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Separation of β-lactoglobulin A, B and C variants of bovine whey using capillary zone electrophoresis

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Abstract

β-Lactoglobulin is a whey protein that affects milk composition and product functionality and which can be present in up to eight genetic variant forms. A free zone capillary electrophoresis method has been developed to separate and identify the β-lactoglobulin A, B and C variants. Three buffer systems [borate, 2-(N-morpholino)-ethanesulphonic acid (MES) and bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bistris)] were examined over a range of pH values and with the addition of the separation buffer modifiers Tween 20 and/or ethanolamine. The most successful combination of these was 50 mM MES at pH 8.0 with the addition of 0.1% Tween 20 which clearly resolved the three variants from both each other and from the other whey proteins even though the MES buffer was acting well outside its pK_a range (pH 5.3–7.3). The retention times and identification of the individual variants were verified by spiking with commercially purified β-lactoglobulin A and B proteins and a β-lactoglobulin AC whey. The method was then used to phenotype β-lactoglobulin in a sample population of New Zealand Jersey cows.

1. Introduction

Genetic variants of the major milk proteins [α_{s1}-, β- and κ-casein and β-lactoglobulin (β-Lg)] are present in the milk produced by New Zealand cows with β-Lg, the major whey protein, present in up to eight variant forms [1]. Although β-Lg A and β-Lg B are the most prevalent variants, the C variant has been observed in some populations of the Jersey breed at frequencies between 0.01 and 0.11 [2–4]. The presence of this variant may affect the milk composition and functional properties of the milk and milk products [5,6]. There are two amino acid differences (Asp-64 and Val-118 are substituted by Gly and Ala, respectively) between the A and B variants, three between the A and C but only one (Gln-59 to His) between the B and C [7,8] (Fig. 1).

In a previous study [9] no C variants were detected in New Zealand Jersey cows using native, non-reducing polyacrylamide gel electrophoresis. DEAE–cellulose ion-exchange chromatography [8], starch gel electrophoresis [10] and chromatofocusing with a Pharmacia polybuffer exchanger column [11] have also been used to separate the three variants but are not practical for phenotyping large cow populations. The A and B variants of β-Lg have also been separated by capillary electrophoresis [12–14] although separation of the C variant has not been reported.

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Position	57	58	59	60	61	62	63	64	65	66 ...	116	117	118	119	120
Variant															
A	Leu	Leu	Gln	Lys	Trp	Gln	Asn	Asp	Glu	Cys ...	Ser	Leu	Val	Cys	Gln
B	Leu	Leu	Gln	Lys	Trp	Gln	Asn	Gly	Glu	Cys ...	Ser	Leu	Ala	Cys	Gln
C	Leu	Leu	His	Lys	Trp	Gln	Asn	Gly	Glu	Cys ...	Ser	Leu	Ala	Cys	Gln

Fig. 1. Amino acid sequence of β-Lg variants (total: 162 amino acids). Differences in amino acid sequence are shaded.

In the present study a capillary electrophoresis method to separate the A, B and C variants of β-Lg has been developed and was then used for the β-Lg phenotyping of a large sample of New Zealand dairy cattle.

2. Experimental

2.1. Chemicals

Sodium tetraborate (borate), 2-(N-morpholino)ethanesulphonic acid (MES), ethanolamine (EA) and polyoxyethylene (20)-sorbitan monolaurate (Tween 20) were obtained from BDH Chemicals (Poole, UK) and bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis-tris) from Sigma (St. Louis, MO, USA). Commercially purified whey proteins (whey protein standards): α-lactalbumin (α-Lac), β-Lg A, β-Lg B and bovine serum albumin (BSA) were supplied by Sigma. Freeze dried whey phenotypic for the β-Lg variants A and C (AC whey) was gratefully received from Mary Christian of the Department of Agriculture, Ellinbank, Victoria, Australia. All reagents were of Analar grade or better and water was purified by reverse osmosis followed by deionization (Milli-Q, Millipore, MA, USA).

2.2. Whey samples

Individual whey samples were prepared from 258 Jersey cows by acid precipitation of casein at pH 4.6 using HCl. The casein was removed by centrifugation and the whey was filtered (0.45 μm) to remove any residual casein precipitate and fat.

2.3. Capillary electrophoresis

Capillary zone electrophoresis (CZE) was performed on an Applied Biosystems 270A-HT CE system (Foster City, CA, USA) using a PE Nelson 900 series interface and a PE Nelson TurboChrom 3.3 software package (Cupertino, CA, USA) for data acquisition and analysis, respectively. The uncoated capillary (72 cm total length, 50 cm effective length and 50 μm internal diameter) was supplied by Applied Biosystems. Samples were injected at the anode using vacuum (17 kPa) for 10 s during method development and 4 s for the whey samples. The separation voltage was 20 kV with detection at 215 nm. Between the injections the capillary was flushed for 2 min (68 kPa) consecutively with 0.1 *M* NaOH, Milli-Q water and buffer to retain separation reproducibility.

3. Results and discussion

AC whey spiked with β-Lg B was initially separated using a 50 m*M* sodium borate buffer at pH 9.0 containing 0.1% EA and 0.1% Tween 20 (Fig. 2) as this buffer system had been used previously to successfully separate α-Lac, β-Lg A, β-Lg B and BSA (unpublished results). The

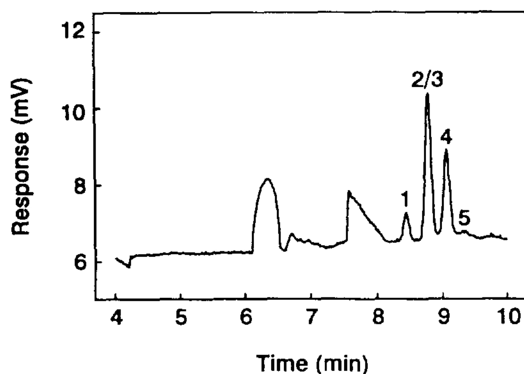

Fig. 2. Initial separation of AC whey and β-Lg B. AC whey and β-Lg B were prepared at 0.2 mg/ml and 0.04 mg/ml in Milli-Q water, respectively. The separation buffer was 50 mM sodium borate, pH 9.0, containing 0.1% ethanolamine and 0.1% Tween 20. Peaks: 1 = α-Lac; 2 = β-Lg C; 3 = β-Lg B; 4 = β-Lg A; 5 = BSA; S = air spike.

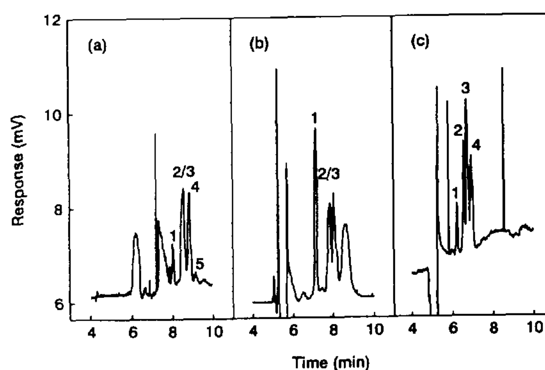

Fig. 3. Effect of buffer on separation of AC whey and β-Lg B. Samples and peaks as in Fig. 2. Buffers: (a) 50 mM sodium borate, pH 8.5, containing 0.1% ethanolamine and 0.1% Tween 20; (b) 50 mM Bistris, pH 7.0; (c) 50 mM MES, pH 8.0.

elution order of the whey proteins was α-Lac, β-Lg B, β-Lg A and BSA. Under these conditions, however, β-Lg C co-eluted with β-Lg B.

A number of different buffers at different pH values and in the presence of the separation buffer modifiers EA and Tween 20 were surveyed to determine the optimal conditions for the separation of β-Lg B and β-Lg C. Buffer pH values were kept above pH 6.0 to increase protonation of the proteins and to thus affect a better separation due to an increased mobility of the β-Lg C.

Fig. 3 shows that of the three buffers investigated 50 mM MES at pH 8.0 resulted in the best separation of the three β-Lg variants. There was a slight separation of β-Lg B and β-Lg C with the sodium borate buffer at pH 8.5 which suggested the complete separation may have been achieved at a lower pH. This was also observed with Bistris buffer at pH 7.0 although in this instance there was also a broad unidentified peak at approximately 10 min.

The MES buffer system was then made up at a number of different pH values (Fig. 4) to determine the optimum pH for separation of the β-Lg variants. Although the pK_a of MES buffer is 6.3 a pH range between 6.0 and 8.5 was investigated based on the results highlighted in Fig. 3. The best resolution of the three β-Lg variants occurred at pH 8.0 although at pH 7.5 there was partial separation of the C and B variants and almost baseline resolution between these and the A variant. Below pH 7.0 there was

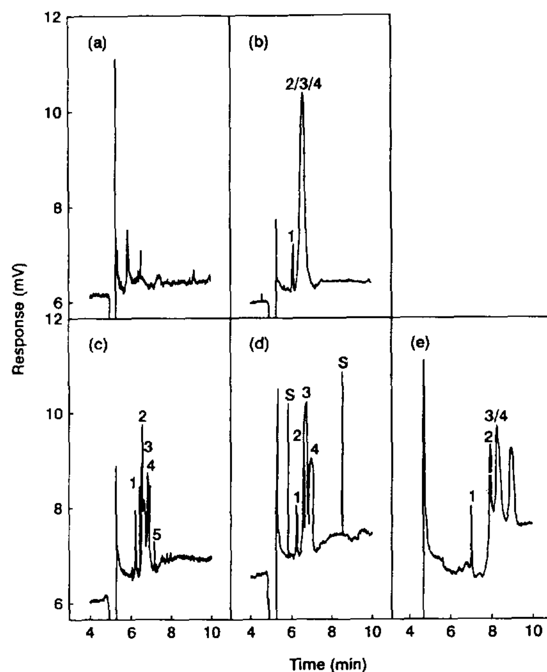

Fig. 4. Effect of buffer pH on separation of AC whey and β-Lg B. Samples and peaks as in Fig. 2. Buffer: 50 mM MES. pH: (a) 6.0; (b) 7.0; (c) 7.5; (d) 8.0; (e) 8.5.

no separation of the β-Lg variants whilst at pH 8.5 the B and A variants were not resolved.

The electrolyte modifiers Tween 20 and EA were then added to the MES buffer in an attempt to further enhance the separation of the β-Lg variants by suppressing protein capillary wall interactions [15]. Fig. 5 shows that both Tween 20 and Tween 20 plus EA resulted in more highly resolved peaks with excellent, near-baseline separation of all three β-Lg variants. A 50 m*M* MES buffer, pH 8.0, containing 0.1% Tween 20 was selected as the most appropriate separation buffer for the separation of β-Lg A, B and C variants in whey.

Positive identification of the peaks obtained from the AC whey by CZE was achieved by spiking the whey with individual whey protein standards (Fig. 6). α-Lac co-migrated with the first protein peak, β-Lg B with the third and β-Lg A with the fourth. Thus by implication β-Lg C was assigned to peak 2. The last minor peak coeluted with a BSA standard (data not shown). In subsequent phenotyping the α-Lac peak was used as an internal marker to correct for peak shifting caused by ion depletion of the cathodal buffer.

The separation method described above was then used to identify the β-Lg phenotypes of a sample population of 258 New Zealand Jersey cows. Examples of the phenotype identification are highlighted in Fig. 7. The frequencies of the different β-Lg phenotypes in the New Zealand Jersey cow population phenotyped is shown in

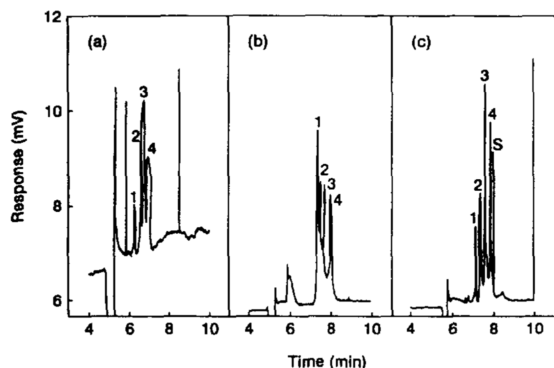

Fig. 5. Effect of separation buffer modifiers on separation of AC whey and β-Lg B. Samples and peaks as in Fig. 2. Buffer: 50 m*M* MES, pH 8.0. Modifier: (a) none; (b) 0.1% Tween 20; (c) 0.1% Tween 20 plus 0.1% ethanolamine.

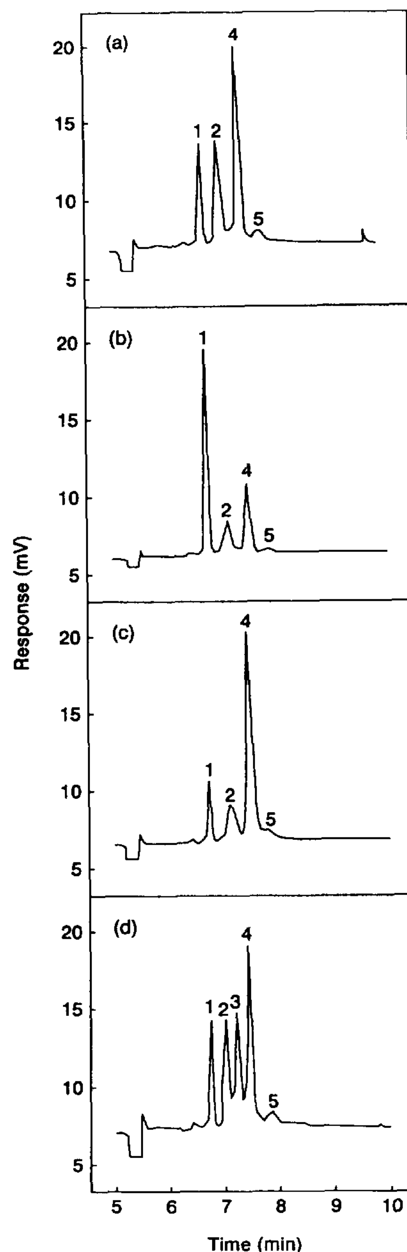

Fig. 6. Verification of AC whey CE peaks by spiking with whey standards. AC whey was prepared at 0.2 mg/ml in Milli-Q water. Individual whey standards were added to the AC whey at 0.14 mg/ml as follows: (a) none; (b) α-Lac; (c) β-Lg A; (d) β-Lg B. Peaks are as in Fig. 2.

Table 1. The A and B alleles were observed in approximately equivalent ratios whilst the C allele had a low frequency of 0.03. No β-Lg CC phenotypes were observed in the sample population.

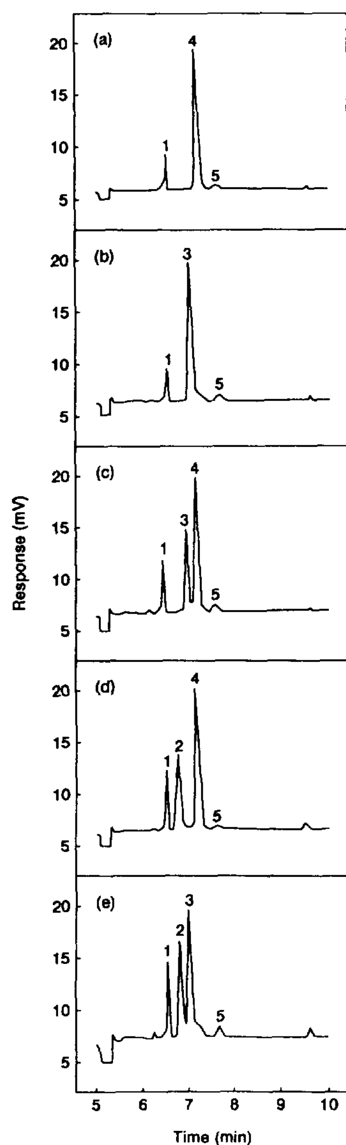

Fig. 7. Electropherograms of whey from individual Jersey cows of different phenotypes. Whey samples were prepared as detailed in Experimental. Electropherograms were aligned using the α-Lac internal standard. The phenotypes are: (a) AA; (b) BB; (c) AB; (d) AC; (e) BC. Peaks are as in Fig. 2.

Table 1
Frequencies of β-Lg phenotypes

Phenotype	Frequency of phenotype
AA	0.225
BB	0.244
AB	0.473
AC	0.023
BC	0.035

Whey samples were collected and analysed as in Experimental. The buffer system was 50 m*M* MES, pH 8.0, containing 0.1% Tween 20.

4. Conclusions

A capillary electrophoretic method based on zone electrophoresis has been developed to separate the β-Lg variants A, B and C in bovine whey. The separation was achieved by exploiting the charge differences that can occur between the variants at certain pH values as a consequence of variant amino acid substitutions. MES buffer (50 m*M*, pH 8.0) gave the best separation of the buffer systems analysed at a pH which was outside its useful pK_a range. The addition of the separation buffer modifier Tween 20 at 0.1% enhanced the resolution of the separation.

In the sample population studied β-Lg C variant containing phenotypes were found to be present at a frequency of approximately 0.06.

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