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# EFFECT OF THE RECOMBINANT CHYMOSINS OF DIFFERENT ORIGINS ON THE QUALITY AND SHELF LIFE OF SOFT CHEESES

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*KEY WORDS:*
*soft cheese, milk-clotting enzymes, recombinant chymosin, proteolysis, bitter taste, rheology, microstructure*

ABSTRACT

The effect of the type (bovine chymosin (Chy-max Extra), camel chymosin (Chy-max M), and modified camel chymosin (Chy-max Supreme)) and applied dose (1500, 2500, or 3500 IMCU/100 kg of milk) of milk-clotting enzyme (MCE) on the proteolysis degree, microstructure, rheological and sensory properties of Crescenza soft cheese was studied. The proteolysis degree of cheeses was directly proportional to the general proteolytic activity (PA) and dose of the MCEs added into milk during the cheese production. With an equal dose of the MCEs added into milk, the highest level of proteolysis was noted in the cheese options produced with recombinant bovine chymosin (Chy-max Extra). There were no statistically significant differences ($p > 0.05$) in the proteolysis degree between the cheese options made with the same doses of the camel chymosin (Chy-max M) and modified chymosin (Chy-max Supreme). After 21 days of storage, the cheeses with Chy-max Extra MCE had the most plastic consistency, while the cheeses with Chy-max Supreme MCE had the hardest and most elastic one. Cheeses produced with Chy-max M MCE occupied an intermediate position in terms of consistency density. Recombinant MCEs based on camel chymosin (Chy-max M) and modified chymosin (Chy-max Supreme) with low level of PA, may be recommended for use in the production of soft cheeses. This will extend the shelf life of cheeses by slowing-down the plasticization rate of their consistency.

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# ВЛИЯНИЕ РЕКОМБИНАНТНЫХ ХИМОЗИНОВ РАЗНОГО ПРОИСХОЖДЕНИЯ НА КАЧЕСТВО И СРОКИ ХРАНЕНИЯ МЯГКИХ СЫРОВ

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АННОТАЦИЯ

Изучено влияние типа (химозин теленка (МФ Chy-max Extra), химозин верблюда (МФ Chy-max M), «модифицированный» химозин верблюда (МФ Chy-max Supreme)) и дозы внесения (1500, 2500 или 3500 IMCU/100 кг молока) молокосвертывающих ферментов на степень протеолиза, микроструктуру, реологические и сенсорные показатели мягкого сыра Кресченца. Степень протеолиза в сырах, была прямо пропорциональна удельной протеолитической активности и дозе МФ, внесенных в молоко при производстве сыра. При равной дозе внесения МФ в молоко, наибольший уровень протеолиза отмечался в вариантах сыров, произведенных с рекомбинантным химозином теленка (МФП Chy-max Extra). Отсутствовали статистически достоверные различия ($p > 0,05$) по степени протеолиза в вариантах сыров, произведенных с одинаковыми дозами химозина верблюда (МФП Chy-max M) и «модифицированного» химозина (МФП Chy-max Supreme). Через 21 сут хранения, консистенция сыров с МФ Chy-max Extra была наиболее пластичной, консистенция сыров с МФ Chy-max Supreme наиболее твердой и упругой. Сыры, изготовленные с МФ Chy-max M, занимали промежуточное положение по плотности консистенции. Рекомбинантные МФ на основе химозина верблюда (Chy-max M) и «модифицированного» химозина (Chy-max Supreme), обладающие низким уровнем ПА, можно рекомендовать для использования в производстве мягких сыров в целях замедления скорости пластификации консистенции сыров в процессе хранения и увеличения за счет этого продолжительности срока сыров.

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## 1. Introduction

Proteolysis is an important part of cheese ripening and occurs as a result of action produced by a milk-clotting enzyme (MCE) and enzymes of starter microorganisms. These components remain active after the completion of cheese production in the cheese bath, which leads to undesirable over-ripening of cheese during the storage that is expressed in the appearance of taste and consistency defects.

In the hard and semi-hard cheeses a considerable part of the MCE is removed from the cheese curd into the whey [1,2]. In the pizza-cheeses (pasta filata) the MCE is mainly inactivated by high temperature applied to the cheese mass for softening before stretching [3].

In the soft cheeses with high moisture content, a significant amount of the MCE is retained in the aqueous phase of cheeses. A greater retention of the MCE in the cheese mass is favored by the low pH of the milk curd set in the process of soft cheese production [4,5]. A combination of high moisture content, low pH, and low salt concentration in the soft cheeses promotes the MCE proteolytic activity (PA) [6,7].

Two approaches can regulate the MCE proteolytic activity in cheeses:

- reduction of the MCE dose;
- use of the MCE with a low PA level.

The MCE dose reduction is associated with such disadvantages as the longer duration of coagulation and the formation of the cheese curd with a higher moisture content. However, negative effects of the MCE dose reduction may be compensated by increasing the milk-clotting activity (MCA) of the MCE with optimized milk coagulation conditions. These conditions include the increased temperature of coagulation (> 34 °C), the milk acidification (below pH 6.6), the enrichment of milk with protein (e. g., with milk powder) and calcium ions ($CaCl_2$) [8,9].

The use of a MCE with a low PA does not require any adjustments of the cheese processing technology. Recombinant bovine and camel chymosin preparations are used as the MCEs with low PA [10,11,12,13,14]. A modified chymosin preparation has recently been put on the market under the brand name Chy-max® Supreme by Chr. Hansen A/S [15]. Modified chymosin is reported to be 95% identical to camel chymosin and has a higher specific MCA level and remains active at a higher pH as compared with the natural camel chymosin [16]. Thanks to these properties, the modified chymosin can induce a rapid coagulation of milk without its prior acidification with a lactic acid starter.

The aim of this paper is to study the effect of the type and the dose of MCEs based on recombinant chymosins of different origin on the proteolysis in soft cheeses and proteolysis-associated processes of cheese structure and consistency changing. The study was conducted in the process of producing soft cheese of the Italian type Crescenza [17]. The choice of Crescenza cheese for the study was associated with two factors. First, the previous studies showed that the shelf life of Crescenza soft cheese could be increased by means of reducing proteolysis in cheese resulted from the use of camel chymosin instead of bovine rennet [10]. Second, a combination of high temperature (38 °C) and low pH level (6.2–6.4 pH units) during milk coagulation in Crescenza cheese technology [18] corresponds to the optimal conditions for the MCE activity and opens up the potential for using a reduced MCE dose without compromising the positivity of milk coagulation and the moisture content of the resulting milk curd.

## 2. Materials and methods

### *2.1. Materials*

In the studies, milk from a single supplier, Agrivolga LLC (Yaroslavl region, Burmasovo village) was used. A lactic acid starter based on direct-to-vat bacterial concentrates (DVS-type) STI®-12 and STI®-14 that contained *Str. thermophilus* strains (Chr Hansen A/S) and MCEs of the following brands (Chr Hansen A/S): Chy-max® Extra 600 Liquid, Chy-max® M 1000, and Chy-max® Supreme 1000 was used in the cheese production.

The qualification of reagents used for analysis was at least “analytical grade”.

### *2.2. Determination of proteolytic activity of milk-clotting enzymes*

The proteolytic activity of milk-clotting enzymes was evaluated according to GOST 34430–2018[1] (Russian National Standard), as applied to weakly acidic proteases (at pH 5.3). Information on the values of the milk-clotting and proteolytic activity of the MCEs used in this study is given in our article [19].

### *2.3. Cheese production*

Information on the process conditions of Crescenza cheese production and composition of the resulting cheeses at the beginning of the storage period is given in [19].

### *2.4. Methods for compositional analysis and proteolysis determination of cheeses*

Chemical composition of cheeses was studied in the course of storage at 7, 14, and 21 days.

The dry matter content in cheeses was determined by heating at a temperature of 102 ± 2 °C according to IDF 4 standard[2]. The protein content in cheeses was determined by Kjeldahl method according to IDF/RM 25 standard[3]. The mass fraction of NaCl in cheeses was determined by Mohr method using titration with $AgNO_3$ [20]. The active acidity of cheeses was measured on a Testo 206-pH2 digital pH-meter (Testo SE & Co. KGaA, Federal Republic of Germany) in a suspension obtained by grinding 10 g of cheese with 10 of distilled water.

The degree of proteolysis in cheeses was evaluated (in percent) as a ratio of the water-soluble nitrogen content (WSN) to the total nitrogen (TN) content in cheeses. Water-soluble nitrogen content was determined in water-soluble extracts of cheeses obtained by Kuchroo and Fox method as modified in [21].

#### *2.4.1. Molecular weight distribution of proteolysis products*

Molecular weight distribution of soluble protein substances was determined in water-soluble extracts of cheeses by high resolution gel filtration using a column Superose 12 10/300 GL (GE Healthcare, Sweden). For analysis, an extract for determination of soluble protein mass fraction was used, additionally filtered through cellulose acetate filters with a pore size of 0.45 µm (Vladipor, Russia). An eluent was 0.05 M $Na_2HPO_4$ + 0.15 M NaCl aqueous solution; the flow rate of the eluent is 0.5 ml/min; detection wavelength was 280 nm. The column was calibrated against the elution time of the protein substances with a known molecular weight: IgG (180 kDa), aldolase (158 kDa), BSA (69 kDa), ovalbumine (43 kDa), β-Lg (36.0 kDa), α-La (14.4 kDa), cytochrome C (12.3 kDa), tryptophan (0.204 kDa). A calibration curve was based on a logarithmic regression model [22]. Molecular weight distribution was calculated as an area proportion of individual peptide fractions in the total area under the chromatogram. The result was expressed as a percent content of peptide fractions within various ranges of the molecular weight [23].

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[1] GOST 34430–2018 Enzyme preparations for food industry. Method for the determination of proteolytic activity. M.: Standartinform, 2018–12 p.

[2] ISO 5534:2004[IDF 4:2004] Cheese and processed cheese — Determination of the total solids content (Reference method).

[3] SO/TS17837:2008[IDF/RM 25:2008] Processed cheese products — Determination of nitrogen content and crude protein calculation — Kjeldahl method.

*2.5. Rheology*

Rheological properties of cheeses were studied using a Weissenberg rheogoniometer, model R-19 (Sangamo Weston Controls Limited, the UK). Testing mode: periodic shearing deformation with a preset frequency and amplitude of oscillations. A combination of "cone-plane" with a diameter of 25 mm was used as working tools. A cone-apex angle was 2°. The linearity of periodic deformation mode was attained at an amplitude of angular displacements of the working tools of $1.1 \cdot 10^{-3}$ rad at a frequency of 3.16 Hz. The temperature of samples in the course of measurements was 21 ± 1 °C. Based on determined primary viscoelastic terms (the storage modulus, G', and loss modulus, G") complex modulus (G*) along with phase angle tangent (tan δ), were calculated [24].

*2.6. Microstructure studies*

The microstructure of cheeses was studied by the method of light microscopy in oblique lighting mode, on micro-sections of cheese with a thickness of 100 ± 10 μm. Photo images were taken with a Canon EOS600D digital camera. Image correction was carried out with Digital Photo Professional software v.4.5 (Canon Inc.).

*2.7. Experimental design and statistical analysis*

The study was conducted on the basis of a full factorial design [25], which included 2 categorial factors varying at 3 levels, i. e. the factor of "MCE brand" (Chy-max Extra, Chy-max M and Chy-max Supreme) and the factor of "MCE dose" (1500, 2500, and 3500 IMCU / 100 kg of milk). The experiments were carried out in triplicate in a randomized order.

The combined effect of experimental factors and cheese storage time on the response variables (pH, degree of proteolysis, and rheological properties) was assessed by modifying the selected full factorial design into a "split-plot" design [26] with introduction of an additional factor, "age".

Statistical processing of the experimental data is carried out using a Statistica® application software (ver. 5.5, StatSoft, the USA).

## 3. Results and discussion

Table 1 shows the results of assessing the effect of factors on response variables by two-way ANOVA.

The ANOVA results given in Table 1 suggest that the effect of experimental factors ("MCE type", "MCE dose", and "cheese age") on the response variables (pH, degree of proteolysis, and rheological properties of cheeses during storage) may have different strength.

Table 1. **Mean sum of squares deviations, level of statistical significance(in parentheses), and $R^2$ values of the ANOVA model for response variables**

| Factor | df | pH | Degree of proteolysis, % | Complex shear modulus (G*), Pa | Loss tangent (tan δ) |
|---|---|---|---|---|---|
| Type | 2 | 0.004 (–) | 67.007 (***) | 71 086 530 (***) | 0.22151 (***) |
| Dose | 2 | 0.015 (**) | 20.673 (***) | 10 168 610 (*) | 0.01103 (–) |
| Age | 2 | 0.063 (***) | 140.266 (***) | 1 037 347 000 (***) | 1.11991 (***) |
| Type*Dose | 4 | 0.002 (–) | 2.202 (***) | 9 041 086 (***) | 0.02317 (**) |
| Type*Age | 4 | 0.001 (–) | 3.512 (***) | 1 734 935 (–) | 0.04677 (***) |
| Dose*Age | 4 | 0.005 (–) | 9.452 (***) | 24 992 840 (***) | 0.04467 (***) |
| Error | 62 | 0.002 | 0.232 | 2 478 696 | 0.00443 |
| $R^2$ | | 0.58 | 0.97 | 0.94 | 0.92 |

df — number of degrees of freedom;
Factor keys: Type — MCE type; Dose — MCE dose; Age — cheese storage time;
Error — percentage of the response variable variation related to an error;
$R^2$ — coefficient of determination for ANOVA model.
Level of statistical significance of the factor effect evaluation (in parentheses): "–" — no significant ($p > 0.05$); "*" — $p < 0.05$; "**" — $p < 0.01$; "***" — $p < 0.001$.

*3.1. Acidity (pH)*

There is a significant effect of "MCE dose" and "age" factors on the change in the pH level of cheeses during storage (Table 1). Depending on the size of effect on the "pH" response variable, the effecting factors are arranged in the following order: "age" > "MCE dose". There is no paired effect of a factor set on the "pH" response variable. The selected set of the ANOVA model factors explains a 58% variation proportion for the "pH" response variable ($R^2 = 0.58$). Figure 1 shows the graphs demonstrating a change in the active acidity of Crescenza cheese options produced with different types and doses of MCEs during the cheese storage.

Data on the pH level in cheeses shown in Figure 1 demonstrates the nature of the effect exerted by "MCE dose" and "cheese age" factors on the pH value. The effect of the "age" factor on pH is associated with cheese pH reduction during storage resulted from transformation of milk sugar into lactic acid by starter bacteria. The graphs given in Figure 1 show that the pH reduction level during the storage of cheeses is approximately the same for all test options (~ 0.1 pH units). At the same time, due to the higher pH level at the baseline, cheeses produced with the maximum MCE dose (3500 IMCU/100 kg of milk) retain a higher pH level until the end of the storage time. This is one of the reasons explaining the effect of "MCE dose" factor on the pH value of cheeses.

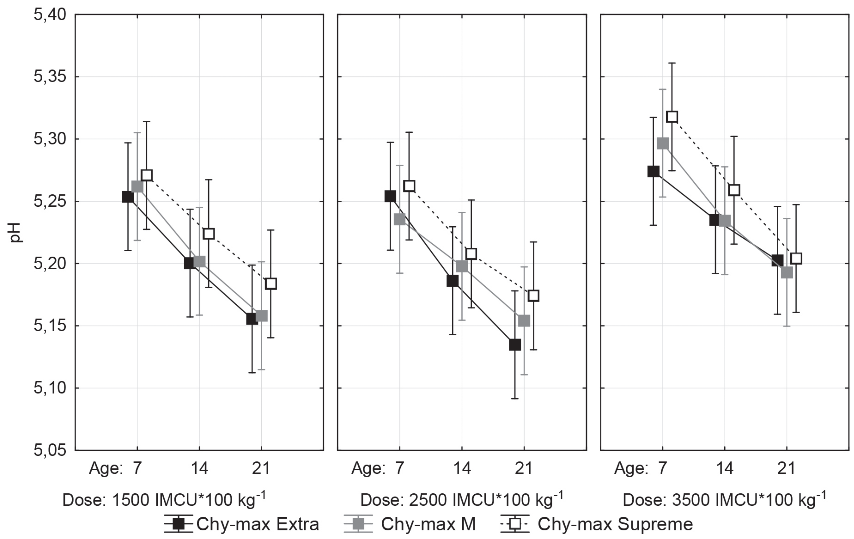


Figure 1. **A behavior pattern of change in the active acidity of Crescenza cheese samples produced with different types and doses of MCEs, during storage of the cheese.**
Keys: Age — the age of the cheese, days; Dose — 1500, 2500, and 3500 IMCU/100 kg — the MCE dose added into milk during cheese production. Data is given as "mean ± confidence interval ($p = 0.05$)".

Another reason explaining the effect of MCE dose on the pH value of cheeses is an effect of MCE dose on the moisture content in cheeses in the beginning of the storage period. Figure 2 shows the dependence of the pH decrease during storage on the dry matter (moisture) content in cheeses.

The data of the graph (Figure 2) clearly demonstrates that cheeses produced with a higher MCE dose of 2500–3500 IMCU/100 kg (Group II) have on average a higher dry matter content (i. e. lower moisture content), than cheeses produced with a low MCE dose of 1500 IMCU/100 kg (Group I). The higher the moisture content in cheeses is, the higher the content of lactose dissolved in the aqueous phase is obtained. The greater amount of available lactose results in a greater formation of lactic acid by a lactic acid starter as well as in a greater decrease in the pH level in cheese.

*3.2. Degree of proteolysis*

The ANOVA results suggest that all the experimental factors exert a pronounced, significant ($p < 0.001$) effect on the degree of proteolysis in cheeses (Table 1). Depending on the size of effect on the "proteolysis degree" variable, the effecting factors are arranged in the following order: "age" > "MCE type" > "MCE dose". The ANOVA model with the selected set of factors describes a variation of the "proteolysis degree" response variable in a significant manner at $R^2 = 0.97$. Figure 3 shows the graphs demon-

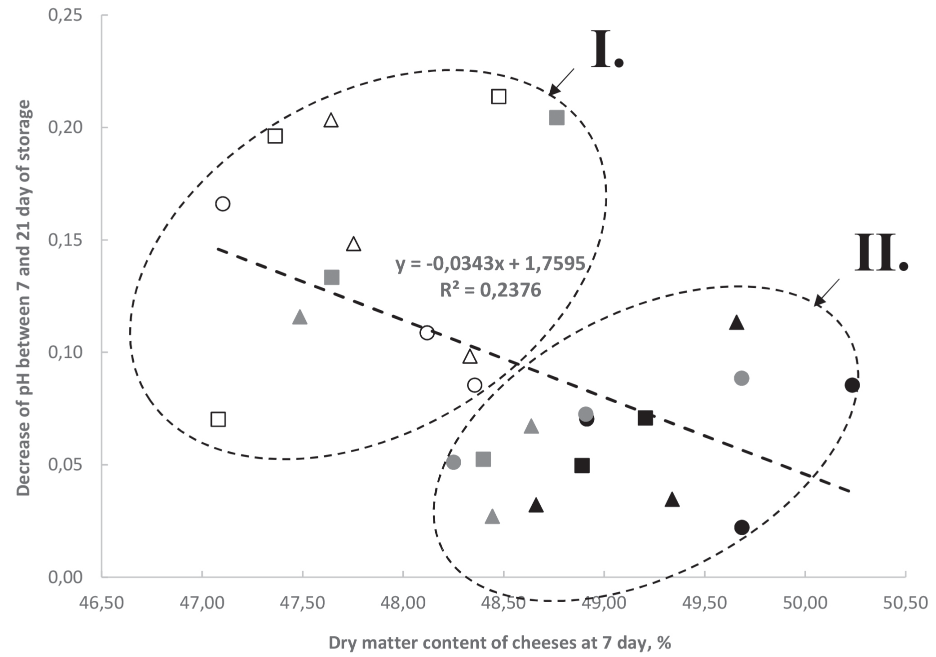


Figure 2. **The relationship between the pH decrease of the cheese (between 7 and 21 days of storage) and the dry matter content in the cheese at the beginning of the storage period (7 days). Groups I and II are distinguished, which have statistically significant differences (p < 0.05) in average dry matter content and in the level of pH decrease. Cheese options are designated by the type and dose of the MCE used in the following manner:** Chy-max Extra at an applied dose of 1500 IMCU/100 kg (□), 2500 IMCU/100 kg (■), 3500 IMCU/100 kg (■); Chy-max M at an applied dose of 1500 IMCU/100 kg (△), 2500 IMCU/100 kg (▲), 3500 IMCU/100 kg (▲); Chy-max Supreme at an applied dose of 1500 IMCU/100 kg (○), 2500 IMCU/ 100 kg (●), 3500 IMCU/100 kg (●).

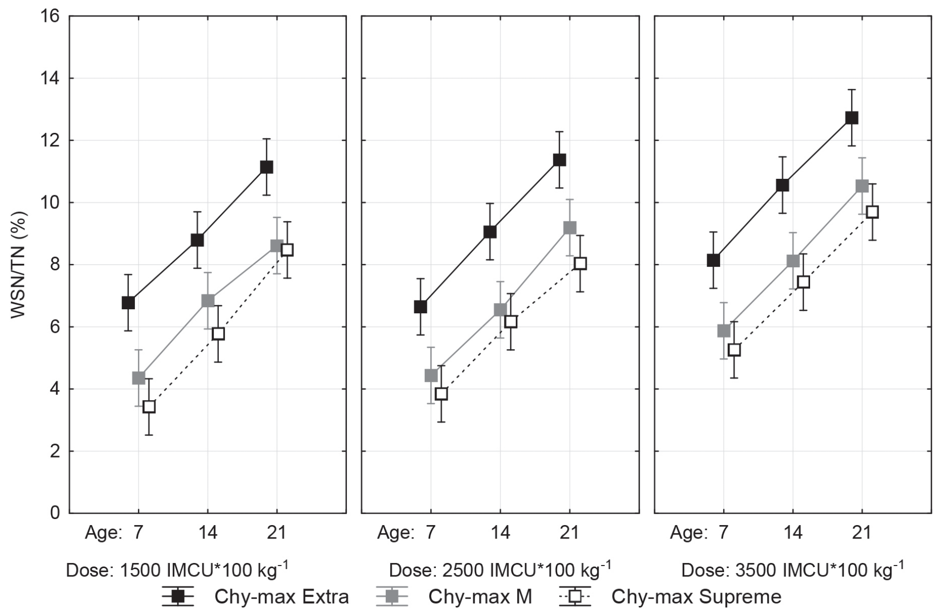


Figure 3. **A behavior pattern of proteolysis in Crescenza cheese samples produced with various types and doses of MCEPs, during storage. Keys: Age — the age of the cheese, days; Dose — 1500, 2500, and 3500 IMCU/100 kg — the MCEP dose added into milk during cheese production. Data is given as "mean ± confidence interval (p = 0.05)"**

strating a change in the proteolysis degree of Crescenza cheese options produced with different types and doses of MCEs during storage.

A behavior pattern of proteolysis plotted in Figure 3 suggests that the "age" exerts the most pronounced effect on the degree of proteolysis in cheeses. A near linear dependence of the proteolysis degree in cheeses on the age is observed. With an increasing dose of MCE added into milk, an increase in the degree of proteolysis is noted. The cheeses produced with the MCE of the same type, but with different doses of the MCE did not show any significant difference ($p < 0.05$) in proteolysis degree at the similar time points throughout the storage period.

With an equal dose of MCEs added into milk, the highest level of proteolysis during storage is noted in the cheese options produced with recombinant bovine chymosin (Chy-max Extra). There is no significant difference in the degree of proteolysis in the cheese options produced with the same doses of the camel chymosin (Chy-max M) and modified chymosin (Chy-max Supreme).

It was found earlier that the studied brands of recombinant chymosins had different levels of nonspecific PA [19]. Figure 4 shows the dependence of the proteolysis degree in cheeses after 21 days of storage on the MCE dose added into milk and calculated as PA.

The data shown in Figure 4 suggests that the degree of proteolysis in cheeses is proportional to the MCE dose added into cheeses and calculated as PA units. Therefore, the higher the PA of the milk-clotting enzyme is and the higher its dose is, the greater degree of proteolysis in cheese is obtained.

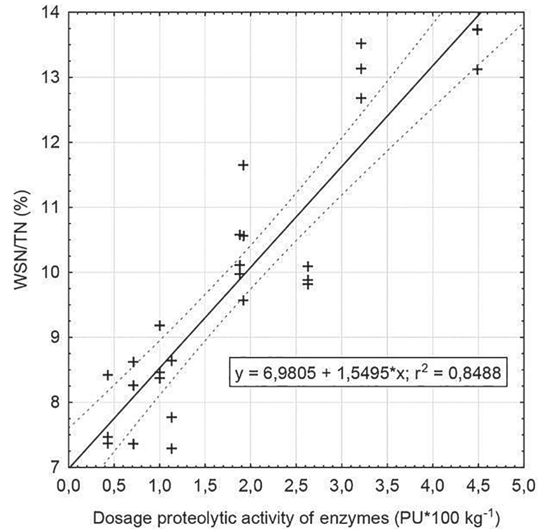


Figure 4. **Dependence of the proteolysis degree in cheeses at storage day 21 on the number of PA units added into milk with a MCE**

*3.3. Molecular weight distribution of proteolysis products*

Along with differences in proteolysis level, the studied MCEs also differ by the specific nature of proteolytic action. Figure 5 shows the diagrams of molecular weight distribution for Crescenza cheese samples produced with different types and doses of MCEs after 21 days of storage.

As compared to Chy-max M and Chy-max Supreme MCEs, Chy-max Extra MCE forms more proteolytic products with a weight of less than 5 kDa, which potentially have a bitter taste [27,28]. In fact, the samples of Crescenza cheese produced with Chy-max Extra MCE, demonstrated the development of bitter taste at the end of the storage time, and the intensity of this taste was proportional to the MCE dose added: from a "slight bitterness" at a dose of 1500 IMCU/100 kg to a "moderately pronounced bitter taste" at a dose of 3500 IMCU/100 kg. At the same time, for cheeses made with Chy-max M and Chy-max Supreme MCEs, even addition of the maximum dose was not associated with development of bitter taste till the end of the storage time. This is in line with the findings obtained by Bansal et al [14] who found that bitter taste developed during ripening in samples of Cheddar cheese produced with Chy-max Extra MCE, but not in samples of cheeses made with Chy-max M MCE.

Milk-clotting enzymes break down caseins mainly to high and medium molecular weight (over 10 kDa) peptides, and some of these peptides are not water-soluble [29,30]. Insoluble products of proteolysis that have passed through a filter with a pore size of 0.45 µm constitute a fraction of turbidity-forming particles in the filtrate, which elutes as a single high peak in the void volume (V0) of the chromatographic column. The turbidity-forming particles also include other insoluble substances, such as fat particles and fragments of fat globule membranes [31]. There was no apparent association between the type or dose of

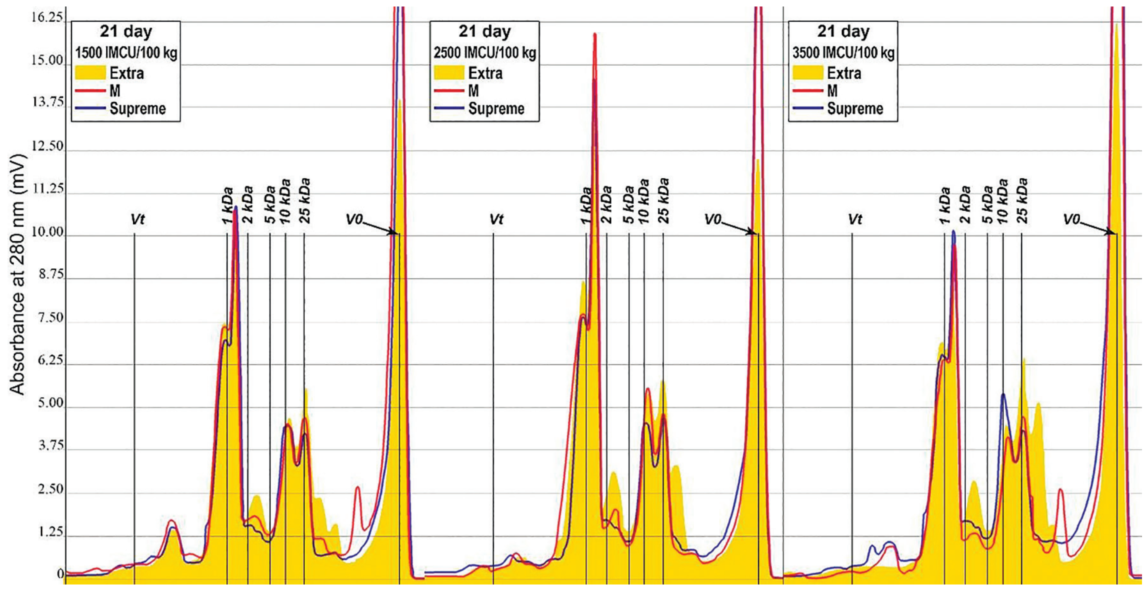


Figure 5. **Molecular weight distribution of water-soluble proteolysis products in Crescenza cheese samples (at the age of 21 days) produced with different types and doses of MCEs. Keys for a MCE brand: Extra — Chy-max Extra; M — Chy-max M; Supreme — Chy-max Supreme. V0 — void column volume, Vt — total column volume**

a MCE and the quantitative content of the fraction of turbidity-forming particles in water-soluble extracts of cheeses.

The chromatogram shows a substantial number of soluble protein substances with a weight of 25 kDa or more (hereinafter we imply a fraction with a molecular weight over 25 kDa, which has a longer retention time than that of the fraction of turbidity-forming particles). The content of this fraction increases with an increase in the MCE dose from 1500 to 3500 IMCU/100 kg for all types of studied MCEs. The Chy-max Extra dose also shows the greatest effect on the content of the fraction with a weight of > 25 kDa. Protein substances of this molecular weight have no taste, therefore, an apparent consequence of the proteolysis characterized by predominant formation of peptides with a molecular weight > 25 kDa is the plasticization of cheese consistency due to degradation of its protein network caused by proteolysis rather than the formation of pronounced taste [32,33,34].

Due to the bond ruptures in the protein matrix that forms a bearing frame of the cheese, a high intensity of cheese proteolysis leads to a loss of the cheese mass cohesion, which is manifested by the plasticization of the consistency (excessively plastic, viscous, spreadable consistency). The association between the proteolysis and the cheese mass structure is confirmed by studies of the microstructure of cheeses produced with MCEs of various types [35,36,37].

### *3.4. Microstructure*

The type and dose of the MCEs used in the production of test cheeses influenced not only the physical and chemical indicators and the degree of proteolysis of cheeses, but also their microstructure. Figure 6 shows the photo images depicting the typical appearance of the microstructure of Crescenza cheese options produced with different types and doses of MCEs at the end of the storage time (21 days).

The fresh cheeses produced with MCEs of various types did not show any difference in the microstructure and were characterized by a heterogeneous structure, which consisted of distinctly separated grains. Besides, there were holes in the cheese structure filled with moisture or air bubbles (photo image not shown). Clear differences in the microstructure of cheeses developed by the end of the storage time.

Cheese mass protein hydration caused by proteolysis occurs during cheese storage. The moisture contained in the boundary layer separating the grains from each other is absorbed by the protein matrix, which results in the disappearance of boundaries between the grains and in the formation of a homogeneous cheese structure. The higher the degree of proteolysis in the cheese is, the greater change in the cheese structure since the beginning of its ripening and storage is observed. Cheeses with a low degree of proteolysis are characterized by a heterogeneous structure with distinctly separated cheese grains. Cheeses with a high degree of proteolysis demonstrate a more uniform, highly dispersed structure due to the disappearance of large grains as a result of their hydration [2,13,35]. Cheese samples produced with Chy-max M and Chy-max Supreme MCEs had a lower degree of proteolysis and a more massive structure of the casein network as compared with cheeses produced with Chy-max Extra MCE.

### *3.5. Rheological properties*

ANOVA results given in Table 1 show that in terms of the effect size exerted on the complex modulus (G*), the effecting factors are arranged in the following order: “age” > “MCE type” >

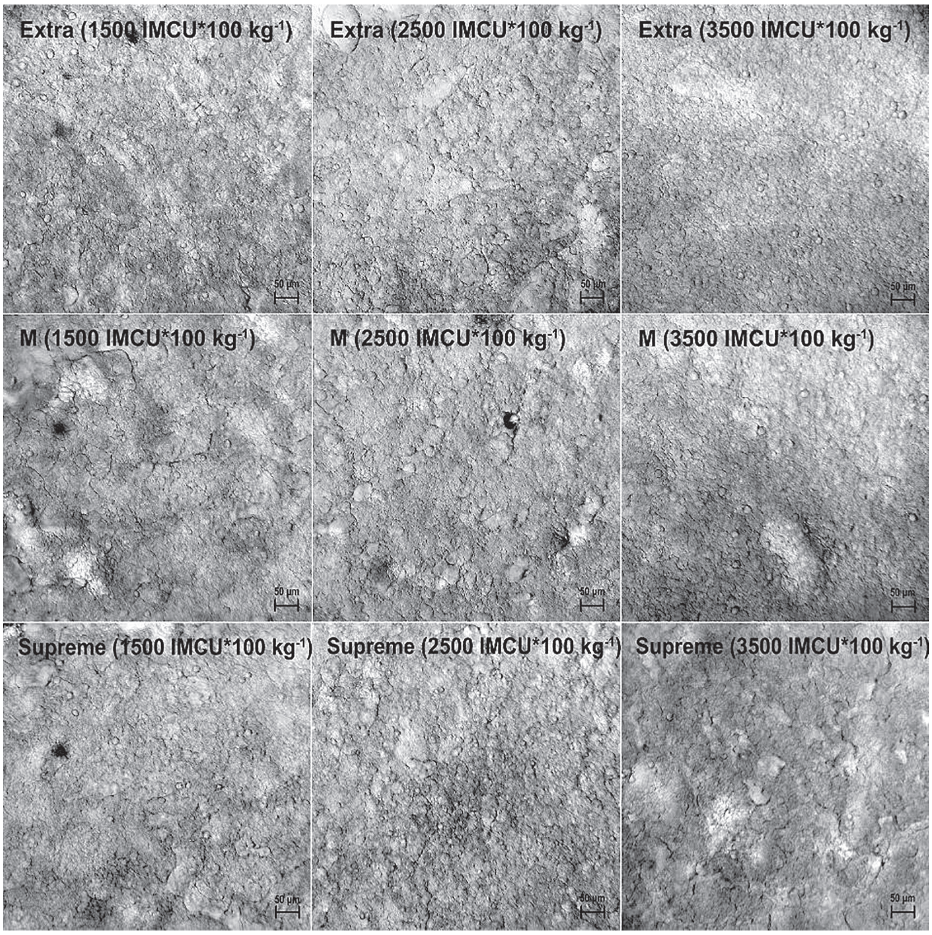


Figure 6. **Microstructure of Crescenza cheese samples produced with different types and doses of MCEs. Keys for the MCE brands: Extra — Chy-max Extra; M — Chy-max M; Supreme — Chy-max Supreme**

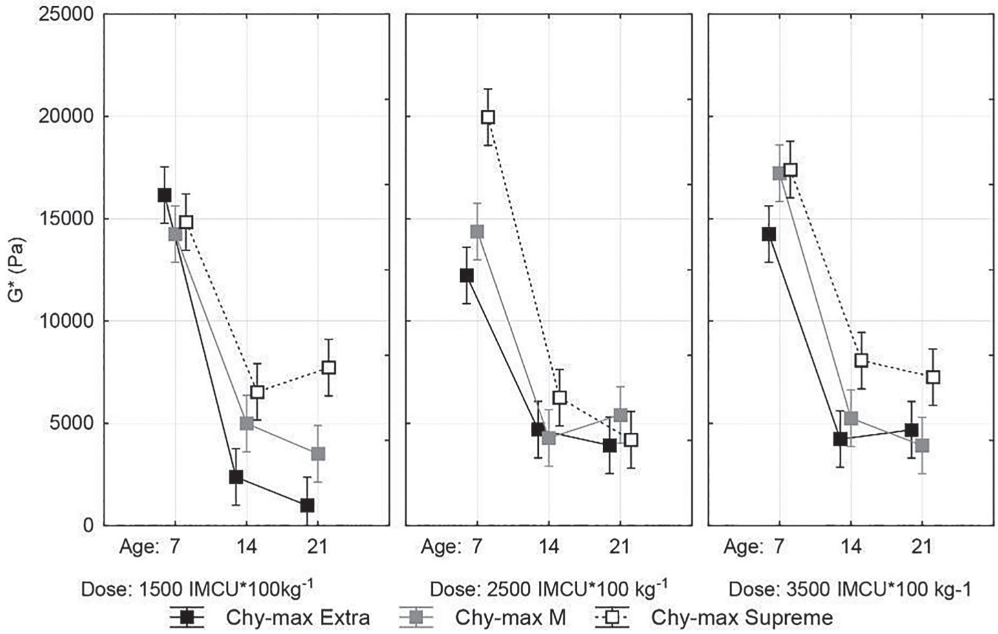


Figure 7. **A behavior pattern of the complex modulus (G*) changing during storage for Crescenza cheese samples produced with various types and doses of MCEs. Keys: Age — the age of the cheese, days; Dose — 1500, 2500, and 3500 IMCU/100 kg — MCE dose added during cheese production. Data is given as "mean ± confidence interval (p = 0.05)"**

"MCE dose". A selected set of factors included in the ANOVA model provides a significant description of the variation of the "complex modulus (*G**)" response variable at $R^2 = 0.94$.

Figure 7 shows a behavior pattern of G* change for various options of the test cheeses during storage.

The complex modulus, *G** is calculated by the following formula [24]:

$$G^* = \sqrt{((G')^2 + (G'')^2)}, \tag{1}$$

where

*G′* is the storage modulus, Pa;

*G″* is the loss modulus, Pa.

The G* modulus reflects the overall reaction of a material to the deformation applied and therefore correlates most closely with the organoleptic estimation of the consistency. Cheeses with hard and elastic consistency are characterized by high *G** values, while cheeses with soft and plastic consistency are characterized by low *G** values [24,38].

The same trend is noted for all cheese options. The *G** value decreases until the 14th day of storage. After that time point, the *G** value remains stable. The *G** value shows no significant changes ($p < 0.05$) between day 14 and day 21 of storage. The decrease in the *G** value from day 7 till day 14 is reflected by the change in cheese consistency, which varies from slightly heterogeneous (which is typical for fresh cheese) to coherent. In the period of time from day 14 till day 21 there are also no changes in cheese consistency in the absence of significant changes in the *G** values.

A clear effect of the MCE type used in the production of cheeses is noted for the rheological properties and sensory assessment of consistency. With the same dose of a MCE, the cheeses produced with Chy-max Supreme MCE have a higher *G** level by the end of the storage time than that of the cheeses made by addition of Chy-max Extra or Chy-max M MCEs. There are no significant differences in *G** values between cheeses produced with the same doses of Chy-max Extra and Chy-max M MCEs throughout the entire storage time.

ANOVA results given in Table 1 show that in terms of the effect size exerted on the loss tangent (tan δ), the effecting factors are arranged in the following order: "age" > "MCE type". There is no direct significant effect of the "MCE dose" factor on the tan δ value. At the same time, a significant effect on the tan δ value is observed for the "MCE dose" factor when it acts in pair with the "age" and "MCE type" factors. The ANOVA model with the selected set of factors describes a variation of the "tan δ" response variable in a significant manner at $R^2 = 0.92$.

Figure 6 shows a behavior pattern of the tan δ change for various options of the test cheeses during storage.

The loss tangent (tan δ) is calculated by the following formula [24]:

$$tg(\delta) = \frac{G''}{G'} \tag{2}$$

Low values obtained for tan δ indicate that gel properties (elasticity, flexibility) are prevailing in the sample. High values indicate the prevalence of liquid properties (fluidity, viscosity) of the sample.

Analysis of the data shown in the graphs (Figure 8) allows us to draw the following conclusions. For the cheeses produced with the same type and dose of the MCE, a significant increase in the tan δ value ($p < 0.05$) is observed between day 7 and day 14 of storage. The changes in the tan δ level of cheeses are insignificant ($p > 0.05$) between day 14 and day 21 of storage. Changes in the tan δ values were accompanied by the change in the sensory assessment of the cheese consistency from "elastic" at 7 days to "plastic" at 21 days.

The tan δ (tan δ > 1) values obtained in this study are higher than the values of tan δ, obtained by other investigators. Alinovi et. al [10] measured the rheological properties of Crescenza cheese and obtained the tan δ values in the range of 0.25–0.33 at a test sample temperature of 4 °C. In the study by Rogers et al [39] evaluating the rheological properties of Cheddar cheese, it was found that the G′ and G″ values obtained for cheeses at 10 °C were higher than those obtained at 25 °C. The high tan δ values obtained in this study may be explained by the composition of cheeses and the measurement conditions. Measurements were taken at a temperature of 22 ± 1 °C, which affected the plasticity of the fatty phase and resulted in a reduced hardness (G' parameter) and an increased fluidity of the cheese mass (G" parameter). This affected the tan δ parameter derived from G' and G".

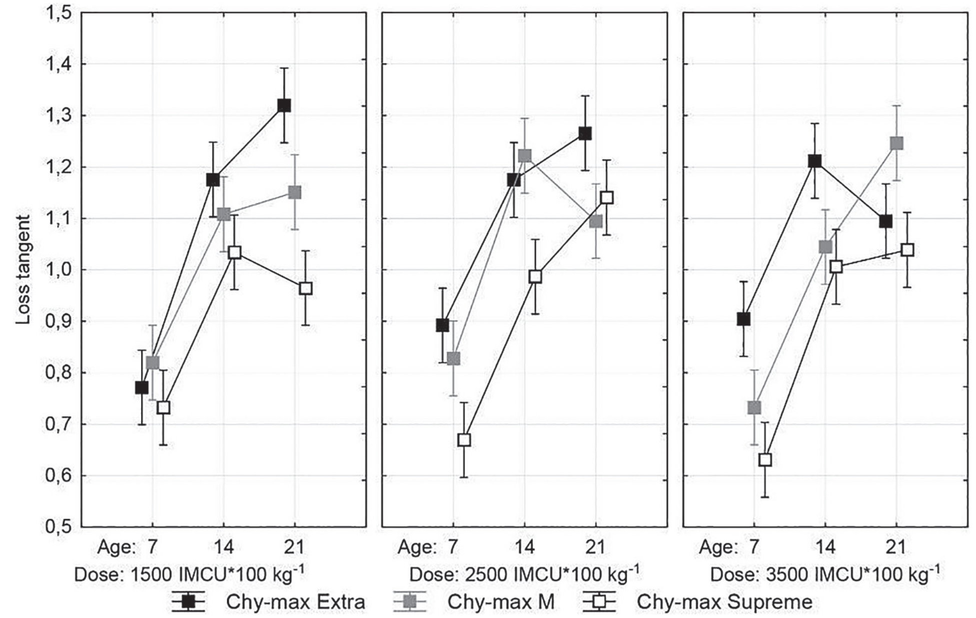


Figure 8. **A behavior pattern of the loss tangent (tan δ) changing during storage for Crescenza cheese samples produced with different types and doses of MCEs. Keys: Age — the age of the cheese, days; 1500, 2500, and 3500 IMCU/100 kg — the MCE dose added during cheese production. Data is given as "mean ± confidence interval (p = 0.05)"**

*3.6. Cheese consistency*

Figure 9 shows the photo images of the appearance of Crescenza cheeses produced with various types of MCEs at the minimum and maximum doses.

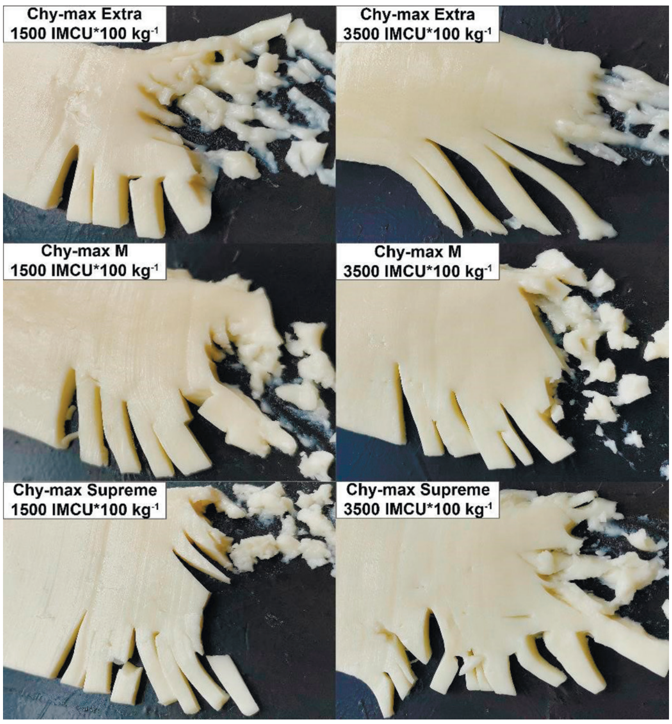


Figure 9. **Structure (appearance) of the test Crescenza cheeses produced with different types and doses of MCEs after 21 days of storage. Cutting was made to show the sliceability of the cheese**

After 21 days of storage, all options of the test Crescenza cheeses maintained the consistency desirable for high-quality cheese. No combination of the studied MCE type and dose showed the formation of viscous, spreadable, knife-sticking consistency of cheese. There were differences in the consistency of cheeses produced with different types and doses of MCEs. Among the cheese options with the same dose of a MCE, the cheeses made with Chy-max Extra MCE had the most plastic consistency, while cheeses produced with Chy-max Supreme MCE had the hardest and most elastic one. The consistency of cheeses made with Chy-max M MCE was distinctly harder than that of cheeses produced with Chy-max Extra MCE, but less hard than that of cheeses made with Chy-max Supreme. Comparison of cheeses produced with the same type of the MCE suggests that cheeses produced with a higher dose of the MCE had the denser, thicker consistency than that of cheeses produced with a lower dose of the MCE. The sensory assessment findings obtained for cheeses were in line with their rheological properties.

## 4. Conclusions

Differences in the type (bovine chymosin (Chy-max Extra MCE), camel chymosin (Chy-max M MCE), modified camel chymosin (Chy-max Supreme MCE)) and the dose (1500, 2500 or 3500 IMCU/100 kg of milk) of MCEs used in production of cheeses led to the significant differences ($p < 0.05$) in the behavior pattern of changes in cheese proteolysis and rheological properties during storage.

The proteolysis degree of cheeses was in direct proportion to the number of PA units added into milk for cheese processing together with a MCE. With an equal dose of the MCEs added into milk, the highest level of proteolysis was noted in the cheese options produced with recombinant bovine chymosin (Chy-max Extra MCE). There were no statistically significant differences ($p > 0.05$) in the degree of proteolysis between the cheese options produced with the same doses of the camel chymosin (Chy-max M MCE) and modified chymosin (Chy-max Supreme MCE).

With the equal doses of MCEs, the cheeses produced with Chy-max Supreme MCE had a higher G* level by the end of the storage time (21 days) than that of the cheeses produced with Chy-max Extra or Chy-max M MCEs. There were no significant differences ($p > 0.05$) in G* values between cheeses produced with the same doses of Chy-max Extra and Chy-max M MCEs throughout the entire storage time.

Microscopic findings confirmed the mechanism of effect exerted by proteolysis on the rheological properties and cheese consistency that was described in the scientific literature. Proteolysis leads to a degradation of the protein matrix that forms a bearing frame of the cheese. An increased degree of proteolysis is associated with the intensified destruction of the protein network; as a result, the elastic properties of the cheese mass characteristic of fresh cheeses decrease, and the plastic properties of the cheese mass typical of ripened cheeses develop. The increased plastic properties of cheeses during storage resulted in an increased loss tangent (tan δ).

By comparing the dependences of the pH level on the moisture content in cheese and of the moisture content in cheese on the type and dose of MCEs used in the production of cheese, a processing method was formulated that allows the manufacturer to adjust the cheese consistency by selecting the type and dose of a MCE. The use of a MCE with a low level of PA (Chy-max M or Chy-max Supreme) at an increased dose (3500 IMCU/100 kg) yields cheeses with a higher dry matter content and a higher pH level. Such cheeses have a dense consistency that changes only slightly by the end of the cheese shelf life.

Recombinant MCEs based on camel chymosin (Chy-max M) and modified chymosin (Chy-max Supreme) which have a low PA level and exert a low proteolysis level may be recommended for use in production of soft cheeses to slow-down the plasticization rate of cheese consistency and hence to increase their shelf life.

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