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Accelerated Maturation of Cheese by Proteolytic Enzymes Produced by *Brevibacterium lines*

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Abstract

Shortening the maturation period has several advantages in reducing the cost of cheese production. Elevated temperature, enzyme addition, modified starter and a slurry method have been used to reduce the period. Successful results in accelerated ripening have been reported by employing enzymes such as proteinases, peptidases, lipase and ß-galactosidase and a mixture of enzymes. In particular, the addition of proteinase in a promising method, though excess amounts of enzymes tend to lead to the development of bitterness and weaken cheese body. Although these shortcomings can be alleviated by the addition of peptidases that release amino acids without affecting the cheese body, such peptidases are not as widely available commercially as are proteinases. The aminopeptidase and serine proteinase which are involved in the maturation of surface ripening of cheese varieties such as Danbo, Limburger and Gruyere have been purified and characterized with a view to applying them to accelerate the ripening of cheese. The effect of aminopeptidase and serine proteinase has been investigated together with the characterization of there enzymes.

Discipline: Biotechnology/ Food
Additional key words: peptidase, proteinase

Introduction

Shortening the maturation period of cheese has several advantages, especially in reducing the cost of cheese production. Recent advances in the analysis of the maturation mechanisms of cheese have led to the development of a method to reduce the maturation period through elevated temperature, as well as by the use of modified starters, semi-liquid slurry and by enzyme addition[16]. Since the first 3 methods are sometimes difficult to control and have resulted in putrefaction, it was reported that the addition of enzymes such as beta-galactosidase[6], lipase[17], proteinase[1,13] and peptidase[14] was a reliable and reproducible method.

During maturation of cheese, components such as proteins, lipids and lactose are metabolized to form flavor compounds by the action of microorganisms and enzymes. Proteins are first hydrolyzed into large peptides and then to smaller peptides and amino acids. Some of the amino acids are further metabolized by microorganisms to form various flavor compounds. By the addition of some proteolytic enzymes, the maturation steps for the formation of peptides and amino acids can be accelerated. However, the addition of proteinase often results in bitterness caused by the release of peptides by the action of proteolytic enzymes[13].

Peptides with some hydrophobic amino acids tend to give a bitter taste. However, further hydrolysis of these bitter peptides leads to the reduction of the bitterness or to the absence of bitterness. Therefore, the use of some peptidases results in the reduction of the maturation period without concomitant bitterness[2,14]. However, the commercial availability of peptidases is very limited. We attempted to identify suitable proteolytic enzymes for cheese maturation.

A bacterium, *Brevibacterium linens*, responsible for surface ripening of cheese varieties[15] such as Danbo, Limburger and Gruyere, was considered to be one of the suitable enzyme sources for accelerating cheese ripening since it is involved in the maturation of surface ripening of cheese and the characteristics of the enzymes it produces are naturally suitable for this purpose.

Production of proteolytic enzymes by *B. linens*

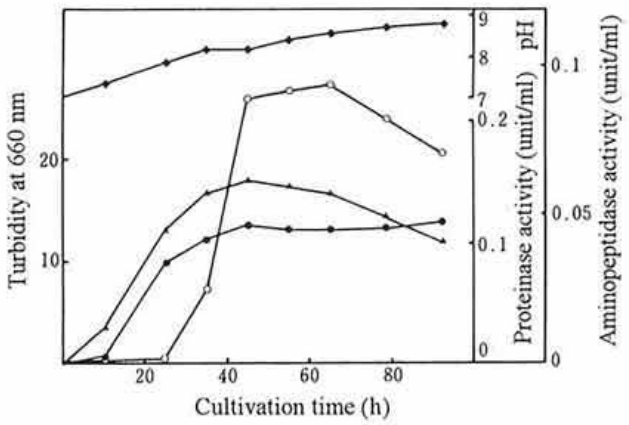
Since this microorganism was reported to produce several types of proteolytic enzymes[4,19], the intracellular and extracellular proteolytic activity was examined. When the 5 strains of *B. linens* were cultured for 24, 48 and 72 h in the medium containing casein, extracellular proteolytic activity was 10–20 times higher than the intracellular one: peptidase and proteinase activities in the extracellular preparation were in the range of 0.03–0.06 and 0.002–0.074 unit/ml whereas those in the intracellular preparation were in the range of 0.001–0.006 and 0.002–0.005 unit/ml, respectively (Table 1).

Table 1. Effect of NaCl on extracellular proteinase and aminopeptidase production by *B. linens*

Medium[a]	NaCl concentration (%)			
	0	0.5	1.5	5
Aminopeptidase activity[b] (unit/ml)				
A	0.060	0.027	0.009	0.002
B	0.092	0.038	0.014	0.003
C	0.087	0.059	0.037	0.003
Proteinase activity[b] (unit/ml)				
A	0.096	0.091	0.079	0.077
B	0.125	0.117	0.101	0.088
C	0.125	0.128	0.115	0.091

a): Media contained glucose 0.5%, yeast extract 0.1%, K_2HPO_4 0.25%, $MgSO_4 \cdot 7H_2O$ 0.02% and the following nutrients (pH7.0): Medium A; tryptone 0.5% and soytone 0.1%, Medium B; tryptone 1% and soytone 0.3%, Medium C; tryptone 2% and soytone 0.9%.

b): Figures show highest aminopeptidase or proteinase activity in the culture filtrate after 24, 48 and 72 h of culture.

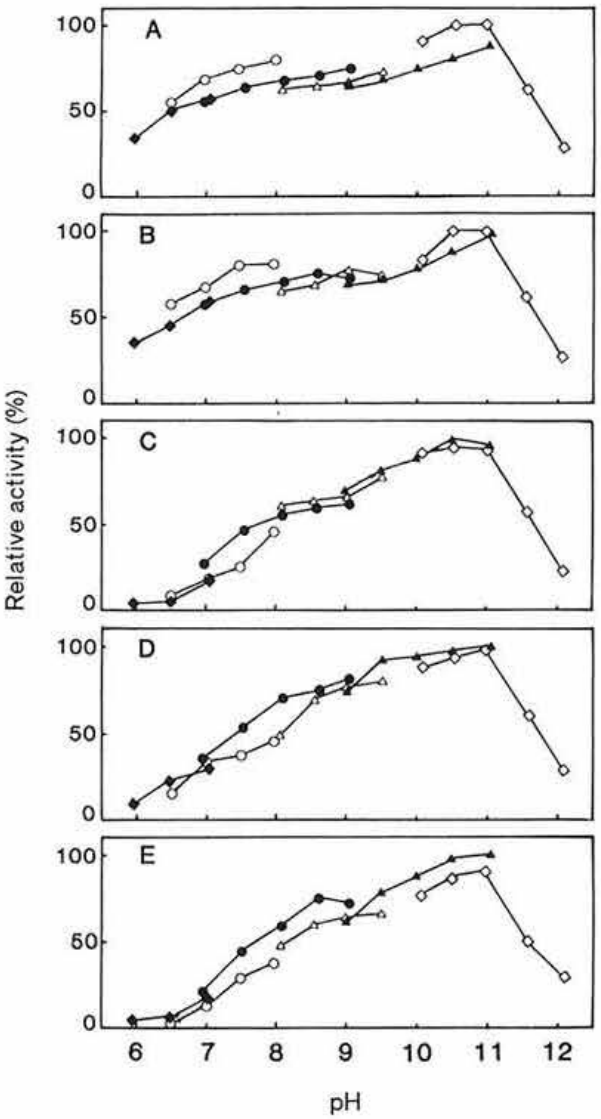


●: Proteinase activity, ○: Aminopeptidase activity,
◆: pH, ▲: Turbidity at 660 nm.

Fig. 1. Time course of cell growth and enzyme (proteinase and peptidase) production by *B. linens*
The microorganism was cultured at 24°C in a medium containing tryptone 1%, glucose 0.5%, soytone 0.3%, yeast extract 0.1%, K_2HPO_4 0.25% and $MgSo_4 \cdot 7H_2O$ 0.02% (pH 7.0).

Optimum medium composition for proteolytic enzyme production by *B. linens*

The tryptone and soytone medium reported by



◆: 2-(N-morpholino)ethanesulphonate,
○: Phosphate, ●: Tris-HCl, △: Borate,
▲: Glycine, ◇: Piperidine.

Fig. 2. Optimum pH for 5 serine proteinases
Enzyme activities were measured by the standard assay in 50 mM of various buffers.

Sorhaug[19] was found to be suitable among the 4 media when tested for production of the extracellular enzymes. Optimum medium composition was found to be as follows: tryptone 1%, K_2HPO_4 0.25%, and $MgSO_4 \cdot 7H_2O$ 0.02%, pH 7.0.

Effect of NaCl on the enzyme production

Aminopeptidase production is strongly affected by the presence of NaCl unlike the proteolytic activity[9]. Since the microorganism is salt-tolerant, reduction of NaCl may have affected the membrane; especially the mechanism of release of peptidase from the cell to the medium.

Relation between growth and enzyme production

Proteinase was produced from the beginning of the logarithmic phase of growth while aminopeptidase production started at the early stationary phase of growth as shown in Fig. 1. A change in the shape from rods to cocci was observed when the production of aminopeptidase was initiated. A similar relationship between the change of shape and yellow pigment production has been reported in this microorganism[18]. Doubling time of growth was calculated to be 2.64 h.

Characteristics of proteinases produced by *B. linens*

Partial purification of one extracellular enzyme[20], and the presence of 3 to 6 extracellular proteinases revealed in electrophoretic zymograms[4] have been reported. Five serine proteinases were purified from the culture filtrate of *B. linens* by a series of column chromatography steps (Table 2) and found to be homogeneous by polyacrylamide gel electrophoresis. All appear to be serine proteinases as they were inactivated by phenylmethylsulphonyl fluoride and not by EDTA or p-chloro-mecuribenzoic acid. They were all active against casein at the alkaline pH of 11.0 as shown in Fig. 2. Specific activity was within the range of 2.66–3.23 unit/mg. Proteinases C, D, and E were more stable at higher temperature and acidic pH than proteinases A and B. Molecular weights estimated by gel filtration were 37,000, 37,000, 44,000, 127,000, and 325,000 for proteinases, A, B, C, D and E, respectively[7].

Characteristics of aminopeptidases produced by *B. linens*

Two aminopeptidases, designated as amino-

Table 2. Purification of the extracellular serine proteinases from *B. linens* F

Step	Proteinase	Volume (ml)	Activity (unit/ml)	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Culture filtrate	A,B,C,D,E	220,000	0.118	0.0129	100	1
DEAE-Sephadex A-50 batch	A,B,C,D,E	12,500	1.67	0.278	80.4	21.6
Ammonium sulphate fractionation	A,B,C,D,E	1,600	9.13	0.590	56.3	47.5
DEAE-Sephadex A-50 chromatography	A,B	354	2.04	0.792	2.78	61.4
	C	906	3.48	0.708	12.1	54.9
	D,E	884	5.50	0.927	18.7	71.9
DEAE-Trisacryl M chromatography	A,B	238	2.25	2.19	2.06	170
	C	421	5.30	2.42	8.60	188
	D,E	377	8.54	2.34	12.4	181
Mono-Q HR5/5 chromatography[a] (Twice)	A	–	–	2.69	(0.47)[b]	209
	B	–	–	2.70	(0.98)	209
	C	–	–	3.23	(6.08)	250
	D	–	–	3.19	(4.59)	247
	E	–	–	2.66	(3.05)	206

One unit of proteinase activity corresponds to the amount of enzyme which releases 1 μmol of tyrosine/min under the specified conditions.

a): Only part of the sample was applied due to the size of the semi-preparative columns.

b): Yields in this step were calculated as if all sample was subjected to the purification although only a portion of sample was applied.

Table 3. Purification of the extracellular aminopeptidases from *B. linens*

Purification step	Volume (ml)	Activity (U/ml)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	10,000	0.0897	0.0308	100	1
Ammonium sulphate fractionation	120	10.1	0.385	136	12.5
DEAE-Sephadex A-50 chromatography	105	7.47	15.3	87.7	498
DEAE-Trisacryl M chromatography	72	3.97	21.3	32.0	692
Mono-Q HR5/5 chromatography[a)]	-	-	48.1	(24.7)[a)]	1,560
Superose-6 HR10/30 gel filtration[a), b)]					
Aminopeptidase A	-	-	52.1	(12.0)[a)]	1,720
Aminopeptidase B	-	-	60.2	(2.19)[a)]	1,950

a): Only a portion of sample was applied in these steps due to the size of the semi-preparative columns. Yields were calculated based on the application of a portion of sample to the column.
b): The aminopeptidase fraction was separated into two fractions, aminopeptidases A and B, in this step.

peptidases A and B, were purified 1,720 and 1,950 fold, respectively, from the culture filtrate of *B. linens* by ammonium sulphate fractionation, a series of column chromatography steps on DEAE-Sephadex, DEAE-Trisacryl M and Mono-Q, and gel filtration on Superose-6, as shown in Table 3. Aminopeptidase A accounted for 85% of the aminopeptidase activity remaining by the end of purification. The purified enzymes were homogeneous based on disc gel electrophoresis. Aminopeptidases A and B showed the same pH optimum of 9.3 and apparent temperature optimum of 40°C, although the former showed a slightly higher pH stability than the latter. Both enzymes were completely inhibited by incubation with EDTA, indicating that they are metalloenzymes as shown in Table 4. The enzymes inactivated by EDTA were reactivated by incubation with

Table 4. Effect of various metal ions on aminopeptidases A and B activities

Metal salt (1 mM)	Relative activity (%)	
	Aminopeptidase A	Aminopeptidase B
$BaCl_2$	98.6	99.4
$CaCl_2$	96.9	93.3
$MgCl_2$	90.4	91.3
$CoCl_2$	32.4	32.0
$AlCl_3$	21.1	18.1
$FeCl_2$	14.8	16.4
$CuCl_2$	8.4	7.5
$MnCl_2$	5.1	5.8
$ZnCl_2$	1.5	1.4
$HgCl_2$	1.2	2.1
$CdCl_2$	0.6	1.2
$SnCl_2$[a)]	9.7	11.1
$NiCl_2$[a)]	2.1	3.4
$PbCl_2$[a)]	0.6	1.2
None	100	100

Enzyme (17 milliunit of aminopeptidase A or B) was preincubated for 1 h at 30°C with the metal salt indicated. After incubation, the activity was measured by the standard assay. Enzyme activity in the absence of metal salts measured by the standard assay was defined as 100%.
a): Precipitate was observed during the enzyme reaction.

Table 5. Reactivation of EDTA-inactivated aminopeptidases A and B by several metal ions

Metal salt	Extent of reactivation (%)	
	Aminopeptidase A	Aminopeptidase B
$CaCl_2$	99.5	98.0
$CoCl_2$	89.3	87.1
$MgCl_2$	47.0	36.8
$ZnCl_2$	36.4	26.7
$MnCl_2$	20.5	33.2
None	0	0

The native enzyme (17 milliunit of aminopeptidase A or B) was incubated with 0.1 mM-EDTA at 0°C for 1 h, then metal salt was added to a concentration of 0.1 mM. After incubation for 30 min at 0°C, the enzyme was assayed. The final concentrations of metal ion and EDTA in the standard assay were 0.04 mM and 0.004 mM, respectively. The activity of the native enzyme which was not treated by EDTA was measured in the absence of metal salts and defined as 100%.

Ca^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, or Mn^{2+} as shown in Table 5. The molecular weights of aminopeptidases A and B were estimated to be 150,000 and 110,000, respectively by gel filtration on Superose 6, and 36,000 and 26,000, respectively, by SDS-PAGE. It was thus assumed that each of the native enzymes occurs as a tetramer. The Km values of aminopeptidases A and B for L-leucine-p-nitroanilide were calculated to be 16.1 and 15.2 mM, respectively[8].

The purified aminopeptidases A and B hydrolyzed a variety of substrates, showing a specificity for N-terminal L-leucine (Table 6). Their substrate specificity differed from that of the aminopeptidase purified by Foissy (1978)[5]. The specificity of the purified aminopeptidase A was not restricted to dipeptides since tripeptide and tetrapeptide substrates were also hydrolyzed. Proline iminopeptidase activity was also confirmed, as the enzyme hydrolyzed L-prolyl-L-methionine and related substrates. This broad substrate specificity of the enzyme, which hydrolyzes peptides containing N-terminal hydrophobic, aromatic and basic amino acids as well as proline, seems most suitable for accelerating cheese ripening.

Effect of proteinases on acceleration of cheese ripening

It is understandable that mainly aspartic, cysteine, or metalloproteinase, with an acidic or neutral rather than alkaline optimum pH should have been used

Table 6. Substrate specificity of aminopeptidase A

Substrate[a]	Relative activity[b]	Substrate[a]	Relative activity[b]
Gly-Leu	<0.1	Tyr-Leu	28.8
-Phe	<0.9	Phe-Ala	<2.5
Ala-Ala	3.6	-Leu	4.3
-Pro	3.6	-Phe	7.9
-Val	6.0	-Phe-Phe	49.1
-Leu	5.0	-Phe-Phe-Phe[a]	21.8
-Phe	11.8	Lys-Ile	2.2
-Lys	11.0	-Phe	5.5
Val-Ala	1.8	His-Ala	2.6
-Pro	<2.9	-Leu	19.8
-Phe	11.2	-Lys	60.0
Met-Gly	0.8	-Phe	25.0
-Pro	<0.4	Arg-Leu	5.5
Leu-NH_2	26.0	Try-Ala	3.7
-Ser	42.7		
-Pro	3.7	Pro-Gly	0.3
-Gly	3.4	-Met	15.2
-Ala	52.9	-Leu	0.9
-Val	91.8	-Tyr	4.9
-Met	408 (435)	-Phe	4.5
-Ile	42.5	-Try	4.6
-Leu	100 (100)		
-Tyr	268 (216)	Glu-*p*NA	<0.02
-Phe	187 (182)	Ala-*p*NA	10.4
-Arg	1,100 (1,364)	Leu-*p*NA	745 (764)
-Try	227 (239)	Lys-*p*NA	39.8
-Leu-Leu	131		
D-Leu-D-Leu	<0.1 (<0.1)		
D-Leu-L-Leu	<0.1 (<0.1)		
L-Leu-D-Leu	<0.1 (<0.1)		

To minimize the experimental error, enzyme concentration (1.5–30 milliunit) and incubation time (5–180 min) were varied depending on the susceptibility of each substrate.

a): All the amino acids were in the L-configuration, except where indicated. Concentration of substrate was 1 mM except for tetraphenylalanine (about 0.4 mM).

b): Rate of hydrolysis relative to that of Leu-Leu, for which 6.7% of the substrate was hydrolyzed after incubation with aminopeptidase A or B (3 milliunit) at 30°C for 10 min. Figures in parentheses refer to aminopeptidase B.

to accelerate the ripening of cheese, because the pH of cheese is around 5. However, the important characteristic of proteinase is not necessarily the optimum pH, but the specificity toward the amino acid residues in casein, particularly for minimizing the release of bitter peptides consisting mainly of aromatic and hydrophobic amino acids. Addition of aspartic proteinase, which attacks these amino acid residues more than metalloproteinase, resulted in a strong bitterness[15].

Proteinase of *B. linens* can be considered to have naturally desirable proteolytic characteristics, since the microorganism is involved in the ripening of several cheese varieties[15]. Two major proteinase fractions of serine proteinase of *B. linens*, C and D-E, which respectively account for 36% and 56% of the total activity in the culture filtrate, were used, even though they were less active and less stable in acidic pH. Compared with the activity measured at the optimum pH of 11.0, the activity at pH 6.2 was 4% for proteinase C, 10% for proteinase D, and 5% for proteinase E (Fig. 2). After incubation at pH 5.2 and 12°C for 24 h, less than 20% of the original activity of proteinases C and D-E was retained. However, the environment in Cheddar cheese in terms of enzyme stability and activity seemed to be quite different from the environment in buffer, as the serine proteinase fractions C and D-E promoted the hydrolysis of casein (Fig. 3). However, quantitative analysis of the serine proteinase activity in the enzyme-treated cheeses was unsuccessful due to the interference in the assay of proteins and peptides and amino acids extracted from cheese together with proteinase. The effect of proteinases C, D-E and Neutrase was indirectly observed as follows: acceleration of caseinolysis, accumulation of peptides soluble in TCA, and development of Cheddar cheese flavor. A statistically significant increase in the

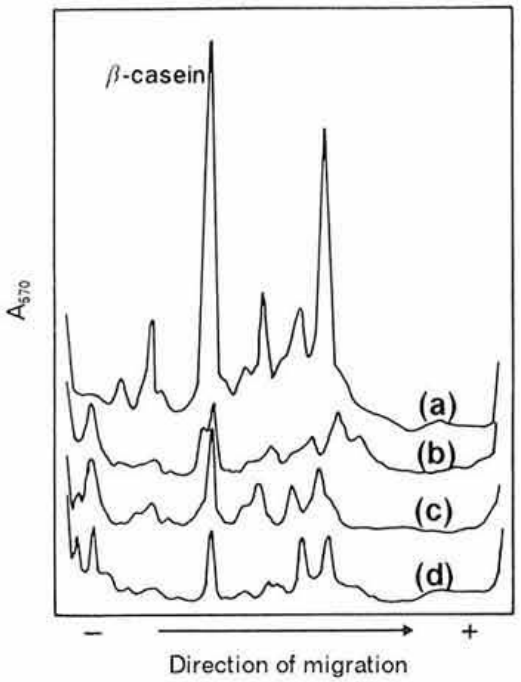


Fig. 3. Polyacrylamide gel electrophoretograms of cheese after 2 months of ripening
(a): Untreated control cheese,
(b): Cheese treated with metalloproteinase (Neutrase; 8.6 unit/kg curd),
(c): Cheese treated with serine proteinase (Pro-C; 26 unit/kg curd); and
(d): Cheese treated with serine proteinase (Pro-D-E; 26 unit/kg curd).
Absorbance at 570 nm.

Table 7. Effect of serine proteinase (Pro-C) of *B. linens* on flavor development of Cheddar cheese ripened at 12°C for 2 months

Amount of proteinase (unit/kg)	Flavor intensity[a]	Bitterness[b]	TCA N[c] (%)
0	2.94	0.1	100
26	3.88**	0.4	125
8.6	3.24	0.4	110
2.9	3.35	0.2	112
8.6 (Neutrase)	3.88**	0.3	125

a): Mean taste panel scores for intensity of Cheddar cheese flavor. Lowest; 0, Highest; 8.
b): Lowest; 0, Highest; 4.
c): Proteolysis is expressed as TCA N (% of control).
** Significantly different mean scores compared with controls at $P<0.01$ ($d(0.05) = 0.47$; $d(0.01) = 0.63$; $df = 64$).

Table 8. Effect of serine proteinase (Pro-D-E) of *B. linens* on flavor development of Cheddar cheese ripened at 12°C for 2 months

Amount of proteinase (unit/kg)	Flavor intensity[a]	Bitterness[b]	TCA N[c] (%)
0	2.94	0	100
26	3.76**	0	152
8.6	3.59*	0	148
2.9	2.76	0	126
8.6 (Neutrase)	3.59*	0	126

a): Mean taste panel scores for intensity of Cheddar cheese flavor. Lowest; 0, Highest; 8.
b): Lowest; 0, Highest; 4.
c): Proteolysis is expressed as TCA N (% of control).
*,** Significantly different mean scores compared with controls at $P<0.05$, and $P<0.01$, respectively ($d(0.05) = 0.59$; $d(0.01) = 0.79$; $df = 64$).

Table 9. Effect of aminopeptidase of *B. linens* on flavor development of Cheddar cheese matured at 12°C for 2 or 3 months

Treatment	Amount of enzyme		Flavor intensity[a] (0–8)	Bitterness (0–4)	SSA-N (%)	TCA-N (%)
	Neutrase (unit/kg)	Peptidase (unit/kg)				
2-month maturation						
C	0	0	3.00 ** –	0	100	100
N	8.58	0	3.69 – **	0	110	119
N*Bl*3	8.58	6.88 (*B. linens*)	4.81 ** **	0	131	154
N*Bl*	8.58	2.30 (*B. linens*)	4.25 * **	0.05	117	120
N*Sl*	8.58	2.57 (*Strep. lactis*)[b]	4.63 ** **	0.05	128	149
3-month maturation						
C	0	0	3.43 ** –	0	100	100
N	8.58	0	4.24 – **	0.1	111	121
N*Bl*3	8.58	6.88 (*B. linens*)	4.76 ** **	0.05	139	157
N*Bl*	8.58	2.30(*B. linens*)	4.38 **	0.05	121	134
N*Sl*	8.58	2.57 (*Strep. lactis*)[b]	4.38 **	0.05	117	135

a): Mean taste panel scores for intensity of Cheddar cheese flavor; *,** Significantly different mean scores compared with treatment N in left column or treatment C in right column at $P<0.05$ and 0.01, respectively.

b): Crude extract of cellular protein of *Strep. lactis* with high exopeptidase activity was used as enzyme source (Law & Wigmore, 1983). Proteolysis is expressed as sulphosalicylic acid (SSA)-soluble N (% control) and trichloroacetic acid (TCA)-soluble N (% control).

typical Cheddar cheese flavor ($P<0.01$) was observed in the cheese containing 26 unit/kg curd of proteinases C (Table 7) and D-E (Table 8) after 2 months of ripening[11]. A nonsignificant difference in casein hydrolysis between serine proteinase and metalloproteinase was observed, although the former was expected to show a broader substrate specificity.

Contamination of proteinases D-E with aminopeptidase was less than 0.1 unit/kg curd and was considered to be negligible, because a peptidase activity of 21 unit/kg curd was required to obtain a synergistic effect on the acceleration of Cheddar cheese ripening in the case of peptidase from *Streptococcus lactis*[14].

Effect of peptidase on acceleration of cheese ripening

Since the availability of food-grade peptidase substantially free from endoproteinase activity is limited commercially, at present, crude preparations rich in peptidase activity, such as heat-treated cells[2] and cell-free extract[14] of cheese starter bacteria have been used for accelerating cheese ripening, and indirect evidence of peptidase action has been reported. By employing substantially purified aminopeptidase from *B. linens*, the synergistic effect of proteinase and peptidase on the acceleration of cheese ripening was confirmed. Sensory analysis of flavor intensity suggested that the maturation period of Cheddar cheese could be reduced from 4–6 months to 2 months[10] by the addition of Neutrase (8.58 unit/kg curd) with the aminopeptidase (6.88 unit/kg curd) as shown in Table 9.

Even though sensitive methods have been developed[3], it is still difficult to detect the added enzyme activity during accelerated ripening of cheese due to the low concentration of the added enzyme (13.9 mg of Neutrase/kg curd in treatment N) and the presence of interfering substances such as amino acids, peptides and proteins. However, both the high affinity of the aminopeptidase to DEAE-Sephadex and the higher activity of the aminopeptidase at pH 8.5 (193 times that at pH 5.2) enable to measure

Table 10. Aminopeptidase activity recovered during cheese maturation at 12°C

Maturation period (months)	Treatment[a]	
	N*Bl*3 (%)	N*Bl* (%)
0	85[b]	93[b]
1	91	89
2	86	92
3	94	87

a): Treatment codes as in Table 9.

b): Aminopeptidase activity was measured twice after extraction of the enzyme from cheese using DEAE-Sephadex. The amount of activity of aminopeptidase added to the curd at the salting stage was expressed as 100%.

the enzyme activity in cheese. It was found that the aminopeptidase which is unstable under acidic conditions remained in a stable form in cheese for at least 3 months (Table 10). It appeared that this environment, which was markedly different from that in buffer, exerted a stabilizing effect on the enzyme. Thus it is possible that other enzymes that are unstable in buffer could be used to accelerate ripening due to a similar stabilizing effect of cheese.

Successful results in the acceleration of ripening of cheese using a combination of Neutrase and a crude enzyme fraction rich in peptidase activity have been reported for the hydrolysis of casein to peptides and the peptidase contributed to the hydrolysis of peptides of medium size to amino acids. It is likely that the bitter peptides released from casein by Neutrase would be further broken by the addition of aminopeptidase as has been reported to occur in heat-treated strain of *Lactobacillus helveticus* in Swedish hard cheese[2] and a cell-free extract of *Streptococcus lactis* in Cheddar cheese[14].

Since the purified aminopeptidase of *B. linens* acts specifically on hydrophobic N-terminal amino acid residues such as leucine, and is inactivated against N-terminal glycyl residues[8], it was expected that the leucine and isoleucine contents would increase in the aminopeptidase-treated cheese. However, molar ratios of amino acids in NBl3 were found to be similar to those of the other cheeses including the control (Table 11). Keeping the natural balance of amino acids similar to that in the control cheese seemed to be important in order to stimulate starter bacteria to convert amino acids further into flavoring substances.

It was confirmed that the aminopeptidase was not distributed evenly in the curd as it was rather difficult to obtain a constant aminopeptidase activity from samples containing as much as 10 g cheese. In contrast to the case of proteinase where both enzyme and substrate have small diffusion constants because of their large molecular size, the problem of uneven distribution of the aminopeptidase will not be serious as both substrates and their products (peptides and amino acids) are likely to migrate to and from the aminopeptidase more easily than enzymes and proteins. Uneven distribution of enzymes can also be overcome by applying a liposome as an enzyme carrier[1,12].

Conclusion

Both serine proteinase and aminopeptidase of *B. linens* can be considered to be a promising enzyme source for accelerating cheese ripening. However, the production of these enzymes should be increased. Culture filtrate of 2.6 l for the proteinase treatment

Table 11. Molar ratios of free amino acids in enzyme-treated cheese after 2 months of maturation

Amino acid[b]	Treatment[a]				
	C	N	N*Bl*3	N*Bl*	N*Sl*
Asp	2.0	1.7	1.8	1.8	1.8
Glu	18.1	17.9	19.3	18.7	18.5
Pro	5.5	4.4	5.4	5.7	6.4
Gly	2.9	3.0	3.2	2.9	3.0
Ala	3.1	3.3	3.7	3.3	3.4
Cys	0.0	0.0	0.0	0.0	0.0
Val	9.1	9.1	9.9	9.2	9.2
Met	1.8	1.9	2.0	1.8	1.9
Ile	2.1	3.2	3.9	3.1	3.1
Leu	20.2	20.9	18.9	18.9	19.0
Tyr	2.6	1.9	1.2	1.4	1.6
Phe	9.3	9.2	8.9	9.0	9.1
His	2.2	3.4	3.5	3.4	3.9
Lys	5.9	5.3	3.5	5.6	5.2
NH_4	14.7	14.1	14.4	14.6	13.3
Arg	0.5	0.7	0.4	0.6	0.6
Total amount (μmol/g cheese)	86.2	98.8	128	116	117

a): Treatment codes as in Table 9.
b): Amounts of Thr, Gln, Ser and Asn were not determined as peaks were unresolved. The amino acid content is expressed as % molar ratios.

at a level of 26 unit/kg or that of 4.9 l for the treatment of aminopeptidase at a level of NBl3 is required to treat 1 kg of curd. Ample scope remains for increasing the proteolytic enzyme production of the strain, by changing the medium composition, culture conditions, and mutation. Furthermore, as it was reported that a combination of proteinase and peptidase for accelerated ripening led to a rapid release of peptides and amino acids with a reduced bitterness, application of crude enzyme preparation of *B. linens*, which naturally contains both serine proteinase and aminopeptidase, would be quite economical.

References

1) Alkhalaf, W. et al. (1988): Liposomes as proteinase carriers for the accelerated ripening of Saint-Paulin type cheese. *J. Food Sci.*, **53**, 1674–1679.
2) Ardo, Y. & Pettersson, H. E. (1988): Accelerated cheese ripening with heat treated cells of *Lactobacillus helveticus* and a commercial proteolytic enzyme. *J. Dairy Res.*, **55**, 239–245.
3) Boudjellab, N., Rolet-Repecaud, O. & Collin, J. C. (1994): Detection of chymosin in cheese by an enzyme-linked immunosorbent assay. *J. Dairy Res.*, **61**, 101–109.
4) Foissy, H. (1974): Examination of *Brevibacterium linens* by an electrophoretic zymogram technique. *J. Gen. Microbiol.*, **80**, 197–207.
5) Foissy, H. (1978): Some properties of aminopeptidase from *Brevibacterium linens*. *FEMS Microbiol. Lett.*, **3**, 207–210.
6) Gooda, E., Bednarski, W. & Poznanski, S. (1983): The protein degradation in Cheddar cheese manufactured from milk treated with lactase. *Milchwissenschaft*, **38**, 83–86.
7) Hayashi, K., Cliff, A. J. & Law, B. A. (1989): Purification and preliminary characterization of five serine proteinases produced by *Brevibacterium linens*. *Int. J. Food Sci. Tech.*, **25**, 180–187.
8) Hayashi, K. & Law, B. A. (1989): Purification and characterization of two aminopeptidases produced by *Brevibacterium linens*. *J. Gen. Microbiol.*, **135**, 2027–2034.
9) Hayashi, K., Cliff, A. J. & Law, B. A. (1990): Culture conditions of for production of proteolytic enzymes. *Nippon Shokuhin Kogyo Gakkaishi*, **37**, 737–739.
10) Hayashi, K., Revell, D. F. & Law, B. A. (1990): Accelerated ripening of Cheddar cheese with the aminopeptidase of *Brevibacterium linens* and a commercial neutral proteinase. *J. Dairy Res.*, **57**, 571–577.
11) Hayashi, K., Revell, D. F. & Law, B. A. (1990): Effect of partially purified extracellular serine proteinases produced by *Brevibacterium linens*. *J. Dairy Sci.*, **73**, 579–583.
12) Kirby, C. J., Brooker, B. E. & Law, B. A. (1987): Accelerated ripening of cheese using liposome-encapsulated enzyme. *Int. J. Food Sci. Tech.*, **22**, 355–375.
13) Law, B. A. & Wigmore, A. S. (1982): Accelerated cheese ripening with food grade proteinases. *J. Dairy Res.*, **49**, 137–146.
14) Law, B. A. & Wigmore, A. S. (1983): Accelerated ripening of Cheddar cheese with a commercial proteinase and intracellular enzymes from starter streptococci. *J. Dairy Res.*, **50**, 519–525.
15) Law, B. A. (1984): Flavor development in cheese. *In* Advances in the microbiology and biochemistry of cheese and fermented milk. Elsevier Appl. Sci. Publ., London, 187–207.
16) Law, B. A. (1984): Accelerated ripening of cheese. *In* Advances in the microbiology and biochemistry of cheese and fermented milk. Elsevier Appl. Sci. Publ., London, 209–228.
17) Lin, J. C. C. et al. (1987): Effect of commercial food grade enzymes on proteolysis and textural changes in granular Cheddar cheese. *J. Food Sci.*, **52**, 620–625.
18) Sneath, P. H. A. (1986): Bergey's manual of systematic bacteriology. 1302.
19) Sorhaug, T. (1981): Comparison of peptide hydrolases from six strains of *Brevibacterium linens*. *Milchwissenschaft*, **36**, 137.
20) Tokita, F. & Hosono, A. (1972): Studies on the extracellular proteases produced by *Brevibacterium linens*. *Jpn. J. Zool. Sci.*, **43**, 39–48.

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