Accelerated Ripening of Ewes' Milk Manchego Cheese: The Effect of Neutral Proteinases

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ABSTRACT

The effect of two commercial preparations of neutral proteinase from *Bacillus subtilis* (Neutrase L and Novozym 257) on chemical, rheological, and sensory characteristics of Manchego cheese made from pasteurized ewes' milk and ripened for 90 d at 12 or 16°C was investigated. Levels of proteinase added to milk were in the range .001 to .004 Anson units/L of milk. Degradation of α_{s1}-casein was not influenced by proteinase level; however, residual β-casein was lower in cheese made with .004 Anson units of proteinase/L. Formation of pH 4.6-soluble *N* in cheese was more influenced by Neutrase L or Novozym proteinase addition than formation of TCA-soluble N or phosphotungstic acid-soluble N. Cheese made with Neutrase L proteinase was more crumbly and less elastic than control cheese, showing lower fracturability and hardness values. Frequency of bitter flavor was not significantly higher for cheese made with Neutrase L or Novozym proteinase than for control cheese. Flavor preference was not influenced by Neutrase L proteinase, but it was slightly impaired by Novozym proteinase. Flavor intensity was more enhanced by Neutrase L proteinase than by Novozym proteinase. Addition of .004 Anson units/L of Neutrase L proteinase would reduce ripening time by 15 d, an increase of 4°C in ripening temperature by 40 d, according to regression equations calculated from experimental data. Therefore, Manchego cheese made from milk to which .004 Anson units/L of Neutrase L proteinase was added would develop in 35 d at 16°C the same flavor intensity as control Manchego cheese held for 90 d at 12°C.

(**Key words**: ripening acceleration, Manchego cheese, ewes' milk)

Abbreviation key: AU = Anson units, NE = Neutrase L, NO = Novozym 257, PTA = phosphotungstic acid.

INTRODUCTION

Manchego cheese is manufactured in central Spain from raw or pasteurized ewes' milk. It is a semi-hard variety according to the ripening period, which may last from 60 d to 1 yr.

Acceleration of Manchego cheese ripening by means of elevated curing temperatures has been recently investigated at our laboratory. Nitrogen—soluble at pH 4.6, in 12% TCA, and in 1% phosphotungstic acid (PTA)—reached, after 4 mo, 26.09, 19.92, and 12.94% in raw milk Manchego cheese ripened at 16°C, respectively, and 21.27, 14.37, and 8.31% if held at 8°C. These values were only 22.02, 16.56, and 8.40% in pasteurized milk Manchego cheese cured at 16°C. After 4 mo, FFA were 11.09 and 16.21 meq/100 g of fat in raw milk cheese ripened at 8 or 16°C and only 9.87 meq/100 g of fat in pasteurized milk cheese cured at 16°C. Flavor intensity of raw milk cheese held at 8°C for 4 mo was reached by raw milk cheese after ripening for 2 mo at 16°C or by pasteurized milk cheese after 3 mo at 16°C (13).

Direct addition of commercial proteinases to cheese milk or curd has been employed to accelerate ripening of cows' milk varieties

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such as Cheddar (8, 9, 15, 16, 21), Edam (18), Saint Paulin (2), Spanish hard cheese (12), and Swedish hard cheese (3). Bitterness was reported as the most common flavor defect in cheeses from milk or curd treated with proteinases (2, 3, 8, 12, 15, 16). Accelerated ripening of Feta cheese, a soft white brined variety traditionally made from ewes' milk, was achieved by addition of heat shocked cultures or microbial proteinases (22). Cheese made from ewes' milk seems to be less susceptible to bitterness than cows' milk cheese (19). Those authors found bitterness in enzymic hydrolysates from cow whole casein more marked than in hydrolysates from ewe whole casein. The reduced probability of bitter flavor defect in ewes' milk cheese makes proteinase addition to ewes' milk or curd a more attractive method than for cows' milk varieties.

Manchego cheese made from pasteurized milk ripened more slowly than raw milk cheese (13); it is highly desirable to improve flavor and ripening rate of the former. The objective of the present work was to investigate the effect of commercial neutral proteinases on the acceleration of the ripening process of Manchego cheese made from pasteurized ewes' milk.

MATERIALS AND METHODS

Neutral Proteinases

Two commercial preparations of a bacterial neutral proteinase from *Bacillus subtilis* were used: Neutrase L (NE, Novo España S. A., Madrid, Spain), a liquid preparation with a declared activity of .5 Anson units (AU)/g (batch number PWN 0088), and Novozym 257 (NO, Novo España S. A.), a solid preparation of the enzyme dispersed on a salt carrier with a declared activity of .01 AU/g (batch number PW2 0100). An Anson unit is defined as the amount of enzyme at 25°C, pH 7.50, and reaction time of 10 min that digests hemoglobin at an initial rate such that the amount of TCA-soluble material liberated per minute gives the same color with phenol reagent as 1 meq of tyrosine. Actual proteolytic activity was measured according to Law and Wigmore (16).

Cheese Manufacture

Pasteurized (15 s at 75°C) ewes' milk from the Instituto Nacional de Investigaciones Agrarias herd was used. The effect of proteinase NE was investigated in two experiments, each consisting of three 100-L vats to which .001, .002 or .004 AU/L had been added and a 100-L control vat without enzyme. Codes for these four vats were NE1, NE2, NE4, and NEC. The same experimental design and enzyme levels were used for proteinase NO, the vats being coded as NO1, NO2, NO4, and NOC, respectively. Milk at 30°C was inoculated (1%) with a coagulated mixed culture of *Lactococcus lactis* INIA 12, 13, 14, and 15 from the Instituto Nacional de Investigaciones Agrarias culture collection. Proteinase preparation (dissolved in 100 ml of water) and 10 ml of animal rennet strength 1:15000 (Hansen, standard, lot number 421213) were added 30 min later. Cheeses were manufactured and salted as previously described (13). Influence of ripening temperature was studied by subdividing cheeses from each vat into two lots, which were ripened at 12 or 16°C for 90 d.

Chemical Analyses

Dry matter, pH, soluble N fractions (pH 4.6-, TCA-, and PTA-soluble N), and residual α_{s1}- and β-caseins were determined in duplicate as previously described (11, 13). The FFA were analyzed in duplicate according to Nuñez et al. (17) and expressed as milliequivalents per 100 g of fat.

Cheese DM Yield

Cheese DM yield (expressed as kg of DM cheese/100 L of milk) was calculated by weighing cheese after 24 h and at weekly intervals throughout ripening, with an accuracy of .1 g, and correcting according to cheese DM value.

Textural Measurements

Fracturability (expressed in newtons), elasticity (expressed in newtons per square millimeter), and hardness (expressed in joules) were determined on six cylinder-shaped samples (17-mm height × 17-mm diameter) from each cheese after 30, 60, and 90 d of ripening, as previously described (13).

Sensory Evaluation

Flavor preference and flavor intensity were evaluated after 30, 60, and 90 d of ripening by

10 trained panelists on 15- to 20-g samples, as previously described (13). The flavor preference scale consisted of a 10-cm vertical line with anchor points at the upper (like extremely) and lower (dislike extremely) ends. The flavor intensity scale had upper (extremely strong) and lower (extremely mild) anchor points. By measuring the distance in centimeters from the mark to the lower anchor point, numerical values were obtained for computation. Apart from this, panelists were requested to evaluate cheese for bitter flavor on a scale of 0 to 2 (0 = not observed, 1 = slightly bitter, 2 = strongly bitter).

Statistical Treatment of Data

Data from NE and NO cheeses were treated separately. Factorial analysis of variance, with proteinase level, temperature, days of ripening, and experiment as main effects, was performed on data obtained at various stages of ripening by means of BMD P8V computer program (BMDP Statistical Software, Los Angeles, CA). Levels of $P > .05$ were considered to be nonsignificant. Multiple linear regression analysis of cheese characteristics on proteinase level, temperature, and days of ripening was performed by means of program BMD P1R (BMDP Statistical Software).

RESULTS AND DISCUSSION

Whey Composition and Cheese Yield

Coagulation time of pasteurized ewes' milk ranged from 32 to 35 min with no significant reduction from NE or NO proteinase levels in the range .001 to .004 AU/L. Reduction of coagulation time by proteinase directly added to cows' milk had been reported by Alkhalaf et al. (1).

Loss of N in whey and reduced cheese yield from direct addition of proteinases in the manufacture of cows' milk varieties have been reported (1, 2, 14, 20). Proteinase addition to ewes' milk had no significant effect on whey N content, which ranged from .233 to .258% in NE cheese-making trials and from .233 to .254% in NO cheese-making trials. Whey DM ranged from 8.38 to 9.87% in NE cheese-making trials and from 7.84 to 9.41% in NO cheese-making trials with no significant differences from proteinase addition. The DM yield of 24-h cheese was not influenced by proteinase addition. Values ranged from 9.60 to 10.72% for NE cheese-making trials and from 10.00 to 10.55% for NO cheese-making trials.

Casein Breakdown

Degradation of α_{s1}-casein throughout maturation on Manchego cheese manufactured with different NE levels was significantly influenced by proteinase level ($P < .05$) and days ($P < .001$) of ripening but not by ripening temperature (Table 1). After 90 d, values for cheese ripened at 12°C ranged from 22.9% for NE4 to 17.2% for NEC and for cheese ripened at 16°C ranged from 17.8% for NE4 to 22.5% for NEC. Breakdown of α_{s1}-casein in proteinase-treated Manchego cheese was not more extensive than in control cheese, in agreement with published data (16, 18) for proteinase-treated Cheddar and Edam cheese. Other workers have reported, however, an enhanced proteolysis of α_{s1}-casein in proteinase-treated cheese (12, 21).

Proteinase level ($P < .05$) and days of ripening ($P < .001$) had significant effects on β-casein breakdown during ripening of NE-treated Manchego cheese (Table 1). Levels of residual β-casein in 1-d-old cheese were considerably lower than the respective values of α_{s1}-casein. Preferential degradation of β-casein by neutral proteinases has been previously reported (6, 16, 18). Values for 90-d cheese ripened at 12°C ranged from 19.7% for NEC to 23.7% for NE1, whereas, in cheese ripened at 16°C, values ranged from 16.5% for NE4 to 25.2% for NEC.

Days of ripening, but not proteinase level or ripening temperature, had a significant ($P < .001$) effect on degradation of α_{s1}-casein in Manchego cheese manufactured with different NO levels (Table 2).

However, β-casein degradation in NO-treated Manchego cheese was significantly ($P < .001$) enhanced by proteinase level, days of ripening, and temperature of ripening (Table 2). Levels in 90-d cheese ripened at 12°C ranged from 24.3% for NO4 to 30.9% for NO1 and for cheese ripened at 16°C ranged from 22.9% for NO4 to 33.9% for NO2.

Soluble N Formation

Formation of pH 4.6-soluble N in NE-treated Manchego cheese was influenced by

TABLE 1. Residual α_{s1}- and β-casein[1] in Manchego cheese manufactured with different levels of Neutrase L proteinase.[2]

Casein fraction	Temperature of ripening	Days of ripening	Anson units/L			
			.004	.002	.001	0
	(°C)					
α_{s1}-Casein	12	1	40.5	34.2	34.8	35.7
		30	27.4	23.0	26.9	21.6
		60	22.3	23.9	17.7	19.7
		90	22.9	21.4	20.0	17.2
	16	30	24.0	24.1	22.1	25.5
		60	19.2	20.5	18.0	21.4
		90	17.9	20.1	18.8	22.5
β-Casein	12	1	23.4	22.2	27.6	24.2
		30	22.5	22.4	28.7	23.8
		60	19.1	25.5	22.8	22.1
		90	21.2	20.7	25.7	19.7
	16	30	20.9	29.6	25.4	31.1
		60	17.7	19.7	22.7	24.9
		90	16.6	19.5	22.9	25.2

[1]Expressed as percentage of α_{s1}- or β-casein levels in milk.

[2]Mean values of two experiments.

proteinase level ($P < .001$), days of ripening ($P < .001$), and temperature ($P < .01$) of ripening (Table 3). After 90 d, pH 4.6-soluble N ranged from 17.39% for NE1 to 24.95% for NE4 in 12°C cheese and from 20.16% for NEC to 27.32% for NE4 in 16°C cheese. Increases up to 50% in pH 4.6-soluble N from proteinase addition have been reported for cows' milk cheeses, although high values were generally associated with bitter flavor (1, 2, 8, 12, 18).

Proteinase level ($P < .01$), days of ripening ($P < .001$), and temperature ($P < .01$) of

TABLE 2. Residual α_{s1}- and β-casein[1] in Manchego cheese manufactured with different levels of Novozym 257 proteinase.[2]

Casein fraction	Temperature of ripening	Days of ripening	Anson units/L			
			.004	.002	.001	0
	(°C)					
α_{s1}-Casein	12	1	51.9	49.8	47.9	48.7
		30	38.3	33.4	34.9	31.5
		60	30.4	29.6	32.1	31.1
		90	25.8	29.2	28.3	28.9
	16	30	30.6	34.7	33.2	34.0
		60	28.3	30.6	28.1	27.4
		90	25.7	31.9	30.2	23.9
β-Casein	12	1	30.3	33.2	34.0	38.6
		30	29.5	30.4	33.5	31.2
		60	27.8	24.5	33.5	32.8
		90	24.3	29.3	30.9	30.7
	16	30	27.9	34.3	37.5	37.2
		60	26.3	22.2	32.4	32.1
		90	22.9	33.9	33.6	20.2

[1]Expressed as percentage of α_{s1}- or β-casein levels in milk.

[2]Mean values of two experiments.

TABLE 3. Soluble N fractions[1] in Manchego cheese manufactured with different levels of Neutrase L proteinase.[2]

N Fraction	Temperature of ripening	Days of ripening	Anson units/L			
			.004	.002	.001	0
	(°C)					
pH 4.6-Soluble N	12	1	8.57	8.16	6.13	6.96
		30	17.20	20.08	14.99	14.27
		60	22.88	18.27	15.78	14.60
		90	24.95	23.93	17.39	18.08
	16	30	19.85	18.73	15.19	13.89
		60	23.93	22.50	16.43	17.22
		90	27.32	25.43	20.98	20.16
TCA-Soluble N	12	1	2.78	2.79	2.32	2.75
		30	8.34	10.16	7.49	8.40
		60	12.48	11.68	10.10	10.43
		90	14.90	14.09	11.08	12.18
	16	30	10.07	10.52	8.81	9.57
		60	14.91	14.64	11.35	11.90
		90	16.44	16.41	12.99	13.55
PTA-Soluble N[3]	12	1	1.85	1.83	1.57	1.62
		30	2.77	3.52	2.50	2.85
		60	4.77	5.22	4.82	4.74
		90	7.41	6.63	4.65	5.06
	16	30	3.92	4.38	3.27	3.37
		60	7.98	8.09	4.79	5.49
		90	8.11	8.16	5.79	5.87

[1]Expressed as percentage of total N.

[2]Mean values of two experiments.

[3]PTA = Phosphotungstic acid.

ripening had significant effects on TCA-soluble N formation in NE-treated cheese (Table 3). After 90 d, values ranged from 11.08% for NE1 to 14.90% for NE4 in 12°C cheese and from 12.99% for NE1 to 16.44% for NE4 in 16°C cheese. A lower amount of TCA-soluble N than of pH 4.6-soluble N was produced in Manchego cheese by NE proteinase, in agreement with previous observations for cows' milk (6). Published data on the increase of cheese TCA-soluble N by proteinases added to milk show a great range, from 10 to 900% (1, 2, 3, 8, 12, 16, 18). Some workers (16) consider a 20% increase in TCA-soluble N as the optimum in order to avoid bitter flavor defects.

The PTA-soluble N formation in NE-treated cheese was influenced by proteinase level ($P < .01$), days of ripening ($P < .001$), and temperature ($P < .01$) of ripening (Table 3). The PTA-soluble N ranged after 90 d from 4.65% for NE1 to 7.41% for NE4 in 12°C cheese and from 5.79% for NE1 to 8.16% for NE2 in 16°C cheese. These increases are similar to those reported for proteinase-treated Swedish hard cheese (3) and lower than increases found for Cheddar cheese made from curd treated with a neutral fungal proteinase (8).

When NO proteinase was added to cheese milk, formation of pH 4.6-soluble N was significantly enhanced by proteinase level ($P < .01$), days of ripening ($P < .001$), and temperature ($P < .001$) of ripening (Table 4). After 90 d, pH 4.6-soluble N ranged from 15.97% for NOC to 20.52% for NO4 in 12°C cheese and from 18.93% for NO1 to 22.06% for NO4 in 16°C cheese.

Proteinase level ($P < .05$), days of ripening ($P < .001$), and temperature ($P < .001$) of ripening showed significant effects on TCA-soluble N formation in NO-treated cheese (Table 4). The TCA-soluble N ranged, after 90 d, from 10.38% for NO1 to 12.63% for NO4 in 12°C cheese and from 11.76% for NO1 to 14.73% for NOC in 16°C cheese.

Formation of PTA-soluble N in NO-treated cheese was influenced by proteinase level ($P < .05$), days of ripening ($P < .001$), and temperature ($P < .01$) of ripening (Table 4). After 90 d, values ranged from 4.50% for NOC to 6.11%

TABLE 4. Soluble N fractions[1] in Manchego cheese manufactured with different levels of Novozym 257 proteinase.[2]

N Fraction	Temperature of ripening	Days of ripening	Anson units/L			
			.004	.002	.001	0
	(°C)					
pH 4.6-Soluble N	12	1	9.29	8.54	8.54	8.44
		30	15.53	13.93	12.53	12.06
		60	14.20	14.89	15.20	14.58
		90	20.52	17.35	17.33	15.97
	16	30	16.76	16.79	14.47	12.79
		60	18.72	16.75	17.40	16.08
		90	22.06	21.13	18.93	19.23
TCA Soluble N	12	1	3.24	2.89	3.44	4.09
		30	8.51	7.20	7.70	7.80
		60	8.95	9.36	9.64	9.99
		90	12.63	11.13	10.38	11.25
	16	30	10.01	8.95	8.58	8.93
		60	12.94	11.29	12.16	11.32
		90	14.58	13.76	11.76	14.73
PTA Soluble N[3]	12	1	1.82	1.56	1.60	1.92
		30	3.42	2.58	2.51	2.85
		60	3.85	3.04	4.33	4.62
		90	6.11	5.53	4.81	4.50
	16	30	3.82	3.34	3.07	3.06
		60	6.13	4.68	4.67	4.50
		90	6.58	5.97	4.83	7.25

[1]Expressed as percentage of total N.

[2]Mean values of two experiments.

[3]PTA = Phosphotungstic acid.

for NO4 in 12°C cheese and from 4.83% for NO1 to 7.25% for NOC in 16°C cheese.

FFA Formation

As expected, FFA formation in NE-treated or NO-treated Manchego cheese was not influenced by proteinase level. Significant effects of days ($P < .01$) and temperature ($P < .05$) of ripening on FFA formation in NO-treated cheese were detected. Mean FFA in 90-d cheese ripened at 12 or 16°C were 8.51 and 10.24 meq/100 g of fat, respectively, in agreement with previous results (13) for Manchego cheese made from pasteurized ewes' milk.

Rheological Changes

Proteinase level ($P < .01$) and days of ripening ($P < .001$), but not ripening temperature, showed significant effects on fracturability of NE-treated Manchego cheese (Table 5). Values after 90 d ranged from 37.00 N for NE4 to 44.29 N for NE1 in 12°C cheese and from 33.90 N for NE4 to 48.16 N for NE1 in 16°C cheese. The NE4 cheese was more crumbly and less elastic than NEC cheese. Softening of cheese texture because of enhanced casein cleavage has been reported for Manchego cheese (13) and other varieties (1, 2, 5, 8, 10, 11, 16, 21).

Elasticity of NE-treated Manchego cheese was only influenced by days of ripening ($P < .001$). Hardness was influenced by proteinase level ($P < .05$) and by days of ripening ($P < .001$). Hardness in 90-d cheese ranged from .411 J for NE4 to .465 J for NE1 in 12°C cheese and from .385 J for NE4 to .492 J for NE1 in 16°C cheese (Table 5).

Texture of NO-treated cheese was not affected by proteinase addition or by ripening temperature. However, days of ripening influenced fracturability ($P < .001$), elasticity ($P < .01$), and hardness ($P < .001$).

Ripening temperature had no effect on rheological characteristics of Manchego cheese in the present work, probably because of the nar-

TABLE 5. Rheological characteristics[1] of Manchego cheese manufactured with different levels of Neutrase L proteinase.[2]

Rheological characteristic	Temperature of ripening	Days of ripening	Anson units/L			
			.004	.002	.001	0
	(°C)					
Fracturability, N	12	30	19.14	20.54	19.85	22.07
		60	33.66	30.75	35.15	33.78
		90	37.00	42.18	44.29	41.45
	16	30	19.50	19.78	22.67	22.11
		60	31.28	31.00	34.93	35.69
		90	23.90	34.01	48.16	43.02
Hardness, F	12	30	.208	.223	.232	.226
		60	.275	.343	.384	.371
		90	.411	.439	.465	.430
	16	30	.218	.226	.271	.236
		60	.355	.336	.406	.397
		90	.385	.417	.492	.451

[1]Six samples per cheese were compressed with an Instron tester (Instron Corp., Canton, MA).

[2]Mean values of two experiments.

row temperature range (4°C) of our experiments. Other workers (10, 13), reporting a significant effect of temperature on cheese texture, worked with temperature ranges of up to 12°C.

Days of ripening was the factor with the most marked influence on cheese texture in the present work. Differences in DM throughout ripening (53.52, 61.62, 67.47, and 68.82% as mean values for 1-, 30-, 60-, and 90-d cheese) may explain the firmer texture of older cheese (Table 5), in spite of increased proteolysis, and in agreement with data previously obtained for cows' and ewes' milk cheese varieties (4, 5, 10, 11, 13).

Sensory Characteristics

Flavor preference of NE-treated cheese was influenced by proteinase level ($P < .01$), days of ripening ($P < .001$), and temperature ($P < .01$) of ripening (Table 6). Proteinase level did

TABLE 6. Sensory characteristics[1] of Manchego cheese manufactured with different levels of Neutrase L proteinase.[2]

Sensory characteristic	Temperature of ripening	Days of ripening	Anson units/L			
			.004	.002	.001	0
	(°C)					
Flavor preference	12	30	6.07	6.04	5.63	5.85
		60	5.75	5.32	5.47	5.58
		90	5.85	6.22	5.39	6.11
	16	30	5.40	5.59	5.01	5.97
		60	5.96	6.38	5.84	5.86
		90	6.42	6.89	6.11	7.03
Flavor intensity	12	30	4.61	4.53	4.54	4.04
		60	5.13	5.70	5.32	4.95
		90	5.93	5.78	5.75	5.49
	16	30	5.75	5.30	5.57	5.48
		60	6.04	5.57	5.99	5.36
		90	7.01	6.53	6.80	6.76

[1]Average scores on a scale from 0 (lower) to 10 (upper) points.

[2]Mean values of two experiments.

TABLE 7. Sensory characteristics[1] of Manchego cheese manufactured with different levels of Novozym 237 proteinase.[2]

Sensory characteristic	Temperature of ripening	Days of ripening	Anson units/L			
			.004	.002	.001	0
	(°C)					
Flavor preference	12	30	5.42	5.59	5.40	5.56
		60	5.48	5.07	5.29	5.52
		90	5.92	6.04	5.69	5.87
	16	30	4.90	5.33	5.56	5.56
		60	6.12	5.64	5.56	6.05
		90	6.19	6.38	6.80	6.93
Flavor intensity	12	30	5.82	5.68	5.71	5.70
		60	6.27	5.99	5.62	5.81
		90	6.57	6.44	5.78	6.58
	16	30	5.97	5.85	6.00	5.95
		60	6.52	6.38	5.92	6.03
		90	6.80	6.39	7.14	7.18

[1]Average scores on a scale from 0 (lower) to 10 (upper) points.

[2]Mean values of two experiments.

not show a consistent trend; mean preference scores were 5.91 for NE4, 6.07 for NE2, 5.57 for NE1, and 6.07 for NEC cheese. Flavor preference increased with days of ripening; mean scores were 5.69, 5.77, and 6.25 for 30-, 60-, and 90-d cheese. Temperature of ripening improved flavor preference of NE-treated cheese; mean scores were 5.77 and 6.04 for 12 and 16°C cheese.

Bitter flavor was rarely detected: in 5, 4, 3, and 2 cases of slightly bitter flavor for NE4, NE2, NE1, and NEC cheese, respectively, out of a total number of 120 score cards per proteinase level. Incidence of slightly bitter flavor in NE-treated cheese and in control cheese was not significantly different, according to a chi-square test.

Proteinase level ($P < .01$), days of ripening ($P < .001$), and temperature ($P < .001$) of ripening showed significant effects on flavor intensity of NE-treated cheese (Table 6). Mean intensity scores according to proteinase level were 5.75 for NE4, 5.45 for NE2, 5.65 for NE1, and 5.35 for NEC cheese. Intensity increased consistently with days of ripening; mean scores were 4.94, 5.45, and 6.25 for 30-, 60-, and 90-d cheese. It also increased at a higher ripening temperature with 5.11 and 5.99 as mean scores for 12 and 16°C cheese.

When NO proteinase was added to milk, flavor preference was only influenced by days ($P < .01$) and temperature ($P < .05$) of ripening (Table 7). Preference increased throughout maturation; mean scores were 5.36, 5.66, and 6.23 for 30-, 60-, and 90-d cheese. Preference score was enhanced by a higher temperature; mean scores were 5.58 and 5.92 for 12 and 16°C cheese.

Flavor was slightly bitter in 8, 7, 7, and 6 cases for NO4, NO2, NO1, and NOC cheese out of 120 score cards per proteinase level. No significant differences in bitter flavor incidence between NO-treated and control cheese were found.

Level of proteinase ($P < .05$), days of ripening ($P < .001$), and temperature ($P < .001$) of ripening showed significant effects on flavor intensity of NO-treated cheese (Table 7). Mean intensity scores as a function of proteinase level were 6.29 for NO4, 6.11 for NO2, 6.04 for NO1, and 6.21 for NOC cheese. Consistent trends were observed for days of ripening (mean scores were 5.84, 6.04, and 6.61 for the 30-, 60-, and 90-d cheese) and for ripening temperature (mean scores were 6.00 and 6.33 for 12 and 16°C cheese).

Proteinase addition to milk enhanced Manchego cheese flavor intensity, especially in the case of NE-treated cheese, without impairing flavor preference, as previously shown for Cheddar and Swedish hard cheese (3, 9, 16). Incidence of bitter flavor in Manchego cheese was low in comparison with cows' milk cheese varieties manufactured with added proteinases (2, 8, 12, 18).

TABLE 8. Multiple linear regression equations of Manchego cheese characteristics on level of Neutrase L proteinase[1] (U), temperature (T), and days of ripening.

		R^2	n
α_{s1}-Casein	= 15.330 + 1353.6 U + 1.097 T − .117 d	.35***	128
β-Casein	= 21.654 − 700.4 U + .312 T − .036 d	.60**	128
pH 4.6-Soluble N	= 4.000 + 1368.3 U + .394 T + .158 d	.76***	128
TCA-Soluble N	= 3.300 + 341.2 U + .011 T + .122 d	.65***	128
PTA-Soluble N	= .224 + 288.5 U + .046 T + .060 d	.62***	128
Fracturability	= 21.683 − 6290.9 U − .042 T + .216 d	.58***	48
Hardness	= .139 − 9.5 U + .001 T + .002 d	.77***	48
Flavor preference	= 4.419 + 1.3 U + .066 T + .009 d	.22*	48
Flavor intensity	= 1.009 + 812 U + .221 T + .072 d	.80***	48

[1]Expressed in Anson units per liter of milk.

*$P < .05$.

**$P < .01$.

***$P < .001$.

Acceleration of Manchego Cheese Ripening

Multiple linear regression equations in Tables 8 and 9 express the feasibility of accelerating Manchego cheese ripening by the addition of neutral proteinases to milk. Data from curd and 1-, 30-, 60-, and 90-d cheese were taken into account for regression equations of residual casein fractions and soluble N fractions. Equations for rheological and sensory characteristics were calculated using data from 30-, 60-, and 90-d cheese.

Breakdown of α_{s1}-casein was not enhanced by NE or NO proteinase addition to milk (Tables 8 and 9). On the contrary, β-casein degradation could be accelerated by NE or NO proteinase. Addition of .004 AU/L would reduce β-casein by 3.21% in NE-treated cheese and by 5.93% in NO-treated cheese, which corresponds to 89 or 297 d of ripening, respectively.

Soluble N fractions fitted well multiple linear regression. Addition of .004 AU/L of NE proteinase should achieve an increase in pH 4.6-soluble N of 5.47%, which corresponds to 35 d of ripening. If .004 AU/L of NO proteinase were added, the increase in pH 4.6-soluble N would only be 2.61%, which

TABLE 9. Multiple linear regression equations of Manchego cheese characteristics on level of Novozym 257 proteinase[1] (U), temperature (T), and days of ripening.

		R^2	n
α_{s1}-Casein	= 13.943 + 872.5 U + 1.723 T − .144 d	.77***	128
β-Casein	= 23.270 − 1482.7 U + .778 T − .020 d	.48***	128
pH 4.6-Soluble N	= 6.133 + 652.4 U − .133 T + .516 d	.72***	128
TCA-Soluble N	= 4.030 + 659.2 U + .002 T + .099 d	.83***	128
PTA-Soluble N[2]	= .918 + 105.2 U + .030 T + .048 d	.82***	128
Fracturability	= 21.135 − 91.1 U + .131 T + .167 d	.19*	48
Hardness	= .223 + .1 U + .002 T + .002 d	.27**	48
Flavor preference	= 3.785 − 36.2 U + .083 T + .014 d	.33***	48
Flavor intensity	= 4.146 + 32.8 U + .082 T + .013 d	.44***	48

[1]Expressed in Anson units per liter of milk.

[2]PTA = Phosphotungstic acid.

*$P < .05$.

**$P < .01$.

***$P < .001$.

corresponds to 22 d of ripening. The TCA-soluble N formation has a weaker response to proteinase addition: .004 AU/L should increase its level by 1.36 or .64% for NE or NO proteinase, which corresponds to 12 or 6 d of ripening, respectively. In the case of PTA-soluble N, .004 AU/L should increase its level by 1.15% or .42% for NE or NO proteinase, respectively, which corresponds to 19 or 9 d of ripening. Fedrick et al. (9) achieved a reduction of 1.0 mo in ripening time of NO-treated Cheddar cheese held at 15°C using TCA-soluble N as a ripening index, compared with control cheese held at the same temperature.

Fracturability would be reduced by .004 AU/L of NE proteinase in 5.16 N and hardness in .039 J. Because fracturability and hardness usually increase during Manchego cheese ripening, this softening of cheese body would be equivalent to retard maturation by 24 or 11 d, respectively. Fedrick et al. (9) reported an acceleration of .5 mo in rheological characteristics for Cheddar cheese vacuum packed in barrier bags with no moisture losses.

Flavor preference would not be influenced by .004 AU/L of NE proteinase. However, it would be slightly impaired by .004 AU/L of NO proteinase, with a decrease of .14 points, equivalent to 10 d of ripening. Flavor intensity would be enhanced by .004 AU/L of NE proteinase or NO proteinase, with increases of .37 or .13 points, respectively, which correspond to 15 or 10 d of ripening.

Flavor intensity in NO-treated Cheddar cheese was accelerated only .2 mo compared with control cheese ripened at 15°C but accelerated 4.1 mo compared with control cheese ripened at 8°C for 6 mo (9). Control Manchego cheese ripened at 12°C for 90 d would show a flavor intensity of 5.64 points according to the regression equation in Table 8. Addition of .004 AU/L of NE proteinase would achieve a 15-d reduction of ripening time, whereas increasing the ripening temperature from 12 to 16°C reduces ripening more, 40 d, according to the same regression equation. Thus, a flavor intensity of 5.64 points would be reached in 35 d if .004 AU/L of NE proteinase were added and cheese was ripened at 16°C. This shortened ripening period would reduce cost considerably with subsequent financial benefit, as shown for Cheddar cheese (7).

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