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Note

Separation of caprine globin chains by reversed-phase high-performance liquid chromatography: evidence for the presence of a silent β^B-globin allele in Sardinian sheep

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Since the polymorphism of sheep and goat haemoglobins (Hbs) was first described in 1955 [1,2] a number of reports have confirmed and extended the original observations, both in domestic and in wild breeds (see Blunt and Huisman [3] and Garrick and Garrick [4] for references). Owing to the presence of different allelic β-globin genes, three Hbs (Hb D, Hb Dmalta and Hb E) have been reported in goats (*Capra hircus*), two (Hb A and Hb B) in sheep (*Ovis aries*), mouflon (*Ovis musimon*) and Barbary sheep (*Ammotragus lervia*), whereas several Hbs due to α-chain variants contribute to complicate the polymorphism of caprine Hbs. To these, specific fetal and embryonic Hbs have to be added. Several species, with a particular chromosomal arrangement of the β-globin gene cluster, also have an additional non-allelic β-globin gene (the β^C gene) which is responsible for the reversible production of the Hb C under physiological stimuli, such as anemia or hypoxia [5,6].

Homozygous and heterozygous animals were identified by electrophoresis on paper or starch block using buffer systems at an alkaline pH, and Hb nomenclature used depended merely on the rate of mobility. Thus, an Hb rapidly moving towards the anode was called Hb A, the next was called Hb B, etc. However, Hbs with similar designations or electrophoretic mobilities do not necessarily have either the same structure or the same physiological properties [3,7]. Progress in the study of such a polymophism has recently been greatly facilitated by the advent of new electrophoretic methodologies, such as conventional isoelectric focusing (IEF) [7–9], IEF in immobilized ultra-narrow pH gradients (IPG) [10–

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12], and gel electrophoresis of dissociated globin chains [7,11]. Thanks to these approaches it became evident that a number of Hb molecules that cannot be separated by conventional starch gel electrophoresis are mixtures of different tetramers [7–12].

Shelton *et al.* [13] recently developed a new reversed-phase high-performance liquid chromatography (RP-HPLC) procedure for the separation of globin chains of human Hb. This widely adopted procedure was extended to the study of several non-human Hbs [14]. In a previous report we described the usefulness of RP-HPLC in the study of the heterogeneity of adult mouflon Hb and in the unambiguous detection of "contaminant" sheep globin genes in the genome of the wild mouflon [7]. In an attempt to contribute further to the knowledge of caprine Hb polymorphism, this report is concerned with the successful extension of HPLC methodology to the study of different species, both at birth and in adult life. Fetal, perinatal and adult globin chains, as well as α and non-α chain variants, of goats, mouflons and sheep can be completely separated. Application of this methodology also allowed the identification of the presence of a previously undescribed silent β-globin variant in sheep of the domestic Sardinian breeds.

EXPERIMENTAL

Reagents and chemicals

Chemicals were obtained from Carlo Erba (Milan, Italy): HPLC-grade acetonitrile (No. 412 411) and trifluoroacetic acid (TFA) (No. 411 561). Other chemicals were reagent grade. High-purity deionized water with a resistance of >15 MΩ was obtained by a Milli-Q system that was fed with a supply of reversed-osmosis purified tap water (Millipore, Bedford, MA, U.S.A.).

Apparatus

The HPLC equipment consisted of two pumps, a controller unit, a dual-channel recorder, and a fraction collector from LKB (Bromma, Sweden), a U6K injector (Waters, Milford, MA, U.S.A.), and an LCI 100 computing integrator (Perkin Elmer, Norwalk, CT, U.S.A.). The column was a 250 mm × 4.1 mm I.D. large-pore Vydac C_4 (The Separations Group, Hesperia, CA, U.S.A.).

Blood samples

Blood samples from newborn and adult goats, wild mouflons and domestic sheep living on the island of Sardinia were collected in EDTA, washed three times with isotonic saline lysed with CCl_4, and diluted with 0.05% KCN to a final concentration of *ca.* 1 g Hb/dl. To determine the Hb phenotype, and to verify the presence, and amount, of Hb F and Hb C at birth, lysates in the concentration range 0.7–1.0 g/dl Hb were analysed by IEF on thin-layer 5% polyacrylamide slab gels in the 6.7–7.7 pH range, as previously described [7,15].

Goats were of the Hb A and Hb D phenotypes and some were the carriers of

two unidentified α chain variants; mouflons were of the Hb B phenotype and of the Hb M phenotype we recently described as physiologically resembling the Hb B of the sheep [7]; sheep were of the Hb A and Hb B phenotypes and were the carriers of the $^{I}\alpha^{8Ser \rightarrow Ala}$ (or $^{I}\alpha^{AIa}$) and the $^{II}\alpha^{113Leu \rightarrow His}$) (or $^{II}\alpha^{His}$) globin chain variants previously characterized [8,16].

RP-HPLC

Globin chain separation followed the RP-HPLC procedure previously described by Shelton *et al.* [13] and by Schroeder *et al.* [14] (see also refs. 17 and 18). Only the modifications to the original procedure, which were necessary because of the chemical properties of the different caprine globin chains, will be described here. Hb solutions were brought to a final concentration of 50–70 μg Hb per 15 μl with developer A (see below) and filtered through 0.45-μm HV filters (Millipore). A 15-μl aliquot of globin solution was then applied to the column. The chromatogram was developed with two linear gradients between developer A (20% acetonitrile–80% water containing 0.1% TFA) and developer B (60% acetonitrile–40% water containing 0.1% TFA) at a flow-rate of 1.0 ml/min at room temperature. The effluent was monitored at 220 nm.

To separate normal and some of the variant globin chains of adult subjects, the first gradient was 48 to 51% developer B in 35 min, and the second was 51 to 60% developer B in 53 min. In the case of newborn samples the second gradient was extended (51 to 63% developer B in 70 min) to allow the optimal separation of the β^{C} and γ chains.

Attempts were also made to efficiently separate the two unidentified (rapidly moving) α chain variants of the goat by means of two very shallow gradients. The more effective was 40 to 42% developer B in 20 min to elute the haem group, followed by 42 to 47.5% developer B in 44 min.

The identity of the different α and non-α globin chains with the chromatographic peaks were easily correlated by comparison of the HPLC pattern with the IEF pattern and with quantitative composition, mobility and hydrophobicity of globins in the acid–urea–Triton gel electrophoresis as previously described [7,19]. Mixing experiments with lysates from different animals also aided the identification of globin chains.

RESULTS

Fig. 1 shows a chromatogram of the lysate of a newborn goat of the Hb A/Hb D phenotype, which was also the carrier of two unidentified α chain variants. Owing to the young age of the animal, both the γ chain of the Hb F and the β^{C} of the Hb C were present, together with small amounts of the adult β^{A} and β^{D} chains. The normal globin chains were completely separated, which allowed their quantitation and isolation. The elution pattern shows the haem group at *ca.* 11 min, followed in 31–36 min by the normal α chain, in 55–57 min by the β^{D} and β^{A}

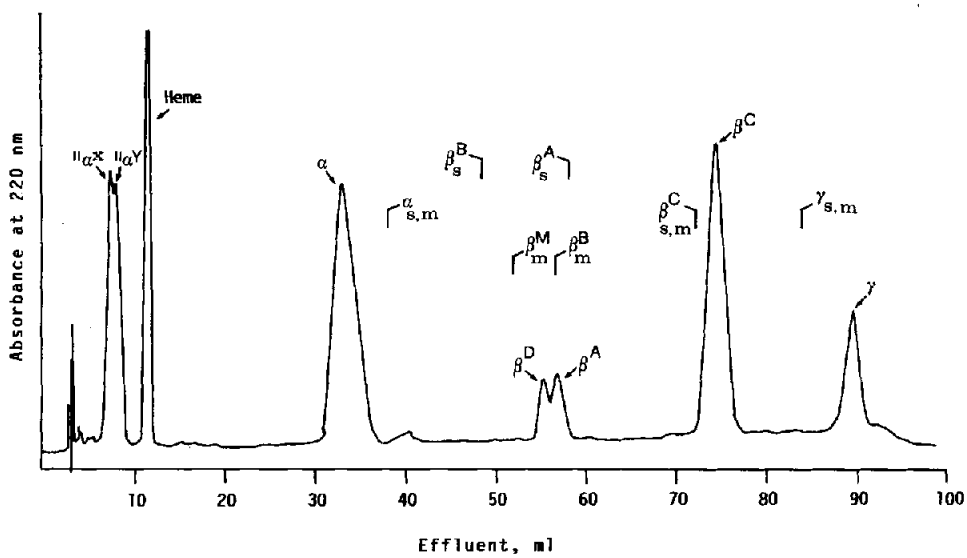

Fig. 1. Separation of the globin chains in a newborn goat Hb AD heterozygote, which is also the carrier of two unidentified $^{II}\alpha$ chain variants (arbitrarily designed as $^{II}\alpha^X$ and $^{II}\alpha^Y$). The positions of sheep (s) and mouflon (m) globin chains are indicated by bars. The gradient was 48% B to 51% B in 35 min, followed by 51% B to 63% B in 70 min.

chains, in 73–78 min by the perinatal β^C chain, and in 88–91 min by the γ chain. Fig. 1 also indicates the positions of mouflon and sheep globin chains eluted by means of the same solvent gradient. No difference was observed in the retention time of the α-chain of mouflon and sheep, even when lysates of the two species were mixed and chromatographed together in order to exclude possible analogies (or differences) determined by technical differences between runs, such as developer preparation, evaporation of solvents, etc. Similarly no difference was observed in the retention time of mouflon and sheep β^C and γ chains. Elution patterns of mouflon α, β^M and β^B chains with respect to sheep α, β^B and β^A chains were as previously described [7]. The two α chain variants of the newborn goat in Fig. 1 emerged as fused peaks immediately before the haem group, representing 35% of the total α chains and indicating that they represent two different $^{II}\alpha$ alleles. These chains were completely resolved with a shallowed gradient, as described below.

Fig. 2 shows the globin chains of a sheep that was electrophoretically identified as an homozygote for the Hb B. The chromatogram is clearly indicative of the presence of two different β chains, one corresponding to the β^B and the other representing a newly observed β^B variant, most probably characterized by a silent substitution with a more hydrophobic residue.

Fig. 3 shows the chromatogram relating to the position of the rather common $^{II}\alpha^{His}$ and $^{I}\alpha^{Ala}$ variants of sheep and of the normal $^{I}\alpha^{Leu}$ chain. Variant globins are

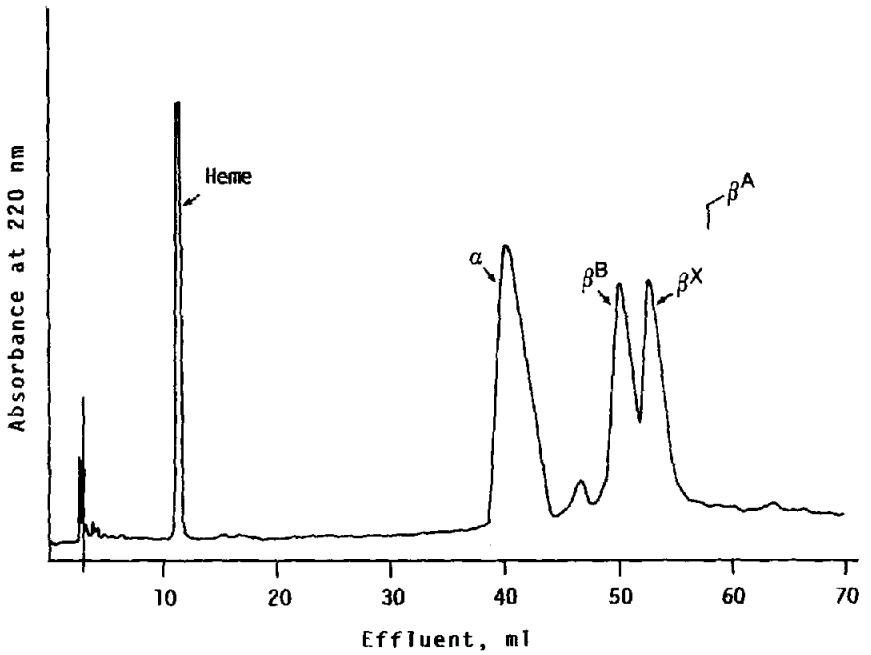

Fig. 2. Separation of the globin chains of a sheep of the Hb BB phenotype indicating the presence of a silent HbβB variant. The position of the β^A chain is indicated by a bar. The gradient was 48% B to 51% B in 35 min, followed by 51% B to 60% B in 53 min.

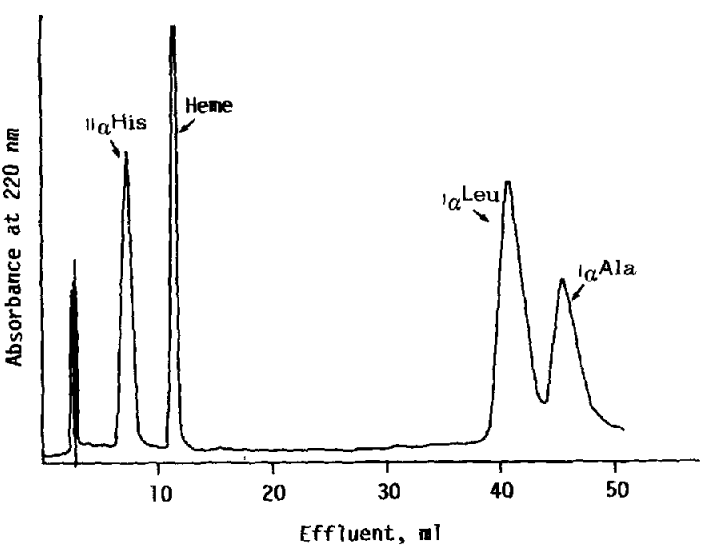

Fig. 3. Positions of the variant $^{II}\alpha^{His}$ and $^{I}\alpha^{Ala}$ globin chains of the sheep with reference to the normal $^{I}\alpha^{Leu}$. The gradient was the same as in Fig. 2.

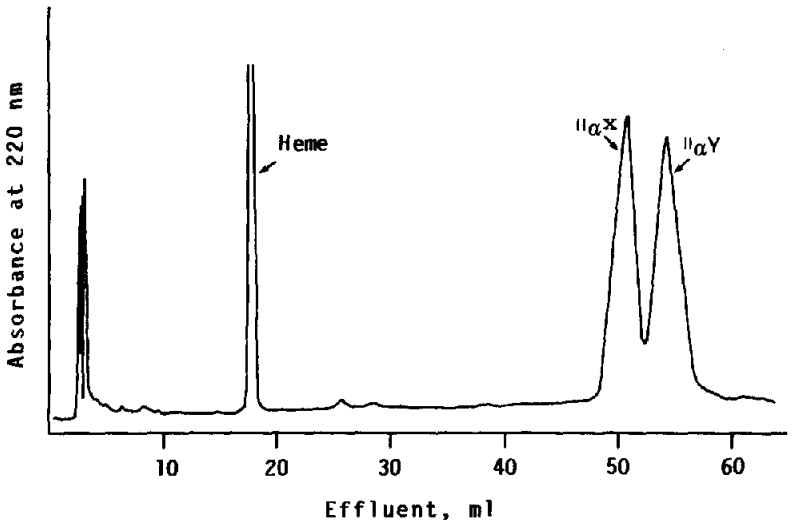

Fig. 4. Complete separation of the two unidentified $^{II}\alpha$-globin chain variants of the newborn goat of Fig. 1. The gradient was 40% B to 45% B in 20 min, followed by 42% B to 47.5% B in 44 min.

usefully separated from the normal one, which allowed quantitative determinations. Percentages obtained were: 49% $^{I}\alpha^{Leu}$, 33% $^{I}\alpha^{Ala}$, and 18% $^{II}\alpha^{His}$.

Fig. 4 shows the result of the separation, obtained by a modification of the gradient slope, of the two α chain variants of the goat which, in Fig. 1, were eluted as fused peaks before the haem. Their relative amounts were approximately the same.

DISCUSSION

This study supports and extends previous observations about the usefulness of the RP-HPLC methodology, first described by Shelton *et al.* [13], in the separation of non-human globin chains and in the study of Hb polymorphism of the caprine species [7,14]. Data indicate that many of the different α and non-α globin chains of caprine species may be advantageously separated, quantitated and eventually isolated. This is of importance in physiological studies, such as the follow-up of the Hb switching in newborn and adult subjects, in biochemical genetics, in the separation and identification of a number of variants, in structural analysis, and so on. The results of this study, which was done on a limited number of randomly selected subjects, also indicate the presence in the Sardinian sheep of, at least, a previously undescribed HbβB allele. This is the case of the β-chain variant, found by chance in a domestic Hb B sheep, which shows an increase in the retention time, with respect to the normal β^{B} chain, suggesting a silent substitution with a more hydrophobic residue. Anaemization of compound hetero-

zygotes Hb B/Hb X sheep was not followed by the appearance of the Hb C, thus suggesting a chromosomal organization of the sheep carrier of the variant similar to that described in the sheep of the B type [5,6]. Electrophoretic and physiological behaviour of this variant resembles that of the HbβE allele described as frequent in India (which is also indistinguishable from Hb B, and of the non-switching haplotype) [20,21]. Preliminary investigations on a number of subjects suggest this variant might be common among Sardinian sheep. As far as we know, there have been no reports about the structure of the β^{E} variant chain, so we could not speculate about possible structural differences or similarities between the chain found in this study and that observed in India. Therefore, we propose to temporarily call our variant as the "HbβE Sardegna". Both the structure of this variant chain and the β-globin gene cluster organization of the carrier sheep are currently under investigation.

Huisman [22], in a recent review of the separation of variant human globin chains by the reversed-phase method, proposed a useful ranking of amino acid residues based on polarities, sizes and pK values at pH values below 3, as determined by the presence of 0.1% TFA in the developers. This ranking is useful in predicting the change in retention time in the presence of a substitution in a polypeptide, and *vice versa* [18,22,23]. With reference to this ranking, the positions in the chromatogram of some of the different globin chains analysed in this study are consistent with their structure and substitutions previously described [3,24,24]. Thus goat β^{D}-chain with His substituted for Asp at position 21 with respect to goat β^{A} has a descreased retention time; sheep γ chain with, among others, three hydrophilic substitutions with respect to goat γ chain, has decreased retention time, and so on. The position of the sheep $^{I}\alpha^{113Leu}$ globin chain, and of the $^{I}\alpha^{8Ser \rightarrow Ala}$ and $^{II}\alpha^{113Leu \rightarrow His}$ variant chains, could be interpreted on the same basis. Quantitative determination of the three α chains, which were found associated in many sheep, was in accordance with previous observations, indicating the duplication of the α chain gene in sheep and the dominant expression of the $^{I}\alpha$ gene with respect to the $^{II}\alpha$ gene [8,9,16]. Similarly, quantitation of the fast-moving α chain variants of the goat (Figs. 1 and 4) indicates a compound heterozygosity conditions for two different alleles of the $^{II}\alpha$ locus.

The possibility of adequately separating normal and variant caprine globin chains by the HPLC methodology will certainly facilitate identification of new variant globin chains and future studies at the polypeptide structural level.

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