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Influence of starter culture on total free aminoacids concentration during ripening of Krk cheese

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Summary

The aim of this study was to determine the influence of microbial (commercial starter) culture on concentration of total free amino groups (amino acids) in cheeses in different ripening stages. Free amino groups were determined by reaction with ninhydrin with cadmium (Cd) in the water soluble cheese extract, and were expressed as the concentration of leucine in cheese dry matter. Changes in concentration of total free amino acids during cheese ripening (0^{th}, 30^{th}, 60^{th}, 90^{th} and 120^{th} day) were monitored. In water soluble extracts of cheese, the presence of free NH_2 groups in all ripening stages was detected, which means smaller peptides and amino acids, whose concentration significantly ($P<0.01$) increased during ripening. Cheeses produced with and without microbial culture resulted in statistically significant differences ($P<0.01$) in content of total free amino acids free on the 90^{th} and 120^{th} day of ripening. Cd-ninhydrin method was found to be suitable for cheese ripening monitoring, as well as for determination of the differences in mature characteristics of cheeses, depending on the production process.

Key words: ewe cheese from the island of Krk, cheese ripening, total free amino acids, microbial culture

Introduction

The main feature of traditional cheese is the use of raw, i.e. thermally unprocessed milk for cheese making. Milk as a raw material influences the specificity (autochthonous quality) of cheeses primarily due to bacteria of lactic acid fermentation naturally present in raw milk, which contribute to the characteristic and recognisable taste, scent and consistency of a certain cheese. Krk cheese is a traditional Croatian island cheese, manufactured from the raw sheep milk. It belongs to the group of hard, full fat cheeses (Figures 1 and 2). The optimum duration of ripening of Krk cheese is 90 days (Antunac et al., 2008). Characteristics of a ripe Krk cheese are: diameter 11.5-15 cm; height 4.5-6 cm; cheese colour varies from light yellow to gold, while a small number of irregularly distributed cheese eyes can be seen on a cut (Figure 3).

Microbial cultures are used in cheese manufacturing with interior bacterial ripening mostly when milk is thermally processed. The primary role of microbial culture is the acidification of curd in a cheese kettle and obtaining a final pH value, while the secondary role can be seen during ripening, particularly during proteolysis as an important factor in developing cheese flavour. Ardö (1997; 2006) mentions that by adding bacteria of the species *Lactobacillus helveticus* into non fatty cheeses, mistakes in flavour

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Figure 1. Krk cheese

Figure 2. Ripening of Krk cheese

and scent can be avoided, since they influence forming of a higher quantity of free amino acids.

Raw milk microflora, the activation of plasminogen into plasmin, the technological manufacturing process, microbial culture type, conditions in a ripening chamber and ripening duration have an important role in the course of proteolysis. On a higher level of proteolysis affects higher temperature, greater amount of retained rennet, activity of the water in cheese, lower salt content and favorable pH value (Kalit et al., 2005).

Enzymes participating in proteolytic processes come from different sources as follows: remaining enzymes of cheese making preparations (chymosine, pepsin or microbial proteinases), milk enzymes (plasmin, lipoprotein lipases, acid phosphatasis, xanthine oxidase), secondary culture enzymes (propionic acid bacteria, *Bacterium linens* strains, moulds), natural milk bacteria and non-starter bacteria microflora. Non-starter bacteria have a significant importance in cheeses manufactured from raw milk, since they replace the starter's activity by their proteolytic activity, thus producing amino acids and peptides of molecular mass similar to those which occur by the starter's activity (Mikulec et al., 2010). In the advanced ripening stage, secondary transformations of decomposition products occur: deamination, decarboxylation and desulphurisation of amino acids, beta oxidation of fatty acids and esterification (Tratnik, 1998).

Figure 3. Cut of mature Krk cheese

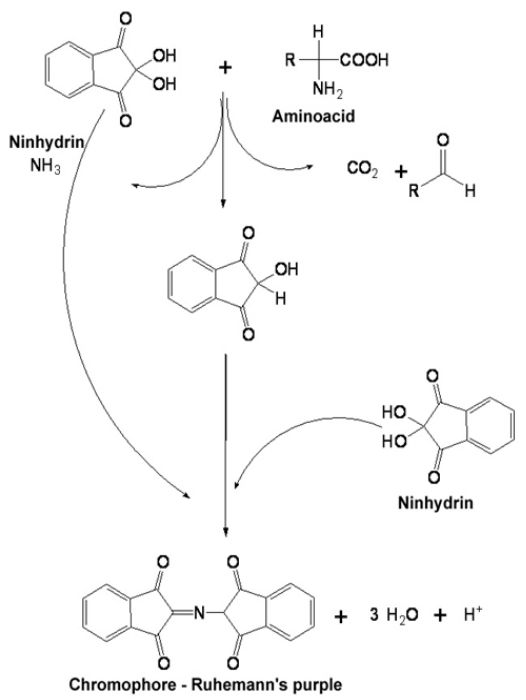


Figure 4. The reaction of ninhydrin with amino acids (taken from: Mikulec, 2010)

Proteolytic changes have a crucial role in forming cheese flavour, since the quantity and ratio of certain amino acids and soluble peptides influence the texture and organoleptic characteristics of cheese (Aston and Dulley, 1982; Kristoffersen, 1985; Law, 1987). It is known that the cheese flavour is concentrated in a fraction soluble in water (peptides, amino acids, organic acids and amines), while the flavour is mostly concentrated in a volatile fraction: organic acids, aldehydes, amines and esters (Mikulec et al., 2010).

Deviations from the standard manufacturing procedures influence measurable ingredients in cheese like: content of water, salt, total and soluble nitrate compounds and pH value (Mikulec, 2010). With the standard method according to Kjeldahl for determining total nitrogen and soluble nitrogen, methods for determining free amino groups by trinitrobenzene sulfonic acid, o-phtaldialdehyde or ninhydrin in substances soluble in water, sulphosalicylic, phospho-wolframic, trichloracetic acid and ethanol are used as a measure of proteolysis and change in the content of peptides and amino acids in cheese (McSweeney i Fox, 1999). The reaction of ninhydrin (2,2-Dihydroxyindane-1,3-dione) which is originally yellow, with free amino acids, results in dark blue or purple known as Ruhemann's purple, which is used for proving the presence of primary and secondary amines. The mechanism of reactions is shown in Figure 4.

The purpose of this paper was to determine the concentration of total free amino acids in Krk cheese manufactured with and without the addition of microbial culture in various stages of ripening and to determine the suitability of Cd-ninhydrin method for monitoring the course of cheese ripening.

Materials and methods

For research purposes, five series (batches) of Krk cheese were manufactured with and without adding microbial culture. Out of 100 L of milk for cheese making, 50 L of milk was treated with microbial culture and 50 L without the addition of microbial culture. The research was made during one lactation of Krk sheep at one family farm from the island of Krk according to the previously described procedure (Pavlinić et al., 2010). Starter culture consisted of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis* and *Lactobacillus helveticus* (EZAL KAZU 300, Sassenage, France). The batch represents a daily cheese manufacturing within which 3 cheeses were manufactured with the addition of microbial culture and 3 cheeses without microbial culture. Cheeses ripened in a ripening chamber with the average air temperature of 15 °C - 18 °C and relative air humidity of 70-80 %.

Figure 5. Sampling cheese

Cheese sampling was done according to the procedure of Licitra et al. (2000). The samples were taken on 0th, 30th, 60th, 90th and 120th day of ripening, after which they were stored in a freezer at the temperature of -80 °C until the laboratory analysis. A cheese drill 13 cm long was used for taking cheese samples. After a sample was taken, a part of a 1 cm sample cut along the rind was returned as a plug on the cheese opening and closed with a wax (Figure 5) and the cheese was returned to a ripening chamber for further ripening.

Determination of free amino groups

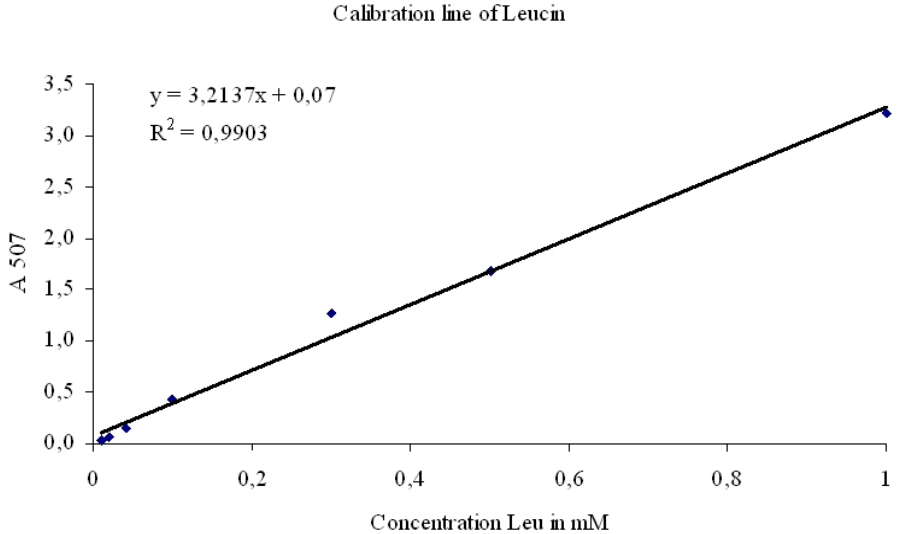
Cheese water extracts were prepared according to the procedure described in the paper by Mayer et al. (1998). Free amino groups of amino acids were determined by the reaction with ninhydrin with cadmium in cheese water extract according to Folkertsma and Fox (1992). The water extract sample (50 μL) was diluted up to 1 mL with distilled water. 2 mL of Cd-ninhydrin reagent was added to a diluted sample. The reagent consisted of 0.8 g of ninhydrin diluted in mixture of 80 mL of 99.5 % ethanol and 10 mL of acetic acid, to which 1 g of $CdCl_2$ was added dissolved in 1 mL of distilled water. The mixture (sample + reagent) was heated up to the temperature of 84 °C for 5 minutes and after cooling to the room temperature, the absorption of light wave 507 nm was measured. Blind trial (1 mL of water + 2 mL of reagents) was measured in the same way. 2 mM initial leucine solution was used for calibrating line, out of which diluted solutions were made (0.01; 0.02; 0.04; 0.08; 0.1; 0.3; 0.5 and 1 mM Leu). Results were expressed as g Leu in 100 g of dry cheese matter. 150 water cheese extracts were analysed (75 cheese extracts manufactured with the additional microbial culture and 75 cheese extracts manufactured without adding microbial culture). Cheeses were analysed in the Reference Laboratory for Milk and Dairy Products of the Department of Dairy Science, Faculty of Agriculture, University of Zagreb.

Statistical data processing

Changes in concentration of total free amino acids during ripening of Krk cheese were statistically analysed by Tukey-Kramer test, in SAS statistical package (1999). The following were calculated in the programme package Microsoft Office Excell (2007): mean values ($\overline{x}$), standard deviations (SD) and standard errors (SE) for cheeses manufactured with and without adding microbial culture on 0th, 30th, 60th, 90th and 120th day of ripening by a descriptive analysis.

Results and discussion

The hydrolysis of peptide/protein connections give amino acids, so their determination can give the insight into the progress of proteolysis during ripening, particularly the release of amino acids and small peptides (Muehlenkamp-Ulate and Warthesen, 1999; Mikulec, 2010). The calibrating line was obtained based on the dependence of leucine concentration and absorption light of wavelength 507 nm,



Graph 1. Dependence of leucine concentration and absorption light of wavelength 507 nm

Table 1. Average concentration of total free amino acids expressed as grams of Leu per 100 g dry matter of cheese in various stages of ripening cheese

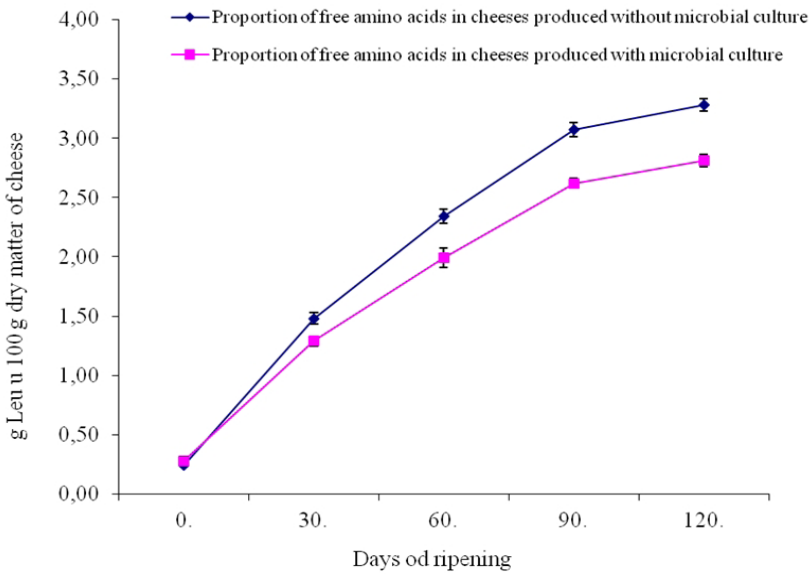
Statistical indicator	Days of rippening	Cheeses without microbial culturee	Cheeses with microbial culture
$\bar{x} \pm SE$	0.	$0.22^{a} \pm 0.01$	$0.28^{a} \pm 0.01$
	30.	$1.48^{b} \pm 0.05$	$1.29^{b} \pm 0.04$
	60.	$2.34^{c} \pm 0.06$	$1.99^{c} \pm 0.08$
	90.	$3.07^{d} \pm 0.06$	$2.62^{e} \pm 0.04$
	120.	$3.28^{d} \pm 0.05$	$2.81^{e} \pm 0.05$

$\bar{x}$ - represents the average concentration of total free amino acids expressed as g Leu 100 g dry matter of cheese
Results express the mean value of the standard error ($\bar{x}$ ± S.E.) of 15 cheeses in various stages of ripening
^{a,b,c,d,e} the mean value in the same row and column of the table with different labels are significantly different ($P<0.05$)

while the linear equation and determination coefficient (R^2) were shown on the Graph 1.

During the ripening of Krk cheese, the concentration of total free amino acids increased, no matter whether cheeses were produced with or without adding microbial culture (Table 1; Graph 2). Nitrogen -containing substances, which include free amino acids, mostly occur by the action of rennet, starter culture and to a smaller extent of peptidase from milk (Kalit et al., 2005). As the result, during the ripening of Krk cheese, the concentration of total free amino acids significantly increased ($P<0.01$) so on the 120th day it was approximately 3.28 g in 100 g of dry cheese matter with the addition of lactic culture, i.e. 2.81 g in 100 g of dry cheese mater without adding lactic culture.

The concentration of total free amino acids on day 0 was insignificantly ($P>0.05$) higher in cheeses manufactured with the addition of microbial culture. It is known that starter bacteria dominate at the beginning of technological manufacturing processes from the initial 10^5-10^7 cfu mL^{-1}, they quickly reach 10^9 cfu mL^{-1} (Cogan, 2002), but the largest number are eliminated in the salting process while the number of Non-Starter Lactic Acid Bacteria (NSLAB) increases during ripening and dominates at the end of the technological process of cheese manufacturing (Hickey et al., 2007). In relation to the total quantity of free amino acids determined on the 120th day of ripening of Krk cheese, the highest increase is visible in the first 30 days of ripening. Thus, the concentration up to day 30 increases by 45.12 % from the total quantity of



Graph 2. Changes in the concentration of total free amino acids in various stages of ripening cheese

amino acids in ripe cheeses manufactured without adding starters, i.e. by 45.91 % in cheeses manufactured with a starter (on day 120). The same was concluded by Freitas et al. (1997) by using Cd-ninhydrin method for monitoring the proteolysis of Picante cheese and Pavia et al. (2000) for Manchego cheese.

In the first 30 days of ripening, there is a sudden increase in casein fraction in Krk cheese (CN) α_{s1}-I-CN and α_{s1}-II-CN by the chymosin activity, after which its hydrolysis into smaller peptides occurs (Mikulec et al., 2008). The same authors determined by electrophoretic technique that the decomposition of β-CN occurs by the plasmin activity which gives smaller γ-CN as main decomposition products and they are visible on a gel only after the 30th day of ripening. It confirms the fact that during proteolysis free amino acids occur, mostly due to the intensive activity of chymosin which is expressed the most in the first three weeks of ripening. Since the primary proteolysis of Krk cheese finishes after the 30th day of ripening, further proteolytic changes are significantly slower.

Secondary proteolysis is caused by the activity of proteinases and peptidases of lactic acid bacteria and non-starter bacteria (Sousa et al., 2001). In Krk cheese samples manufactured without adding microbial culture, proteolytic changes in the first 60 days were more intensive in relation to cheeses manufactured with the addition of microbial culture, although statistically significant changes were not determined. A decreased share of water in cheese might have influenced the decrease in proteolytic chymosin activity after 60 days (Grappin et al., 1985).

Proteolytic changes during the ripening of Krk cheese manufactured with and without microbial culture were monitored by chromatographic (HPLC) technique (Pavlinić et al., 2010). The mentioned research established significant changes in the concentration of total peptides/amino acids during ripening for both cheese groups, which corresponds to the results of this research.

In the first 60 days of ripening of Krk cheese no statistically significant differences were determined ($P>0.05$) in the concentration of the total free amino acids regarding the manufacturing method. However, statistically significant differences ($P<0.01$) were determined in the concentration of total free amino acids between the 90th and 120th day of ripening, depending on the use or non use of the microbial culture. Significant differences in the concentration of total free amino acids can be caused by a higher quantity of NSLAB which prevail at the end of cheese ripening (Hickey et al., 2007). Since no significant changes were determined in the concentration of total free amino acids in both cheese groups between the 90th and 120th ripening day, it can be concluded that the optimum duration of ripening of Krk cheese is 90 days, which corresponds to previous researches of Antunac et al. (2008) and Mikulec et al. (2010). From the above it can be concluded that there is no need for using microbial culture in the manufacturing of Krk cheese if the cheese ripening lasts 90 or more days.

Regarding the obtained results, which correspond to Folkertsma and Fox (1992), Freitas et al. (1997) and Pavia et al. (2000), it can be concluded that Cd-ninhydrin method is suitable for monitoring cheese ripening and that it can also determine statistically significant differences in ripe cheeses regarding the differences in the technological manufacturing process. Pavia et al. (2000) also concluded that this method can be used for determining the degree of ripeness of Manchego cheese and as such it can replace the method according to Kjeldahl. Cd-ninhydrin method proved to be suitable for determining the degree of ripeness of Krk cheese. By determining the composition of various length peptides and catabolic products of amino acid decomposition, together with obtained results, further criteria for distinguishing Krk cheese from other similar traditional cheeses can be suggested.

Conclusion

Cadmium-ninhydrin method for determining the concentration of total free amino acids proved to be efficient for monitoring the ripening of Krk cheese and for determining differences in the concentration of total free amino acids in a mature cheese. Results correspond to results of similar researches, so this method can be used for monitoring the ripening of other cheeses.

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Utjecaj mikrobne kulture na koncentraciju ukupnih slobodnih aminokiselina tijekom zrenja Krčkog sira

Sažetak

Cilj rada bio je odrediti utjecaj dodatka mikrobne (komercijalne, starter) kulture na koncentraciju ukupnih slobodnih amino skupina (aminokiselina) u sirevima u različitim fazama zrenja. Slobodne amino skupine određivane su reakcijom s ninhidrinom uz kadmij (Cd) u vodenom ekstraktu sira, te su izražene kao koncentracija leucina u suhoj tvari sira. Praćene su promjene koncentracije ukupnih slobodnih aminokiselina tijekom zrenja Krčkog sira (0., 30., 60., 90. i 120. dan). U vodenim ekstraktima sira u svim fazama zrenja detektirano je prisustvo slobodnih NH_2 skupina, odnosno amina, aminokiselina i manjih peptida, čija se koncentracija značajno ($P<0,01$) povećavala tijekom zrenja. U sirevima proizvedenim sa i bez dodatka mikrobne kulture utvrđene su značajne razlike ($P<0,01$) u koncentraciji ukupnih slobodnih aminokiselina 90. i 120. dana. Kadmij-ninhidrinska metoda se pokazala prikladnom za praćenje tijeka zrenja Krčkog sira, kao i za utvrđivanje razlika u koncentraciji ukupnih slobodnih aminokiselina zrelih sireva ovisno o tehnološkom procesu proizvodnje.

Ključne riječi: Krčki ovčji sir, zrenje sira, slobodne aminokiseline, mikrobne kulture

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