Mathematical Models for Combined High Pressure and Thermal Plasmin Inactivation Kinetics in Two Model Systems

D. Borda,¹ A. Van Loey,² C. Smout,² and M. Hendrickx²

¹Department of Food Bioengineering, Faculty of Food Science and Engineering, University Dunarea de Jos, 111 Domneasca Street, 800201 Galati, Romania

²Laboratory of Food Technology, Department of Food and Microbial Technology, Katholieke Universiteit Leuven, Kasteelpark Arenberg 22, B-3001 Heverlee, Belgium

ABSTRACT

The combined high-pressure thermal inactivation kinetics of plasmin was studied in 2 model systems. The first system contained both plasmin and plasminogen, whereas, in the second system, all plasminogen was converted into plasmin, with urokinase, before the inactivation studies. High-pressure treatments were conducted in the range of 300 to 800 MPa combined with temperatures from 30 to 65°C. Under all conditions of pressure and temperature (isobaric-isothermal) studied, for both systems, first-order inactivation was observed. A third-degree polynomial model (derived from thermodynamic principles) successfully described the temperature and pressure dependence of the inactivation rate constant over the entire experimental domain. The antagonistic effect and the stabilization effect observed above a threshold pressure value of 600 MPa were thought to be related to the disruption of disulfide bonds in plasmin and plasminogen.

(Key words: plasmin, high pressure, kinetic, mathematical model)

Abbreviation key: HP = high pressure.

INTRODUCTION

Over the last few years, a number of researchers (Scollard et al., 2000; Huppertz et al., 2002; Garcia-Risco et al., 2003) have studied the high-pressure stability of milk constituents. The recent interest in developing high pressure (HP) treatment applications for dairy products is related to potential benefits including the reduction of the microbiological load while maintaining the sensory and nutritional quality of the products (Lopez-Fandino et al., 1996; Trujillo et al., 2000; O'Reilly et al., 2001). Among the major advantages, the limited thermal degradation of nutrients (such as

vitamins), the preservation of color, and the reduction of additives used in thermal processes can be mentioned.

Generally, HP treatment at room temperature allows inactivation of vegetative bacterial cells in the range 300 to 600 MPa, whereas microbial spores are more pressure-stable, and inactivation requires HP (typically above 700 MPa) in combination with elevated temperature (typically above 70°C) (Hendrickx et al., 1998; Heinz and Knorr, 2001). Hence, considering the specific properties of milk constituents and the problems associated with dairy product inocuity, a combined HP mild-temperature treatment could be a more appropriate approach. Some of these applications include shelf-life extension of milk, the acceleration of cheese ripening, or the design of novel dairy products with new or improved functional properties. In this context, understanding the complex phenomena that take place during combined HP/thermal treatments requires further study. Detailed analysis of pressure-temperature inactivation kinetics for several milk components in model systems can help to apply such treatments to real systems.

Plasmin (EC 3.4.21.7) is a native milk proteinase that can play an important role in coagulation and ripening of many cheese varieties and in influencing the stability of UHT milk. This enzyme is very important in regulating casein hydrolysis in dairy products and readily hydrolyzes β -casein, α_{s2} -casein and, more slowly, α_{s1} -casein. Plasmin is an alkaline serine proteinase with a pH optimum of 7.5 (Fox and McSweeney, 1996). As part of a complex system comprising plasminogen (representing the zymogen), plasminogen activators, plasminogen activator inhibitors, and plasmin inhibitors, plasmin exhibits interesting behavior during HP thermal inactivation. Plasminogen, the inactive form of plasmin, can be converted into plasmin either in the presence of urokinase or tissue type activators (Nielsen, 2003).

In a previous study (Borda et al., 2004), we investigated combined thermal and HP inactivation of plasmin in a model system containing plasminogen and plasmin, and the kinetics in terms of inactivation rate constants and activation energies was reported.

Received May 9, 2004.

Accepted August 19, 2004.

Corresponding author: D. Borda; e-mail: daniela.borda@ugal.ro.

The aim of the current research was to quantify the combined thermal and HP inactivation kinetics of the plasmin system, after conversion of plasminogen into plasmin with commercially available urokinase, and to compare the results obtained with those for the system containing both plasminogen and plasmin.

At the same time, the second objective was to develop mathematical models for the combined pressure-temperature inactivation of plasmin in the 2 systems, namely a plasmin system with both plasminogen and plasmin present, and a system in which all plasminogen was converted into plasmin with urokinase. Furthermore, we tried to relate differences in the proposed models to possible differences in structural changes that occur during HP thermal inactivation in these 2 systems.

MATERIALS AND METHODS

Experimental Conditions

Two plasmin systems were considered for detailed kinetic studies: (1) a diluted plasmin system 1:3 (vol/vol) in phosphate buffer containing both plasminogen and plasmin and (2) a system in which, before the inactivation experiments, plasminogen was converted into plasmin with commercially available urokinase. Both systems were isolated from milk based on the procedure described by Metwalli et al. (1998).

To prepare the system with plasminogen converted into plasmin, 100 μL of plasmin system (containing both plasmin and plasminogen) was mixed with 100 μL of urokinase solution (1000 Ploug U/mL) and 1000 μL of 0.05 M phosphate buffer, pH 6.6, and then incubated for 2 h at 37°C.

Isobaric-isothermal (pressure/temperature) treatments were conducted in multivessel (8 vessels of 8 mL) HP equipment (Resato, Roden, The Netherlands) with a maximum pressure of 1000 MPa, as described previously (Borda et al., 2004).

After the thermal or combined HP/thermal treatment, the remaining plasmin activity was measured by fluorescence spectrophotometry using 1 mM N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin as a substrate, based on a modified procedure described in the literature (Richardson, 1983). The procedure detects the fluorescent intensity of the reaction product between plasmin and coumarin peptide, 7-amino-4-methyl coumarin.

As a basis to formulate a plasmin inactivation model for the system containing both plasminogen and plasmin, 58 different pressure/temperature combinations in the 0.1 to 800 MPa pressure range and 30 to 90°C temperature range were considered. Formulation of an appropriate mathematical model for plasmin inactivation,

in the system where plasminogen was converted into plasmin with urokinase, was based on 43 pressure-temperature combinations in the same range.

Data Analysis

The thermal HP inactivation of plasmin in both systems could be described by first-order kinetics. In case of constant extrinsic/intrinsic factors the first order model can be integrated to obtain:

$$\ln \frac{A}{A_0} = -k_{obs}t \quad [1]$$

where A_0 is the initial activity (at $t = 0$) and A the residual activity at time t , after the treatment.

The temperature dependence at constant pressure of the plasmin inactivation rate constant (k_{obs}) could be described by the Arrhenius equation:

$$\ln k_{obs} = \ln k_0 - \left[\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_0} \right) \right] \quad [2]$$

where k_0 is the inactivation rate constant at reference temperature T_0 , E_a is the activation energy, and R is the universal gas constant ($R = 8.314 \text{ J/mol K}$). The activation energy at constant pressure was estimated using a linear regression analysis.

To express the pressure-temperature dependence of the inactivation rate constant of enzymes, a thermodynamically based equation is frequently used (Smeller and Heremans, 1997; Heremans, 2002; Ludikhuyze et al., 2002).

Using the Eyring transition state theory, this thermodynamic model can be converted into a kinetic model to obtain equation 3, which is frequently used to describe the HP thermal denaturation of proteins in general and enzymes in particular (Heremans and Smeller, 1998; Van Loey et al., 2002):

$$\ln(k_{obs}) = \ln(k_0) + \frac{\Delta S_0^\ddagger}{RT}(T - T_0) + \frac{\Delta C_p^\ddagger}{RT} \left(T \ln \frac{T}{T_0} - 1 \right) + T_0 - \frac{\Delta V_0^\ddagger}{RT}(P - P_0) - \frac{\Delta \kappa^\ddagger}{2RT}(P - P_0)^2 - \frac{\Delta \zeta^\ddagger}{RT}(P - P_0)(T - T_0) \quad [3]$$

where $\Delta C_p^\ddagger$ is the heat capacity change ($T \delta \Delta S / \delta T$)_P, $\Delta \zeta^\ddagger$ is the thermal expansibility factor ($\delta \Delta V / \delta T$)_P = $(-\delta \Delta S / \delta P)$ _T, and $\Delta \kappa^\ddagger$ is the compressibility factor ($\delta \Delta V / \delta P$)_T.

Table 2. Activation energies (E_a) for pressure-temperature inactivation of the plasmin system, after conversion of plasminogen with urokinase.

Pressure (MPa)	E_a (kJ/mol)
300	126.69 ± 20.23^a (0.95)
400	79.71 ± 1.96 (0.99)
500	85.29 ± 3.78 (0.98)
600	81.17 ± 5.81 (0.97)
700	79.59 ± 7.71 (0.95)
800	90.64 ± 5.58 (0.98)

^aStandard error of regression.

pressure could be expressed by the Arrhenius equation (equation 2). The activation energies were estimated using a linear-regression approach (Table 2).

From this table, it can be seen that the activation energy changes with pressure and at elevated pressures the activation energy is lower than at atmospheric pressure. This indicates that the inactivation-rate constant is less temperature sensitive in the 30 to 65°C range, at elevated pressures.

Comparison Between the Combined Pressure and Temperature Plasmin Inactivation in the System With and Without Urokinase

The inactivation rate constants of the system containing plasminogen and plasmin in the pressure range 300 to 800 MPa and temperatures between 30 and 65°C are summarized in Table 3 (results from Borda et al., 2004).

Interpolated iso-rate contour plots on a P,T diagram are shown in Figure 1 for both plasmin systems. Figure 1A shows the iso-rate contour plot for plasmin inactivation in a system containing plasmin and plasminogen. An antagonistic effect followed by a stabilization effect is observed at high pressures at all temperatures studied (30 to 65°C). The antagonistic effect at 600 MPa requires an increase in temperature in order to achieve the same inactivation rate constant as for pressures below 600 MPa. Up to 600 MPa, the synergistic effect allows a pressure increase to be combined with a temperature decrease, to obtain the same inactivation-rate constant. The results obtained for the plasmin system converted with urokinase (Figure 1B) showed a similar stabilization effect, but in this case the antagonistic effect is less pronounced at all temperatures studied (30 to 65°C). In the graphical presentation (Figure 1B), the bending of the iso-rate contour lines to the right side of the P/T diagram at 600 MPa is less pronounced.

From the results obtained, 600 MPa can be considered (for both systems studied) a threshold value at which the synergistic effect of pressure and temperature is hindered. A possible explanation for this behavior

Table 3. Estimated rate constants of plasmin inactivation in the system with plasminogen and plasmin, phosphate buffer (pH 6.6).

Pressure (MPa)	Temperature (°C)						
	30°C	35°C	40°C	45°C	50°C	55°C	60°C
	$k \times 10^{-2}$ per min						
300	...	...	...	...	...	0.208 ± 0.0003 ^a	0.568 ± 0.0005
350	...	...	...	...	0.394 ± 0.0003	0.737 ± 0.0007	2.264 ± 0.0014
400	0.560 ± 0.0005	0.648 ± 0.0004	0.823 ± 0.0006	1.23 ± 0.0010	2.257 ± 0.0023	3.983 ± 0.0031	7.059 ± 0.0033
450	...	...	...	...	2.364 ± 0.0024	10.14 ± 0.0084	16.379 ± 0.011
500	0.955 ± 0.0004	1.584 ± 0.0005	1.698 ± 0.0008	2.82 ± 0.0030	10.28 ± 0.0098	20.79 ± 0.0066	21.079 ± 0.015
550	...	...	...	...	12.14 ± 0.0103	...	...
600	1.087 ± 0.0006	1.725 ± 0.0010	1.751 ± 0.0007	2.610 ± 0.0030	13.72 ± 0.0044	17.83 ± 0.0111	25.384 ± 0.016
700	0.864 ± 0.0004	1.567 ± 0.0009	1.362 ± 0.0011	1.734 ± 0.0023	7.726 ± 0.0089	13.95 ± 0.0234	23.90 ± 0.0317
750	...	...	2.059 ± 0.0008	2.962 ± 0.0027	...	...	...
800	0.751 ± 0.0004	1.418 ± 0.0007	1.557 ± 0.0008	2.548 ± 0.0047	8.818 ± 0.0069	14.68 ± 0.0104	15.91 ± 0.0246

^aStandard error of regression.

Figure 1. Iso-rate contour for the experimental values obtained through combined high-pressure thermal inactivation of plasmin in 2 model systems: (A) System containing plasmin and plasminogen in phosphate buffer pH 6.6.; (B) Plasmin system converted with urokinase in phosphate buffer pH 6.6. The inner and outer lines represent k values of 0.06 and 0.3/min, respectively.

ior at 600 MPa and temperatures between 30 and 65°C can be related to the nonreversible disruption of the proteins' structure, and reassociation of the disrupted proteins (Tedford and Schaschke, 1999). Upon combined pressure and temperature action, a cleavage of the disulfide bonds that are stabilizing the plasmin and plasminogen structure might take place. As a result, plasmin can dissociate into subunits and the plasminogen structure will be disrupted.

The absence of plasminogen in the system converted with urokinase and the fewer disulfide bonds stabilizing

Figure 2. Predicted iso-rate contour diagram for combined high-pressure temperature inactivation: (A) of the plasmin system (phosphate buffer pH 6.6) obtained by replacing the estimated parameters in equation 7; (B) of the plasmin system converted with urokinase (phosphate buffer pH 6.6) obtained by replacing the estimated parameters in equation 8. The inner and outer lines represent k values of 0.06 and 0.3/min, respectively.

the plasmin structure, as compared with the system with plasminogen and plasmin, resulted in less dramatic changes above 600 MPa, so no antagonistic effect was noticed and only the stabilization phenomenon was observed. Only further in-depth structural studies will allow confirmation of this hypothesis.

Figure 3. Correlation between the natural logarithm of the experimental k -values of the isobaric-isothermal inactivation of (A) the plasmin system (phosphate buffer pH 6.6) and the natural logarithm of predicted k -values according to equation 7, and (B) the plasmin system (phosphate buffer pH 6.6) converted with urokinase and the natural logarithm of predicted k -values according to equation 8.

Mathematical Models for Inactivation-Rate Constant vs. Pressure and Temperature

In a first attempt, the second-degree polynomial (combining equations 3 and 4) was used to describe the pressure-temperature dependence of the plasmin inactivation-rate constants. For both systems, the second-degree polynomial was not suitable to describe the

inactivation-rate constant as function of pressure and temperature (results not shown).

To model the data presented in Figure 1A and B, and to formulate a general inactivation model, the thermodynamic model (equation 3) was expanded including third-degree terms using a Taylor series development for a function of 2 variables (P, T) resulting in equation 5:

$$\begin{aligned} \Delta G = & \Delta G_0 + \frac{\delta \Delta G}{\delta T} (T - T_0) + \\ & \frac{\delta \Delta G}{\delta P} (P - P_0) + \frac{1}{2} \frac{\delta^2 \Delta G}{\delta T^2} (T - T_0)^2 + \\ & \frac{1}{2} \frac{\delta^2 \Delta G}{\delta P^2} (P - P_0)^2 + \frac{\delta^2 \Delta G}{\delta P \delta T} (T - T_0)(P - P_0) + \\ & \frac{1}{6} \frac{\delta^3 \Delta G}{\delta P^3} (P - P_0)^3 + \frac{1}{2} \frac{\delta^3 \Delta G}{\delta P \delta T^2} (P - P_0)(T - T_0)^2 + \\ & \frac{1}{6} \frac{\delta^3 \Delta G}{\delta T^3} (T - T_0)^3 + \frac{1}{2} \frac{\delta^3 \Delta G}{\delta P^2 \delta T} (P - P_0)^2(T - T_0) \end{aligned} \quad [5]$$

Equation 5 can be transformed into a linearized equation (equation 6), so that multilinear regression analysis can be applied.

If we carry out the following substitutions:

$$\begin{aligned} \ln(k) = a \quad X_1 = -\frac{(P - P_0)}{RT} \quad X_2 = \frac{(T - T_0)}{RT} \\ X_3 = -\frac{(P - P_0)^2}{2RT} \quad X_4 = \frac{(T - T_0)^2}{2RTT_0} \\ X_5 = -\frac{(P - P_0)(T - T_0)}{RT} \quad X_6 = -\frac{(P - P_0)^3}{6RT} \\ X_7 = -\frac{(P - P_0)(T - T_0)^2}{2RT} \quad X_8 = -\frac{(T - T_0)^3}{6RT} \\ X_9 = -\frac{(P - P_0)^2(T - T_0)}{2RT} \end{aligned}$$

we obtain:

$$\ln(k_{obs}) = a + AX_1 + BX_2 + CX_3 + DX_4 + EX_5 + FX_6 + GX_7 + HX_8 + IX_9 \quad [6]$$

By using multiple linear regression analysis we can perform multivariate tests across the multiple dependent variables and select the number of significant variables in the model. The “forward” selection procedure in the REG multiple linear regression analysis (SAS, 2001) allows one to start with no variables in the model,

Table 4. Estimated parameters for plasmin inactivation in different plasmin systems: I) plasmin system containing both plasmin and plasminogen using equation 7; and II) plasmin system containing plasmin only (after conversion of plasminogen with urokinase) using equation 8.

Plasmin system containing plasmin and plasminogen			Plasmin system containing plasmin only		
Parameter	Estimated value	SE (%)	Parameter	Estimated value	SE (%)
a	-4.196	0.13	a	-4.3487	0.0927
A	-22.59	2.93	A	-20.668	1.9915
B	165.14	36.59	B	190.249	25.426
C	-0.08511	0.016	D	3042.113	896.342
D	4860.29	1128.75	E	-0.40838	0.1083
E	-0.345	0.149	F	0.00066	0.00014
F	0.0008	0.00013	I	-0.0005	0.00058
$R^2 = 0.86; M_w = 7$			$R^2 = 0.93; M_w = 7$		

and for each independent variable, it will calculate the *F*-statistic that evaluates whether the contribution of the variable to the model is significant. Based on this selection procedure, for the plasmin system containing both plasminogen and plasmin, equation 6 was reduced to:

$$\ln(k_{obs}) = a + AX_1 + BX_2 + CX_3 + DX_4 + EX_5 + FX_6 \quad [7]$$

Hence, one extra term $(P - P_0)^3$ was finally introduced in the second-degree polynomial approach. The simulated iso-rate plot has now a similar shape as the experimental iso-rate plot and showed the same bending (Figure 2A) at pressures above 600 MPa.

For the plasmin system converted with urokinase, the same approach was used obtaining equation 8:

$$\ln(k_{obs}) = a + AX_1 + BX_2 + DX_4 + EX_5 + FX_6 + IX_9 \quad [8]$$

As compared with equation 7 (presenting the plasmin-plasminogen mixture), equation 8 (presenting the behavior of plasmin only) has an extra term in $(P - P_0)^2(T - T_0)$ and one term less, representing the $(P - P_0)^2$ dependence of the inactivation rate constant. The predicted iso-rate contour (Figure 2B) does not have an elliptical shape because a third-degree term is included. The predicted shape is obviously closer to the experimental iso-rate contour and allows taking into account the stabilization phenomenon for pressures above 600 MPa.

In addition to the corrected correlation coefficient for the model, R^2 , the Mallows' M_w statistic was computed for each model selected; M_w is a measure for the total sum of squared errors and is defined as:

$$M_w = \left(\frac{SSE_p}{s^2} \right) - (N - 2p) \quad [9]$$

where s^2 is the mean error sum of squares for the full model, SSE_p is the error sum of squares for a model with *p* parameters including the intercept, *N* is the number of observation, and *p* is the number of parameters including the intercept.

The best situation occurs when M_w is very close to *p*. For equations 7 and 8 the M_w value was equal to *p*.

The plots of the residuals (differences between experimental and predicted inactivation rate constants) as a function of temperature and pressure showed an unbiased scattering for both cases. The random distribution of the residuals indicates good agreement between the proposed model and the experimental data.

Based on equations 7 and 8, parity charts representing the difference between the natural logarithm of predicted inactivation rate constants and experimental values were plotted, providing a good fit of the experimental data compared with the predicted data (Figure 3A, B). The deviation from the bisector can be considered an indicator for the accuracy of the proposed model. The estimated parameters and associated standard errors, as well as some statistical information on the quality of the fit, are summarized in Table 4.

CONCLUSIONS

Plasmin displays a different HP and thermal inactivation behavior in presence or absence of plasminogen. Different mathematical models were proposed for plasmin inactivation in a system containing plasminogen and plasmin and for a system containing plasmin only, after conversion of plasminogen by urokinase. The models were mutually compared. The antagonistic and stabilization effects present above 600 MPa and temperatures from 30 to 65°C were related to the presence of disulfide bond, which stabilize plasmin and plasminogen structure, and their disruption. However, detailed structural studies are needed to confirm this hypothesis.

This effect is of interest for further studies as well as for the formulation of models for HP thermal inactivation.

vation of plasmin in systems containing β -casein and β -lactoglobulin.

ACKNOWLEDGMENT

The authors wish to thank the European Commission (QLK1-CT-2000-60014) and the Fund for Scientific Research-Flanders (FWO) for their financial support.

REFERENCES

- Borda, D., Indrawati, C. Smout, A. Van Loey, and M. Hendrickx. 2004. High pressure thermal inactivation kinetics of a plasmin system. *J. Dairy Sci.* 87:2351–2358.
- Fox, P. F., and P. L. H. McSweeney. 1996. Proteolysis in cheese 74. *Food Rev. Int.* 12:457–509.
- Garcia-Risco, M. R., I. Recio, E. Molina, and R. Lopez-Fandino. 2003. Plasmin activity in pressurized milk. *J. Dairy Sci.* 86:728–734.
- Heinz, V., and D. Knorr. 2002. Effects of high pressure on spores. Pages 77–113 in *Ultra High Pressure Treatments of Foods*. M. Hendrickx and D. Knorr, ed. Kluwer Academic/Plenum Publishers, New York, NY.
- Hendrickx, M., L. Ludikhuyze, I. Van den Broeck, and C. Weemaes. 1998. Effects of high pressure on enzyme related to food quality. *Trends Food Sci. Technol.* 9:197–203.
- Heremans, K. 2002. The effects of high pressure in biomaterials. Pages 22–46 in *Ultra High Pressure Treatments of Foods*. M. Hendrickx and D. Knorr, ed. Kluwer Academic/Plenum Publishers, New York, NY.
- Heremans, K., and L. Smeller. 1998. Protein structure and dynamics at high pressure. *Biochim. Biophys. Acta* 1386:353–370.
- Huppertz, T., A. L. Kelly, and P. F. Fox. 2002. Effects of high pressure on constituents and properties of milk. *Int. Dairy J.* 12:561–572.
- Lopez-Fandino, R., A. V. Carrascosa, and A. Olano. 1996. The effects of high pressure on whey denaturation and cheese-making properties of raw milk. *J. Dairy Sci.* 79:1929–1936.
- Ludikhuyze, L., A. Van Loey, Indrawati, S. Denys, and M. Hendrickx. 2002. Effects of high pressure on enzymes related to food quality. Pages 115–160 in *Ultra High Pressure Treatments of Foods*. M. Hendrickx and D. Knorr, ed. Kluwer Academic/Plenum Publishers, New York, NY.
- Ly-Nguyen, B., A. Van Loey, C. Smout, S. E. Ozcan, D. Fachin, I. Verlent, S. Vu Truong, T. Duvetter, and M. Hendrickx. 2003. Mild-heat and high pressure inactivation of carrot pectin methylesterase: A kinetic study. *J. Food Sci.* 4:1377–1383.
- Metwalli, A. M., H. H. de Jongh, and M. J. S. van Boekel. 1998. Heat inactivation of bovine plasmin. *Int. Dairy J.* 8:47–56.
- Morlid, E. 1981. The theory of pressure effects on enzymes. *Adv. Protein Chem.* 34:93–166.
- Nielsen, S. S. 2003. Plasmin system in milk. Pages 929–934 in *Encyclopedia of Dairy Sciences*. Vol. II. H. Roginskin, J. W. Fuquay, and P. F. Fox, ed. Academic Press, Elsevier Science, London, UK.
- O'Reilly, C. E., A. L. Kelly, P. M. Murphy, and T. P. Beresford. 2001. High pressure treatment applications in cheese manufacture and ripening. *Trends Food Sci. Technol.* 12:51–59.
- Richardson, B. C. 1983. The proteinase of bovine milk and the effect of pasteurization on their activity. *Rev. N.Z. J. Dairy Sci. Technol.* 18:233–245.
- Scollard, P. G., T. P. Beresford, P. M. Murphy, and A. L. Kelly. 2000. Barostability of milk plasmin activity. *Lait* 80:609–619.
- Smeller, L. 2002. Pressure-temperature phase diagrams of biomolecules. *Biochim. Biophys. Acta* 1595:11–29.
- Smeller, L., and K. Heremans. 1997. Some thermodynamic and kinetic consequences of phase diagram of protein denaturation. Pages 54–58 in *High Pressure Research in the Biosciences and Biotechnology*. K. Heremans, ed. Leuven University Press, Leuven, Belgium.
- Tedford, L. A., and C. J. Schaschke. 1999. Induced structural change to β -lactoglobulin by combined pressure and temperature. *Biochem. Eng. J.* 5:73–76.
- Trujillo, A. J., M. Capellas, M. Buffa, C. Royo, R. Gervilla, X. Felipe, E. Sendra, J. Saldo, V. Ferragut, and B. Guamis. 2000. Application of high pressure treatment for cheese production. *Food Res.* 33:311–316.
- Van Loey, A., Indrawati, C. Smout, and M. Hendrickx. 2002. Inactivation of enzymes: From experimental design to kinetic modeling. Pages 49–58 in *Handbook of Food Enzymology*. J. Whitaker, A. Voragen, and D. Wong, ed. Marcel Dekker, Inc., New York, NY.