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Enzyme Activity in Spanish Goat's Cheeses*

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ABSTRACT

Specific enzyme activity has been studied in Spanish goat's cheese. Neutral protease, aminopeptidase, and carboxypeptidase activity was analysed during the ripening of two Spanish goat's cheeses. One cheese was Majorero cheese from the island of Fuerteventura; the other was a soft cheese mould-ripened by P. candidum *on the surface. The trend in neutral protease activity was rather similar in both cheeses over the ripening period. Protease activity was higher in Majorero cheese and higher on the surface than inside the mould-ripened cheese.*

Aminopeptidase activity also increased in both cheeses over the entire ripening period, but it was higher in the mould-ripened cheese than in the Majorero cheese.

Carboxypeptidase activity was not recorded in either the Majorero or the soft cheese.

INTRODUCTION

Hydrolysis of cheese proteins is one of the most important phenomena taking place during ripening, and it gives rise to the texture and the bouquet of the cheese.

This proteolysis is the result of the action of the protease existing in the milk and the rennet (Gripon *et al.*, 1975) and the enzymes produced by

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microorganisms (Desmazeaud & Gripon, 1977) during growth (exocellular enzymes) or freed on bacterial cell lysis (endocellular enzymes).

The enzyme systems in specific microorganisms of importance in cheese technology have been studied on a number of occasions (El Soda *et al.*, 1983; Singh & Sharma, 1983). However, there have been few studies on enzyme activity in cheese extracts (Lenoir & Auberger, 1982; Spettoli & Zamorani, 1985), and ripening has normally been described on the basis of the data obtained from proteolytic microbe activity on substrates other than cheese and hence subject to different conditions. Extrapolation of such data may not always be an adequate approach for the evaluation of proteolysis during cheese ripening, and it may therefore be appropriate to study actual enzyme activity in cheese.

The present study deals with protease, aminopeptidase, and carboxypeptidase activity in the soluble extracts of two Spanish cheeses made from pasteurized goat's milk.

MATERIALS AND METHODS

Industrially manufactured goat's cheeses were employed. One cheese was a semi-hard cheese, Majorero, from Fuerteventura in the Canary Islands, considered one of the most typical Spanish goat's cheeses. The other was a soft cheese with surface flora (mould-ripened) comparable with French cheeses, made in the Spanish province of Cáceres. Both cheeses were made from pasteurized milk to which $CaCl_2$ (0·1–0·2 g/l), mesophilic starter, and commercially available rennet were added.

Coagulation temperature in the manufacture of the Majorero cheese was initially 30°C, subsequently rising to 37°C at a rate of approximately 1°C every 5 min. The curd was separated from the whey, pressed in a mould for 2 h, and then salted for 40 h. The cheeses were ripened for 90 days at a temperature of 10–12°C and a relative humidity (RH) of 85–87%. Cheese weight was 2–4 kg each.

Coagulation time in the production of the soft cheese that underwent surface ripening by moulds was 18–20 h at 22°C. The curd (150 g) was separated from the whey, placed in moulds 15-cm high by 5-cm in diameter, and salted for 1 or 2 h, after which it was sprayed with a solution of *P. candidum* spores. The cheeses were ripened for 15 days at 10–12°C and an RH of 75–87%. They were then stored for 15 days in a cold store at 0–4°C, which is considered adequate for marketing.

Samples for microbiological, physico-chemical, and enzyme analysis were taken from the salted cheeses (M-1 and G-1), at different times during ripening, and also, in the case of the mould-ripened cheese, during storage.

The samples of Majorero cheese ripened for 5, 30, 60, and 90 days have been designated M-2, M-3, M-4 and M-5, respectively, in the Tables. The samples of mould-ripened cheese after 7 and 15 days of ripening were designated G-2 and G-3, respectively, and the samples taken after 7 and 15 days of storage were designated G-4 and G-5.

Microbiological analysis

Ten grams from the interior of the curd or cheese were homogenized in 90 ml of 2% sodium citrate that had been heated to 45°C. In the soft, mould-ripened cheese, the surface was analysed as well; surface samples measured 5 × 5 cm, to a depth of 5 mm. Starting with this initial solution, a series of decimal dilutions were prepared in Ringer's solution (1/4), and these were inoculated on media suitable for the counts: Plate Count Agar (PCA) (DIFCO) for total germs, incubated at 30°C for 72 h, and SMCA (Standard Methods Caseinate Agar) (Martley *et al.*, 1970), also incubated at 30°C for 72 h.

Physico-chemical analysis

The pH, soluble nitrogen (SN), and non-protein nitrogen (NPN) in the inside of both cheeses were analyzed as described by Juárez *et al.* (1983). The pH on the surface of the mould-ripened cheese was also measured.

Enzyme analysis

Enzyme extraction

The enzyme extract from the cheeses was obtained by homogenizing 20 g of sample from the interior of the cheese or from the surface of the mould-ripened cheese in an Ultra-Turrax macerator for 3 min with 50 ml of 0·01M citrate buffer at 4°C and a pH of 6·0. The homogenate was centrifuged at 12 000 *g* for 10 min, and the sediment was washed twice with buffer. The supernatant was filtered and topped up to 100 ml with buffer. The enzyme activity in these soluble cheese extracts was then analyzed.

Determination of neutral protease activity

Neutral protease activity was determined according to the method of Paquet & Gripon (1980) on isoelectric casein at a final concentration of 2% in 0·1M phosphate buffer at a pH of 6·0. The reaction mixture was incubated for 20 min, and the reaction was then stopped with TCA (final concentration 2·5%). The peptide and aromatic amino acid contents in the filtered supernatant were read by spectrophotometry, measuring absorbance at 280 nm.

Determination of aminopeptidase activity
The method of Desmazeaud & Juge (1976) was applied using a substrate of L-leucine-*p*-nitroanilide in 0·066M phosphate buffer at a pH of 7·0 (final concentration 0·03%). After incubation for 60 min the reaction was stopped by adding TCA (final concentration 12%), and absorbance in the filtrate at 410 nm was measured.

Determination of carboxypeptidase activity
A substrate of benzoyl-L-tyrosine-*p*-nitroanilide (1·5 mM) in a 0·1M phosphate buffer at a pH of 7·0 was used according to the method of Hayashi (1980). Incubation time for the reaction mixture was 10 min at 25°C, after which absorbance at 410 nm in the supernatant obtained after precipitation with $HgCl_2$ (0·01M) was measured.

RESULTS AND DISCUSSION

Neutral protease activity

Neutral protease activity in the soluble extracts from the Majorero cheese and mould-ripened cheese during ripening and during storage of the latter is presented in Table 1. The higher level of protease activity taking place in the Majorero cheese is reflected in the higher soluble nitrogen/total nitrogen (SN/TN) proteolysis index for this cheese as compared with that for the

TABLE 1
Neutral Protease and Aminopeptidase Activity in the Soluble Extracts of Majorero and Mould-Ripened Cheese during Ripening

Sample	*Proteinase activity (absorb. 280/g/h)*		*Aminopeptidase activity (absorb. 410/g/h)*	
M-1	0·73		0·07	
M-2	0·88		0·15	
M-3	0·97		0·16	
M-4	1·50		0·16	
M-5	1·49		0·17	
	Surface	*Interior*	*Surface*	*Interior*
G-1	0·62	0·55	1·12	—
G-2	0·70	0·60	1·20	0·07
G-3	0·79	0·64	1·57	0·32
G-4	0·96	0·84	1·60	0·40
G-5	0·99	0·88	1·61	0·41

TABLE 2
Soluble Nitrogen/Total Nitrogen (SN/TN) and Non-Protein Nitrogen/Soluble Nitrogen (NPN/SN) during the Ripening of Majorero and Mould-Ripened Cheese

Sample	*SN/TN* (%)	*NPN/SN* (%)
M-1	17·9	21·4
M-2	27·2	43·2
M-3	30·0	42·6
M-4	33·7	52·2
M-5	30·0	58·5
G-1	5·2	46·6
G-2	5·6	59·6
G-3	10·6	75·0
G-4	13·4	76·0
G-5	19·1	77·5

mould-ripened cheese, shown in Table 2. The trends for protease activity in the extracts from the rind and interior of the mould-ripened cheese were similar, but the values were higher for the surface extract than for the extract from inside the cheese (0·62 in the fresh cheese and 0·99 after 28 days on the surface; 0·55 in the fresh cheese and 0·88 after 28 days in the interior of the cheese). The results agree, in a qualitative sense, with those reported by Lenoir & Auberger (1982) for Camembert cheese extracts.

Most of the microbial proteolytic enzymes present in cheese come from the lactic bacteria added in the starter and from the action of moulds in blue cheeses or cheeses with surface flora (Miranda & Gripon, 1986). The results of the analysis of the proteolytic flora in the cheeses are shown in Table 3. In the Majorero cheese the proteolytic flora increased through 30 days of ripening (5×10^5 cfu/g); this flora then declined, disappearing after 90 days of ripening. There was also an increase in the inside of the mould-ripened cheese through the first 14 days of ripening (3×10^4 cfu/g), followed by a decrease, with this flora disappearing after 28 days. The lower number of microorganisms inside this cheese as compared to the Majorero cheese can be attributed to its lower pH (Table 4). The proteolytic bacteria increased during ripening and storage on the surface of the mould-ripened cheese, reaching 5×10^6 cfu/g after 28 days. The increase was most pronounced through 14 days as a result of the rise in the pH brought about by mould growth.

Comparing the protease activity of the cheese extracts and the trends for proteolytic bacteria during ripening, it would appear that the increased protease activity observed was due, not only to the enzymes present in the

TABLE 3
Total Germs and Proteolytic Flora during the Ripening of Majorero and Mould-Ripened Cheese

Sample	*Total germs (cfu/g)*		*Proteolytic flora (cfu/g)*	
M-1	$2{\cdot}7 \times 10^8$		$2{\cdot}0 \times 10^4$	
M-2	$5{\cdot}1 \times 10^7$		$4{\cdot}0 \times 10^4$	
M-3	$3{\cdot}6 \times 10^8$		$5{\cdot}0 \times 10^5$	
M-4	$6{\cdot}1 \times 10^7$		$9{\cdot}0 \times 10^1$	
M-5	$2{\cdot}1 \times 10^8$		—	
	Surface	*Interior*	*Surface*	*Interior*
G-1	$8{\cdot}2 \times 10^8$	$1{\cdot}7 \times 10^8$	$4{\cdot}0 \times 10^3$	$8{\cdot}0 \times 10^2$
G-2	$1{\cdot}8 \times 10^8$	$1{\cdot}6 \times 10^8$	$3{\cdot}0 \times 10^4$	$7{\cdot}0 \times 10^3$
G-3	$4{\cdot}3 \times 10^8$	$2{\cdot}4 \times 10^8$	$2{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^4$
G-4	$1{\cdot}0 \times 10^8$	$5{\cdot}4 \times 10^8$	$7{\cdot}0 \times 10^6$	$2{\cdot}0 \times 10^1$
G-5	$1{\cdot}2 \times 10^8$	$3{\cdot}6 \times 10^8$	$5{\cdot}0 \times 10^6$	—

residual rennet, but also to the exocellular microbial enzymes produced during microbial growth and to the endocellular enzymes freed on microbial cell lysis.

An increase in aminopeptidase activity was detected on the L-leucine-*p*-nitroanilide substrates in the extracts from both cheeses throughout ripening and, in the mould-ripened cheese, storage (Table 1). Higher levels of activity were observed at the surface of the mould-ripened cheese than in the interior or in the Majorero cheese. The activity in the soluble extracts from

TABLE 4
pH during the Ripening of Majorero and Mould-Ripened Cheese

Sample	*pH*	
M-1	6·04	
M-2	5·65	
M-3	5·63	
M-4	5·58	
M-5	5·74	
	Surface	*Interior*
G-1	4·36	4·36
G-2	4·36	4·31
G-3	7·17	5·35
G-4	7·12	4·70
G-5	7·45	5·44

the Majorero cheese remained at similar low levels throughout ripening and at the end of the period was 9·4 and 2·4 times lower than the activity recorded in the extracts from the surface and the interior of the mould-ripened cheese, respectively. The increased aminopeptidase activity at the surface of the mould-ripened cheese was mainly due to the proteolytic activity of the moulds and this explains the higher ratios of non-protein nitrogen/soluble nitrogen (NPN/SN) recorded compared to those for the Majorero cheese (Table 2).

No carboxypeptidase activity was detected on the benzoyl-L-tyrosine-*p*-nitroanilide substrate in either of the cheeses studied at any time during the ripening period.

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