dilution of 1:1000 (v/v). Details on the methodology for the revelation of the antibodies attached to the well are given under Materials and Methods.

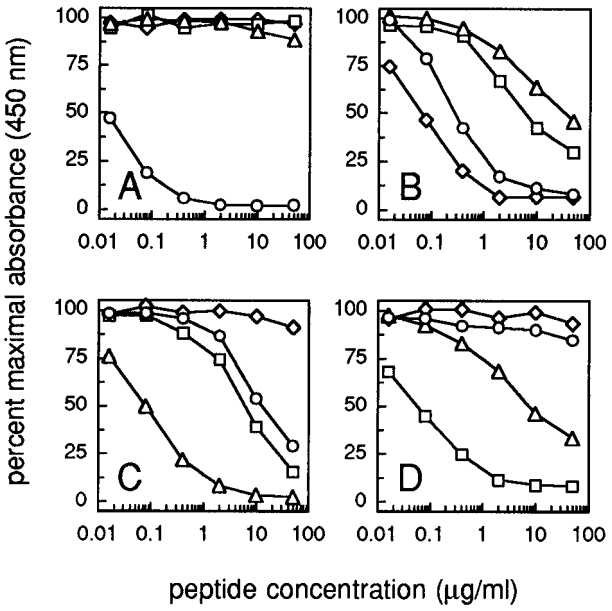

Figure 2. Reactivity of anti-N11L (A), anti-E7P (B), anti-E8E (C), and anti-E8Q (D) antisera toward N11L (○), E7P (◇), E8E (△), and E8Q (□) peptides in competitive ELISA tests. Antisera were tested in microtiter plates coated with the respective peptide antigens by using 1 μg/mL N11L, 20 μg/mL E8Q and E7P, and 10 μg/mL E8E as standard coating solutions. Antisera were used at the following final dilutions: anti-N11L, 1:8000 (v/v); anti-E8E and anti-E7P, 1:4000 (v/v); and anti-E8Q, 1:2000 (v/v). Antibody revelation methodology was performed as described under Materials and Methods.

competitive assays were carried out. Antisera were diluted to an optimized titer and incubated together with increasing quantities of whole casein from different species in microtiter plates previously coated with appropriate amounts of the corresponding peptide antigens (Figure 3).

Among the four casein species, only bovine casein was able to prevent anti-N11L and anti-E8E from being

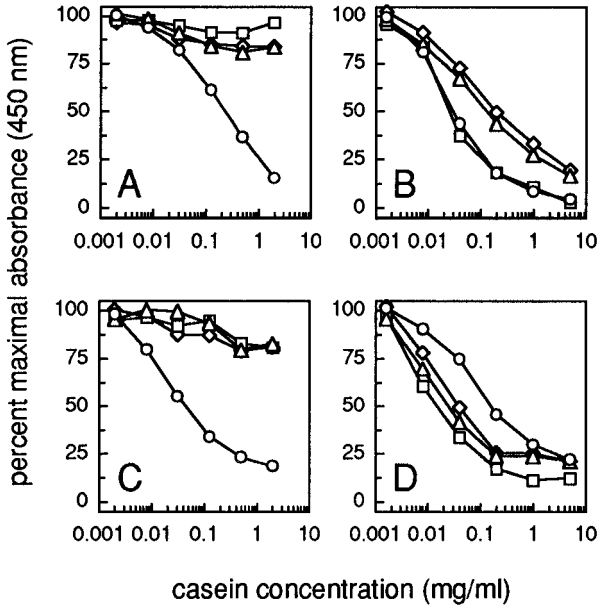

Figure 3. Reactivity of anti-N11L (A), anti-E7P (B), anti-E8E (C), and anti-E8Q (D) antisera toward bovine (○), caprine (◇), ovine (△), and water buffalo (□) whole caseins in competitive ELISA tests. Antisera were tested in microtiter plates coated with the respective peptide antigens by using 1 μg/mL N11L, 20 μg/mL E8Q and E7P, and 10 μg/mL E8E as standard coating solutions. Antisera were used at the following final dilutions: anti-N11L, 1:16000 (v/v); anti-E8E and anti-E7P, 1:8000 (v/v); and anti-E8Q, 1:4000 (v/v). Antibody revelation methodology was performed as described under Materials and Methods.

coupled with the peptide antigen immobilized onto the solid support (Figure 3A,C). Whole casein samples from bovine as well as those from the other species interacted well with both anti-E8Q and anti-E7P (Figure 3B,D). This means that only anti-N11L and anti-E8E specifically recognized bovine casein at variance with the other antisera.

Antiserum Specificity in Immunoblotting Analysis. To establish the specificity of antipeptide antibodies against the parent proteins, immunoblotting of the electrophorized caseins was carried out.

Isoelectric focusing patterns of whole casein from the four species were stained either with Coomassie Brilliant Blue G-250 or with the four antipeptide antibodies as primary antibodies. The distinct bands detected in bovine casein by staining with anti-N11L (Figure 4B) and anti-E8E (Figure 4D) did not seem to correspond to any of the main bovine α_{s1}-casein components stained with Coomassie Brilliant Blue G-250 (Figure 4A). Immunoblotting by using anti-E7P revealed in bovine casein the same components recognized by anti-N11L and anti-E8E; in addition, anti-E7P reacted with some other bands along either the ovine or caprine and water buffalo α_{s1}-casein fraction (Figure 4C). Taking into account the complexity of electrophoretic patterns of individual ovine casein samples (Chianese et al., 1996) and the fact that ovine α_{s1}-casein is composed of a mixture of components containing either 199 or 191 amino acid residues (Ferranti et al., 1995), the bands revealed by anti-E7P may correspond to those relative to the 199 residue long components. Obviously the 191 residue α_{s1}-casein components could not be detected by anti-E7P owing to the deletion of the E7P peptide in the short chain. Anti-E8Q did not detect any band migrating within the area of α_{s1}-casein from different species (Figure 4E). However, a number of unidentified bands within the area of β- and κ-casein were revealed.

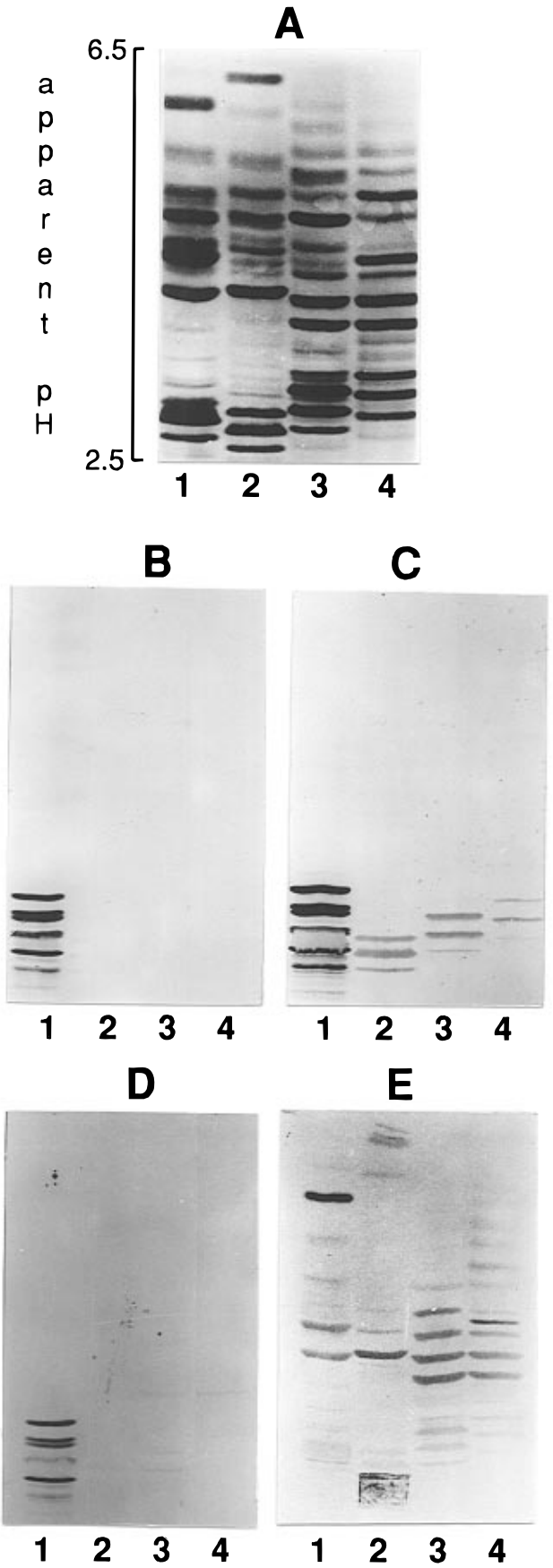

Figure 4. Isoelectric focusing and immunoblotting of 100 μg amounts of whole caseins from cow (lane 1), water buffalo (lane 2), sheep (lane 3), and goat (lane 4) milks: (A) Coomassie Brilliant Blue G-250 stain; immunodetection using as primary antibodies (B) 1:1000 (v/v) anti-N11L, (C) 1:1000 (v/v) anti-E7P, (D) 1:1000 (v/v) anti-E8E, and (E) 1:500 anti-E8Q; 1:2000 (v/v) diluted horseradish peroxidase labeled goat anti-rabbit IgG antibodies were used as secondary antibodies.

Control analysis by disc-PAGE showed that these components did not belong either to β-casein or to κ-casein (results not shown). Further studies are needed to identify the origin of these protein components, which only in the case of ovine species have been demonstrated to correspond to nonallelic forms of α_{s1}-casein (P. Ferranti, 1996, personal communication).

PAGIF and Immunoblotting of Individual Bovine Casein Samples. In Figure 5A the Coomassie Brilliant Blue G-250 patterns of some individual casein samples containing α_{s1}-casein B and C variants are shown. Surprisingly, immunodetection of the reactive anti-N11L bovine components gave a similar pattern regardless of the sample analyzed and the α_{s1}-casein variant (Figure 5B). Five or six bands occurred of similar intensity, whereas one (or two) prominent band(s) was (were) revealed by Coomassie Brilliant Blue G-250, according to the presence of the α_{s1}-casein variants B and C, as described by Addeo et al. (1983). This suggests that bands revealed by anti-N11L were only minor α_{s1}-casein components occurring in every bovine milk sample, irrespective of the amino acid substitution Glu^{192} (B) → Gly^{192} (C) (Grosclaude et al., 1972).

Immunoblotting of PAGE-Separated Caseins. To identify the molecular casein species functioning as a target for anti-N11L, PAGE analysis by disc-PAGE at pH 8.6 of the casein samples from different species was also performed.

By using a such technique, a sharp distinction was thus definitively ascertained between the α_{s1}-casein Coomassie Brilliant Blue G-250 stained and the anti-N11L immunostained components. The two molecular species showed clearly a distinct electrophoretic mobility at alkaline pH, as the Coomassie Brilliant Blue G-250 stained patterns (Figure 6A) were compared with those obtained by immunoblotting with anti-N11L (Figure 6B). In fact, this latter specifically detected some minor components migrating clearly ahead of the major bovine α_{s1}-casein bands, whereas polyclonal antibodies raised against FPLC-purified α_{s1}-casein (Chianese et al., 1992) were able to recognize both the fastest migrating bovine components and the main α_{s1}-casein either in bovine or in the other species (Figure 6C).

To determine the molecular weight of the anti-N11L reactive protein species, the SDS−PAGE analysis of bovine casein samples was carried out. In agreement with the previous findings (Trieu-Cuot and Gripon, 1981), α_{s1}-casein and α_{s2}-casein were found to comigrate, the anodic mobility being slower than that of β-casein (results not shown). By using anti-N11L as primary antibodies, one faint band having about 21 kDa apparent molecular mass was specifically stained (results not shown). This means that the α_{s1}-casein fraction actually contains lower molecular mass components showing a different antigenicity with respect to the higher molecular mass α_{s1}-casein components.

DISCUSSION

In indirect ELISA assays, a very limited cross-reactivity of antipeptide antibodies toward closely similar peptides has been observed. However, a large variety of factors could lead to a defective recognition of peptides by antibodies. By using competitive ELISA assays, based on the inhibition of the ELISA reaction, the systematic errors concerning the indirect ELISA format have been overlooked. Furthermore, competitive ELISA assays allowed the sensitivity and the precision of the antigen detection to be improved since the binding

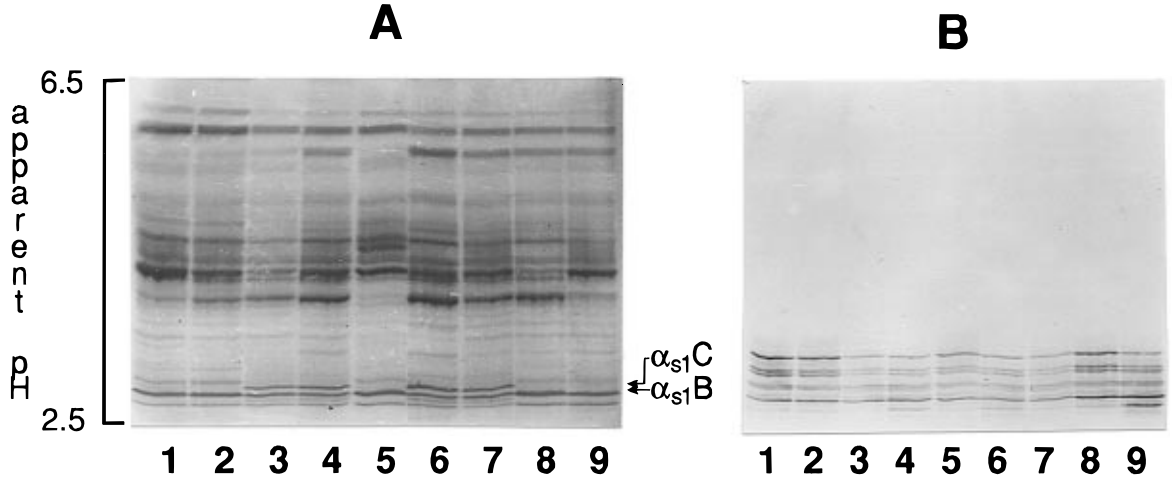

Figure 5. Individual bovine whole caseins (100 μg amounts of each sample) separated through isoelectric focusing on polyacrylamide gel and blotted onto nitrocellulose: (A) Coomassie Brilliant Blue G-250 stain; (B) immunodetection with 1:1000 (v/v) diluted anti-N11L antisera as primary antibodies and secondary antibodies as indicated for Figure 4.

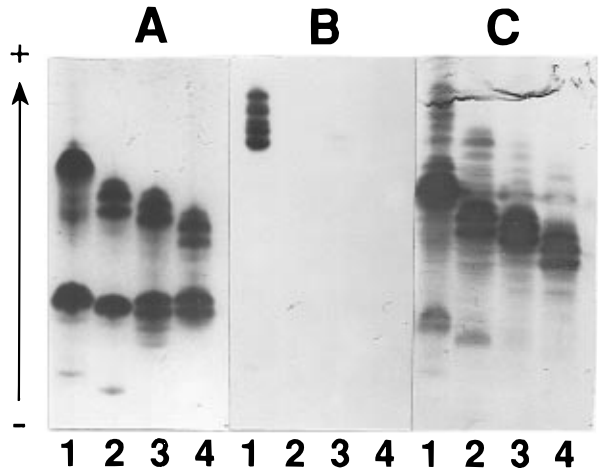

Figure 6. Disc-PAGE at pH 8.6 in denaturing conditions of bovine (lane 1), water buffalo (lane 2), sheep (lane 3), and goat (lane 4) whole caseins. Sample loaded was 50 μg of protein. (A) Coomassie Brilliant Blue G-250 stained gel; immunodetection using 1:1000 (v/v) dilutions of (B) anti-N11L and (C) polyclonal anti-α_{s1}-casein sera as primary antibodies and secondary antibodies as indicated for Figure 4.

of even low-avidity antibodies to the antigen was effectively forced by acting on the antigen concentration. On the contrary, by using the indirect ELISA procedure, owing to the limited binding capacity of the solid support, usually around 300 ng/cm^2 (Harlow and Lane, 1988), a further increase of the antigen concentration over certain limits was ineffective.

The synthetic E8E, E8Q, and E7P peptides, having a common N-terminal sequence linked to the same carrier protein, presumably present a similar orientation in the conjugate. Therefore, the differences among them should be located on the extremity opposite to that engaged in the linkage with the carrier. According to this hypothesis, the immunodominant regions of a peptide in the peptide−carrier conjugate are distal to the point of attachment to the carrier, as suggested by Schaaper et al. (1989). The most apparent similarity in raising immune response was shown by the peptides E8E and E8Q, which carry a different net charge but nonetheless share a common moiety relating to the carboxyl group in the lateral chain. In addition, the E7P peptide, which is lacking in one single C-terminal amino acid as compared with both E8E and E8Q peptides, did not cross-react with either anti-E8E or anti-E8Q. On the other hand, it could be excluded that the length of the peptide used as immunogen determines the specificity of the antibodies since the N11L peptide, in which two amino acids are added to the N-terminal of the E8E peptide, was recognized by anti-E8E but not by anti-E8Q.

Since our antisera had required some unavoidable structural features to work on similar peptides, a great deal of stringency was expected also toward the parent proteins. In fact, ovine, caprine, and water buffalo α_{s1}-caseins, containing E7P and E8Q peptides, were recognized only by anti-E7P and anti-E8Q, whereas no reactivity was shown at all by both anti-N11L and anti-E8E.

It would seem that the substitution of a single amino acid residue at site 148 (Q or E) drastically affects the protein recognition, as suggested by Rolland et al. (1995), even if the same difference had not prevented peptide recognition, as already pointed out. Indeed, bovine casein gave a positive response to anti-E8E, anti-E7P, and anti-N11L as well as to anti-E8Q, although its α_{s1}-fraction did not contain the integral E8Q peptide.

However, it should be borne in mind that the simplicity of the ELISA technique conceals a large potential for misinterpretation of results when samples do not contain pure antigen, as in the case of whole casein where the presence of nonallelic forms (Ferranti et al., 1995; P. Ferranti, personal communication, 1996) might interfere. In this case, the overall signal might be due to a combination of the reaction between antibodies and some molecular species displaying different degrees of affinity.

Immunoblotting has allowed us to visualize what the antibodies effectively detect when antisera and caseins interact.

The striking cow specificity of anti-N11L and anti-E8E assessed by ELISA experiments was found to be due to the strong affinity of the antibodies toward a set of at least five distinct protein bands. Unexpectedly, these antibodies did not recognize the major bovine α_{s1}-casein components, as confirmed by PAGE analysis at alkaline pH. The molecular species detected by anti-E8E and anti-N11L were shown to carry other epitope structures peculiar to bovine α_{s1}-casein. They coexisted with the well-known major form and apparently did not exhibit any polymorphism, as does the major α_{s1}-casein.

Further studies are required to understand if this group of bands corresponds to degraded forms of α_{s1}-

casein or to quite independent forms, produced from different mRNAs. At present these components occurring in all individual bovine caseins, even in freshly secreted milk, seem to be nonallelic forms of α_{s1}-casein. Surely the conformation of the α_{s1}-casein-like forms lets the target region be attained by our antibodies, whereas that of the major α_{s1}-casein component does not, even if the target stretch is contained in the α_{s1}-casein chain. Most probably, the conformation of α_{s1}-casein from the other species is similar to that of bovine, namely preventing the access of antibodies to the specified region. Thus, both the α_{s1}-casein-like reactive forms occurring only in bovine casein and the conformational constraints acting on all α_{s1}-caseins might contribute together to generating the observed cow-specificity.

On the basis of a hydrophilicity scale, the 139−149 α_{s1}-casein region had already been predicted by Hopp and Woods (1983) to be poorly exposed at the protein surface and therefore hardly accessible to the antibodies.

Actually, anti-E7P recognized bovine anti-N11L positive bands as well as the main ovine, caprine, and water buffalo α_{s1}-casein bands. Likely, anti-E7P antibodies have higher avidity since they not only detect the more reactive bovine α_{s1}-casein forms but are also able to reach their target in the less accessible regions of α_{s1}-casein. The anti-E7P binding activity toward ovine α_{s1}-casein could enable us to measure out by ELISA tests the amount of the ovine α_{s1}-casein long form in individual samples and ascertain if a variable ratio between the long and the short forms affects milk quality.

Anti-E8Q antibodies failed to act on either α_{s1}-casein major components from the four species or the bovine α_{s1}-casein reactive form. The activity shown by anti-E8Q toward whole caseins from different species in ELISA assays was not clarified by the immunoblotting analysis, owing to the interaction of the antibodies with different protein bands migrating in the β- and κ-casein area, where no α_{s1}-casein bands have been found until now.

The strategy using synthetic peptides for predetermining specificity of antibodies toward a given protein epitope has revealed both a conceptual simplicity and a practical convenience. This approach has allowed us to determine the existence of a novel form of α_{s1}-casein in bovine milk, even though bovine casein had in the past been the subject of very detailed studies (Swaisgood, 1992). It is the unique target of two antibody preparations, which can be used for detecting adulteration with bovine milk of ovine, caprine, and water buffalo dairy products. Indeed, such a use of the antibodies against bovine α_{s1}-casein fragment 140−149 has been already proposed to control ovine (Rolland et al., 1993) and caprine (Rolland et al., 1995) products; however, this stemmed from the erroneous statement that the recognized protein was bovine α_{s1}-casein as a whole in its complexity and not minor components of the α_{s1}-casein, as resulted from this study.

Throughout this work it was confirmed once more that the ELISA results, even those which appear obvious, obtained from assays on impure antigens must be supported by a control technique, such as immunoblotting.

ABBREVIATIONS USED

PBS, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4; TEAP, 0.1% triethylammonium phosphate buffer, pH 3.0; FAB-MS, fast atom bombardment mass spectrometry; MH^+, quasi-molecular ion mass value observed in FAB-MS spectrum; KLH, keyhole limpet hemocyanin; ELISA, enzyme-linked immunosorbent assay; disc-PAGE, vertical polyacrylamide gel electrophoresis; PAGIF, isoelectric focusing on thin-layer polyacrylamide gel; N11L, the peptide NQELAYFYPEL, identical to the sequence 139−149 of bovine α_{s1}-casein; E8E, the peptide ELAYFYPE, identical to the sequence 141−148 of bovine α_{s1}-casein; E8Q, the peptide ELAYFYPQ, identical to the sequence 141−148 of ovine, water buffalo, and caprine α_{s1}-casein; E7P, the peptide ELAYFYP, identical to the common sequence 141−147 of α_{s1}-casein from different species; anti-N11L, antisera raised to the N11L peptide conjugated to KLH; anti-E8E, antisera raised to the E8E peptide conjugated to KLH; anti-E8Q, antisera raised to the E8Q peptide conjugated to KLH; anti-E7P, antisera raised to the E7P peptide conjugated to KLH.

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