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Thermal resistance of β-galactosidase in dehydrated dairy model systems as affected by physical and chemical changes

L. Burin[a,*], M.P. Buera[a], G. Hough[b], J. Chirife[a]

[a]*Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina*
[b]*Instituto Superior Experimental de Tecnología Alimentaria, H. Yrigoyen 931, (6500) 9 de Julio, Buenos Aires, Argentina*

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Abstract

The objective was to study the remaining activity of the enzyme β-galactosidase in dehydrated dairy systems and its relationship with simultaneous chemical (i.e. non-enzymatic browning, NEB) and physical changes (structural collapse) at temperatures from 70 to 105 °C. The presence of milk proteins had a structure-stabilizing effect, which was not reflected by enzymic stabilization or NEB prevention. Although the remaining enzyme activity was correlated with physical and chemical changes in some of the systems exposed at 22% R.H., these changes were not parallel to the macroscopic changes in some of the anhydrous systems. The single lactose systems were highly collapsed at all the temperatures analyzed, but enzymic inactivation and NEB were dependent on the storage temperature rather than on the degree of collapse. Chemical and physical changes were not correlated to the remaining activity. Changes which occur at a molecular level may not be related to the changes at a supramolecular level (such as those derived from glass transition or collapse). © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Enzyme stability; β-galactosidase; Dehydrated dairy products; Non enzymatic browning: Collapse; Glass transition

1. Introduction

The development of new nutritional preparations requires enzyme stabilization, (lactase, proteases) in the dry state (Kim, Chung, Lee, Choi, & Kim, 1999). In multicomponent systems, such as dairy products, several chemical and physical changes may occur upon storage, which may affect protein structure and functionality. Powders and other dehydrated preparations may contain amorphous solids, which can undergo a glassy to rubbery transition when stored at temperatures above the glass transition temperature (T_g). Physical changes (stickiness, collapse, crystallization and aroma retention) have been described as temperature-time-moisture-dependent phenomena occuring above a collapse temperature (Chirife, Karel, & Flink, 1973; To & Flink, 1978; Tsourouflis, Flink, & Karel, 1976) and may cause problems in production and storage of dehydrated food powders (Lazar, Brown, Smith, & Lindquist, 1956; White & Cakebread, 1966). Structural changes above T_g result from a decrease in viscosity and resultant flow, because the liquid-like material above T_g has a tendency to minimize its volume (Karel, Anglea, Buera, Karmas, Levi, & Roos, 1994).

The glassy state of the amorphous matrix in which the reactants are embedded was considered an important cause, responsible for the diffusion-limited rates of chemical reactions that take place at low moisture content (Karel, Anglea, Buera, Karmas, Levi, & Roos, 1994). Non-enzymatic browning (NEB) is frequently cited as one of the main deteriorative reactions taking place in dehydrated systems, and it is susceptible to diffusional limitations. The glass transition may influence chemical reactions if the mechanism has a mobility-dependent step and the size and structure of the reactants is such that their translational diffusion is coupled to the matrix viscosity (Champion, Blond, & Simatos, 1997). As the moisture content is increased, the matrix is plasticized by water, the glass–rubber transition occurs, the diffusional limitations disappear, and an increase in the reaction rate should be noticeable (Buera et al., 1995). As a result, degradation pathways would be expected to depend strongly on the storage temperature relative to T_g (Karel, Buera, & Roos, 1993; Levine & Slade, 1992)

* Corresponding author. Tel. +54-11-4794-3344; fax: +54-11-4576-3366.
E-mail address: pilar@di.fcen.uba.ar (L. Burin).

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as long as the deterioration mechanisms are mobility-dependent.

Several workers have tried to relate the thermal inactivation of enzymes in nearly anhydrous sugar-containing model matrices with the glass transition phenomena (Buera, Ross, Moreno, & Chirife, 1999; Cardona, Schebor, Buera, Karel, & Chirife, 1997; Mazzobre, Buera, & Chirife, 1997a, 1997b; Rossi, Buera, Moreno, & Chirife, 1997; Terebiznik, Buera, Pilosof, 1997, 1998, Schebor, Buera, & Chirife, 1996; Schebor, Burin, Buera, Aguilera, & Chirife 1997), peptide stability (Bell 1997) and colour formation in the glassy state (Schebor, Buera, Karel, & Chirife, 1999). It was observed that the formation of a glassy system is not the only condition for assessing chemical stability. It thus remains necessary to analyze the interrelations between physico-chemical changes and structure in complex dairy systems.

The objective of the present work was to study thermal inactivation of the enzyme β-galactosidase in dehydrated dairy model systems and its relationship with chemical (i.e. NEB) and physical changes (manifested as structural collapse or shrinkage), as a consequence of the storage at different temperatures. Conveniently, formulated dairy model systems, having the same glass transition temperatures, but different structural stabilities allowed the analysis of chemical stability in relation to collapse and T_g separately.

2. Materials and methods

2.1. Preparation of model systems

The model systems were designed to contain different proportions of milk components.

Amorphous matrices were obtained by freeze-drying solutions containing 20% (w/w) total solids. The systems were composed of:

1. lactose (L) (Mallinckrodt, NY, LA, St. Louis, USA);
2. lactose + sodium caseinate (Sigma Chemical Co., St. Louis, MO, USA) 50:50 (L + C);
3. skim milk powder (M: 52% lactose, 29% casein and 6% whey proteins, dry basis), bought at a local supermarket;
4. sweet cheese whey powder (W: 72% lactose and 13% proteins, dry basis) was provided by SANCOR Cooperativas Unidas, Ltda., Bs. As., Argentina.

The aqueous solutions were cooled over an ice-bath and the enzyme β-galactosidase, from *A. oryzae* (Sigma Chemical Co., St. Louis, MO, USA) was added to each solution. The amount of enzyme added was 270 units/g of matrix (one unit is the enzyme quantity to hydrolyze 1 mmole of lactose/h at 37 °C and pH 4.5). Previous studies indicated that this amount allowed sensitive detection of changes upon heating. Aliquots of 1 ml of each model solution were placed in 4-ml vials and immediately frozen using liquid air. A Stokes freeze-dryer, model 21 (F.J. Stokes Company, Equipment Div., Pennsalt Chem. Corp., Philadelphia, PA), was used which operated at a −40 °C condenser plate temperature and a chamber pressure of less than 13 Pa during 48 h. After freeze-drying, the samples were transferred into vacuum desiccators, for 1 week at 26 °C over M_g ClO_4 for "zero" moisture content, or over saturated salt solution of KCH_3COO, for 22% relative humidity (R.H.).

2.2. Determination of moisture content

The water contents of the samples after 1 week over M_g ClO_4 or KCH_3COO atmospheres were determined by difference in weight before and after drying in a vacuum oven at 96 or 70 °C for 48 h, respectively. These conditions had been proved to be adequate to assess constant weight.

2.3. Heat treatment

The model systems were stored in forced air convection ovens at selected temperatures (70, 75, 80, 85, 95 and 105 °C). Vials containing "zero" moisture samples were left open. Samples previously humidified at 22% R.H. were hermetically sealed, using Teflon tape around their top and cups.

At suitable intervals two samples were removed from the oven and the remaining activity of the enzyme and color development were determined as described below.

2.4. β-galactosidase activity

After heat treatment, 1 ml of citrate buffer (pH 4.4, 0.1 M) was added to each sample and the systems were kept in an ice bath until complete dissolution was achieved. Then, 1 ml of 15% (w/w) lactose in citrate buffer was added and the vials were incubated for 60 min at 37 °C. After incubation, the samples were exposed to boiling water for 6 min to inactivate the enzyme. Lactose hydrolysis was determined by measuring the amount of glucose formed. An enzymatic method, based on the oxidation of glucose by glucose oxidase to gluconic acid and hydrogen peroxide, was employed. Two replicates of each sample were analyzed and, as two samples were taken from the oven at each time, the average of four measurements was reported for each storage time. The amount of lactose hydrolyzed by samples without thermal treatment (L_0) was considered to correspond to 100% β-galactosidase activity; the amount of lactose hydrolyzed after heat treatment (L_t) was referred to L_0, and the remaining activity (RA) was expressed as :

$$RA = 100 \times (L_t/L_0)$$

2.5. Collapse measurement

Collapse was achieved in the 22% R.H. samples as a consequence of exposure to moisture/temperature at which $T > T_g$, and suitable time conditions. The degree of collapse was determined semi-quantitatively by calculating sample volume from measured height and diameter of the samples. The degree of collapse was expressed as:

$$1 - \frac{d_t^2 \times h_t}{d_c^2 \times h_c} = \text{degree of collapse}$$

where d_t = diameter of the sample after thermal treatment; d_c = initial diameter of the sample before thermal treatment; h_t = height of the sample after thermal treatment; h_c = initial height of the sample before thermal treatment

The average of both samples was reported.

2.6. Browning

The systems were reconstituted with 2 ml of water, and 0.6 ml of this suspension was used for colour determination.

Colorimetric measurements were performed with a HunterLab 5100 Colour Difference Meter (Hunter Associates Laboratory, USA) using a 1.6-cm diameter aperture and an illumination mode that illuminated the aperture area from the bottom of the sample. A white background was placed on the top of the sample holder. The CIE tristimulus values, X, Y, Z, were obtained directly from the instrument.

Colour functions were calculated for illuminant D and a 10° observer. Browning index ($BR = 100 (\times -0.31)/0.172$), where $x = X/(X + Y + Z)$, was found to be an adequate measure of non-enzymatic browning reactions (Buera & Resnik, 1990). The average of five determinations was reported for each sample.

2.7. Glass transition temperatures

Glass transition temperatures of the different model systems were determined by differential scanning calorimetry (onset values) using a Mettler TA4000 Thermal Analysis System with TA72 software calibrated with the melting point of indium (156.6 °C), lead (327.5 °C) and zinc (419.6 °C). Duplicate samples in open pans were stored in vacuum desiccators over saturated salt solutions. After 10 days, the pans were hermetically sealed and scanned from at least 40 °C below the glass transition temperature to at least 40 °C above the glass transition temperature. An immediate rescan was run for each sample to verify the endothermic baseline shift associated with the glass transition. The onset temperature of the change in heat capacity was considered as the glass transition temperature. An empty aluminium pan was used as reference.

Table 1
Water content and T_g obtained for the model systems equilibrated at different relative humidities

SYSTEM	R.H. (%)	Water content (% d.b.)	T_g, onset value (°C)
L	0	~0	92.8
	22	5.0	47.6
L + C	0	~0	99.4
	22	6.3	45.0
M	0	~0	95.5
	22	5.8	48.1
W	0	~0	94.2
	22	4.6	50.0

3. Results and discussion

Table 1 shows the water content and the T_g values obtained for the freeze-dried systems. The T_g values for each moisture condition were fairly similar, being between 93 and 100 °C for the anhydrous systems, and between 45 and 50 °C for the systems stored under 22% R.H. Jouppila and Roos (1994) reported T_g values for amorphous lactose and skim milk that were almost the same as those in the present study. It is to be noted that both casein and whey proteins, as high molecular weight compounds, should increase the T_g values of relatively low molar mass compounds, such as lactose. The T_g of "dry" lactose is around 100 °C (Jouppila & Roos, 1994), while that of anhydrous casein and sodium caseinate is around 140 °C (Kalichevsky, Blanshard, & Tokarczuk, 1993). However, the T_g values of present dairy models (which are mixtures of lactose and caseinate/whey proteins) are very close to those of lactose (Jouppila & Roos, 1994). It is possible that lactose and sodium caseinate exist in different immiscible phases, DSC detecting the carbohydrate-rich phase in the caseinate:lactose 1:1 sytems. Kalichevsky et al. (1993), working with casein: lactose (10:1) systems, demonstrated that the addition of sugars to sodium caseinate had little effect on the T_g value of the caseinate, which suggests that both components behave as immiscibly ones, the T_g of carbohydrate-rich systems (as those analyzed in the present work, Table 1) being governed by lactose T_g, and in caseinate-rich systems, by caseinate T_g. The phase separation (manifested as two T_gs) could be observed by Kalichevsky et al. (1993) by DMTA measurements of casein:fructose (2:1) samples.

The remaining β-galactosidase activity was analyzed in anhydrous and 22% R.H. samples at all the selected storage temperatures. Fig. 1a, b shows the kinetics for β-galactosidase inactivation in the anhydrous and exposed to 22% R.H. matrices at 85 and 75 °C, respectively. The loss of enzymatic activity during thermal

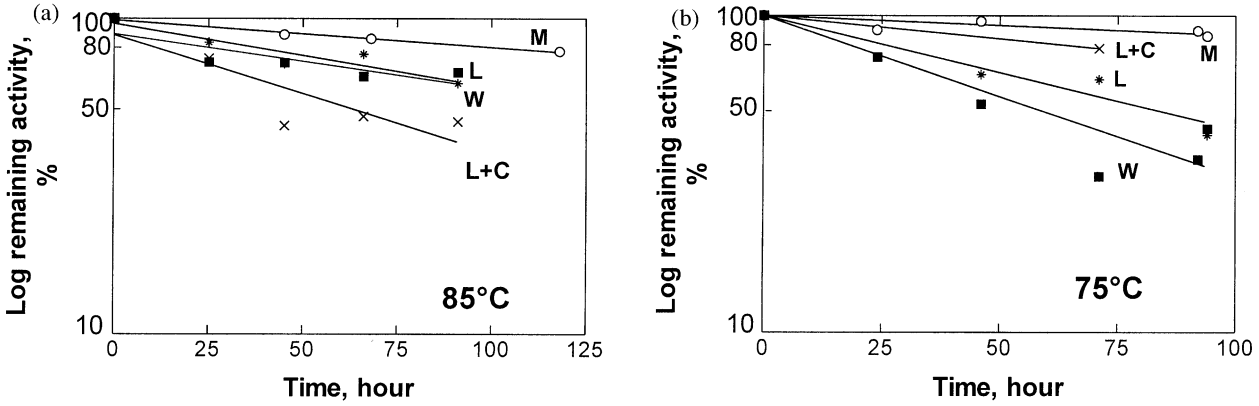

Fig. 1. Remaining activity of the enzyme as a function of storage time at 85 or 75 °C for (a) anhydrous systems and (b) systems exposed to 22% R.H., respectively.

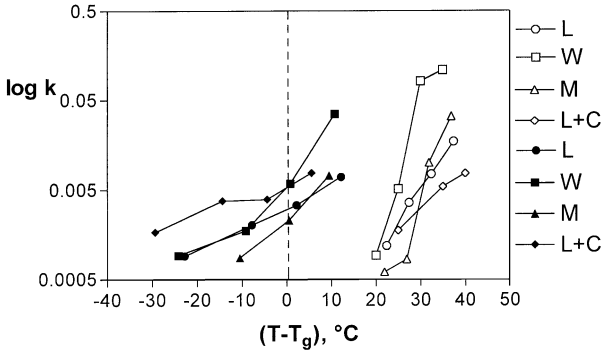

Fig. 2. Log of k (the rate of remaining activity of the enzyme) as a function of $(T-T_g)$ in all systems studied (open symbol: exposed at 22% R.H., and filled symbol: anhydrous systems).

treatment was found to follow first-order reaction kinetics. Similar kinetics rate-order was reported by Mazzobre et al. (1997a) for thermal inactivation of β-galactosidase in low moisture maltodextrin, PVP and trehalose systems. The pseudo-first order rate constants (k) were calculated for each storage temperature and their relative error was estimated to be between 9 and 14%. Fig. 2 shows the constant of the remaining activity kinetics (k) of the enzyme as a function of $(T-T_g)$ for all systems studied. As shown in Fig. 2, enzyme inactivation was observed at $T-T_g < 0$ (anhydrous systems). This was also recently observed by several authors, for different enzymes in matrices stored at $T < T_g$ conditions (Mazzobre et al., 1997a; Schebor et al., 1997; Terebiznik et al., 1998). Enzyme inactivation may involve a change in the active site, which can be due to protein denaturation (unfolding and/or hydrophobic aggregation) or to blockage of specific active groups by the formation of covalent links (amino-carbonyl condensation or oxidation) that modify and/or make the active site of the enzyme inaccessible to its substrate. Mahoney and Wilder (1989) identified the presence of sulphydryl groups involved in the β-galactosidase active site. Taragano and Pilosof (2001) found a large loss of pectin lyase activity in a dehydrated extract at temperatures below the onset of denaturation, and showed that inactivation by denaturation is not the only mechanism for pectin lyase activity loss.

Since the anhydrous systems were treated at temperatures below and above their glass transition temperature, they are adequate models for determining whether changes in temperature dependence of the kinetic rates of reaction are manifested in the region of temperature close to T_g. If a change in reaction mechanism due to the change in physical state of the system, towards more favourable reaction conditions occurs above T_g, a sharp increase in the reaction rate would be expected (Levine & Slade, 1992). The results shown in Fig. 2 demonstrate the lack of a sharp reaction rate increase in the temperature dependence of the thermal inactivation kinetics of β-galactosidase and $T-T_g$ of the matrix.

None of the anhydrous systems presented shrinkage after 24 h, probably because the T_g value of the anhydrous samples, as determined by DSC (95 °C), was close to the highest storage temperature analyzed (105 °C). It is well known that the collapse of an amorphous material is dependent on the difference between the storage temperature and the T_g of the matrix $(T-T_g)$ (Bonelli, Schebor, Cukierman, Buera, & Chirife et al., 1997; Levi & Karel, 1995). On the other hand, during the enzyme inactivation of the samples exposed at 22% R.H. ($T-T_g$ values >20 °C) temperature-dependent collapse was noted and also browning development. Structural changes observed for the 22% R.H. systems due to the lowered viscosity above the T_g could result in acceleration of the enzymatic activity loss. Fig. 3 (a) shows the relationship between remaining enzymic activity and degree of collapse for the systems exposed to 22% R.H.

For the systems containing proteins, remaining activity decreased as the degree of collapse increased, and a steep decrease of remaining enzymic activity was observed for samples at a degree of collapse of 0.5 or higher. For the lactose system, however, almost total collapse of the matrix was measured at all temperatures

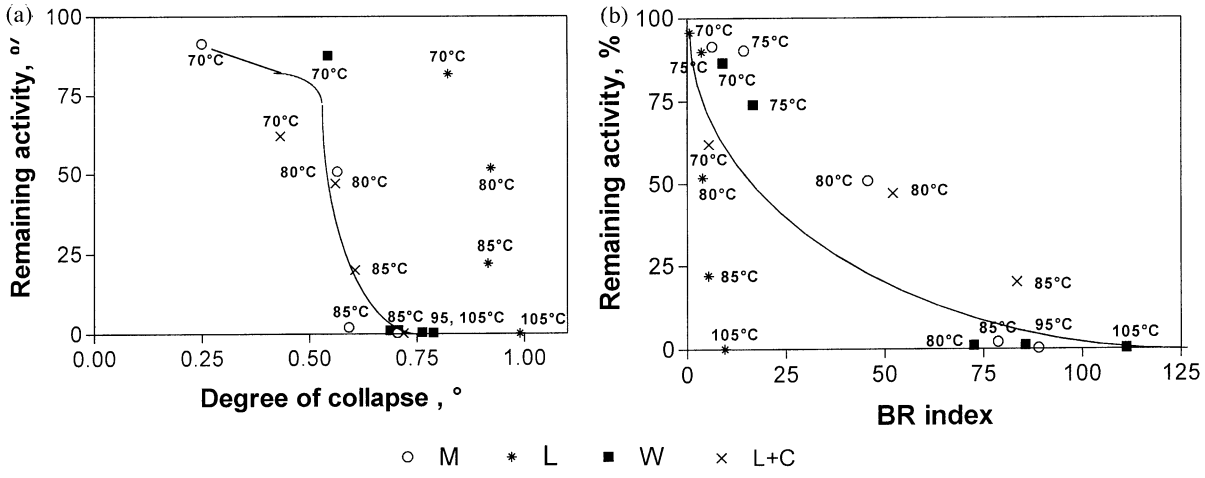

Fig. 3. Remaining activity of the enzyme as a function of (a) degree of collapse and (b) BR index after one day of storage of the systems exposed to 22% R.H.

studied, and remaining activity seemed to be dependent only on storage temperature; thus, collapse did not affect remaining activity for the lactose system.

It is interesting to note that all the systems exposed at 22% R.H. had approximately the same T_g value (Table 1), which was between 45 and 50 °C. However they showed a different degree of collapse (Fig. 3a). The observed differences in the collapse value in the L, compared to that of M and W systems at 70 °C were probably due to the molecular interactions of lactose (the main amorphous component) with the different soluble and insoluble proteins, although their $(T-T_g)$ values were similar. The observed degree of collapse, at a given observed temperature, was dependent on the composition of the system. At 70° C, for example, the lactose system (L) was highly collapsed, and the degree of collapse was in the order L > W > L + C > M. The incorporation of proteins (systems W, L + C and M), had a structure-stabilizing effect without altering the T_g value significantly. Moreover, the M system (containing both casein and whey proteins) was the most stable to collapse at all temperatures studied and it is possible that both casein and whey proteins interact, perhaps forming a network, to provide this observed effect [as pointed out by Aguilera (1995), although for fully hydrated systems]. Netto, Desobry, and Labuza (1998) reported that casein hydrolysates showed less physical change when compared to whey protein hydrolysates at higher R.H. (56 and 65%) and high $(T-T_g)$ (around 40 °C). They explained the observed differences based on Peleg's (1997) classification of powders, for which whey protein hydrolysates was considered a cohesive powder, by active interparticle forces that can cause stickiness and caking. Casein hydrolysates were considered as noncohesive powders, which have negligible interparticle interaction and have free-flowing properties.

The Maillard reaction is an important factor to consider during storage in enzyme stabilization of dehydrated systems (Colaço et al., 1994; Schebor et al., 1997; Terebiznik et al., 1998). Fig. 3b shows the relationship between the remaining activity and BR index, after one day of storage at all temperatures studied, in the systems exposed to 22% R.H. A relationship between remaining activity and color development was observed for the systems L + C, W and M. The systems with a lactose matrix (L) again showed behaviour which was different from the general trend. Although colour development in lactose system was negligible, remaining activity of the enzyme diminished as the storage temperature increased, indicating a temperature-specific effect.

In the temperature range studied, the systems M and L + C, which presented the most stable physical structure had a relatively high rate of enzymatic activity loss and browning development (Figs. 2 and 3). Although L systems were the most collapsed matrices at all measured temperatures, their relative degree of BR index was low. However, the rate of enzyme inactivation in these systems was close to that of the lactose/protein systems. The W systems presented an intermediate degree of collapse (between that of L and L + C/M systems) and the browning development rate was parallel to the enzyme inactivation rates in these systems. As observed by Nelson and Labuza (1994), in some systems, and due to the porosity associated with glassy systems or the collapse and crystallization associated with supercooled liquid systems, glassy matrices could be fairly reactive and supercooled liquid matrices could be stable to chemical reactions. Therefore, stability to chemical reactions would be a function of the state of the matrix, as well as several other composition-dependent properties. This was also pointed out by Tsimidou and Biliaderis (1997) where, for lipid oxidation, it was

Table 2
Percent variance explained by the first two PLS factors

Dimension	RA	Collapse	Colour	Temperature	$T-T_g$
PLS 1	75	49	36	92	94
PLS 2	4	49	45	3	2

not clear whether the limited free volume associated with the glassy state is sufficient to restrict small molecules such as oxygen from diffusing through the amorphous matrix. And, once in the supercooled liquid state, where structural collapse of the metastable matrix might occur, the expected increase in reaction rates, as a result of higher reactant mobility could have been counteracted by the high density of the collapsed samples.

To correlate remaining activity (RA = Y variable) with collapse, NEB, T and $T-T_g$ (X variables), multiple linear regression could have been used (Montgomery, 1991). However, this method is adequate if the number of samples is much higher than the number of variables, and if the X variables are not correlated with each other. Stepwise multiple regression could be used to select a sub-group of the X variables; but in this case the complete overview of the relationships in the data would be lost. Partial least squares regression (PLS; Hough, 2000; Martens & Martens, 1986) is a multivariate regression method that handles multicollinearity and small sample sets. PLS combines the X variables onto a reduced number of factors or latent variables that can be regarded as the main "harmonies" between the X variables. The correlation coefficients of the original X and Y variables with the A components can be calculated. A biplot which includes both these correlation coefficients and the samples scores on the A components can be used to present PLS results. Genstat's (Numerical Algorithms Group, Oxford, UK) PLS procedure was used for calculations. The first two PLS dimensions accounted for most of the variance in the data as shown in Table 2. Thus a system with one dependent variable (RA) and four independent variables (collapse, colour, T and $T-T_g$) can be explained by only two dimensions. The correlation coefficients of RA and the X-variates with the first two PLS factors are presented graphically (Fig. 4), together with the X-PLS scores for each one of the samples. Variables that are highly correlated with a common PLS dimension are highly correlated with each other; e.g. RA, T and $T-T_g$ are all highly correlated with PLS 1 and are therefore all highly correlated. Samples that are close on one PLS dimension have similar values for the variables explained by that dimension; e.g. M80 and L + C80 are close on PLS 2 and this corresponds to them having similar values for NEB and C, the difference in these last variables is explained by PLS 2.

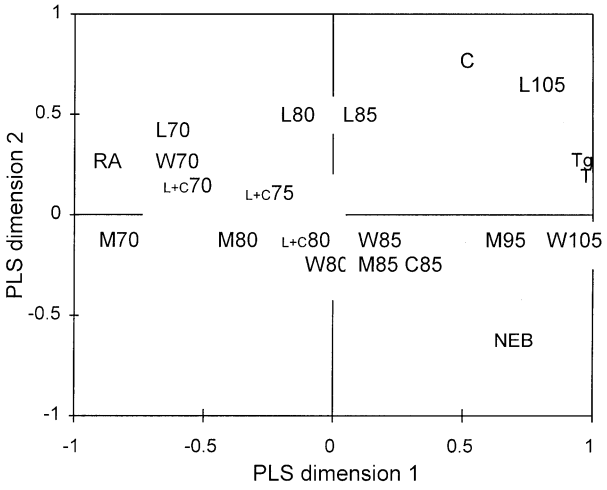

Fig. 4. Correlation coefficients of remaining activity (RA), nonenzymatic browning (NEB), collapse (c), storage temperature (T) and $T-T_g$ (T_g); with the first two PLS dimensions. PLS scores for samples at different storage temperatures are: L: lactose, L + C: lactose + casein, M: milk powder, W: whey powder.

From Figs. 3 and 4 and from the PLS analysis, it can be concluded that:

1. RA is highly correlated with storage temperature: as storage temperature increased, RA decreased.
2. Collapse and colour were correlated to RA, but not as highly as T was correlated to RA.
3. Lactose is the system that differs most from the others due to its having high collapse and low colour for all storage temperatures.
4. Difference in collapse values for the different systems can be better observed at a storage temperature of 70 °C than at higher temperatures.

The chemical (NEB and enzymatic inactivation) changes in some of the systems were not parallel to the macroscopic changes. As pointed out by Chinachoti (1997), different type of analysis provide information reflecting different levels of distance or time dimensions.

The behaviour of conveniently formulated model systems allowed anlysis of structure–property relationships separately in relation to chemical stability. The results of the present paper illustrate that the extent of macroscopic changes (structural shrinkage or collapse), related to long range structural relaxations, depends on the compositions of the systems. For the particular dairy systems studied, the presence of milk proteins had a structure stabilizing effect: for a given ($T-T_g$) value, the

systems containing proteins presented less shrinkage than the systems of a lactose matrix), without conferring chemical (towards NEB or enzyme inactivation) stability. On the other hand, a highly collapsed lactose matrix was more stable towards browning development than un-collapsed lactose–protein matrices. When selecting components for a given formulation, it should be noted that systems with the same T_g value may have different structural stabilities, according to their compositions, and that macroscopic stability cannot be related to chemical stability when systems of different matrix compositions are compared.

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