

Amino Acid Analysis of the β -Lactoglobulins from Individual Cows of Two Phenotypes

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Received September 5, 1964

Amino acid analyses were performed on β -lactoglobulin samples prepared from individual dairy cows, 24 of which were homozygous for β -lactoglobulin A and 9 for β -lactoglobulin B. No differences were found in individual contents of any amino acids other than the previously known β -A- β -B difference amino acids. The total analyses agreed well with previously published analyses on β -A and β -B obtained from pooled milk of many animals.

Studies of dairy cattle family groups have indicated that the two β -lactoglobulin genetic variants (A or B) are determined by a single pair of allelic genes with no dominance (2). This conclusion was based on the difference in electrophoretic mobility on paper, and shortly thereafter the information on the aspartic acid-glycine and the valine-alanine differences became available (3-5). The latter residues could not be expected to cause any electrophoretic difference,¹ nor would other neutral amino acid substitutions be discovered by this technique. Genetic calculations (2) based on

¹ Hydrophobic residues of greatly different side-chain dimensions (such as valine and alanine) might, however, cause differences in conformation stability, rates of denaturation, etc. Such a difference, in fact, exists between β -A and β -B (6), and it is undoubtedly significant that the pH 8.7 electrophoretic separation (1) is impossible in the Tiselius cell (1°C) or on paper in the cold, but works only when the electrophoresis is done at room temperature. Under these conditions, the paper strips become quite warm and the β -lactoglobulins are exposed to highly denaturing conditions.

these experiments could not rule out such a possibility, nor the possibility that cows producing β -A (rapidly moving on the paper) might indeed carry the difference-valine of β -B. Furthermore, the occurrence of two amino acid changes as the result of a single gene change was quite puzzling. A possibility would be that two independent loci were involved. In this connection, the analyses (4) of β -lactoglobulins A and B, which were performed on proteins isolated from the pooled milk of a number of homozygous animals, required rather more rounding off of the valine-alanine difference than that of the aspartic-glycine difference to arrive at the whole-number difference in each case of two residues per molecule. This also could indicate a two-loci state of affairs, with one or more individuals in the pool having abnormal Asp-Gly and Val-Ala ratios. The genetic implications of these possibilities have been discussed previously (7).

On the basis of the above, amino acid analyses were performed on β -lactoglobulins from as many individual cows as possible including all of the still-available animals

whose milk had been pooled to prepare the large batches of β -A and β -B previously analyzed (3, 4), and including a daughter-dam pair of known homozygous phenotype (by electrophoretic criteria alone) and a number of daughters of proven homozygous bulls (2). The amino acid composition of the pooled β -A and β -B (given in Ref. 4) could

then serve as a base against which any significant individual differences could be checked.

EXPERIMENTAL

Samples of milk from each of 30 Holstein and 3 Ayrshire-Holstein crossbred cows in the Beltsville experimental herd (24 typed as A/A, 9 as

TABLE I
MOLE RATIOS OF RESIDUES OF 24-HOUR HYDROLYSES^a

Animal No.	Gly	Val	Ala	Thr	Ser	Glu	Pro	½ Cys	Met	Ileu	Leu	Tyr	Phe
<i>β-Lactoglobulin A, 16 Asp</i>													
445	3.0	9.5	13.7	7.6	6.4	24.5	7.9	4.3	3.9	7.9	21.1	3.7	3.7
3477	3.1	8.8	13.8	.5	.5	.5	8.2	3.8	3.7	8.1	21.6	.6	.7
3645	3.2	9.6	13.8	.6	.5	.8	8.1	3.3	3.4	8.2	21.2	.5	.9
3653	3.1	8.6	13.7	.6	.6	.2	8.8	3.4	3.5	7.6	20.8	.5	.6
3653 ^b	3.1	9.2	14.0	.6	.4	.5	8.3	4.2	3.9	8.4	21.2	.6	.7
3655	3.2	8.7	13.7	.6	.5	.6	8.2	3.5	3.7	8.1	21.3	.7	.8
3666	3.1	8.2	13.9	7.5	6.4	24.3	8.4	3.4	3.7	7.7	21.0	3.6	3.6
3678	3.1	8.3	13.5	.6	.7	.0	7.5	3.5	3.6	7.3*	20.8	.4	.6
3679	3.2	9.6	14.1	.6	.5	.4	8.4	2.9	3.6	7.9	21.1	.8	.8
3691	3.0	9.0	13.5	.5	.4	.4	7.9	4.0	3.8	7.8	20.4	.7	.7
3822	2.4*	9.0	13.7	.6	.4	.6	8.3	3.0	3.8	8.5*	21.2	.7	.8
3825	3.1	9.1	13.4	.5	.4	.2	7.8	4.1	4.0	7.8	20.5	.6	.7
3828	3.1	8.4	14.7*	7.7	6.5	24.3	9.0*	3.5	3.7	7.4	20.8	3.6	3.7
3836	3.1	8.8	14.6*	.6	.6	.6	8.5	3.9	3.6	7.7	20.7	.6	.8
3853	3.0	8.0*	13.5	.4	.6	.5	8.2	3.2	3.8	7.6	21.1	.6	.6
3855	3.2	8.9	13.8	.4	7.0*	.1	8.2	3.6	3.8	7.5	20.6	.5	.8
3856	3.1	8.7	13.9	.6	6.5	.8	7.5	3.7	4.0	8.2	21.4	.8	.7
3856 ^b	3.2	8.8	14.0	.4	.4	.3	8.7	3.0	3.6	8.2	21.0	.6	.7
3870	3.1	9.5	13.7	7.6	6.4	24.4	7.4*	4.0	3.7	8.4	21.2	3.7	3.9
3873	3.1	9.4	13.5	.6	6.5	.3	8.0	4.3	3.6	8.0	20.9	.6	.9
3876	3.1	9.8*	14.0	.6	6.2*	.4	8.3	4.6	3.9	8.3	21.3	.7	.8
3880	3.2	8.7	13.8	.6	6.5	.4	9.1*	3.5	3.9	8.1	21.1	.7	.8
3893	3.0	9.3	13.5	.6	.5	22.9*	8.0	3.4	4.0	8.0	21.1	.6	.8
3818	3.2	8.2	13.8	.6	.6	24.4	8.2	3.8	3.6	7.6	20.9	.7	.8
4001	3.1	8.7	13.8	7.6	6.5	24.8	8.2	3.7	4.0	8.3	21.3	3.7	3.7
Average ^c	3.11	8.91	13.74	7.56	6.49	24.43	8.18	3.66	3.75	7.95	21.02	3.63	3.74
<i>β-Lactoglobulin B, 15 Asp</i>													
3812	4.2	8.3	14.8	7.6	6.6	24.2	7.8	4.5	3.8	7.7	20.6	3.5	3.7
4000	4.0	7.1	14.7	7.6	6.6	24.4	8.4	3.6	3.7	7.5	20.9	3.7	3.7
3887	4.0	7.3	14.6	7.6	6.6	24.3	8.4	2.9	3.8	7.8	21.0	3.6	3.7
Average ^c	4.06	7.56	14.70	7.60	6.60	24.30	8.20	3.66	3.77	7.66	20.83	3.60	3.70

^a Ratios based on aspartic acid.

^b Duplicate analyses

^c Starred (*) values excluded. These values are greater than the standard deviation in every case and vary from the average by >3% (alanine), >1.2% (serine), >4% (proline), >5% (leucine), and >10% (valine).

TABLE II
MOLE RATIOS OF RESIDUES OF 96-HOUR HYDROLYSES^a

Animal No.	Gly	Val	Ala	Ser	Glu	Pro	Ileu
<i>β-A Lactoglobulin, 16 Asp</i>							
3603	3.1	10.9	13.9	4.9	24.5	7.9	9.7
3666	3.2	9.9	14.0	5.1	24.5	8.2	9.8
3678	3.1	9.8	13.9	4.2	24.4	7.9	9.7
3822	3.1	10.1	13.9	4.7	24.2	8.3	9.9
3836	3.1	10.2	14.1	4.8	24.4	8.4	9.9
3853	3.0	9.9	13.7	4.8	24.3	7.8	9.6
3873	3.1	10.0	14.0	4.8	24.5	8.3	9.7
3880	3.1	10.1	14.1	4.8	24.5	8.4	9.9
3893	3.2	10.1	14.1	5.0	24.6	8.2	9.9
3818	3.1	10.0	13.7	5.0	24.4	8.0	10.1
3828	3.1	10.9	13.8	4.4	24.5	8.2	9.8
3828	3.0	9.6	13.7	5.4	24.7	8.2	9.8
3828	3.0	9.6	13.3	5.3	24.6	7.6	9.6
Average ^b	3.09	9.94	13.86	4.87	24.47	8.10	9.79
Pooled β-A ^c	3.07	9.78 ^d	14.10	6.35 ^d	24.30	8.48	9.63
<i>β-B Lactoglobulin, 15 Asp</i>							
3813	4.0	9.0	14.5	6.3	24.8	8.5	9.6
3881	4.2	9.0	14.8	6.3	25.0	8.8	9.4
3887	4.0	8.8	14.1	6.2	24.9	8.7	9.5
3890	4.0	8.7	14.4	6.2	25.0	8.6	9.6
3896	3.9	8.5	14.2	6.4	24.8	8.9	9.6
4003	4.1	8.9	14.8	6.3	25.0	8.9	9.8
416	4.0	8.8	14.4	6.3	25.1	9.0	9.7
Average ^b	4.02	8.81	14.46	6.27	24.94	8.78	9.59
Pooled β-B ^c	3.88	9.16 ^d	14.77	5.87 ^d	24.28	8.31	9.62

^a Ratios based on aspartic acid.

^b Individual values shown rounded off to nearest tenth. Average calculated on unrounded values.

^c Data of Ref. (4) corrected to 18,000 molecular weight and based on aspartic acid.

^d Extrapolated to zero (serine) and infinite (valine) hydrolysis time.

B/B) were used, and the β-lactoglobulins were prepared by the method of Aschaffenburg and Drewry (8). The proteins were recrystallized six times from NaCl solutions, and 1-mg samples (±10%) of lyophilized crystals were hydrolyzed at 110°C with 6.7 M HCl. The hydrolyzates were analyzed² on an automatic amino acid analyzer of the type described by Spackman *et al.* (9).

The animals selected were related as follows: animal 3477 was the dam of 3853, by H-41, who also sired 3822, 3828, 3836, 3855, and 3856; H-22 had four daughters represented: 3653, 3666, 3678, and 3679; H-10 sired 3825, 3876, and 3818; P-17 sired

3870, 3893, and 4001; "Fobes" sired 3880, 4000, and 3881; and an Ayrshire bull "Preferred" sired 3887, 3890, and 3896. Related animals were selected because it was considered to increase the chances, if any, of finding electrophoretically "hidden" substitutions.

RESULTS AND DISCUSSION

The amino acid analysis of the 24-hour hydrolyzates³ is given in Table I, where the

³ In order to economize on analyzer time, and since the pooled β-lactoglobulin samples previously analyzed (4) had been hydrolyzed 24, 72, and 96 hours to check amino acid destruction, it was decided to hydrolyze for one period of time only, and to recheck any samples showing variations at a different hydrolysis time.

² Analysis for the basic amino acids was not done, since differences in these residues would have been noticed in the screening experiments.

micromolar values obtained have been converted to molar ratios with aspartic acid residues as the basis—16 residues in the β -A polypeptide chain and 15 in the 18,000 molecular weight subunit of β -B (4, 5, 10). Because of destruction of cystine, analytical variations for this amino acid are not considered significant. The individual starred analyses were considered to be sufficiently deviant to warrant repetition at 96-hour hydrolysis time, particularly for animals 3822 and 3828, which showed deviations in more than one amino acid. The 96-hour hydrolyses are shown in Table II, where for reasons of brevity, individual values are omitted for threonine, $\frac{1}{2}$ cystine, methionine, leucine, tyrosine, and phenylalanine, since none of these showed any significant variation at either 24- or 96-hour hydrolysis (with the exception of additional cystine destruction at the longer time).

Both Tables I and II show clearly the aspartic acid-glycine and the valine-alanine differences of one residue per subunit. While the valine and alanine differences between β -A and β -B reported previously (4) were 0.6–0.7 residues, the present values provide even better evidence for a full residue difference. The high and low values given by certain individual cows at 24-hour hydrolysis time were analytical errors, since upon repetition, no significant variation from the down-column average, or from the analysis of the pooled lactoglobulins can be seen. The differences (β -A to β -B) in the averages for proline after 96-hour hydrolysis (Table II) cannot be explained; it should be noted, however, that no such difference is seen at 24-hour hydrolysis (Table I) nor was seen in our previous communication (4). In the same way, the apparent difference of one residue of serine per chain in Table II is seen not to be real by comparison with the essentially identical (24-hour) values given for this residue (Table I). Linear extrapolation of both β -A and β -B values of serine to zero hydrolysis time give 6.75 and 7.02 residues per molecule, respectively, which agrees well with the literature values of 14 residues of serine per 36,000 molecular weight (4, 5, 11). It is of interest to note that our earlier work on the two variants (4) has also shown a significantly greater destruc-

tion of serine in β -A upon prolonged hydrolysis. Speculation about the cause of this difference is, however, not warranted at this time.

CONCLUDING REMARKS

Among the 33 homozygous dairy cattle examined, no significant differences in electrophoretically inactive amino acids have been found. None of the animals of either type make β -lactoglobulins differing measurably in analysis from that of a pooled sample. Within the limits of the analytical technique (9), the differences in aspartic-glycine content and alanine-valine content between β -A and β -B are one residue per subunit of 18,000 molecular weight, and so far as is known, these differences always occur together. A single repeatable deviation from this regular pattern would provide a definite indication of the existence of two loci controlling the synthesis of proteins differing from each other in two separated sequence positions (7). The situation is further complicated by the recent discovery (12) of a third electrophoretic variant, β -C, in which there appears to be an additional single amino acid difference from β -B (13), and now three compositional differences from β -A. More information is needed on the β -lactoglobulins, and particular attention should be paid to the minor and exotic breeds of cattle, where ancestors of these much-studied proteins may well be found.

ACKNOWLEDGMENTS

The authors would like to thank Drs. S. N. Timasheff and W. G. Gordon for useful discussions.

REFERENCES

1. ASCHAFFENBURG, R., AND DREWRY, J., *Nature* **176**, 218 (1955).
2. PLOWMAN, R. D., TOWNEND, R. E., KIDDY, C. A., AND TIMASHEFF, S. N., *J. Dairy Sci.* **42**, 922 (1959).
3. GORDON, W. G., BASCH, J. J., AND KALAN, E. B., *Biochem. Biophys. Res. Commun.* **3**, 672 (1960).
4. GORDON, W. G., BASCH, J. J., AND KALAN, E. B., *J. Biol. Chem.* **236**, 2908 (1961).
5. PIEZ, K. A., DAVIE, E. W., FOLK, J. E., AND GLADNER, J. A., *J. Biol. Chem.* **236**, 2912 (1961).

6. GOUGH, P., AND JENNESS, R., *J. Dairy Sci.* **45**, 1033 (1962).
7. TIMASHEFF, S. N., AND TOWNEND, R., *J. Dairy Sci.* **45**, 259 (1962).
8. ASCHAFFENBURG, R., AND DREWRY, J., *Biochem. J.* **65**, 273 (1957).
9. SPACKMAN, D. H., STEIN, W. H., AND MOORE, S., *Anal. Chem.* **30**, 1190 (1958).
10. TOWNEND, R., KIDDY, C. A., AND TIMASHEFF, S. N., *J. Am. Chem. Soc.* **83**, 1419 (1961).
11. STEIN, W. H., AND MOORE, S., *J. Biol. Chem.* **178**, 79 (1949).
12. BELL, K., *Nature* **195**, 705 (1962).
13. KALAN, E. B., GREENBERG, R., WALTER, M., AND GORDON, W. G., *Biochem. Biophys. Res. Commun.* **16**, 199 (1964).