Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 99 (2006) 45–50
Food Chemistry
www.elsevier.com/locate/foodchem

A method for the large-scale isolation of β-casein

Thom Huppertz [a,*], Jean-Baptiste Hennebel [a,b], Therese Considine [a], Shakeel-Ur-Rehman [a], Alan L. Kelly [a], Patrick F. Fox [a]

[a] Department of Food and Nutritional Sciences, University College Cork, Cork, Ireland
[b] Ecole Nationale Supérieure Agronomique de Montpellier, Montpellier, France

Received 14 February 2005; accepted 13 July 2005

Abstract

A method for the large-scale isolation of β-casein from renneted skim milk was developed. The curd from renneted skim milk was dispersed in hot ($\geqslant$70 °C) water to inactivate residual chymosin. The heated curd was subsequently recovered by centrifugation, resuspended in water and incubated at 5 °C, during which β-casein dissociated from the curd; the suspension was centrifuged and the aqueous phase lyophilised. The isolated protein consisted mainly of β-casein, containing a minor amount of γ-caseins and traces of other caseins. Unless chymosin was fully inactivated by heating, some β-casein was hydrolysed at the Leu_{192}–Tyr_{193} bond. The yield of β-casein increased with incubation time, up to ~20% of the β-casein present in the milk after 24 h at 5 °C. Reducing milk pH to 5.5 or 6.0, prior to renneting, caused a high level of contamination with α_s-caseins. This isolation procedure can be easily scaled-up to an industrial process and the β-casein-depleted curd may be used for the manufacture of rennet casein or processed cheese.

Keywords: β-Casein; Isolation; Milk

1. Introduction

Industrial-scale production of whole casein from milk has been ongoing for at least 100 years. Initially, isolated casein was used for industrial purposes, such as glues, paper glazing or synthetic fibres but, since the 1960s, caseins have gained importance as food ingredients and are now one of the principal food proteins. Applications of caseins in dairy products include cheese analogues, powdered coffee creamers, yoghurt and milk beverages; caseins are also used in a wide range of non-dairy foods (beverages, dessert-types, bakery, pasta, confectionery and meat products) and pharmaceutical and health-care products (see Mulvihill & Ennis, 2003; Southward, 1989).

More recently, industrial demand for purified individual caseins, particularly β-casein, has developed. Due to its relatively low molecular mass, flexible structure, moderate charge, absence of intra-molecular covalent bonds and the fact that it does not form inter-molecular disulphide bonds, β-casein has all the key characteristics of an excellent surface-active agent and polymeric stabiliser (Dickinson, 2003). β-Casein may also be added to milk to improve the strength of the rennet-induced coagulum (Ali, Andrews, & Cheeseman, 1980a; Yun, Ohmiya, & Shimizu, 1982); it may be used in infant formula (Murphy & Fox, 1991a) or as a starting material for the production of bioactive peptides (FitzGerald & Meisel, 2003; Gobetti, Minervini, & Rizzello, 2004; Maubois, 1984).

* Corresponding author. Tel.: +353 21 490 1412; fax: +353 21 427 0213.
E-mail address: t.huppertz@ucc.ie (T. Huppertz).

0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.07.025

Since the middle of the 20th Century, wide varieties of methods have been developed to purify β-casein, using differential precipitation (Hipp, Groves, Custer, & McMeekin, 1952; Igarashi, 1995, 1999), gel chromatography (Nakahori & Nakai, 1972; Nakai, Toma, & Nakahori, 1972), ion-exchange chromatography (Cayot, Courthaudon, & Lorient, 1992; Christensen & Munksgaard, 1989; Leaver & Law, 1992) or chromatography on hydroxyapatite (Donnelly, 1977). However, these methods have the disadvantage of limited possibility for scaling-up, as well as the fact that the isolated β-casein often needs further purification prior to use as a food ingredient.

Several other methods, potentially large-scale, have been developed to isolate β-casein from caseinate at a low temperature, e.g., from sodium caseinate by filtration through filter paper (Famelart, Hardy, & Brulé, 1989), microfiltration (Famelart & Surel, 1994) or ultrafiltration (Murphy & Fox, 1991b) or from cold-renneted calcium caseinate (Allen, McAuliff, & Donnelley, 1985); however, extensive hydrolysis of β-casein by chymosin is observed using the latter method (Murphy & Fox, 1991b). Ward and Bastian (1996) modified the method of Allen et al. (1985) by renneting a calcium caseinate solution at 30 °C, holding at 4 °C for up to 48 h to dissociate β-casein, followed by separation of the β-casein-depleted coagulum and warming the supernatant to 30 °C to precipitate β-casein. Although Ward and Bastian (1996) claimed that no significant hydrolysis of β-casein occurs during this procedure, studies in our laboratory have shown extensive hydrolysis of β-casein.

Hence, there appears to be a need for a procedure for the isolation of β-casein in which hydrolysis of β-casein by chymosin is avoided. The objective of this study was to develop a large-scale method for the isolation of β-casein directly from rennet-coagulated skim milk, rather than from calcium caseinate. The principle of the developed method is the increased solubility of β-casein at low temperatures, as initially observed by Sullivan et al. (1955) and Payens and Van Marwijk (1963), and the observation, by McGann and Pyne (1960), that incubation of rennet-coagulated milk at a low temperature leads to dissociation of β-casein from the curd.

2. Materials and methods

2.1. Isolation of β-casein

The experimental procedure for isolation of β-casein is summarised in Fig. 1. Aliquots (200 ml) of pasteurised skimmed bovine milk, obtained from CMP Dairies (Cork, Ireland), were warmed to 30 °C; rennet (1 μl ml^{-1}; Maxiren 180, DSM Food Specialties, Delft, The Netherlands) was added and milk held for 15 min at 30 °C. The coagulum was cut, centrifuged at 5000*g* for 15 min at 30 °C, and the whey decanted off. A volume of demineralised water (50–100 °C), equal to that of the whey, was added to the curd and the mixture held at the temperature of the water for 5 min, followed by centrifugation at 5000*g* for 15 min at 5 °C; the supernatant was discarded. The curd was macerated using a mortar and pestle and suspended in demineralised water (5 °C) to a volume similar to that of the original milk sample. The suspension was held at 5 °C for up to 24 h and then centrifuged at 5000*g* for 15 min at 5 °C. The supernatant was filtered through Whatman No. 113 filter paper and lyophilised. A small portion of the curd was also lyophilised. All experiments were performed in triplicate on individual milk samples.

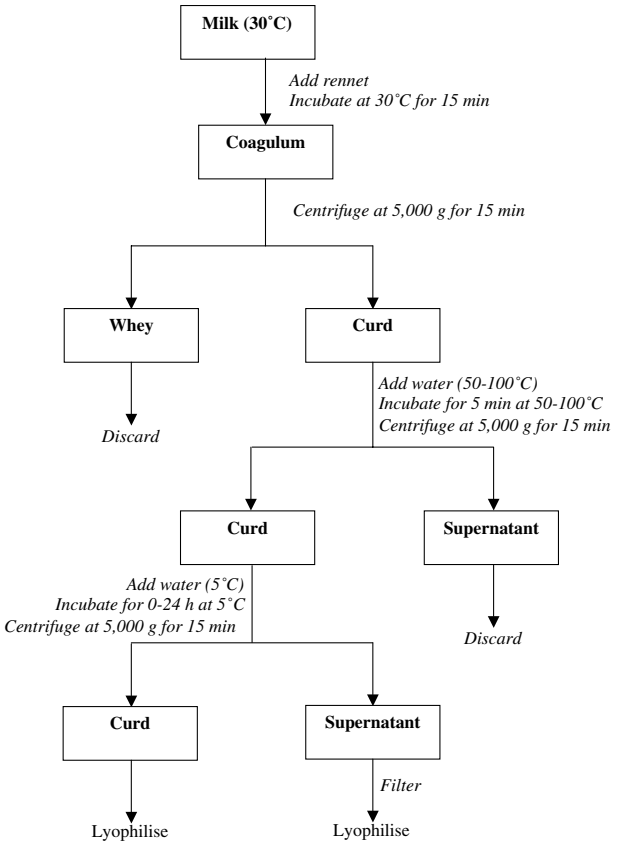

Fig. 1. Flow diagram of the procedure for the isolation of β-casein from renneted pasteurised skim milk.

2.2. Analytical methods

For determination of the yield of β-casein, the protein content of the filtered supernatant after cold-incubation, as well as the casein content of the original milk (i.e., total protein – pH 4.6-soluble protein), was determined in triplicate by the Kjeldahl method (IDF, 1986); the yield of the isolated fraction was expressed as g protein 100 g^{-1} of casein in milk.

The purity of the isolated β-casein was assessed by urea polyacrylamide gel electrophoresis (urea–PAGE;

Andrews, 1983), followed by staining, according to the method of Blakesley and Boezi (1977); lyophilised samples were dissolved in sample buffer at a level of 5 mg ml^{-1}.

3. Results

An electrophoretogram of the β-casein fraction isolated from curd heated at 50, 60, 70, 80, 90 or 100 °C, followed by incubation at 5 °C for 24 h, is shown in Fig. 2; in all samples, the principal protein was β-casein, with minor amounts of γ-caseins and only traces of α_{s1}-casein. Fractions isolated from curd heated at 50 or 60 °C (Fig. 2, lanes 1 and 2) contained some β-casein f1–192, which was not detected in the fraction isolated from curd that had been heated at 70–100 °C (Fig. 2, lanes 3–6). Adjustment of milk pH to 5.5 or 6.0, prior to renneting, resulted in considerable contamination of the isolated fraction with α_s-caseins (data not shown).

Incubation time had little effect on the level of γ-caseins in the isolated protein fraction (Fig. 3), but the trace-level of α_{s1}-casein in these fractions appeared to increase with treatment time. The electrophoretogram of the curd heated at 70 °C, followed by incubation for up to 24 h at 5 °C and subsequent lyophilisation (Fig. 4) showed a gradual decrease in the level of β-casein over time, with little noticeable effect of incubation time on the other casein fractions. The yield of isolated β-casein increased gradually with treatment time (Fig. 5); the rate of extraction, i.e., grammes of protein extracted h^{-1}, decreased with increasing incubation time. After 24 h of

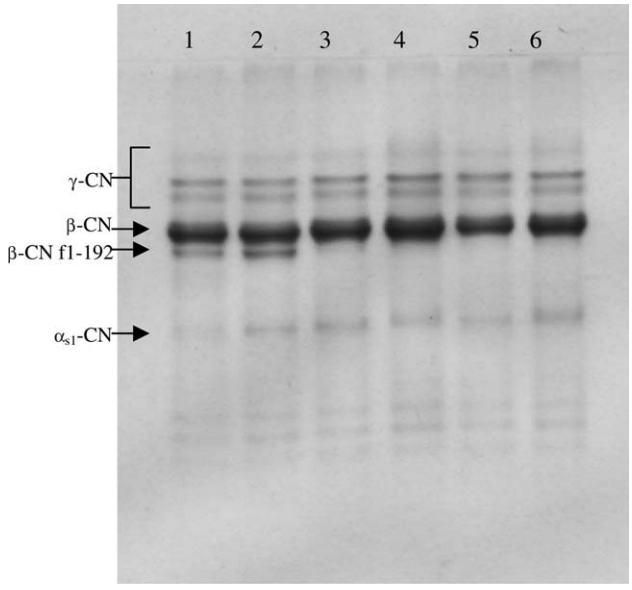

Fig. 2. Urea–PAGE electrophoretogram of lyophilised supernatants prepared by extracting rennet curd heated at 50 (lane 1), 60 (lane 2), 70 (lane 3), 80 (lane 4), 90 (lane 5) or 100 °C (lane 6) for 5 min, with demineralised water at 5 °C for 24 h.

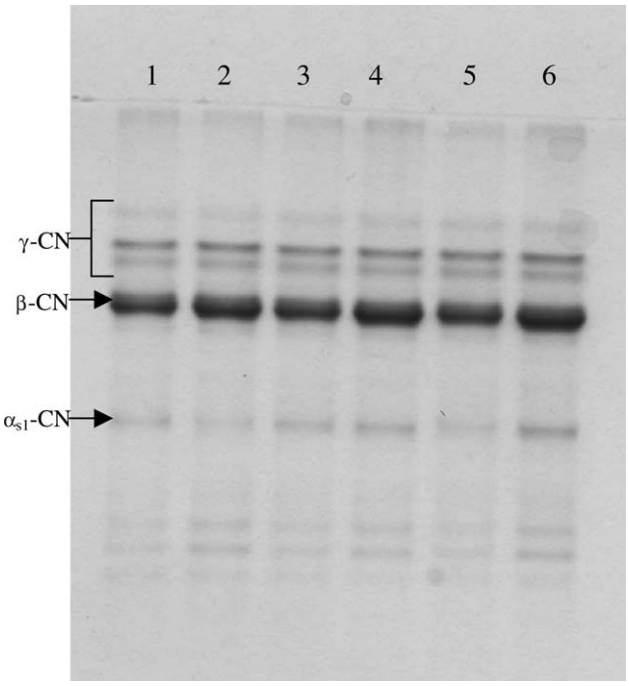

Fig. 3. Urea–PAGE electrophoretogram of lyophilised supernatants prepared by extracting rennet curd, heated at 80 °C for 5 min, with demineralised water at 5 °C for 1h (lane 1), 2h (lane 2), 4h (lane 3), 6h (lane 4), 8h (lane 5) or 24h (lane 6).

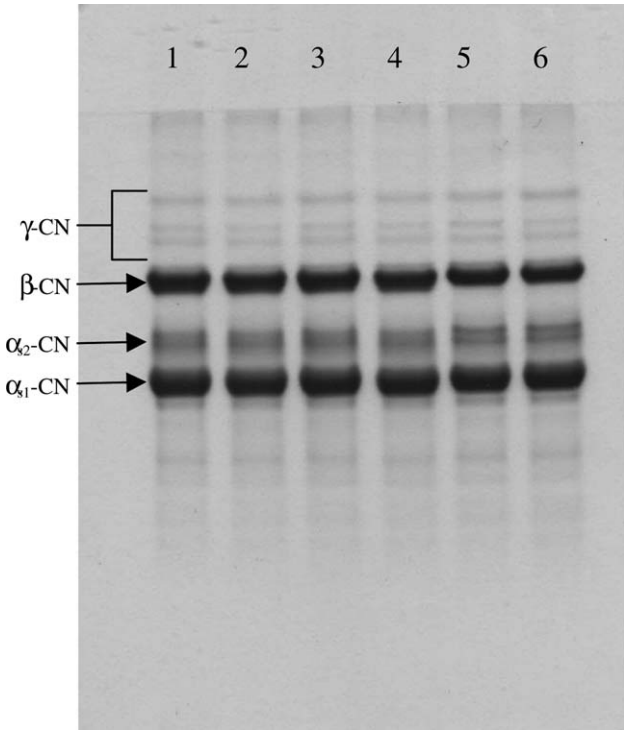

Fig. 4. Urea–PAGE electrophoretogram of curd heated at 80 °C for 5 min, followed by extraction with demineralised water at 5 °C for 1h (lane 1), 2h (lane 2), 4h (lane 3), 6h (lane 4), 8h (lane 5) or 24 h (lane 6) and subsequent lyophilisation.

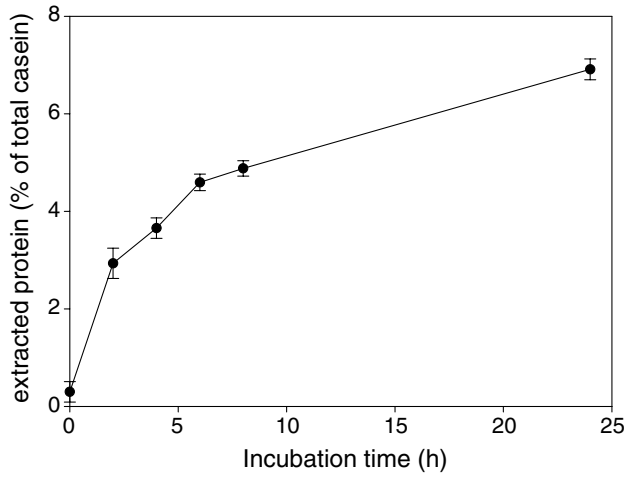

Fig. 5. Influence of incubation time at 5 °C on the level of casein, expressed as a % of total casein, extracted from curd heated at 80 °C for 5 min. Values are means of data from triplicate experiments on individual milk samples, with the standard deviation indicated by vertical error bars.

incubation, ~7% of total casein was isolated, which, assuming that this is pure β-casein, and that β-casein represents 35% of total casein (Fox, 2003), indicates the extraction of ~20% of total β-casein.

4. Discussion

The results presented in this communication suggest that, using the procedure described, large quantities of pure β-casein can be prepared. The fact that some β-casein f1–192 was observed in the extract from curd heated at 50 or 60 °C (Fig. 2) indicates that chymosin was not inactivated at these temperatures, since β-casein f-1-192 results from the β-casein from chymosin (Visser & Slangen, 1977). Data on heat-induced inactivation of chymosin in cheese systems are not available but, in liquid media, complete inactivation of chymosin is observed at a temperature >60 °C (Harper & Lee, 1975; Hyslop, Swanson, & Lund, 1979; Thunell, Duersch, & Ernstrom, 1979); the thermoresistance of chymosin increases with the total solids content of the media (Daemen, 1981). From the results of this study (Fig. 2), it appears that inactivation at a temperature $\geqslant$70 °C is required to prevent the hydrolysis of β-casein during isolation.

The isolation procedure described in this communication is based on the selective solubilisation of β-casein at low temperatures. On cold storage of milk, a large increase in the level of non-sedimentable β-casein occurs, whereas non-sedimentable levels of other caseins increase only slightly (Ali, Andrews, & Cheeseman, 1980b; Dalgleish & Law, 1988; Davies & Law, 1983; Downey & Murphy, 1970; Law, 1996; McGann & Pyne, 1960; O'Connor & Fox, 1973; Pierre & Brule, 1981; Roefs, Walstra, Dalgleish, & Horne, 1985; Rose, 1968). Solubilisation of β-casein, on storage, has been related to the weakening of hydrophobic bonds at low temperature (Law, 1996; Pierre & Brule, 1981) as well as increased solubility of β-casein at low temperature (Payens & Van Marwijk, 1963; Sullivan et al., 1955), leading to an equilibrium between micellar and monomeric soluble casein (Pierre & Brule, 1981). The increased contamination of the isolated β-casein with α_s-caseins when milk was acidified to pH 5.5 or 6.0, prior to renneting (data not shown), is in agreement with increased dissociation of micellar α_{s1}-casein at a lower pH (Dalgleish & Law, 1988).

The fact that the proportion of the γ-caseins does not increase with incubation time (Fig. 3) suggests that the level at which they are present in the isolated fraction is determined primarily by their level in the curd prior to cold-extraction. γ-Caseins are products of the hydrolysis of β-casein by plasmin, which is optimally active at 37 °C (Bastian & Brown, 1996; Kelly & McSweeney, 2003). At 5 °C, very little plasmin-induced hydrolysis of β-casein is observed (Crudden, Fox, & Kelly, 2005; Guinot-Thomas, Al Ammoury, Le Roux, & Laurent, 1995; Huppertz, Fox, & Kelly, 2004). Thus, plasmin-induced hydrolysis may be expected prior to milking, as well as during rennet-induced coagulation of the milk, but it is unlikely to occur during extraction at 5 °C. The presence of γ-caseins in the β-casein fraction may not present a major problem, as their properties, which are quite similar to β-casein, also make them good emulsifiers and stabilisers (Caessens, Gruppen, Visser, Van Aken, & Voragen, 1997, 1999; Wilson, Mulvihill, Donnelley, & Gill, 1989). However, if required, γ-caseins may be removed from the β-casein fraction, potentially by ethanol-induced precipitation of β-casein, as described by Igarashi (1989, 1995).

The yield of β-casein after 24 h was ~7% of total casein (Fig. 5), which corresponds to ~20% of total β-casein in milk, which is considerably lower than that reported by Ward and Bastian (1996), i.e., 55% of total β-casein after 24 h. However, caution is warranted with the latter figure, since these authors calculated yield from the dried weight of precipitated casein; first, some minerals may be present in the β-casein precipitate, and second, it is unlikely that the drying method used by Ward and Bastian (1996), i.e., storage in a desiccator, would remove all moisture from the precipitate. Thus, the actual yield may be lower than that reported by Ward and Bastian (1996). A yield of ~20% of total β-casein after 24 h of extraction (Fig. 5) is somewhat lower than the level of β-casein which dissociates from casein micelles in milk on cold storage for 24 h, e.g., ~25–40% (Ali et al., 1980b; Downey & Murphy, 1970; Law, 1996; O'Connor & Fox, 1973; Roefs et al., 1985). This may be due to the fact that dissociation of β-casein is

hindered by the curd matrix, particularly in the core of the curd particles.

In conclusion, using the method described in this communication, large quantities of β-casein can be isolated from rennet-coagulated pasteurised skim milk, which is cheaper than the sodium or calcium caseinate used in previous studies. Furthermore, chymosin-induced hydrolysis of β-casein, which caused problems in some previously described methods, is avoided in this method. Other fractions obtained in the isolation procedure may be used for the manufacture of other products. For example, the whey obtained on centrifugation of the coagulum (see Fig. 1) can be used for the manufacture of whey protein concentrate or isolate, and the extracted, partially β-casein-depleted, curd (Fig. 4) may be used in the manufacture of processed cheese or rennet-caseinate.

Some aspects of the described procedure for isolation of β-casein may be of interest for future studies to optimise the procedure, e.g. the potential for scale-up of the method. The studies presented in this communication show that gramme quantities of pure β-casein can readily be obtained from litre quantities of milk, but some modifications may be required in scale-up to a process using hundreds or thousands of litres of milk; aspects of this process that may require special attention are the method and rate of separation of the whey or supernatant from the curd and the efficiency of heat treatment for inactivating the chymosin when higher quantities of curd are used. The potential use of pepsin instead of chymosin, as a coagulant, may also be of interest, because the former is more easily inactivated than the latter (O'Keeffe, Fox, & Daly, 1977) so a lower inactivation temperature may be applied. Furthermore, optimisation of the yield of β-casein may be studied with respect to the influence of the properties of the medium used for extraction, namely, the volume per weight of curd and the ionic strength of the medium. Finally, further purification of the isolated protein, if required, may be achieved using selective ethanol-induced precipitation, as previously used by Igarashi (1989, 1995).

References

Ali, A. E., Andrews, A. T., & Cheeseman, G. C. (1980a). Factors influencing casein distribution in cold-stored milk and their effects on cheese-making parameters. *Journal of Dairy Research, 47*, 383–391.

Ali, A. E., Andrews, A. T., & Cheeseman, G. C. (1980b). Influence of storage of milk on casein distribution between micellar and soluble phases and its relationship to cheese-making parameters. *Journal of Dairy Research, 47*, 371–382.

Allen, E. M., McAuliff, A. G., & Donnelley, W. J. (1985). Simplified approaches to casein fractionation. *Irish Journal of Food Science and Technology, 9*, 85 (1 page).

Andrews, A. T. (1983). Proteinases in normal bovine milk and their action on the caseins. *Journal of Dairy Research, 50*, 45–55.

Bastian, E. D., & Brown, R. J. (1996). Plasmin in milk and dairy products: an update. *International Dairy Journal, 6*, 435–457.

Blakesley, R. W., & Boezi, J. A. (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry, 82*, 580–581.

Caessens, P. W. J. R., Gruppen, H., Visser, S., Van Aken, G., & Voragen, A. G. J. (1997). Plasmin hydrolysis of β-casein: foaming and emulsifying properties of the fractionated hydrolysate. *Journal of Agricultural and Food Chemistry, 45*, 2935–2941.

Caessens, P. W. J. R., Visser, S., Gruppen, H., Van Aken, G. A., & Voragen, A. G. J. (1999). Emulsion and foam properties of plasmin derived β-casein peptides. *International Dairy Journal, 9*, 347–351.

Cayot, P., Courthaudon, J. L., & Lorient, D. (1992). Purification of α_s-, β- and κ-caseins by batchwise ion-exchange separation. *Journal of Dairy Research, 59*, 551–556.

Christensen, T. M. I. E., & Munksgaard, L. (1989). Quantitative fractionation of casein by precipitation or ion exchange chromatography. *Milchwissenschaft, 44*, 480–484.

Crudden, A., Fox, P. F., & Kelly, A. L. (2005). Factors affecting the hydrolytic action of plasmin in milk. *International Dairy Journal, 15*, 305–313.

Daemen, A. L. H. (1981). The destruction of enzymes and bacteria during the spray-drying of milk and whey: the thermoresistance of some enzymes and bacteria in milk and whey with various total solids contents. *Netherlands Milk and Dairy Journal, 35*, 133–144.

Dalgleish, D. G., & Law, A. J. R. (1988). pH-induced dissociation of bovine casein micelles. I. Analysis of liberated caseins. *Journal of Dairy Research, 55*, 529–538.

Davies, D. T., & Law, A. J. R. (1983). Variation on the protein composition of bovine casein micelles and serum casein in relation to micellar size and temperature. *Journal of Dairy Research, 50*, 67–75.

Dickinson, E. (2003). Interfacial, emulsifying and foaming properties of milk proteins. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry. Proteins* (3rd ed., Vol. 1, pp. 1229–1260). New York: Kluwer Academic/Plenum Publishers.

Donnelly, W. J. (1977). Chromatography of milk proteins on hydroxyapatite. *Journal of Dairy Research, 44*, 621–625.

Downey, W. K., & Murphy, R. F. (1970). The temperature-dependent dissociation of β-casein from bovine casein micelles and complexes. *Journal of Dairy Research, 37*, 361–372.

Famelart, M. H., Hardy, C., & Brulé, G. (1989). Etude des facteurs d'extraction de la caséine β. *Lait, 69*, 47–57.

Famelart, M. H., & Surel, O. (1994). Caseinate at low temperatures: calcium use in β-casein extraction by microfiltration. *Journal of Food Science, 59*, 548–553.

FitzGerald, R. J., & Meisel, H. (2003). Milk protein hydrolysates and bioactive peptides. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry 1: Proteins* (3rd ed., pp. 675–698). New York: Kluwer Academic/Plenum Publishers.

Fox, P. F. (2003). Milk proteins: general and historical aspects. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry. Proteins* (3rd ed., Vol. 1, pp. 1–48). New York: Kluwer Academic/Plenum Publishers.

Gobetti, M., Minervini, F., & Rizzello, C. G. (2004). Angiotensin I-converting-enzyme-inhibitory and antimicrobial bioactive peptides. *International Journal of Dairy Technology, 57*, 173–190.

Guinot-Thomas, P., Al Ammoury, M., Le Roux, Y., & Laurent, F. (1995). Study of proteolysis during storage of raw milk at 4 °C: effect of plasmin and microbial proteinases. *International Dairy Journal, 5*, 685–697.

Harper, W. J., & Lee, C. R. (1975). Residual coagulants in whey. *Journal of Food Science, 40*, 282–284.

Hipp, N. J., Groves, M. L., Custer, J. H., & McMeekin, T. L. (1952). Separation of α-, β-, and γ-casein. *Journal of Dairy Science, 35*, 272–281.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2004). Proteolysis and plasmin activity in high pressure-treated bovine milk. *Lait, 84*, 297–304.

Hyslop, D. B., Swanson, A. M., & Lund, D. B. (1979). Heat-inactivation of milk clotting enzymes at different pH. *Journal of Dairy Science, 62*, 1227–1232.

Igarashi, Y. (1989). A method for determination of γ-casein and its use for investigating proteolysis in bovine milk. *Journal of Dairy Research, 56*, 619–629.

Igarashi, Y. (1995). An improved procedure for the preliminary fractionation of milk proteins. *International Dairy Journal, 5*, 305–310.

Igarashi, Y. (1999). Separation of caseins by chemical procedures. *International Dairy Journal, 9*, 377–378.

IDF. (1986). Milk. Determination of nitrogen content (Kjeldahl method) and calculation of crude protein content. *Standard 20A*. Brussels: International Dairy Federation.

Kelly, A. L., & McSweeney, P. L. H. (2003). Indigenous proteinases in milk. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry. Proteins* (3rd ed., Vol. 1, pp. 495–522). New York: Kluwer Academic/Plenum Publishers.

Law, A. J. R. (1996). Effects of heat treatment and acidification on the dissociation of bovine casein micelles. *Journal of Dairy Research, 63*, 35–48.

Leaver, J., & Law, A. J. R. (1992). Preparative-scale purification of bovine caseins on a cation-exchange resin. *Journal of Dairy Research, 59*, 557–561.

Maubois, J. L. (1984). Separation, extraction and fractionation of milk protein components. *Lait, 64*, 485–498.

McGann, T. C. A., & Pyne, G. T. (1960). The colloidal phosphate of milk. III. Nature of its association with casein. *Journal of Dairy Research, 27*, 403–417.

Mulvihill, D. M., & Ennis, M. P. (2003). Functional milk proteins: production and utilization. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry 1: Proteins* (3rd ed., pp. 1175–1228). New York: Kluwer Academic/Plenum Publishers.

Murphy, J. M., & Fox, P. F. (1991a). Functional properties of α_s-/κ- or β-rich casein fractions. *Food Chemistry, 39*, 211–228.

Murphy, J. M., & Fox, P. F. (1991b). Fractionation of sodium caseinate by ultrafiltration. *Food Chemistry, 39*, 27–38.

Nakahori, C., & Nakai, S. (1972). Fractionation of caseins directly from skimmilk by gel chromatography. 1. Elution with sodium dodecylsulfate. *Journal of Dairy Science, 55*, 25–29.

Nakai, S., Toma, S. J., & Nakahori, C. (1972). Fractionation of caseins directly from skimmilk by gel chromatography. 2. Elution with phosphate buffers. *Journal of Dairy Science, 55*, 30–34.

O'Connor, P., & Fox, P. F. (1973). Temperature-dependent dissociation of casein micelles from the milk of various species. *Netherlands Milk and Dairy Journal, 27*, 199–217.

O'Keeffe, A. M., Fox, P. F., & Daly, C. (1977). Denaturation of porcine pepsin during Cheddar cheese manufacture. *Journal of Dairy Research, 44*, 335–343.

Payens, T. A., & Van Marwijk, B. W. (1963). Some features of the association of β-casein. *Biochimica et Biophysica Acta, 71*, 517–530.

Pierre, A., & Brule, G. (1981). Mineral and protein equilibria between the colloidal and soluble phases of milk at low temperature. *Journal of Dairy Research, 48*, 417–428.

Roefs, S. P. F. M., Walstra, P., Dalgleish, D. G., & Horne, D. S. (1985). Preliminary note on the change in casein micelles caused by acidification. *Netherlands Milk and Dairy Journal, 39*, 119–122.

Rose, D. (1968). Relationship between micellar and serum casein in bovine milk. *Journal of Dairy Science, 51*, 1897–1902.

Southward, C. R. (1989). Use of caseins and caseinates. In P. F. Fox (Ed.), *Developments in dairy chemistry 4: Functional proteins* (pp. 173–244). London: Elsevier Applied Science Publishers.

Sullivan, R. A., Fitzpatrick, M. M., Stanton, E. K., Annino, R., Kissel, G., & Palermiti, F. (1955). The influence of temperature and electrolytes upon the apparent size and shape of α- and β-casein. *Archives of Biochemistry and Biophysics, 55*, 455–468.

Thunell, R. K., Duersch, J. W., & Ernstrom, C. A. (1979). Thermal inactivation of residual milk clotting enzymes in whey. *Journal of Dairy Science, 62*, 373–377.

Visser, S., & Slangen, K. J. (1977). On the specificity of chymosin (rennin) in its action on bovine β-casein. *Netherlands Milk and Dairy Journal, 31*, 16–30.

Ward, L. S., & Bastian, E. D. (1996). A method for isolating β-casein. *Journal of Dairy Science, 79*, 1332–1339.

Wilson, M., Mulvihill, D. M., Donnelley, W. J., & Gill, B. P. (1989). Surface active properties at the air–water interface of β-casein and its fragments derived by plasmin hydrolysis. *Journal of Dairy Research, 56*, 487–494.

Yun, S. E., Ohmiya, K., & Shimizu, S. (1982). Role of β-caseins in milk curdling. *Agricultural and Biological Chemistry, 46*, 443–449.