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ARTICLE *in* INTERNATIONAL DAIRY JOURNAL · FEBRUARY 1998

Impact Factor: 2.01 · DOI: 10.1016/S0958-6946(98)00026-0

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Int. Dairy Journal 8 (1998) 99–104

Printed in Great Britain
0958-6946/98/$19.00 + 0.00

Comparison of the Effect of Heating on the Thermal Denaturation of Nine Different β-Lactoglobulin Preparations of Genetic Variants A, B or A/B, as Measured by Microcalorimetry

C. Holt[a]*, R. Waninge[b], P. Sellers[c], M. Paulsson[d], R. Bauer[e], L. Øgendal[e], S. P. F. M. Roefs[f], P. van Mill[f], C. G. de Kruif[f], J. Léonil[g], J. Fauquant[g] and J. L. Maubois[g]

[a] *Hannah Research Institute, Ayr, Scotland KA6 5HL, UK*
[b] *Department of Food Engineering, Lund University, P.O. Box 124, S-221 00 Lund, Sweden*
[c] *Department of Physical Chemistry 2, Lund University, P.O. Box 124, S-221 00 Lund, Sweden*
[d] *Department of Food Technology, Lund University, P.O. Box 124, S-221 00 Lund, Sweden*
[e] *Department of Mathematics and Physics, Royal Veterinary and Agricultural University, Thorvaldsenvej 40, DK 1871 Frederiksberg, Denmark*
[f] *NIZO, Postbus 20, 6710 Ede, The Netherlands*
[g] *INRA-Laboratoire de Recherche de Technologie Laitière, 65 Rue de Saint Brieuc, 35042 Cédex, France*

(Received for publication 2 April 1998)

ABSTRACT

Three samples each of the pure A and B genetic variants together with three samples of the mixed A/B variants of β-lactoglobulin were studied by differential scanning microcalorimetry. All the samples were characterised with respect to whey protein composition and shown to be almost pure β-lactoglobulin. Other analyses showed only minor amounts of other contaminants but variable degrees of lactolation ranging from <1.9 to 10.4 mol%. Thermograms were recorded at about 3 mg β-lg mL^{-1} in a 10 mM P_i buffer pH 7, with the ionic strength of milk, using a scan rate of 60°C h^{-1} on both Microcal MC-2 and MCS calorimeters and reasonable agreement was found for the denaturation temperature measured on the two instruments. The biggest source of variation in the thermograms of samples was due to the genetic variant. For the single variants, other differences were in the same order as the variability seen in repeat preparations of the same sample. A slightly greater variation was found among the A/B samples where the one with the lowest degree of lactolation was most stable. However, dialysis of the sample against the P_i buffer almost completely eliminated the difference. Qualitatively, all thermograms appear to be the sum of 4 processes centred at temperatures of approximately 55, 77, 105 and 125°C. Compared to the A variant the thermogram of the B variant shows a less prominent shoulder at about 55°C. Also, the broad feature at about 105°C is reduced and the peak at 125°C much enhanced.

INTRODUCTION

An important use of whey proteins recovered from milk in the course of the manufacture of cheese and other dairy products is as functional ingredients in food, where the function is usually to increase viscosity or water retention or bring about gelation or help to stabilise emulsions and foams. Reproducible food functionality is sought by the manufacturer and consumer but not enough is known about the causes of variation in the performance of different whey proteins or whey protein fractions. Although sample purity, composition and extent of denaturation are known to affect thermograms of whey proteins, some indications from the literature are that even in carefully prepared samples of β-lactoglobulin (β-lg) in high purity, there are unexplained differences between investigators (Brownlow, 1994). Likewise, in a comparison of aggregation kinetics measured by light scattering, a commercial preparation of β-lactoglobulin was found to differ appreciably from 5 other samples prepared in the same institute (Hoffmann *et al.*, 1996). It was of interest to see, therefore, whether there is a general problem of lack of reproducibility in the denaturation behaviour of β-lactoglobulin samples from different sources when comparisons are made under carefully controlled conditions. Altogether, 9 samples were examined and compared using microcalorimetry to measure thermal denaturation.

* Corresponding author.

MATERIALS AND METHODS

Preparation sourcing and characterisation of samples

The pure β-lg variants A and B, each from homozygous cows and the mixed AB samples were obtained from 4 sources, namely, the Hannah Research Institute (hriA, hriB), NIZO (nizoA, nizoB, nizoAB) and the INRA-Laboratoire de Recherche de Technologie Laitière (inraAB). Detailed information is available on the preparation and purity of each of these samples. We

also employed samples from Sigma Chemical Co. (sigmaA type L7880, lot 13H7020; sigmaB type L 8005, lot 13H7150; sigmaAB, type L3908, lot 75H7080) but information on the source and method of preparation of these samples is not available. The sigmaA and sigmaB samples were found to have 33 and 28 mol%, respectively, complexed to P_i in the ratio 1 mol (mol β-lg monomer)$^{-1}$ of P_i, as revealed by electrospray ionisation mass spectroscopy (ESIMS). The proportion could be reduced considerably by dialysis against a large excess of water acidified with trifluoroacetic acid to pH 3.0 for 24 h at 4°C and the P_i was eliminated almost completely by 72 h, demonstrating that the association was ionic rather than covalent.

hri samples

These were prepared from the milk of individual cows homozygous for either the A or B variant. The raw milk was cooled to room temperature immediately after milking and a crude (~90%) sample of β-lg obtained by fractional precipitation with $(NH_4)_2SO_4$, following the method of Armstrong *et al.* (1967). Subsequent purification by gel filtration on a Sephadex G 75 column (Pharmacia Biotech) followed by exhaustive dialysis against distilled water and freeze drying yielded a product estimated by PAGE to be at least 95% pure β-lg A or B. A chloroform-methanol extraction of the freeze dried material contained only traces of lipid-like material, estimated by GC-MS to be <0.2 mol (mol β-lg monomer)$^{-1}$.

nizo samples

The method of preparation involves a combination of ultrafiltration, diafiltration and freeze drying, following the procedure of Maubois (1987). Raw milk from a homozygous herd was thermized and centrifuged. Then, by cross-flow microfiltration, caseins and the remaining fat were removed. The synthetic whey was desalted by dia-ultrafiltration. Alpha-lactalbumin was precipitated at pH 3.8 and filtered off. Denatured beta lactoglobulin was removed at pH 4.6. The permeate was dia-filtered and concentrated in a solution of pH 6.7 and freeze dried. The percentages of the dry weight as β-lg, ash and lipid-like substances were, respectively 99, 0.25 and 0.13 (nizoA); 98, 0.3 and 0.11 (nizoB) and 92, 2.0 and n.d (nizoAB).

inra samples

A clarified whey was prepared from raw milk from a local dairy and used as the starting material for the isolation of the β-lg by the method of Fauquant *et al.* (1988), as modified by Léonil *et al.* (1997) to reduce lactolation. The method is essentially cross flow microfiltration to remove micellar casein and other large particles followed by ultrafiltration and diafiltration against water to produce a retentate with a protein to total solids ratio of 0.978. Residual casein was removed by centrifugation after acidification to pH 4.6 with 3 M citric acid. In the next stage, β-lg was separated from α-lactalbumin by causing the latter to aggregate at pH 3.8, (where the protein is thermally unstable in the apo-form) by heating to 56°C for 30 min and the precipitate removed by centrifugation. The freeze dried product was characterised by PAGE, reverse phase HPLC (RPHPLC) and ESIMS and shown by conventional chemical analyses to comprise 98.3% dry matter, 94.9% M protein (TNx6.38) 0.5% NPN, 0.4% ash and 2% citric acid.

RPHPLC

All the samples were analysed for protein content by reverse phase high pressure liquid chromatography as described by Hoffmann *et al.* (1996) and the results are summarised in Table 1. As can be seen from Table 1, all the samples are of high purity in terms of the proportion of β-lg in the chromatogram. In some samples of the single genetic variant there appears to be a low level of absorbance in the region of the other variant for reasons that are not clear. In Table 1, both of these peaks are added together to give the total β-lg.

Lactolation

Previous work (Maubois *et al.*, 1995; Léonil *et al.*, 1997) has shown that β-lg is modified by covalent binding of a lactose residue as soon as milk is heated above the cow's normal temperature of 37°C. The formation of the complex manifests itself by a M + 324 Da peak in the electrospray mass spectrum and the extent of lactolation was shown to increase with the time and temperature of heat treatment. The degree of lactolation was measured on all of the samples by the total ion current in ESIMS (Léonil *et al.*, 1997) and the results are summarised in Table 2.

Table 1. RPHPLC Analysis of 9 Samples of β-Lg. Values are % Total Absorbance at 214 nm

Sample	α-la[a]	bsa[b]	Ig[c]	β-lgA	β-lgB	Total β-lg
sigmaA	0	0	0	85.7	14.3	100
hriA	0.7	0	0	99.3	0	99.3
nizoA	0.9	0	0	97.2	1.9	99.1
sigmaB	0	0	0	4.7	95.3	100
hriB	0	0.8	0	2.8	96.4	99.2
nizoB	1.8	0	0.6	0	97.6	97.6
sigmaAB	0	0	0	47.5	52.5	100
nizoAB	2.1	0	0.6	50.7	46.5	97.2
inraAB	0.6	0	0	50.6	48.8	99.4

[a] α-Lactalbumin.
[b] Bovine serum albumin.
[c] Immunoglobulins.

Table 2. Protein Concentration of a Solution Containing 3 mg Freeze Dried Sample Per g Solution and Mol% Lactolation of the Samples Used in the Survey Determined by ESIMS

Sample	Genetic variant	Concentration (mg g^{-1})	Protein % of freeze dried weight	Lactolated β-lg, mol% total β-lg
sigmaA	A	2.48	82.7	7.2
hriA	A	2.77[a]	92.0[a]	3.2
nizoA	A	2.76	92.1	10.0
sigmaB	B	2.53	84.4	7.5
hriB	B	2.61[a]	87.0[a]	4.4
nizoB	B	2.83	94.3	8.7
sigmaAB	A + B	2.66	88.6	10.4
nizoAB	A + B	2.36	78.8	10.2
inraAB	A + B	2.43	80.8	<1.9

[a] Mean of two preparations.

Buffer P and preparation of the solution

The composition of buffer P is 60 mM NaCl, 5.7 mM NaH_2PO_4, 4.3 mM Na_2HPO_4, which, when made up, gives a pH of 6.7. The pH and ionic strength of the buffer are approximately the same as those of milk. The disadvantage of using a phosphate buffer is the evidence of a relatively tight binding of the ion to the protein so that its presence may influence the physicochemical properties other than through the pH and ionic strength.

The samples were stored as a powder at −20°C or lower, over dry silica gel. For buffer P, the solution was made up by weighing out the approximate amount of dry powder and making up to the required weight of solution using the buffer. The concentration of each solution was checked by measuring the absorbance at 280 nm using a specific extinction coefficient of 0.96 $L\,g^{-1}cm^{-1}$ and the % protein in the freeze dried material determined (Table 2).

Size distribution by HPLC

Buffer P was filtered through a 20 nm filter before use. To prepare a stock solution, 30 mg of the freeze dried β-lg sample was dissolved in 2.5 mL of the buffer and dialysed against a further 1000 mL, with stirring at 4°C for about 8 h. The 1000 mL was changed and the dialysis continued for 16 h. After opening the dialysis bag, the β-lg was filtered twice through a 200 nm filter, the concentration determined and adjusted to 10 $mg\,mL^{-1}$ with buffer P.

The HPLC column was a Waters Protein-PAK 300SW, run at a flow rate of 1 $mL\,min^{-1}$ and concentration in the eluate was measured with a Wyatt/Optilab 903 refractive index detector. The injected volume was 50 μL of the 10 $mg\,mL^{-1}$ stock solution and the eluent was buffer P + 0.01% sodium azide.

Chromatograms were recorded for the hriA, hriB, nizoA, nizoB, nizoAB and inraAB samples only and the results are shown in Fig. 1. The β-lg dimer is eluted between 8.3 and 10 min with a partially resolved shoulder at about 10 min found in some of the chromatograms for the monomer; the amplitude of the dimer peak is about unity so the total amount of aggregated material in any of the samples is only a few %. Peaks at about 5.8 min correspond to the aggregates produced by heating at 67.5°C, those at about 7.5 min are pentamers or hexamers and at about 8.3 min trimers or tetramers elute.

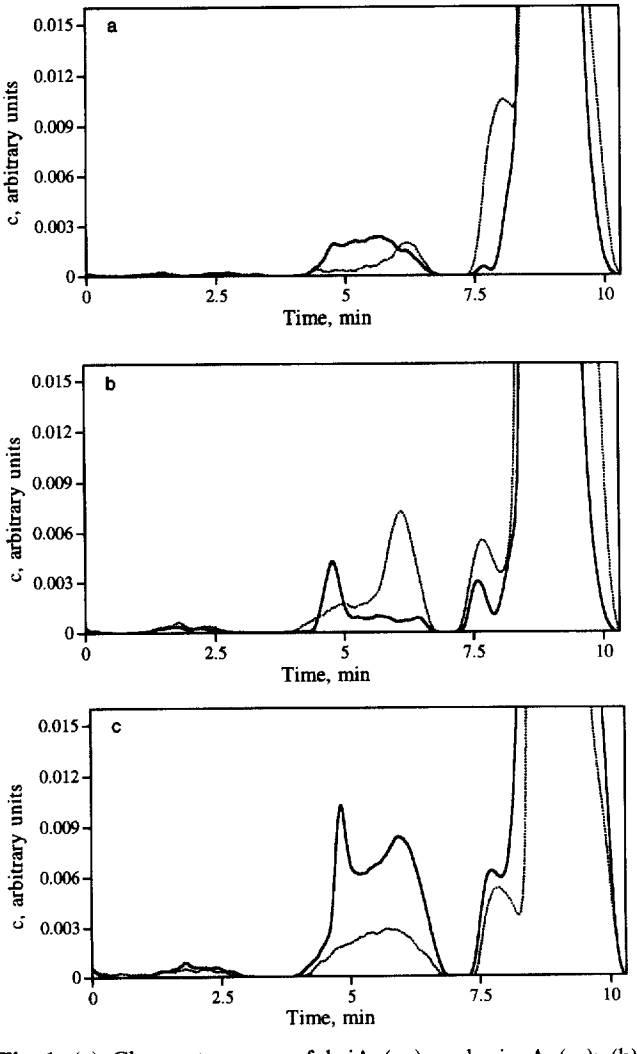


Fig. 1. (a) Chromatograms of hriA (—) and nizoA (····); (b) chromatograms of hriB (—) and nizoB (····); (c) chromatograms of nizoAB (—) and inraAB (····).

Microcalorimetry

Two different calorimeters were employed, both manufactured by Microcal Inc. of Ma, USA: the MC-2 model in the University of Lund and the MCS model at the Hannah Research Institute. Samples were prepared as described above to give a protein concentration of about 3 $mg\,g^{-1}$, using buffer P with the addition of 0.02% NaN_3 as preservative. After dissolving, the solution was filtered through a 1 μm (MCS) or 0.2 μm (MC-2) filter and the pH recorded again. Actual concentrations were determined from the UV absorbance at 280 nm. Thermograms were scanned from 25 to 115°C (20–135°C for the MCS calorimeter) at 60°C min^{-1} with buffer P as reference. A baseline was also recorded with water in the sample and reference cells.

RESULTS AND DISCUSSION

MC-2 thermograms

Figure 2 shows the thermograms of the three samples of the B genetic variant. The absence of a clearly established baseline makes quantitation of the thermograms hazardous so we will limit the interpretation to the position of the main maxima (T_2) and qualitative observations on the shape of the thermogram. The general features of the thermogram are in accordance with a previous study of thermograms at similar concentrations and scan rates (Qi *et al.*, 1995) in showing a main peak at 70–80°C and a subsidiary, somewhat variable, feature at about T_3 = 105°C, though it is close to the upper temperature limit. Compared to higher concentrations the main peak is asymmetric and broad but qualitatively at least, the main peak appears less broad than with the A variant at the same concentration and a shoulder at about T_1 = 55°C is less clearly seen. the positions of the maxima found using the MC-2 calorimeter with 8 of the 9 samples are summarised in Table 2 and compared with the findings from the MCS instrument.

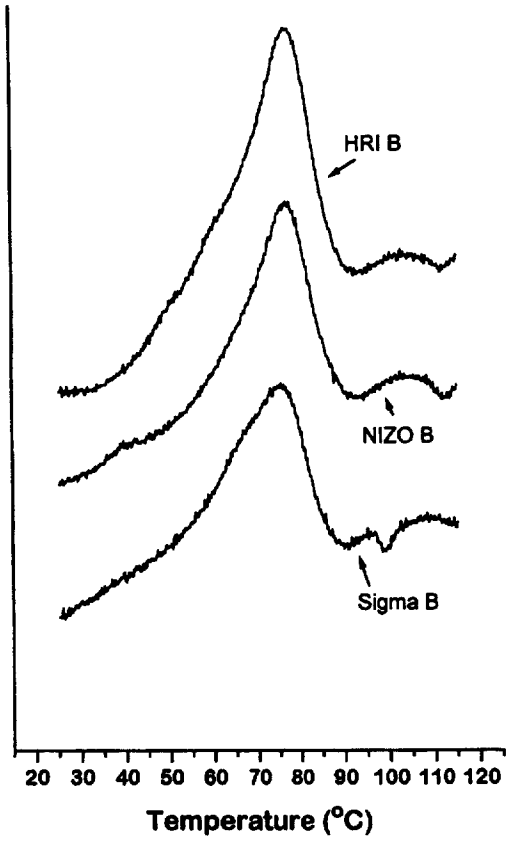
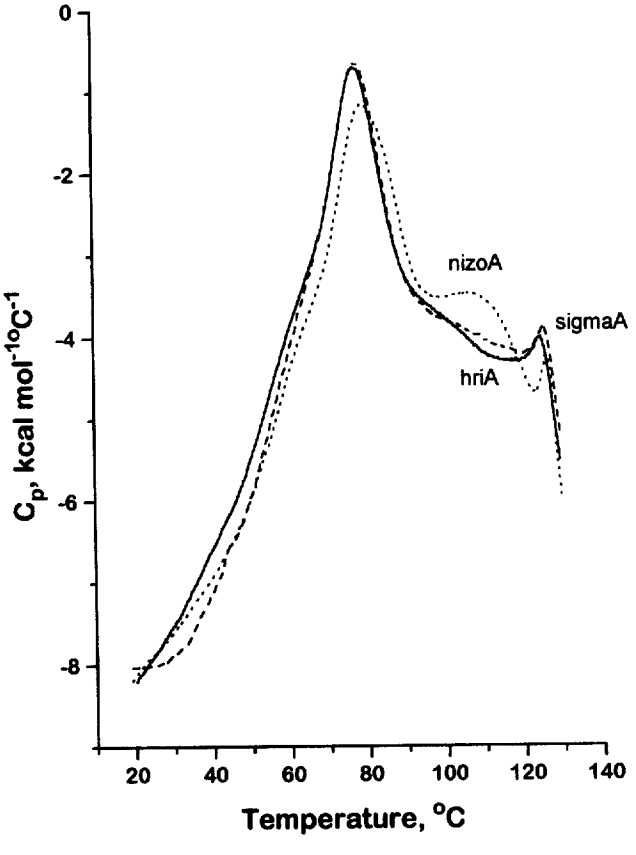


Fig. 2. Thermograms of the three preparations of the B variant recorded on the MC-2 calorimeter at concentrations of 3.0 (hriB), 3.1 (nizoB) and 2.7 mg mL^{-1} (sigmaB). Spectra have been separated on the y axis for clarity.



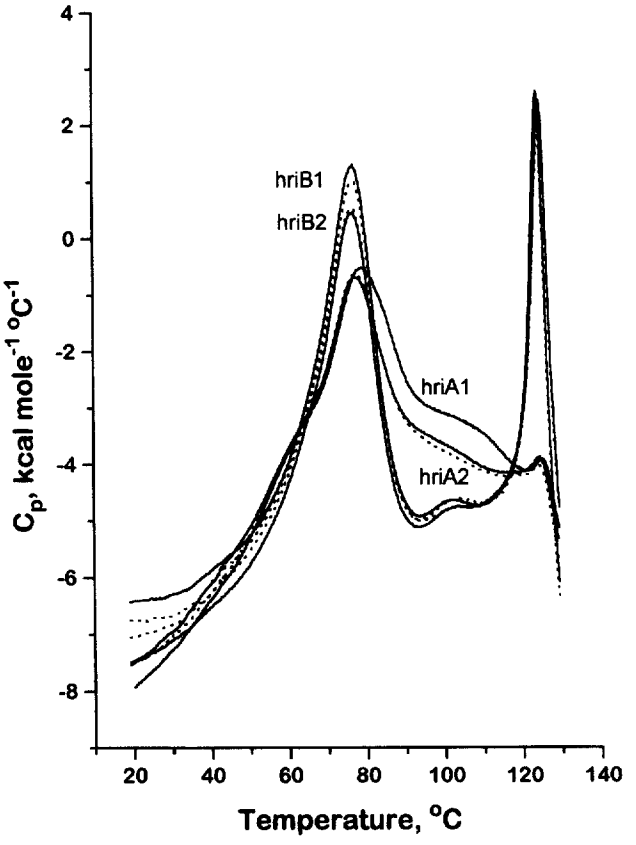


Fig. 4. Comparison of thermograms of hriA and hriB and demonstration of variability in repeated preparations of the β-lg from the same individual cow by the same method. For each sample, replicate thermograms are given as solid and dotted lines and except at the start of the scan, the replicate scans are in excellent agreement. The two B variant preparations show two well separated peaks with good agreement of the T_2 and T_4 values. With the A variant, the T_4 peak is much reduced and, in the example shown, the T_2 values are reproducibly different for reasons that are not yet clear.

MCS thermograms

Figure 3 shows the thermograms of the three samples of the A variant. Although the position of the baseline is still not established, the extended temperature range of these measurements allows another thermal feature to be seen: a small maximum at about $T_4 = 125$°C. Similar measurements have been made on the B variants and in Fig. 4 a comparison is made of some typical A and B thermograms. The most striking feature is that in spite of similar T_2 values, the shapes of the thermograms are quite different. Besides the less prominent shoulder at $T_1 = 55$°C, the B variant shows a weaker feature at about T_3 and a much stronger peak at T_4. Such differences are a strong indication that the course of thermal denaturation is radically different in the two variants and some insight into their very different propensities for fouling dairy heat exchangers may be gained.

Thermograms of the A/B samples are more variable between sources than the pure variants but in general

Fig. 3. Thermograms of the three preparations of the A variant recorded on the MCS calorimeter at concentrations of 2.68 (hriB), 2.83 (nizoB) and 2.53 mg mL^{-1} (sigmaB).

Table 3. Positions (°C) of 3 of the 4 Gross Features Found in Thermograms of 9 Samples of β-Lg

Sample	MC-2		MCS		
	T_2	T_3	T_2	T_3	T_4
sigmaA	77.9	~100	77.6	—	125.5
hriA	79.3	—	77.6	—	124.1
nizoA	78.9	~107	79.1	105	126.0
sigmaB	76.0	~108	76.2	—	124.3
hriB	77.2	~103	76.4	102	123.9
nizoB	77.3	~104	77.2	104	125.5
sigmaAB	76.4	—	76.2	—	123.3
nizoAB	76.9	—	76.9	—	124.1
inraAB	78.3	—	80.6	108	128.4
inraAB, dialysed		—	78.1	—	124.5

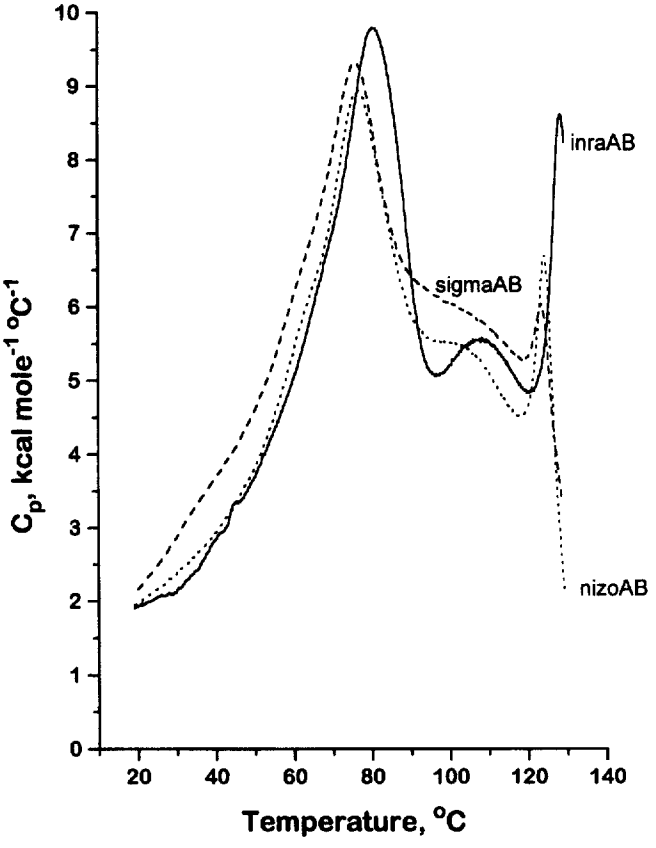


Fig. 5. Thermograms of the three preparations of the AB samples recorded on the MCS calorimeter at concentrations of 2.66 (sigmaAB), 2.36 (nizoAB) and 2.43 mg mL^{-1} (sigmaAB). The inraAB sample, which had the highest T_2 and T_4 values and a clear maximun at T_3, was converted into a thermogram much like the other 2 samples by overnight dialysis against buffer P.

shape are intermediate between the pure A and B thermograms. The inraAB sample differs from the other 2 in having higher T_2 and T_4 values and a more prominent T_3 but these differences were almost completely removed when the sample was first dialysed for 24 h against buffer P before recording the thermogram. Table 3 gives the comparison of peak positions found by the MC-2 and MCS calorimeters. In general, there is a very satisfactory agreement between instruments, considering that T_2 is very sensitive to concentration in this range (Qi *et al.*, 1995) and the concentrations used were not exactly the same. Thus, calorimetric measurements of T_2 made at high concentrations on a Perkin Elmer DSC-7 calorimeter were previously shown to agree with those recorded with an MC-2 calorimeter (Qi *et al.*, 1995) and this concordance is now extended to the MCS calorimeter.

CONCLUSIONS

Thermograms of the β-lg samples are most noticeably different because of the proportions of the A and B genetic variants. Thermograms can be decomposed into 4 thermal features with maxima at about 55, 77, 105 and 125°C. Compared to the A variant, the shoulder at 55°C is less noticeable in the B variant and the peak at 125°C is much stronger, apparently at the expense of the shoulder or peak at about 105°C.

In one sample (inraAB), dialysis was sufficient to bring the thermogram into much closer agreement with the nizoAB and sigmaAB samples in spite of the differences in degree of lactolation. Thermograms of the pure variants were in good agreement in spite of differences in degree of lactolation between the samples.

The present findings contrast with a recent light scattering study (Hoffmann *et al.*, 1996) showing marked differences between sigma and nizo preparations of the same kind as were studied here.

ACKNOWLEDGEMENTS

The sample survey was carried out as part of European Commission-funded concerted action on the Molecular Basis of the Aggregation, Denaturation, Gelation and Surface Activity of Whey Proteins (MADGELAS), CT96-1202. All other members of the MADGELAS group are thanked for their co-operation in the preliminary sample survey, of which this paper forms a part. The HRI work was supported by the Scottish Office Agricultural, Environmental and Forrestry Department. The NIZO research was supported by the Dutch Ministry of Economic Affairs through the programme IOP-Industrial Proteins and by the dairy companies Coberco-Research Deventer and Friesland Dairy Foods Beilen. E. Little (HRI) and G. Henry (INRA-LRTL), are thanked for skilled technical assistance and D. Mollé (INRA-LRTL) for mass analysis.

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