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Bovine β-Lactoglobulin E, F and G of Bali (Banteng) Cattle, *Bos (Bibos) javanicus*

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Abstract

An electrophoretic examination is made of samples of milk from eight Bali (banteng) cattle, *Bos (Bibos) javanicus*, in the Northern Territory of Australia. There are two new electrophoretically distinct bovine β-lactoglobulins, designated E and F, present in these samples. It is shown by amino acid analysis and tryptic peptide mapping of isolated samples that: (1) both E and F differ from the B variant of domestic cattle (*Bos taurus*) by +1 Gly, −1 Glu at residue 157 or 158; and (2) the F variant has the additional charge differences from E and B due to +1 Tyr, −1 Asp at residue 129 or 130. It is also shown by studies of peptides produced by the action of *Staphylococcus aureus* strain V8 protease and by more recent amino acid analyses that (1) the Gly/Glu substitution is at residue 158; (2) there is an additional Bali variant, designated β-lactoglobulin G, differing from E by +1 Met, −1 Ile at residue 78, but having the same mobility as E; and (3) there is an hitherto undetected neutral residue substitution of +1 Ser, −1 Pro at position 50 in the F variant. The relation of these variants to other known β-lactoglobulin variants of the *Bos* genus is discussed.

Introduction

The banteng, *Bos (Bibos) javanicus*, is a species of wild oxen indigeneous in historic times to parts of south-east Asia and the islands of Borneo and Java. Banteng have long been domesticated in Java and Bali, the domesticated form being known as Bali (or Balinese) cattle. The presence of a population of feral Bali cattle on the Cobourg Peninsula, Northern Territory of Australia, is well known. These animals are descended from cattle imported to the early military settlements over a century ago (Letts 1962, 1964; Calaby 1975). A tame herd of these animals has been under study by Letts and others at the Beatrice Hills Experiment Station of the Northern Territory Administration. We were fortunate to have the opportunity to type electrophoretically samples of the milk of eight of these animals and to fractionate milk from two of them in the course of our comparative studies of β-lactoglobulin and α-lactalbumin from various species.

On the basis of electrophoretic mobility differences we proposed that there were two new variants of ruminant β-lactoglobulin present in the milk of Bali cattle. We designated these new variants β-lactoglobulin E and F in descending order of mobility. This conclusion was generally supported by initial studies of their amino acid compositions and tryptic peptide maps (see Bell and McKenzie 1976; McKenzie 1976). However, there were some puzzling features of the amino acid analyses. In particular there were low values for proline in the F variant; also there were some apparent, but not clear-cut, differences in the methionine and isoleucine contents of the E protein according to whether it was isolated from milk of an

electrophoretically typed E animal or an animal typed EF. Recent studies of peptides obtained by the action of *Staphylococcus aureus* strain V8 protease have enabled us to show that the F variant includes a neutral substitution and also that there is indeed a third variant, designated G. This is the first example of a genetic variant in ruminant β-lactoglobulins in which the substitution (G *v.* E) involves solely neutral amino acid residues. In the present paper we describe the occurrence and properties of the three variants, especially in relation to other known β-lactoglobulins.

Experimental

Materials

All reagents were of analytical grade, except that ammonium sulfate was enzyme grade (Schwarz-Mann, New York) and HCl, NH_3 and distilled water were re-distilled in Pyrex glass. Imidazole was purified by recrystallization from benzene. The hydrolysed starch used in gel electrophoresis was from Connaught Laboratories, Toronto, Ont. Trypsin TPCK was from Worthington Biochemical Corp., Freehold, New Jersey. *S. aureus* strain V8 protease was from Miles Laboratories Research Products Division. Fluorescamine used in fluorescent detection of peptides was the Roche reagent Fluram (Hoffmann-La Roche, Basle).

Milk samples for typing were flown to Brisbane where they were skimmed and subjected to electrophoresis. Samples for fractionation were cooled and flown direct to Canberra, where they were subjected, within 1 h of arrival, to the first stage of fractionation.

Whey Protein Fraction

The 'whey' protein fraction was isolated from the milk samples by ammonium sulfate precipitation according to the method of Armstrong *et al.* (1967). It was held as ammonium sulfate paste at 2°C until further fractionated or used in analytical electrophoresis. Prior to fractionation it was dialysed against 0·05 M imidazole–0·043 M HCl buffer, pH 6·3 (20°C).

Electrophoresis

Starch-gel electrophoresis of skim milk samples for typing was made with gels 6 mm thick by the method of Bell (1967). However, commercial hydrolysed starch from Connaught Laboratories, was used instead of hydrolysed starch prepared from Australian potato starch (Drug Houses of Australia, Sydney) except in one experiment (see Results). The concentration of Connaught hydrolysed starch in the gel was 135–150 g dm^{-3}, adjusted according to the properties of each Connaught batch. The buffer system was an H_3BO_3–NaOH system (gel buffer: 0·030 M H_3BO_3–0·015 M NaOH, pH 8·7, gel pH *c.* 8·5; electrode buffer: 0·3 M H_3BO_3–0·075 M NaOH, pH 8·5). Samples were applied and gels stained as described previously (Bell 1967).

Electrophoresis of 'whey' protein fractions was carried out on gels 1·5 mm thick by the method described by McKenzie (1971).

Immunoelectrophoresis

Immunoelectrophoresis was performed, as described by Bell and McKenzie (1967), by using rabbit antisera to bovine β-lactoglobulin AB, bovine α-lactalbumin B (Antibodies Inc., Davis, California) and bovine serum.

Amino Acid Analysis

Samples were hydrolysed in 6 M HCl in the presence of a drop of phenol to minimize loss of tyrosine. Early analyses were made with a Beckman 120 B amino acid analyser, using two-column procedures and manual integration of peaks. Later analyses were made with a model 120 C analyser, with more highly purified reagents, better procedures for evacuation of hydrolysis tubes, and automatic integration of peaks. In the most recent work, a modified 120 C analyser using a single-column procedure with a sensitivity of 1 nmol, automatic sample injection and a Beckman model 126 data system were employed.

Peptide Studies

(i) *Tryptic peptides*

The protein was dissolved in formic acid (98–100%) and oxidized with freshly prepared performic acid under the conditions of Hirs (1967), except that the final solution was not diluted prior to lyophilization. After re-solution and a further lyophilization the oxidized protein was suspended in NH_4HCO_3 buffer (0·063 M, pH 8) and digested with 2% (w/w) of TPCK-trypsin at 37°C for 4 h and lyophilized. Those tryptic peptides, which were readily soluble in dilute acetic acid at pH 4, were applied to Whatman 3 MM paper and subjected to electrophoresis at pH 4·7 (pyridine 25 cm^3 and acetic acid, 25 cm^3, diluted to 1 dm^3 with water), 40 V cm^{-1}, 1·5 h, followed by ascending chromatography in butanol–acetic acid–pyridine–water (15 : 3 : 10 : 12 v/v) for 18 h. The peptides were detected by ninhydrin or, more recently, by their fluorescence after spraying with Fluram.

(ii) S. aureus *V8 protease peptides*

The protein was dissolved in 8 M urea (pH 9, Tris-HCl buffer) reduced with 2-mercaptoethanol and then alkylated with iodoacetamide (Crestfield *et al.* 1963). The carboxamidomethylated protein was suspended in NH_4HCO_3 buffer (0·063 M, pH 8) and digested with 2% by weight of *S. aureus V8* protease at 37°C, 4 h (Drapeau 1977). The soluble peptides were subjected to peptide mapping on Whatman 3 MM paper as for the tryptic peptides above.

Ultracentrifugation

The protein was dialysed against 0·12 M sodium acetate–0·04 M acetic acid, pH 4·65, and the protein concentration determined after removal from the dialysis sac by ultraviolet absorption at 278 nm (Bell and McKenzie 1967*a*). Sedimentation velocity measurements were made in a 12-mm single sector (4°) cell with Kel F centrepiece at 56000 rpm. An An-D rotor was used in a Beckman Spinco model E ultracentrifuge with automatic speed control, RTIC unit and schlieren optics.

Results

Electrophoresis of Skim Milk Samples

Milk samples from eight Bali (banteng) cows were examined electrophoretically using the NaOH–H_3BO_3–starch-gel system, pH 8·5. Two new β-lactoglobulin bands were found in the samples examined. None had bands of similar mobility to the bovine genetic variants A, B, C, and Droughtmaster that had been previously studied in these laboratories. Typical patterns showing the new bands, designated β-lactoglobulin E and F in descending order of mobility, are compared in Fig. 1 with those of samples containing known variants [except the D variant, discovered by Grosclaude *et al.* (1966) in Montbéliarde cattle, and not available to us].

The E band migrated ahead of Droughtmaster, but behind B and C, while F migrated behind Droughtmaster. In this buffer system, using Connaught starch gel, the B and C variants were not resolved. However, when hydrolysed starch gel prepared from DHA starch was used, the B and C variants were resolved and the E band moved behind the C band, but still ahead of the Droughtmaster band. The order of mobility observed in the Connaught gels is maintained on paper electrophoresis at pH 8·6 (barbiturate buffer system—see Bell *et al.* 1981*a*).

On immunoelectrophoresis both protein bands E and F showed reactions of identity with antiserum to bovine β-lactoglobulin AB and could not be qualitatively distinguished from other variants by this method.

In the eight samples of milk examined from Bali cows, three had β-lactoglobulin band E, one had band F, and four had bands E and F. The E and F bands stained with equal intensity when occurring together. This observation is in accordance with

the hypothesis that the mechanism of inheritance is by co-dominant alleles, as is the case for other ruminant β-lactoglobulins.

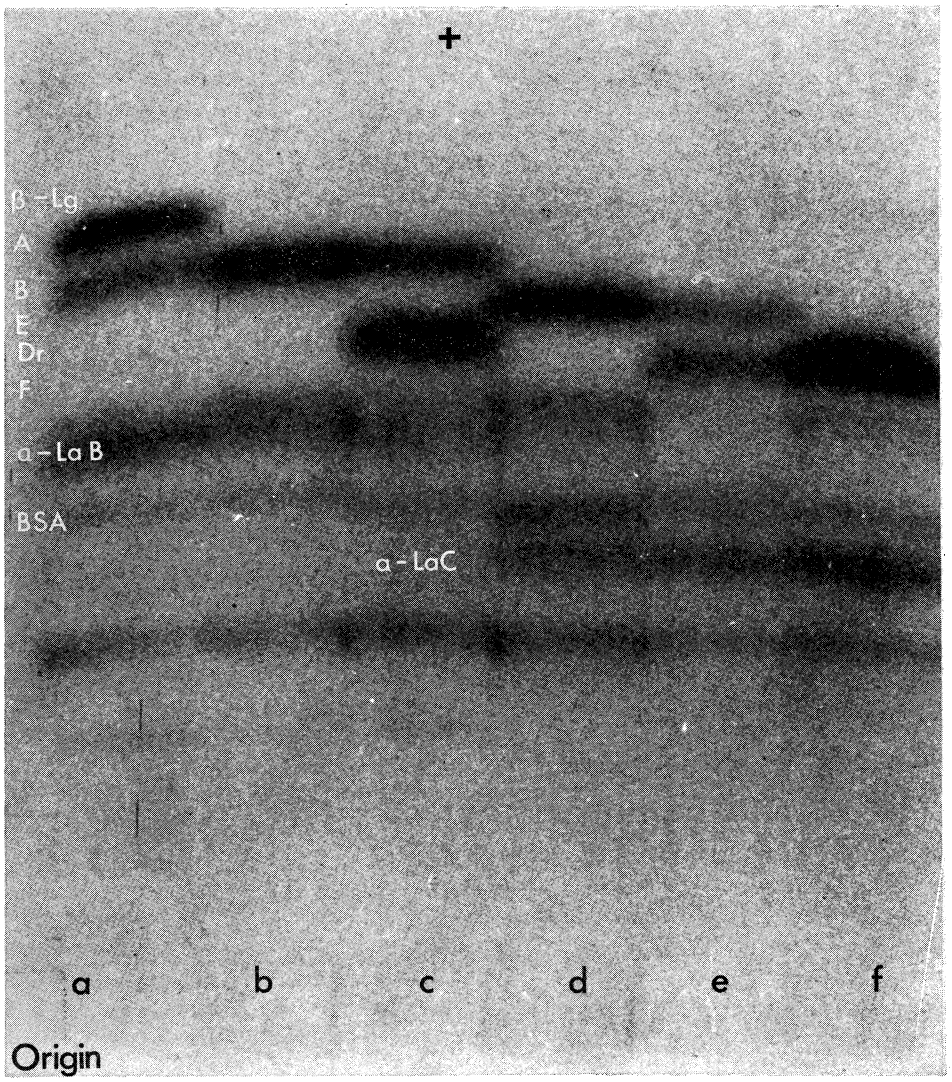

Fig. 1. Starch-gel electrophoretic patterns of skim milk samples in the $NaOH–H_3BO_3$ buffer system, pH 8·5. (*a*) Jersey milk sample, β-lactoglobulin type AB; (*b*) Jersey, β-lactoglobulin type BC; (*c*) Droughtmaster, β-lactoglobulin type BDr; (*d*) Bali (banteng), β-lactoglobulin type E; (*e*) Bali, β-lactoglobulin type EF; (*f*) Bali, β-lactoglobulin type F. All the Bali samples show the α-lactalbumin C variant. β-lactoglobulin B and C and α-lactalbumin A and B are not resolved in the $NaOH–H_3BO_3$ Connaught gel system used. BSA, bovine serum albumin.

We shall show below that there is a third variant, designated β-lactoglobulin G, that is not resolved from E in electrophoresis.

All of the eight samples had the same α-lactalbumin variant, designated C, which was of lower mobility than the other known variants A and B. α-Lactalbumin C is discussed further in Bell *et al.* (1981*a*).

Isolation of Proteins

Ammonium sulfate 'whey' protein from a single cow (No. 166) that had been electrophoretically typed as β-lactoglobulin E, was dialysed against imidazole-HCl buffer, pH 6·3 (20°C), and subjected to gel filtration on Sephadex G75 at 20°C by the method of Armstrong *et al.* (1970). A typical elution profile is shown in Fig. 2.

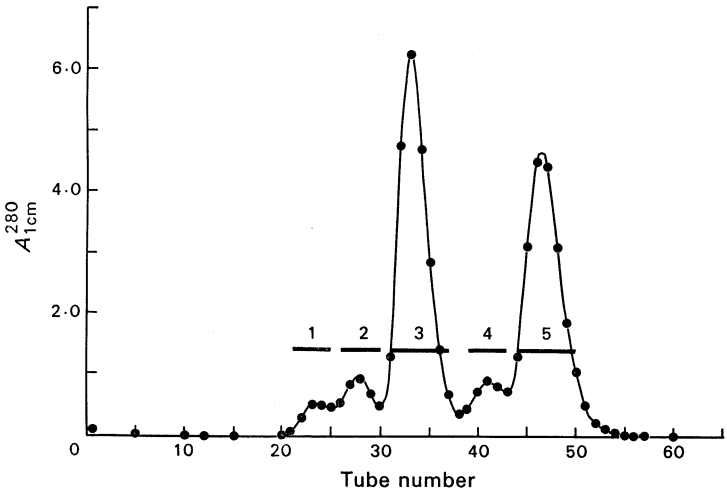

Fig. 2. Elution profile of gel filtration of total ammonium sulfate 'whey' protein from milk of cow No. 166 (typed as β-lactoglobulin E by electrophoresis) on Sephadex G75 at 20°C in imidazole-HCl buffer, pH 6·3. Each fraction discussed in the text is identified by a bar and numeral.

The resolution of the pattern is qualitatively similar to that for gel filtration of 'whey' proteins of *B. taurus* (Western dairy breeds) and *B. indicus* (Zebu) (Armstrong *et al.* 1970). The fractions 1–5 shown in Fig. 2 were examined by starch-gel electrophoresis, and had the following composition:

Fraction 1: Iron-binding proteins and probably some galactosyl transferase [UDP-D-galactose: *N*-acetylglucosamine β-4-galactosyl transferase (EC 2.4.1.38)].

Fraction 2: Bovine serum albumin and probably some galactosyl transferase.

Fraction 3: Primarily β-lactoglobulin.

Fraction 4: Minor slow components of α-lactalbumin (Hopper and McKenzie 1973), and some contamination with other proteins, such as serum albumin.

Fraction 5: α-Lactalbumin, main component and minor fast component (FC), with some slight contamination with other proteins.

Notable differences from *B. taurus* and *B. indicus* fractions were that fraction 3 (β-lactoglobulin) appeared to be 'cleaner' in the *B. javanicus* and the ratio of peak areas for β-lactoglobulin : α-lactalbumin in the total whey profile was smaller for the *B. javanicus* samples.

Fraction 3 was concentrated, dialysed against imidazole buffer, and subjected to ion-exchange chromatography on DEAE-Sephadex A25, at 2°C. It was eluted with a linear gradient of NaCl. This chromatography was repeated until a concentrated solution of the main peak gave a single band on starch-gel electrophoresis. This

Table 1. Amino acid composition of preparations of tryptic difference peptides I, II and III

Results are expressed in terms of those values set in italic font and taken as unity. The highest level of contamination of any of the residues in each peptide is shown in square brackets. The values for B shown in parentheses are derived from the known sequence of the B variant

Residue	Peptide I prepn			Peptide II prepn			Peptide III prepn				
	EG(166)	F(171)	B	EG(166)	F(171)	(B)	G(166)	E(166)	E(171)	F(171)	B
Lys		[0·15]		*1*	*1*	(1)	*1*	*1*	*1*	*1*	*1*
His	0·91	0·83	0·88								
Arg											
Cysteic acid	0·95	0·78	0·87								
Asp	1·25	1·16	0·95	1·90	1·01	(2)					
Thr	0·98	0·94	0·95	0·36	0·71	(1)	[0·09]				
Ser	0·82	0·94	0·96	[0·38]	[0·26]						
Glu	3·30	3·22	3·96	3·18	3·04	(3)					
Pro	0·84	0·76	0·64	0·68	1·01	(1)	0·95	1·03	1·05	1·04	1·04
Gly	*1*	*1*	[0·13]						[0·02]	[0·05]	[0·03]
Ala	[0·08]			1·08	1·01	(1)	1·00	1·02	1·01	1·00	1·00
Val				0·84	1·04	(1)	0·94	0·98	0·93	0·96	0·97
Met. sulfone							1·05	[0·08]			
Ile	1·08	1·08	*1*				0·03	0·96	0·99	1·03	1·00
Leu	1·49	1·85	2·1	1·20	1·02	(1)					
Tyr				0·00	0·93						
Phe	1·01	1·05	1·1				0·96	0·98	0·96	0·98	0·99
Nanomoles/ residue	33·3	33·4	23·3	5·0	12·5		38·2	35·1	51·6	35·3	49·8
Residue Nos.[A]		149–162			125–135				78–83		

[A] Numbering of residues in protein chain sequence for the peptide.

preparation of β-lactoglobulin E from the apparently homozygous cow No. 166 was identified as β-lactoglobulin EG(166), for reasons that will be discussed below.

The other cow from which samples suitable for fractionation were made available was cow No. 171, which was heterozygous, by electrophoresis, in β-lactoglobulin E and F. The total whey protein from this cow gave an essentially similar elution pattern on gel filtration to that of cow No. 166. Fraction 3 was, of course, a mixture of β-lactoglobulin E and F (together with minor impurities). After concentration and dialysis, this fraction was subjected to ion-exchange chromatography on DEAE-Sephadex A25 at 2°C. The β-lactoglobulin was contained in two partially resolved major peaks. The leading peak consisted mainly of β-lactoglobulin E and the trailing peak, β-lactoglobulin F. Appropriate fractions were taken and separately rechromatographed on DEAE-Sephadex A25 until each showed a single E and F band respectively. These preparations were identified as β-lactoglobulin E(171) and β-lactoglobulin F(171).

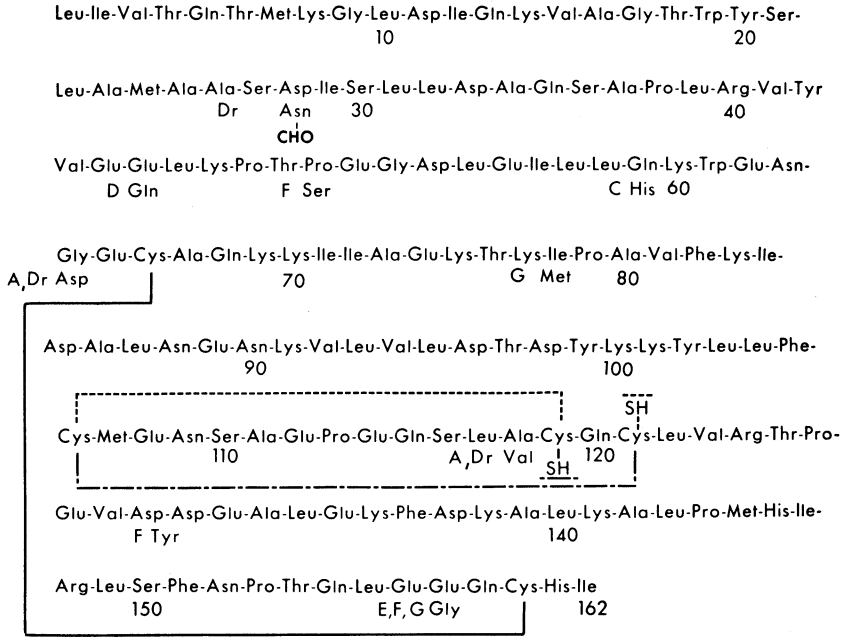

Fig. 3. Sequence of bovine β-lactoglobulin B (Braunitzer *et al.* 1973) showing the substitutions of the following variants: A (Braunitzer *et al.* 1973); C (H. A. McKenzie and D. C. Shaw, personal communication); D (Brignon and Ribadeau-Dumas 1973); Droughtmaster (H. A. McKenzie, W. H. Murphy and D. C. Shaw, personal communication, quoted by Bell and McKenzie 1976 and McKenzie 1976); E, F and G (present work). The sequence has the following modification from that of Braunitzer *et al.* (1973): residues 155–156 have been altered from Leu-Gln to Gln-Leu, in accordance with Grosclaude *et al.* (1976) and Préaux *et al.* (1979). The proposal of Grosclaude *et al.* (1976) that residue 11 is Asn instead of Asp has not been substantiated by our work and that of Préaux *et al.* (1979). The variant D_{yak} (not shown) isolated by Grosclaude *et al.* (1976) has the substitution Gly for Glu at residue 158. It is assumed that the alternate –SH/–SS positions proposed by McKenzie *et al.* (1972) apply to all variants.

Initial Amino Acid Analysis

It was clear from the initial analyses performed in 1971–72 (cited by McKenzie 1976 and Bell and McKenzie 1976) that both β-lactoglobulin EG(166) and E(171),

as well as F(171), differ from bovine β-lactoglobulin B by +1 Gly, −1 Glu residue. Also β-lactoglobulin F(171) differs from E(171) and EG(166) by +1 Tyr which could be matched with −1 Asp or possibly −1 Pro. On the basis of those analyses the proline content of F(171) was less than a full residue different and the values obtained for some other residues did not unequivocally indicate whether or not there were other differences. Likewise there appeared to be partial differences in the methionine and isoleucine contents of the putative E protein when preparations EG(166) and E(171) were analysed.

Table 2. Amino acid composition of preparations of *S. aureus* protease difference peptides I, II and III
Results are expressed in terms of those values set in italic font and taken as an integral number. The highest level of contamination of any of the residues in each peptide is shown in square brackets

Residue	Peptide I prepn		Peptide II prepn			Peptide III prepn	
	EG(166)	B	G(166)[A]	E+G(166)	(B)	F(171)	E(171)
Lys			2·97	2·78	(3)	*1*	*1*
His	1·04	0·89					
Arg				[0·21]			
CMCys	0·93	0·78					
Asp			*2*	*2*	(2)		
Thr			1·16	1·13	(1)	0·81	0·96
Ser						1·00	
Glu	1·11	1·18	1·32	1·22	(1)	1·05	1·06
Pro			1·02	1·09	(1)	0·99	1·89
Gly	0·98	0·02	[0·17]				
Ala	[0·04]	[0·22]	2·20	2·10	(2)	[0·07]	[0·07]
Val			0·99	1·00	(1)		
Met			0·85	0·41	(0)		
Ile	*1*	*1*	1·11	1·58	(2)		
Leu			1·07	1·24	(1)	0·94	0·95
Tyr							
Phe			0·94	1·07	(1)		
Nanomoles/ residue	87·8	67·7	18·8	195·6		19·8	31·9
Residue Nos	158(159)–162			75–89		46–51	

[A] Methionine sulfoxide form separated from mixture of methionine (G) and isoleucine (E) forms of peptide.

Tryptic Peptides

Comparison of tryptic peptide maps of β-lactoglobulin preparations EG(166) and F(171) with that of bovine β-lactoglobulin B indicated a single charge difference peptide for the EG(166) preparation. The amino acid composition of the difference peptides (peptide I in Table 1) compared with the sequence of the B variant (Fig. 3) indicated that the tryptic peptide contains the *C*-terminal residues 149–162 and there is a substitution of Gly for Glx. On the basis of electrophoretic mobility difference it was concluded that the residue substituted was Glu and not Gln. Therefore, EG(166) and F(171) have a glycine residue at position 157 or 158 in the sequence shown in Fig. 3.

While the F(171) protein had this substitution, its map also revealed a second peptide with a charge difference. The map of preparation EG(166) did not show this difference from that of β-lactoglobulin B. Comparison of the composition of this

peptide (peptide II in Table 1) with that of protein EG(166) (or the B variant) indicated a Tyr/Asp substitution (F : EG or B). By examination of the sequence of β-lactoglobulin B it is apparent that the tryptic peptide consists of residues 125–135 and the mutation is at position 129 or 130.

No further differences were detected at that time in analyses of the soluble tryptic peptides separated on the maps which had been stained with ninhydrin; nor was there any appreciable difference between the compositions of the peptides precipitated at pH 4 (due to limited solubility in dilute acetic acid at pH 4) from tryptic digests of EG(166) and F(171).

S. aureus *V8 Protease Peptides*

Examination of the peptides produced by *S. aureus V8* protease cleavage of the protein chain after glutamic acid residues enabled the Gly/Glu substitution in EG(166) and F(171) to be unequivocally located at residue 158. The *C*-terminal peptide was produced by cleavage after Glu 157 and contained one residue of glycine (peptide I in Table 2). The cleavage in β-lactoglobulin B is after Glu 158 to give a shorter peptide.

Apart from the difference peptides noted above, the map of the *S. aureus* digest of preparation EG(166) had an extra peptide compared with that of the B variant. Study of this and surrounding peptides revealed that the EG(166) sample was heterogeneous within a peptide spanning residues 75–89 (peptide II in Table 2). The sample consisted of equal proportions of two components, in one of which Met substituted for one of the Ile residues. The peptides had the same electrophoretic mobility. However, the methionine sulfoxide form of the peptide, designated G(166), was partially resolved by chromatography in butanol–acetic acid–pyridine–water (15 : 3 : 10 : 12 v/v) from the forms containing methionine or isoleucine, designated E+G(166).

In order to locate the position of this Met/Ile substitution, peptides were cleaved from performic acid-oxidized protein by the action of trypsin. Maps of these tryptic peptides were examined using Fluram for detection, and the heterogeneity of β-lactoglobulin EG(166) was confirmed. The methionine sulfone peptide [G(166)] was clearly resolved from the isoleucyl peptide [E(166)] chromatographically and analysed for residues 78–83, indicating a substitution of Met for Ile at position 78 (peptide III in Table 1). This substitution was *N*-terminal in the tryptic peptide and hence was largely lost in analyses of our earlier work when ninhydrin was used for detection.

Neutral Substitutions

While the earlier tryptic peptide studies and amino acid analyses had clearly indicated the mutations that lead to charge differences between the electrophoretically different E and F variants, the amino acid analyses of the proteins had left some doubt as to whether there were other substitutions (not involving charge difference). The recent mapping experiments have shown that the β-lactoglobulin EG(166) is heterogeneous.

Accordingly this preparation was re-analysed, using more stringent conditions than the earlier analyses. The results shown in Table 3 confirmed that protein EG(166) differed also from protein E(171) by +0·5 Met, −0·5 Ile.

It is concluded that the EG(166) preparation must consist of equal parts of variant E and a further variant, designated β-lactoglobulin G. Variant G differs from E by +1 Met, −1 Ile at position 78. Thus cow 166 is heterozygous in β-lactoglobulin (i.e. EG).

Table 3. Amino acid compositions of Bali cattle β-lactoglobulin preparations

Values for the individual residues are expressed as ratios to glycine taken as 5. This value was chosen so that the total number of residues is *c.* 162, consistent with the number of residues in the single-chain monomer of all known ruminant β-lactoglobulins. The values for threonine and serine have been corrected for hydrolytic losses. Values for valine and leucine did not increase significantly with time of hydrolysis. Isoleucine values did increase: results given below for preparations EG and E are 96 h hydrolyses. Only 22 h hydrolyses were performed for preparation F, a corrected value would be *c.* 9·8

Residue	Prepn EG(166)	Prepn E(171)	Prepn F(171)	Residue	Prepn EG(166)	Prepn E(171)	Prepn F(171)
Lys	14·8	14·9	14·5	Ala	15·1	15·1	15·1
His	1·9	1·9	1·9	½Cys[A]	5·0	4·8	(4·6)
Arg	2·9	2·9	2·9	Val	8·9	8·9	8·9
Asp	14·9	15·0	13·9	Met[A]	4·4	3·9	(3·6)
Thr	7·8	8·0	7·6	Ile	9·6	9·9	9·1
Ser	7·0	7·0	7·4	Leu	22·0	22·0	21·7
Glu	23·8	23·9	23·3	Tyr	3·9	3·9	4·9
Pro	7·8	7·8	6·9	Phe	4·0	4·1	3·9
Gly	*5*	*5*	*5*	Trp[B]	2·2	—	2·0

[A] Determined as cysteic acid and methionine as methionine sulfone on oxidized sample. Values in parenthesis are earlier analyses and these are not as accurate as later analyses. All known ruminant β-lactoglobulins have two cystine (four half-cysteine) and one cysteine residues per monomer. The present analyses and peptide studies for E, F, G are consistent with these values.
[B] Determined by ultraviolet absorption method.

Examination of the tryptic peptides covering residues 78–83 of the β-lactoglobulin E(171) and F(171) preparations indicated that E(171) was solely the E form (containing Ile at residue 78). Likewise F(171) had only Ile at residue 78 (peptide III in Table 1).

The recent analyses given in Table 3 clearly indicate that, in addition to +1 Tyr and −1 Asp, F(171) had one less residue of proline than EG(166) or E(171). In order to try to locate which proline might be missing, a fresh map of a tryptic digest of F(171) was compared with that of E(171). Analyses revealed each of the proline-containing peptides and showed that F(171) had a peptide in the same position and comparable yield to one from E(171) which clearly represented residues 41–60. The peptide from F(171) had the characteristic high content of Glu and Leu but had only one residue of Pro compared to two residues in E(171) and the Ser was higher than the level of contamination seen in E(171). The earlier maps of F and E had shown this feature, but, due to the peaks being more spread-out, the low colour yield for Pro and a higher level of contamination by Ser and other amino acids, the significance was not realized at that time. This peptide contained two residues of Lys because the Lys. Pro sequence at position 47–48 was resistant to cleavage by trypsin. The fact that F(171) gave the same peptide in equivalent yield means that Pro (position 48) must still occur and hence Pro at position 50 was involved in the difference. The substitution in F(171) was shown to be +1 Ser, −1 Pro when a peptide from a

S. aureus strain V8 protease digest of F(171) gave the composition shown under peptide III in Table 2. This peptide had a lower chromatographic mobility than the equivalent one from E(171) containing two residues of Pro and no Ser (residues 46–51).

In summary, the E, F and G variants all differ from the B variant by +1 Gly, −1 Glu at residue 158. The G variant has a further difference from B (or E or F) of +1 Met, −1 Ile at residue 78. However, the F variant has two further differences from B, namely +1 Tyr, −1 Asp at residue 129 or 130 and +1 Ser, −1 Pro at residue 50.

Ultracentrifugation

The A variant is the sole β-lactoglobulin, so far discovered, that undergoes appreciable octamerization (tetramerization of the dimer) in acetate buffer at pH 4·7 and low temperature (Timasheff and Townend 1962; McKenzie 1971). In view of the nature of the amino acid residue differences betweeen the E, F and G variants compared with the A variant it seemed unlikely that they would undergo this polymerization. Nevertheless, sedimentation velocity measurements were made for the preparation from cow 166, now known to be β-lactoglobulin EG. Measurements were made at 9 and 18 g dm^{-3} at 3°C. The schlieren patterns at both concentrations were symmetrical by visual examination, there being no indication of the bimodal pattern typical of the A variant (McKenzie *et al.* 1967). The $S_{20,w}$ values were 2·84 S and 2·71 S for the respective concentrations. These values are in satisfactory agreement with those observed at equivalent concentrations for the C variant dimer which does not polymerize under these conditions (Bell and McKenzie 1967*a*).

Discussion

β-Lactoglobulin is the dominant whey protein in the milk of all ruminants that have been examined, namely the cow, sheep, goat and red deer (McKenzie 1971; Jenness 1974, 1979; McDougall and Stewart 1976). Since the original demonstration of the occurrence of the two common genetic variants, A and B, in domestic breeds of cattle by Aschaffenburg and Drewry (1957), further variants have been found in cattle and two variants have been found in ovine milk (Bell and McKenzie 1967*b*). Prior to the present work on milk of Bali cattle, *Bos* (*Bibos*) *javanicus*, and of independent work by Grosclaude *et al.* (1976) on the milk of the yak, *Bos* (*Poephagus*) *grunniens*, all studies of milk had been directed towards the milk of members of the subgenus *Bos*, of the Bovinae. It is now possible to compare the β-lactoglobulins of these three subgenera.

A summary is given in Table 4 of the known bovine β-lactoglobulin variants and their occurrence. The numbers of amino acid residues we believe to be in the peptide chain of each variant are shown in Table 5.

The variants are listed in Table 4 in order of decreasing electrophoretic mobility at pH 8·5. The variants listed in the left-hand half of Table 4 are those that have been studied in our laboratories and we have established this order. It has not been possible for us to study the two variants, D and D_{yak}, shown in the right-hand half, because of Australian Government quarantine regulations. However, we have deduced their mobility for our conditions of electrophoresis in the following way.

Table 4. Known genetic variants of bovine β-lactoglobulin

Variant	Occurrence	Variant	Occurrence
A	*Bos taurus*, *Bos indicus*[A]		
B	*Bos taurus*, *Bos indicus*[A]		
C	Jersey (Nguni?)[B]		
E,G	Bali[C]	D, D_{yak}	Montbeliarde, Simmental,
Dr	Droughtmaster[D]		Jersey; Yak[E]
F	Bali[C]		

[A] Variants A and B found in all *Bos taurus* and *Bos indicus* breeds so far examined (Aschaffenburg and Drewry 1957; Aschaffenburg 1968).
[B] Bell (1967), Bell and McKenzie (1967*a*); Aschaffenburg (1968).
[C] Present work.
[D] Bell *et al.* (1970).
[E] Grosclaude *et al.* (1966, 1976).

Table 5. Comparison of number of amino acid residues in bovine β-lactoglobulin variants of several subgenera and species of the genus *Bos*

Results are based on the sequence determination of Braunitzer *et al.* (1973) and studies of Bell *et al.* (1968) and McKenzie (1971) for variants A, B and C, of Brignon *et al.* (1969) and Brignon and Ribadeau-Dumas (1973) for variant D, of Bell *et al.* (1970) for the Droughtmaster (Dr) variant, on present work for variants E, F and G, and on the work of Grosclaude *et al.* (1976) for the D_{yak} variant. The amino acid residues are listed in three groups: the first being the polar residues Asp+Asn and Glu+Gln, the second being the 'basic' polar residues (His, Arg, Lys) and the third being the neutral polar (except Asn, Gln) and non-polar residues. Within each group the residues are listed in order of increasing hydrophobic (decreasing hydrophilic) character based on the hydrophobicity indices of Bigelow (1967). Values expressed as number of residues per single chain molecule, molecular weight 18 000

Residue	*Bos* (*Bos*) *taurus* variants				*Bos* (*Bos*) *indicus*	*Bos* (*Bibos*) *javanicus* variants			*Bos* (*Poephagus*) *grunniens*
	A	B	C	D	variant Dr[A]	E	F	G	variant D_{yak}
Asp+Asn	16	15	15	15	16	15	14	15	15
Glu+Gln	25	25	24	25	25	24	24	24	24
His	2	2	3	2	2	2	2	2	2
Arg	3	3	3	3	3	3	3	3	3
Lys	15	15	15	15	15	15	15	15	15
Gly	3	4	4	4	3	5	5	5	5
Ser	7	7	7	7	7	7	8	7	7
Thr	8	8	8	8	8	8	8	8	8
Ala	14	15	15	15	14	15	15	15	15
½Cys	5	5	5	5	5	5	5	5	5
Met	4	4	4	4	4	4	4	5	4
Val	10	9	9	9	10	9	9	9	9
Leu	22	22	22	22	22	22	22	22	22
Ile	10	10	10	10	10	10	10	9	10
Pro	8	8	8	8	8	8	7	8	8
Phe	4	4	4	4	4	4	4	4	4
Tyr	4	4	4	4	4	4	5	4	4
Trp	2	2	2	2	2	2	2	2	2
Carbohydrate					+				

[A] Separated from milk of *Bos taurus* × *Bos indicus* cross.

On the basis of amino acid composition, peptide and sequence studies it would appear that the β-lactoglobulin variant, D_{yak}, isolated from milk of the yak by Grosclaude *et al.* (1976) is identical with the E variant that we have isolated from the milk of Bali cattle. Thus it would be expected that the mobility of β-lactoglobulin D_{yak} would be the same as that of β-lactoglobulin E in any electrophoretic system.

Although the variant D_{yak} differs in amino acid composition from the D variant, their net charge is the same at pH 8·5, and Grosclaude *et al.* (1976) found that these two variants had the same mobility in their electrophoretic system. Hence, it may be reasonably assumed that the E (also G), D and D_{yak} variants have the same mobility under our conditions of electrophoresis. However, it should be emphasized that Thompson (1970) was not able to resolve satisfactorily the D variant in alkaline acrylamide gels from the B variant and was only able to resolve D from A, B and C in alkaline urea–starch gels (in the presence of 2-mercaptoethanol) using the conditions of Grosclaude *et al.* (1966).

Proposed amino acid sequences for the several variants are compared in Fig. 3. It can be seen that the C, D, E and D_{yak} variants differ from the common B variant by single amino acid residue substitutions. Variants G and A differ from B in two replacements. In contrast the F variant has a total of three residue differences from B.

β-$\text{Lactoglobulin}_{\text{Droughtmaster}}$ (Dr) is unique among the variants in having a carbohydrate moiety attached. It differs from B by three substitutions: two are similar to those of variant A, but the third consists of the substitution of Asn for Asp at residue 28, the Asn having a carbohydrate moiety attached [H. A. McKenzie, W. H. Murphy, and D. C. Shaw, quoted by Bell and McKenzie (1976), and also unpublished data]. In our original study of this variant we believed it only differed from A in having a carbohydrate moiety (Bell *et al.* 1970). It is now apparent that it is a true genetic variant since it has this amino acid residue difference from A.

All of these variants exist near the iso-ionic point (*c.* pH 5·2) as dimers of two non-covalently linked polypeptide chains of 162 residues (Droughtmaster having also the carbohydrate moiety on each chain). This can be contrasted with the β-lactoglobulins of milk of the pig and horse, where the molecular weight indicates that these proteins occur as the monomer. Also they do not contain any sulfhydryl residues (see Bell *et al.* 1981*b*, 1981*c*).

The D, D_{yak}, E and G variants are unusual among the known β-lactoglobulin variants in that they have the same net charge at pH 8·5. In comparison with the B variant, D and D_{yak} have the substitution of an uncharged amino acid residue for a charged residue (Glu) at different chain positions. Variant D has Gln substituted for Glu at position 45 and D_{yak} has Gly instead of Glu at position 158.

In contrast the G variant differs from the E variant in having a Met/Ile substitution at position 78. This is the first example of two variants of bovine β-lactoglobulin differing from one another solely by the substitution of one uncharged amino acid residue for another uncharged residue. It is an example of how 'electrophoretically silent' variants may be differentiated by peptide and sequencing studies. One other such example in milk protein studies is the detection of the β-casein B_Z of the milk of *Bos indicus* (Zebu) (Aschaffenberg 1968).

Our recent detection of the substitution of Ser for Pro in the F variant, compared with B, is further confirmation of the need to make studies of the type we have described, if neutral substitutions are to be located.

In the following paper (Bell *et al.* 1981*a*) additional new variants for milk proteins of Bali cattle are found and a comparison is made of the β-lactoglobulins, α-lactalbumins, α_{s1}-, β-, and κ-caseins of milk of three subgenera of the genus *Bos* and of α-lactalbumins and β-lactoglobulins in the genera *Bos* and *Bubalus*.

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