Journal of Dairy Research (2004) **71** 496–499. © *Proprietors of Journal of Dairy Research 2004*
DOI: 10.1017/S0022029904000494 *Printed in the United Kingdom*

Susceptibility of plasmin and chymosin in Cheddar cheese to inactivation by high pressure

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Received 11 July 2003 and accepted for publication 26 November 2003

Keywords: High pressure, plasmin, chymosin, cheese.

High pressure (HP) processing has attracted considerable interest in dairy research in recent years; its effects on cheese have been reviewed by O'Reilly et al. (2001). HP treatment of cheese may influence several of its properties, e.g., for hard and semi-hard cheeses, such as Cheddar and Gouda, the most notable effects are on the texture and rate of ripening. HP treatment increased elasticity and improved organoleptic properties of Gouda cheese (Kolakowski et al. 1998). Messens et al. (2000) reported that, immediately after treatment at 400 MPa, Gouda cheese was less rigid and solid-like and more viscoelastic than untreated cheese. HP treatment also enhances the functional properties of Mozzarella cheese (Johnston & Darcy, 2000; O'Reilly et al. 2002).

Yokohama et al. (1992) reported more extensive proteolysis in Cheddar cheese HP-treated at 50 MPa for 3 d at 25 °C than in controls ripened conventionally for 6 months. However, at least 10 times more starter was used in the manufacture of the Cheddar cheese used by Yokohama et al. (1992) than in conventional manufacture. O'Reilly et al. (2000) also found that HP treatment of conventionally-manufactured Cheddar cheese at 50 MPa for 3 d at 25 °C accelerated proteolysis, but to a lower extent than that reported by Yokohama et al. (1992). According to O'Reilly et al. (2000), enhanced proteolysis in cheese treated at 50 MPa was due mainly to an increased level of hydrolysis of α_{s1}-casein by chymosin. Treatment of hard goats' milk cheese at 50 MPa for 3 d had little effect on proteolysis, but treatment at 400 MPa for 5 min considerably increased secondary proteolysis, i.e. conversion of peptides to amino acids (Saldo et al. 2002). In Gouda cheese, ripening indices were not influenced significantly by HP treatment (Kolakowski et al. 1998; Messens et al. 1999).

Plasmin (EC 3.4.21.7) and chymosin (EC 3.4.23.4) are two key enzymes in the ripening of many cheese varieties, including Cheddar, and differences in the ripening of HP-treated cheeses may be due to effects of HP on the activity of these enzymes. In milk, plasmin activity is reduced at pressures >400 MPa (Scollard et al. 2000a, b; Garcia-Risco et al. 2000, 2003; Huppertz et al. 2003); chymosin activity in undiluted rennet is also reduced at >400 MPa (Malone et al. 2003). To date, HP-induced inactivation of these enzymes in cheese has not been studied.

In this study, the effect of HP on plasmin and chymosin in Cheddar cheese was studied to establish a possible source of influence of HP treatment on cheese ripening, i.e. through changes in the activity of key enzymes.

Materials and Methods

Cheese supply and high pressure treatment

Portions of 14 d-old Cheddar cheese (~25 g), obtained from a local manufacturer (Carbery Milk Products, Ballineen, Ireland), were placed in polyethylene bags and vacuum packaged. Samples were then stored at 8 °C for not longer than 16 h and equilibrated at 8, 20 or 30 °C for 2 h prior to HP treatment at these temperatures.

High pressure treatment

HP treatment was performed using a Stansted Fluid Power Iso-Lab 900 High Pressure Food Processor (Stansted Fluid Power, Stansted, Essex, UK), using a 90:10 mixture of ethanol and castor oil as the pressure transmitting medium. Treatment was carried out at 200 to 800 MPa for 0–60 min; rates of pressure increase and decrease were 300 MPa/min. The temperature of the vessel was controlled thermostatically at 8, 20 or 30 °C throughout treatment.

Determination of enzyme activity in cheese

For determination of the level of residual plasmin activity, finely-grated cheese (100 mg) was dispersed in 1 ml 20 g/l trisodium citrate solution by continuous agitation at 37 °C for 30 min, followed by centrifugation at 2000× ***g*** for 5 min. Plasmin activity in the fat-free subnatant was determined using the substrate N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (Sigma

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https://doi.org/10.1017/S0022029904000494 Published online by Cambridge University Press

Table 1. Effect of treatment at 200–800 MPa for 0–60 min at 8, 20 or 30 °C on residual plasmin activity in 14 day-old Cheddar cheese, expressed as a percentage of the mean value for untreated cheese (100%)

Values are means of data from triplicate experiments on individual cheese samples ± standard deviation

Treatment temperature (°C)	Treatment time (min)	Residual plasmin activity (% of control)			
		200 MPa	400 MPa	600 MPa	800 MPa
8	30		101·8 ± 5·5[a]	102·2 ± 4·7[a]	96·2 ± 5·7[a]
	60		99·4 ± 4·6[a]	98·9 ± 3·4[a]	95·9 ± 2·7[a]
20	0†	101·5 ± 3·9[a]	99·2 ± 5·4[a]	101·0 ± 4·7[a]	103·6 ± 2·7[a]
	15	103·7 ± 6·4[a]	97·2 ± 4·3[a]	90·4 ± 4·2[b]	88·9 ± 6·2[b]
	30	100·8 ± 3·8[a]	102·8 ± 5·1[a]	91·5 ± 3·7[b]	88·7 ± 3·7[b]
	60	99·7 ± 4·7[a]	101·1 ± 4·7[a]	93·0 ± 4·7[b]	86·2 ± 1·6[b]
30	30		92·3 ± 5·2[a]	68·5 ± 3·7[c]	66·3 ± 5·5[c]
	60		85·6 ± 4·6[b]	55·7 ± 4·9[d]	51·5 ± 4·1[d]

[a,b,c,d] Values without a common superscript were significantly different ($P < 0·05$)
† Treatment for 0 min indicates immediate release of pressure after reaching the desired value

Table 2. Effect of treatment at 200–800 MPa for 0–60 min at 8, 20 or 30 °C on residual chymosin activity in 14 day-old Cheddar cheese, expressed as a percentage of the mean value for untreated cheese (100%)

Values are means of data from triplicate experiments on individual cheese samples ± standard deviation

Treatment temperature (°C)	Treatment time (min)	Residual chymosin activity (% of control)			
		200 MPa	400 MPa	600 MPa	800 MPa
8	30		98·7 ± 2·2[a]	011·7 ± 2·5[b]	10·1 ± 1·6[b]
	60		97·5 ± 3·3[a]	11·6 ± 1·1[b]	7·4 ± 2·0[c]
20	0†	100·7 ± 6·1[a]	95·6 ± 4·7[a]	100·2 ± 5·5[a]	13·4 ± 5·0[b]
	15	101·0 ± 5·5[a]	96·8 ± 6·0[a]	6·7 ± 2·2[c]	3·5 ± 3·1[c]
	30	98·2 ± 3·9[a]	98·7 ± 3·7[a]	3·8 ± 4·5[c]	4·7 ± 4·2 [c]
	60	95·9 ± 1·5[a]	99·5 ± 6·0[a]	6·8 ± 2·6[c]	3·6 ± 3·4[c]
30	30		98·7 ± 3·7[a]	6·0 ± 1·1[c]	6·9 ± 2·0[c]
	60		98·9 ± 3·2[a]	4·5 ± 3·4[c]	5·4 ± 2·4[c]

[a,b,c] Values without a common superscript were significantly different ($P < 0·05$)
† Treatment for 0 min indicates immediate release of pressure after reaching the desired value

Chemical Co., St. Louis, MO, USA; Richardson & Pearce, 1981).

For determination of the level of residual chymosin, 50 mg of finely grated cheese was dispersed in 1 ml of 0·1 M-trisodium citrate by continuous agitation at 37 °C for 30 min; these dispersions were centrifuged at 2000× ***g*** for 5 min and the fat-free subnatant was used for further analysis. Residual chymosin activities in cheese dispersions were determined using a 1 mg/ml aqueous solution of the synthetic heptapeptide Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu (Bachem AG, Bubendorf, Switserland). This substrate solution (30 µl) was added to 200 µl 0·1 M-sodium formate buffer, pH 3·2, containing 0·5 g sodium azide/l, and the reaction was initiated by the addition of 70 µl of the cheese dispersion. The mixture was then incubated at 37 °C for 24 h and the reaction terminated by heating at 80 °C for 15 min. Heated samples were centrifuged at 14 000× ***g*** for 15 min, prior to analysis of 100 µl of the supernatant by reversed-phase high performance liquid chromatography, as described by Hurley et al. (1999).

Statistical analysis

All experiments were repeated three times on individual cheese samples. Statistical analysis was performed using a randomised block design, using Minitab version 12 (Minitab Ltd., Coventry, UK). The effect of HP treatment on plasmin or chymosin activity in Cheddar cheese was examined using the General Linear Model technique, with Tukey's pairwise comparisons at a 95% confidence level.

Results and Discussion

Effect of high pressure treatment on plasmin activity in cheese

HP treatment of Cheddar cheese for up to 60 min at 800 MPa at 8 °C or up to 400 MPa at 20 °C did not inactivate plasmin, but treatment for 15–60 min at 600–800 MPa at 20 °C caused slight inactivation (≤15%;

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Table 1). These results are in agreement with those of Scollard et al. (2000a). On treatment at 30 °C, greater inactivation of plasmin occurred at pressures >400 MPa; e.g. treatment at 800 MPa for 60 min at 30 °C reduced plasmin activity by almost 50% (Table 1). Increased HP-induced inactivation of plasmin in milk with increasing treatment temperature was reported by Garcia-Risco et al. (2000). In milk, inactivation of plasmin occurs at pressures ≥400 MPa (Scollard et al. 2000a, b; Garcia-Risco et al. 2000, 2003; Huppertz et al. 2003); treatment at 600 MPa for ≥10 min reduced plasmin activity by ≥70% (Scollard et al. 2000b; Huppertz et al. 2003). Differences between HP-induced inactivation of plasmin in Cheddar cheese (Table 1) and milk (Scollard et al. 2000a, b; Garcia-Risco et al. 2000, 2003; Huppertz et al. 2003), indicate that plasmin is considerably more baroresistant in cheese than in milk, which may be due to various factors. Scollard et al. (2000a) showed that, in buffer systems, the presence of β-lactoglobulin (β-lg) makes plasmin considerably more susceptible to HP-induced inactivation. Since Cheddar cheese contains very little β-lg, HP-induced inactivation of plasmin in cheese via interaction with β-lg should be lower than in milk. Thermal inactivation of plasmin in whey decreases as the pH of the whey is reduced (Crudden & Kelly, unpublished data) and the pH of Cheddar cheese (~5·2) is considerably lower than that of milk (~6·7); however, the influence of pH on HP-induced denaturation of plasmin has thus far not been studied.

Effects of high pressure treatment on chymosin activity in cheese

Treatment of Cheddar cheese for a period ranging from 0–60 min at a pressure ≤400 MPa or at 600 MPa for 0 min at 20 °C did not inactivate chymosin (Table 2). This is consistent with the report of Messens et al. (1999), who concluded, based on gel electrophoretograms, that chymosin activity in Gouda cheese was not affected by treatment at 50–400 MPa for 20–100 min. At a pressure ≥600 MPa, a dramatic reduction in chymosin activity occurred, e.g. loss of >90% of activity after treatment at 600 or 800 MPa for 15–60 min (Table 2). Treatment temperature had little effect on HP-induced inactivation of chymosin in Cheddar cheese; similar levels of chymosin remained after treatment for 30 or 60 min at 400, 600 or 800 MPa at 8, 20 or 30 °C (Table 2).

HP-induced inactivation of chymosin at a pressure >400 MPa, as observed in Cheddar cheese in this study (Table 2), was also observed in undiluted rennet (Malone et al. 2003), although less inactivation occurred in the latter medium, e.g. <50% after treatment at 800 MPa for 5 min at 25 °C. As the mechanism for HP-induced inactivation of chymosin is not known, it is not possible to establish the exact nature of differences between HP-induced inactivation of chymosin in cheese and undiluted rennet.

Conclusions

In conclusion, plasmin in Cheddar cheese was highly resistant to HP treatment at 8 or 20 °C, whereas chymosin was inactivated at a pressure >400 MPa. Greater HP-induced inactivation of plasmin was observed when treatment temperature was increased to 30 °C; however, treatment temperature had only a small effect on HP-induced inactivation of chymosin. In Cheddar cheese, plasmin appeared to be more resistant to pressure than chymosin, as is also observed on thermal processing in liquid media. The limited effect of treatment at ≤400 MPa on plasmin and chymosin in Cheddar cheese indicates that ripening of cheese treated at such pressures should not be influenced significantly by changes in the activity of these enzymes. However, several studies have reported increased proteolysis in cheese HP-treated at up to 400 MPa (Yokohama et al. 1992; O'Reilly et al. 2000, 2003; Saldo et al. 2002); this may possibly be due to enhanced proteolytic action of chymosin and/or plasmin against their substrates while under pressure. Further research is required to establish whether HP can influence the activity of other enzymes in cheese, e.g. bacterial proteases and lipolytic and glycolytic enzymes.

This research was funded under the Food Institutional Research Measure (FIRM) which is administered by Irish Government under the National Development Plan 2000–2006.

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