Use of a single-strand conformation polymorphism analysis to perform a simple genotyping of bovine κ-casein A and B variants

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Summary. We propose an alternative method for casein genotyping, generally carried out using polymerase chain reaction followed by restriction fragment length polymorphism analysis. Application of the single-strand conformation polymorphism technique detects nucleotide changes in the fragment amplified by means of polymerase chain reaction and thus avoids the use of restriction enzymes. A 453 bp fragment from exon IV of κ-casein has been amplified. The two variants (A and B), found with the highest frequencies in most bovine breeds and included in some dairy cattle selection schemes, can be discriminated using single-strand conformation polymorphism analysis of heat denatured fragments in acrylamide–bis-acrylamide (100:1) gels followed by silver staining. κ-Casein genotyping is therefore simplified, although variants A and E on the one hand, and B and C on the other, are not distinguishable with this technique.

Caseins (α_{s1}, α_{s2}, β and κ) are the major milk proteins in mammals; in solution they combine with calcium phosphate to form loosely ordered aggregates, termed micelles, which sequester up to 5% of their dry weight as Ca^{2+}. The role of κ-casein is to stabilize these micelles.

The two best studied and first described genomic variants of κ-casein are κCn^{A} (Thr^{136}, Asp^{148}) and κCn^{B} (Ile^{136}, Ala^{148}) (Neelin, 1964; Schmidt, 1964; Woychik, 1964). Later, four additional genetic variants have been identified electrophoretically: $\kappa Cn^{C/D}$ (Di Stasio & Merlin, 1979; Mariani, 1983; Seibert *et al.* 1987), κCn^{E} (Erhardt, 1989), κCn^{F} (Ikonen *et al.* 1996) and κCn^{G} (Erhardt, 1996).

Alleles A and B determine great differences in production traits (e.g. milk yield and casein, protein and fat contents) and cheesemaking properties (such as coagulation time, curd firmness and rate of coagulation) of milk (see Lin *et al.* 1992). These alleles are universally distributed among *Bos taurus* and *Bos indicus*, their frequencies being very similar in most breeds, except in those highly selected for yields of milk (Holstein Friesian) or milk fat (Jersey), where there are differences in the frequency of variant B: 0·028 *v.* 0·879 (Hoogendoorn *et al.* 1969) or 0·32 *v.* 0·77 (McLean *et al.* 1984).

Bovine genotypes for these two variants have acquired enough importance to justify their inclusion in cattle selection programmes. Traditionally, individual genotyping has been effected by Southern restriction fragment length polymorphism (RFLP: Levéziel *et al.* 1988, with *Hind*III; Rando *et al.* 1988, with *Pst*I; Rogne *et al.* 1989, with *Hinf*I or *Hind*III) or by polymerase chain reaction (PCR)–RFLP

(Denicourt *et al.* 1990, with *Taq*I and *Hind*III: Medrano & Aguilar-Córdova, 1990, with *Hinf*I). Digestion products are then resolved in agarose gels and show different patterns according to the possible genotypes: AA, AB and BB.

In this paper we describe an improved way to genotype κ-casein, reducing costs and time: the single-strand conformation polymorphism (SSCP) technique (Orita *et al.* 1989). This technique is based on the fact that the migration of short single-stranded fragments under non-denaturing conditions is different according not only to their length but also to their sequence (Beier, 1993).

materials and methods

Blood samples

Fifteen cows of the Asturiana beef cattle breed (see Mason, 1988), were used, and DNA was extracted following the 'salting-out' procedure described by Miller *et al.* (1988).

Amplification

Two primers reported by Alexander *et al.* (1988), based on the sequence of κ-casein cDNA (Gorodetskiĭ & Kaledin, 1987) were used to perform the PCR reactions. A 453 bp fragment of exon IV of bovine κ-casein was amplified: this exon encodes 94 % of the mature protein and contains the two nucleotide substitutions responsible for the differences between A and B variants. The composition of the primers is as follows.

Primer κCn forward: $^{5'}$TGT GCT GAG TAG GTA TCC TAG TTA TGG$^{3'}$

Primer κCn reverse: $^{5'}$GCG TTG TCT TCT TTG ATG TCT CCT TAG$^{3'}$

Total DNA (250 ng) was used in a 50 μl PCR reaction volume containing 50 mM-Tris-HCl, pH 9·0, 20 mM-$(NH_4)_2SO_4$, 2 mM-$MgCl_2$, 1 unit (as defined by the manufacturer) Tfl DNA polymerase from Epicentre (Ecogen, E-28015 Madrid, Spain), 200 μM-dNTP from Bioprobe (Ecogen) and 50 pmol of each primer, covered by a thin layer of mineral oil. Following a hot start (94 °C for 5 min), 30 cycles were carried out (94 °C for 1 min, 65 °C for 1 min, 72 °C for 2 min) ending with a 5 min final extension at 72 °C in a thermocycler (MJ Research Inc., Watertown, MA 02172, USA). At this stage, samples were processed by restriction and SSCP analyses.

Restriction analysis

A sample of the amplification product (10 μl) was digested with 6 units *Hinf*I (Pharmacia Biotech, E-08190 Barcelona, Spain) at 37 °C overnight. Restriction fragments were revealed on an agarose gel at 40 g/l (NuSieve 3:1 Agarose; FMC, DK-2665 Vallensback, Denmark) subjected to electrophoresis in TBE 1× buffer (90 mM-Tris-borate–2 mM-EDTA, pH 8·0; Sambrook *et al.* 1989) at 55 V constant voltage for 3 h. After staining with ethidium bromide, gels were visualized under u.v. light.

A 100 bp ladder has been used as a molecular marker; in order to assure the discrimination of the non-digested and the 426 bp fragments, a complete, non-digested amplified fragment was loaded (lanes 2 and 14 in both gels) as well as a 426 bp fragment obtained from bovine α_{s2}-casein (lanes 3 and 15 in Fig. 1*a*; lanes 3 and 14 in Fig. 1*b*). The 100 and 27 bp bands were too weak to be visible in the Figure.

Single-strand conformation polymorphism analysis

Amplification products were also resolved by SSCP without any previous treatment. Several factors were tested in order to improve the sensitivity of the technique and the sharpness of the bands (Spinardi *et al.* 1991): the ionic strength of the buffer (0·5×, 1× or 1·5×), the presence or absence of glycerol (100 g/l), the acrylamide : bis-acrylamide ratio (100 : 1 or 19 : 1) and the running temperature (6, 10 or 20 °C).

A sample (4 μl) of each PCR reaction was removed, diluted 3-fold in loading buffer (5·5 mM-EDTA, pH 8·0 containing (g/l) formamide 42·5, xylene cyanole 0·6, bromophenol blue 0·6), heat denatured, snap chilled on ice and resolved on polyacrylamide gels. (Each gel contained 4·158 g polyacrylamide and 0·046 g *N*, *N*′-methylene-bis-acrylamide to give a ratio of 100 : 1 in a final volume of 35 ml; or 3·99 g polyacrylamide and 0·2 g *N*, *N*′-methylene-bis-acrylamide for a ratio of 19 : 1, in the same final volume.)

Electrophoresis was carried out using an SE-400 Hoefer gel system (Hoefer Scientific Instruments, San Francisco, CA 94197, USA) for 14 h at 200 V constant voltage and the bands were silver stained according to the method described by Bassam *et al.* (1991).

RESULTS

Restriction fragment length polymorphism analysis

Fig. 1 shows the *Hinf*I digestion fragments of the individuals analysed: the three possible genotypes (AA, AB and BB) were present. The pattern originated by this enzyme included a 27 bp fragment (not visible in the Figure) in all individuals. The homozygotes AA (lane 5 in Fig. 1*a*, *b*) had another restriction target that led to a pattern of 326 and 100 bp fragments (this was very weak and not visible in the Figure), while BB homozygotes (lanes 6, 11, 12 and 13 in Fig. 1*a*, lanes 4 and 11 in Fig. 1*b*) gave a single 426 bp fragment. Consequently, the heterozygotes AB provided an intermediate pattern of both homozygotes, i.e. the 426, 326, 100 and 27 bp fragments.

Single-strand conformation polymorphism analysis

Fig. 2 shows the polyacrylamide SSCP gel, using the best combination of factors (see below). When the same samples were resolved, PCR–RFLP and SSCP patterns were in complete agreement. The SSCP gel gave two bands in homozygotes and four in heterozygotes, as DNA migrated here in a single-stranded form. The sensitivity of SSCP for detecting changes in the nucleotide sequence depended on several factors. The best results were obtained when TBE 1× was used: at 20 °C the bands were sharper than at lower temperatures, and addition of glycerol did not improve the definition of the bands and delayed its migration across the polyacrylamide. The two acrylamide ratios were used to test the influence of the size of the pore on the migration of the fragment (the smaller the ratio, the larger the pore). Better and sharper bands for a maximum size of 453 bp were obtained when the acrylamide : bis-acrylamide ratio was 100 : 1 rather than 19 : 1. This is in agreement with Savov *et al.* (1992), who reached the same conclusion working with a 425 bp fragment: they failed to discriminate between homozygotes and heterozygotes using the 19 : 1 ratio, but were successful with a 29 : 1 ratio.

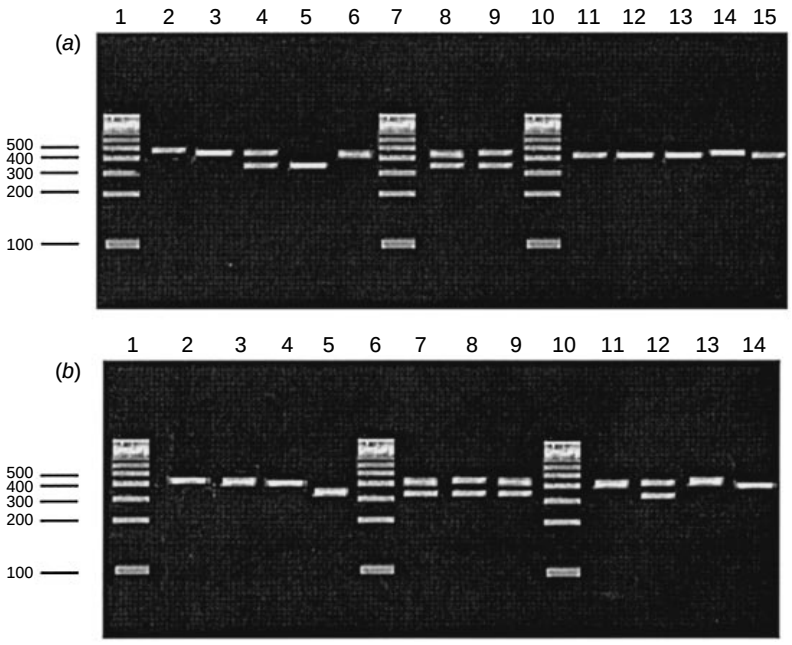

Fig. 1. Restriction patterns of the 453 bp κ-casein fragment after digestion with *Hinf*I. (*a*) Lanes 1, 7 and 10, 100 bp ladder; lanes 2 and 14, non-digested polymerase chain reaction (PCR) products; lanes 3 and 15, 426 bp α_{s2}-casein PCR fragment; lane 5, homozygote AA; lanes 4, 8 and 9, heterozygotes AB; lanes 6, 11, 12 and 13, homozygotes BB. (*b*) Lanes 1, 6 and 10, 100 bp ladder; lanes 2 and 13, non-digested PCR products; lanes 3 and 14, 426 bp α_{s2}-casein PCR fragment; lane 5, homozygote AA; lanes 7, 8, 9 and 12, heterozygotes AB; lanes 4 and 11, homozygotes BB.

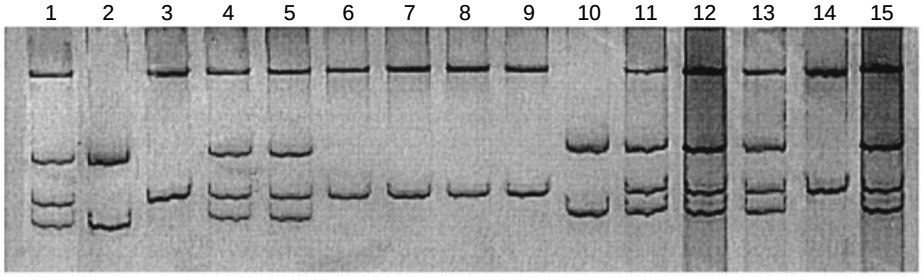

Fig. 2. Single-strand conformation polymorphism analysis of the 453 bp κ-casein fragment in an acrylamide–bis-acrylamide (100:1) silver-stained non-denaturing gel. Lanes 2 and 10, homozygotes AA; lanes 1, 4, 5, 11, 12, 13 and 15, heterozygotes AB: lanes 3, 6, 7, 8, 9 and 14, homozygotes BB. Although specific polymerase chain reaction products, the number of bands is two in homozygotes and four in heterozygotes as DNA migrates here in a single-stranded form.

Discussion

Milk protein types are associated with quantity and quality of milk (Hoogendorn *et al.* 1969). It is therefore not surprising that milk protein types in Jersey, for example, differ from those in Holstein, as a consequence of selection pressure. As a result, over the last 10 years κ-casein genotyping has been introduced into many selection programmes, as additional information for the evaluation of dairy sires.

At present, only variants A and B are recorded for each individual, although others have been described: variant C/D (variant D has been demonstrated to be

identical to C) and variant E, which have been reported to be rare in most breeds (Erhardt, 1989), with the exception of the Finnish Ayrshire breed, where variant E is quite common (0·38 (Velmala *et al.* 1995), 0·307 (Ikonen *et al.* 1996)). For the recently described variants F and G, there is no information yet available about their frequencies in different breeds. Some confusion has arisen as nucleotide substitutions affecting variants A and E on one hand, and B and C on the other, do not alter the targets of the restriction enzymes conventionally used (see above). Correct genotyping of all κ-casein alleles by PCR–RFLP requires at least the four enzymes *Hae*III, *Hha*I, *Hind*III and *Mae*II (Prinzenberg *et al.* 1996).

The application of the technique described here does not make it possible to discriminate A from E or B from C. Despite this, breeders have taken the option to genotype A and B which are at the moment the only two variants for which correlations with production traits regarding milk and milk fat yields have been described (McLean *et al.* 1984; Lin *et al.* 1992). This does not exclude the possibility that future work correlating milk properties with the other variants will make their systematic genotyping as interesting as for A and B.

At present, genotyping of A and B variants is carried out by means of PCR–RFLP using restriction enzymes. The SSCP technique we apply here is a useful tool, which allows breeders to characterize all the individuals under evaluation more easily, quickly and cheaply. More research may make it possible to determine which variant (A, B, C, E, F, G) is present by a single analysis. SSCP and related techniques are very likely to resolve these future prospects and our laboratory is currently working along these lines.

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