DIFFERENT APPROACHES IN ANALYZING CHYMOSIN PURITY

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ABSTRACT

Chymosin is a specific proteolytic enzyme found in rennet, and is the key enzyme in cheese production classified in the aspartic endopeptidases (EC 3.4.23.4). The aim of this study was to determine the purity of different commercially available chymosins and its equivalents using electrophoretic and chromatographic techniques. Chymosins produced by the company Chr. Hansen, CHY-MAX 200 and CHY-MAX Plus, CHY-MAX PowderExtra NB, as well as Maxiren 1800 Granulate from the company DSM, Sirnik from SZR – Travnik, Kraljevo and Planika from Mikroprocessing, Bileca were used as materials for this study. The purity level of the commercially available enzymes was analyzed using electrophoretic (sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE) and chromatographic (Rapid Resolution Liquid Chromatography or RRLC) techniques. Results showed no presence of undeclared protein fractions due to inappropriate purification process in the samples except for CHY-MAX M 200 which had two protein fractions, most likely as a result of a polymorphism. All the CHY-MAX and Maxiren samples have chymosin as the active component (36 kDa), except for Planika and Sirnik which have a natural protease from *R. miehei*. Chromatographic analysis showed that beside the active component (chymosin), the preservative sodium benzoate was present in varying concentrations in all but CHY-MAX PowderExtra NB.

Keywords: chymosin, purity, chromatography, electrophoresis.

INTRODUCTION

Cheese production is one of the first examples of biotechnological enzyme uses (Szecsi, 1992). It is assumed that the firstly created cheese was an unexpected result of transporting and/or storing milk in the stomachs of animals, especially young ruminants (Tamime, 1993). With the development of mankind, the cause of milk coagulation was found to be the presence of the proteolytic enzymes chymosin and pepsin and this enzyme complex got the name rennet (Fruton, 2002). As time from the discovery of cheese passed, its popularity and demand were constantly growing, which led to the shortage of chymosin. This shortage made man look for other alternative enzymes that will have the ability to clot milk.

The first alternative enzymes for clotting milk came from plants like *Malva sylvestris*, *Capparis spinosa*, *Urtica dioica* and *Cynara scolymus* (Fox et al., 2004). Beside the plant alternatives, the development of microbiology allowed for the selection and usage of microorganisms (mainly fungi) which contain natural proteases that could cause coagulation of the milk as well. The most widely used microbial proteases come from the fungus *Rhizomucor miehei*. The common problem caused by using natural rennet, plant and microbial proteases for cheese productions is their unspecific proteolytic activity that causes bitter tastes in the ripened cheeses to occur. Also another negative side of these enzymes is the lower yield (randman) of cheese during production (Agboola et al., 2004).

Chymosin (36 kDa) which is naturally found in rennet, is a very specific proteolytic enzyme that recognizes and cleaves the amino acid sequence of κ-casein between the 105^{th} and 106^{th} amino acid. This cleavage causes the other milk proteins (αs1, αs2 and β-casein) to be exposed to the calcium ions in the milk, and they create insoluble complex called calcium paracaseinate, which is in fact cheese (Gastaldi et al., 2003).

Today, due to the development of genetic engineering the chymosin gene is isolated and cloned into microorganisms like bacteria (*Escherichia coli*), yeast (*Kluyveromyces lactis*) and mold (*Aspergilus niger*) who later serve as living factories for chymosin production (El-SohaimyElsayed et al., 2010; Pfizer central research 1988., Groton, CT, USA, 1988; Weslowski-Louvel et al., 1996).

Enzyme purity is a key factor in cheese production, because if it isn't proper, it can have very negative implications on the final cheese product. The improper purification techniques can result in the presence of other unwanted enzymes which may have nonspecific proteolytic activities that cause bitter tastes to occur in

the cheese. The presence of other organic or nonorganic compounds such as additives or preservatives can lead to false information about the enzyme activity or can have inhibiting effect on the milk clotting enzyme - chymosin. This would make them unsuitable for the production of ripened cheeses and will result in lower yield of cheese. It is the reason why we decided to estimate the purity of different commercial products containing chymosin, present at the market of Western Balkan countries.

MATERIAL AND METHODS

Material

The commercial solutions containing enzymes used in this study were as follow: CHY-MAX® M 200 which contains recombinant camel chymosin and CHY-MAX® Plus and CHY-MAX® Powder Extra which contain recombinant bovine chymosin from the company Chr. Hansen; Maxiren® 1800 granulate which contains recombinant bovine chymosinfrom the company DSM. Beside these internationally recognizable brands, the chymosin solutions Sirnik from SZR – Travnik, Kraljevo and Planika from Mikroprocessing, Bilekawhich are chymosin analogs from microbial origin (*Rhizomucormiehei*) were used as well.

Methods

The purity level analysis of the commercial enzymes was done using electrophoretic and chromatographic techniques.

Electrophoresis

Electrophoresis is a commonly used technique in which charged molecules (in this case proteins) are separated using homogenous electrical field. In this study, sodium dodecyl sulfate polyacrylamide gel electrophoresis or SDS-PAGE with 12,5 % resolving gels was used. SDS-PAGE is a denaturing type of electrophoresis because it uses β-mercaptoethanol that reduces all the disulfide bonds in the protein structure, while the sodium dodecyl sulfate bonds to all the protein regions, breaks all the non-covalent bonds and gives the proteins a negative charge.

The liquid samples: CHY-MAX® M 200, CHY-MAX® Plus, Sirnik and Planikawere treated with equal amount of 2X reducing buffer, and the solid samples: CHY-MAX® Powder Extra and Maxiren® 1800 granulate were firstly dissolved in distilled and deionized water (10 mg sample in 1 mL water), and after that were treated with the same amount of 2X reducing buffer. All the treated samples were than boiled at 100 °C for 4 minutes in order to achieve full denaturation. For the electrophoretic analysis two protein standards were used: high range (220-53 kDa) and full range (245-5 kDa). The gels were prepared following the laboratory manual for SDS-PAGE reagents and gel formulas (Wick, 2010).

Chromatography

The chromatographic methods are one of the most powerful and widely used analytical methods. They can be used for separation, identification and purification of different chemical compounds in complex mixtures. The high performance liquid chromatography (HPLC) is the most widely used type of chromatography because of its many advantages like much shorter run time and high accuracy. Rapid resolution liquid chromatography (RRLC) was used for this study.

The chromatographic analyses were performed on Agilent 1260 Infinity Rapid Resolution chromatograph, using Poroshell 120 EC-C18 (50 mm x 3 mm; 2,7μm). The solid state samples were prepared by measuring 0,020 g of CHY-MAX® Powder Extra and 0,018g of Maxiren® 1800 granulate and dissolving them each in 10 mL 0,01 % HCOOH. The liquid samples were prepared by adding 200 μL from CHY-MAX® M 200 and CHY-MAX® Plus and 1.5 mL from Sirnik and Planika and adding them in 10 mL 0.01 % HCOOH. The samples Sirnik and Planika were additionally diluted 4 times by taking 100 μL of the first dilution and adding 300 μL of 0.001 % HCOOH. The sample elution was done using a mobile phase made of 0.01 % HCOOH and CH_3CN (90/10; *V/V*).

RESULTS

Electrophoretic results

In this study 12.5 % SDS-PAGE was used, for which the running conditions for protein samples (applied sample amount and current intensity) were optimized.

The first electrophoretic gel (Fig. 1) contains the samples CHY-MAX PowderExtra NB 1 % (10 mg/mL), Maxiren 1800 Granulate 1 % (10 mg/mL), CHY-MAX Plus, CHY-MAX M 200, Sirnik and Planika each with applied amount of 15 μL. The duration of the electrophoresis was 1 hour and 45 minutes, using 100 V and 52 mA.

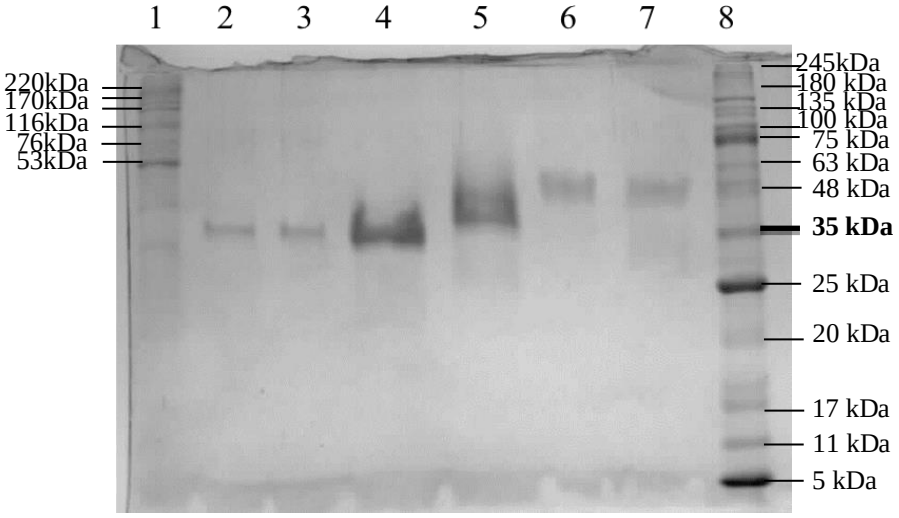

Figure 1. 12.5 % SDS-PAGE analysis: 1 - High range protein standard 2.5µL; 2 - CHY-MAX PowderExtra NB 1 % 15 µL; 3 –Maxiren 1800 Granulate 1 % 15 µL; 4- CHY-MAX Plus 15 µL; 5- CHY-MAX M 200 15 µL; 6- Sirnik 15 µL; 7- Planika 15 µL; and 8 – Full range protein standard 2.5 µL

The second electrophoretic gel (Fig. 2) shows the samples CHY-MAX PowderExtra NB 1 % (10 mg/mL), Maxiren 1800 Granulate 1 % (10 mg/mL), CHY-MAX Plus, CHY-MAX M 200 with applied amount of 15 µL and Sirnik and Planika with applied amount of 10 µL. The electrophoresis was performed under the same conditions as described previously.

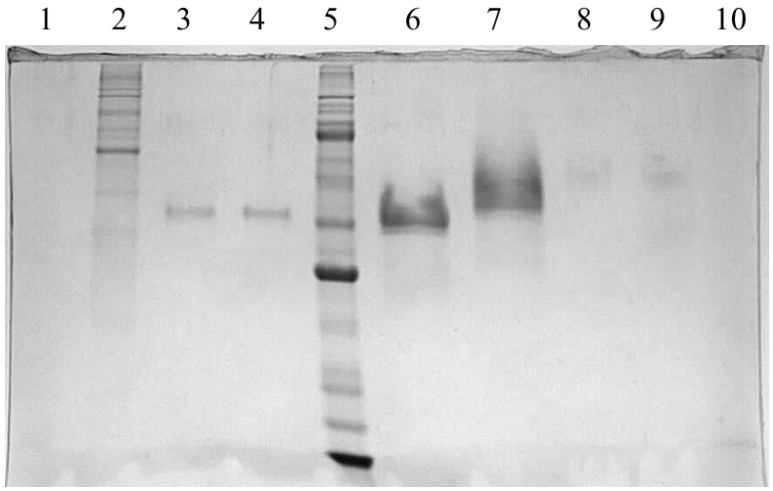

Figure 2. 12.5 % SDS-PAGE analysis: 2 - High range protein standard 2.5 µL; 3 - CHY-MAX PowderExtra NB 1 % 15 µL; 4 – Maxiren1800 Granulate 1 % 15 µL; 5 - Full range protein standard 2.5 µL; 6 - CHY-MAX Plus 15 µL; 7 - CHY-MAX M 200 15 µL; 8 - Sirnik 10 µL; 9 - Planika 10 µL

On a figure 3 is shown the electrophoresis gel with the samples in different concentrations as follow: CHY-MAX PowderExtra NB 5 % (50 mg/mL), Maxiren 1800 Granulate 5 % (50 mg/mL), CHY-MAX Plus, Sirnik and Planika apllied in 15 µL each, while the sample CHY-MAX M 200 is applied with 15 and 10 µL. The analysis runtime was 2 hours, on 100 V and 82 mA.

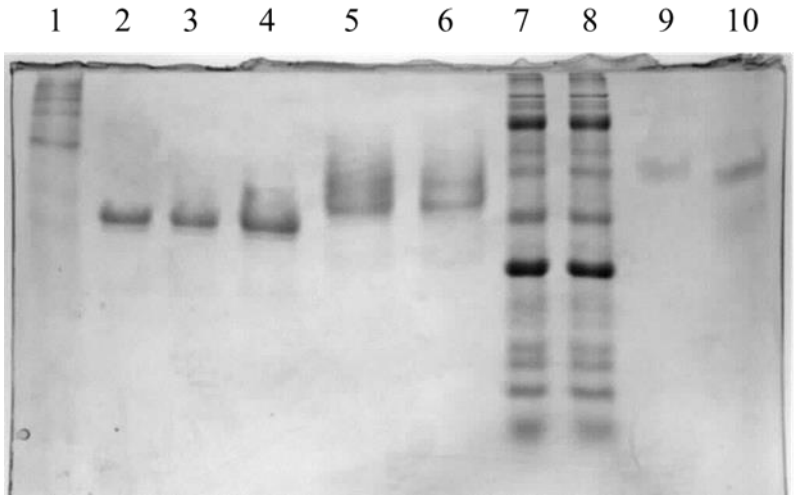

Figure 3. 12.5 % SDS-PAGE analysis: 1 - High range protein standard 2.5 µL; 2 - CHY-MAX PowderExtra NB 5 % 15 µL; 3 - Maxiren1800 Granulate 5 % 15 µL; 4- CHY-MAX Plus 15 µL; 5 - CHY-MAX M 200 15 µL; 6 - CHY-MAX M 200 10 µL; 7 - Full range protein standard 2.5 µL; 8- Full range protein standard 2.5 µL; 9 - Sirnik 15 µL; 10 - Planika 15 µL

On the gel in Figure 4 were run the following samples: CHY-MAX PowderExtra NB 5 % (50 mg/mL), Maxiren 1800 Granulate 5 % (50 mg/mL), CHY-MAX Plus и CHY-MAX M 200 applied in lower amounts in order to get a better resolution of the protein fractions. The duration of the electrophoresis was 2 hours using the electricity with 100 V and 82 mA.

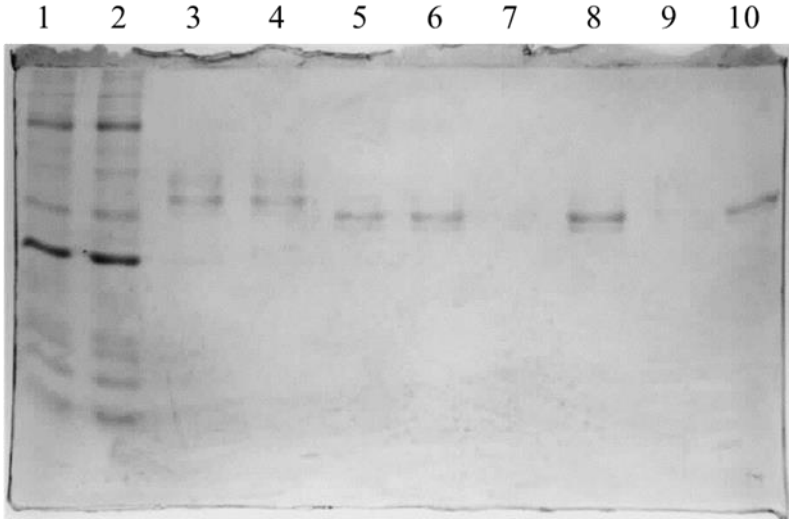

Figure 4. 12.5 % SDS-PAGE analysis: 1 - Full range protein standard 1.5 µL; 2- Full range protein standard 1.5 µL; 3 - CHY-MAX M 200 2.5 µL; 4 - CHY-MAX M 200 2.5 µL; 5 - CHY-MAX Plus 2.5 µL; 6 - CHY-MAX Plus 2.5 µL; 8 - CHY-MAX PowderExtra NB 5 % 2.5 µL; 10 - Maxiren 1800 Granulate 5 % 2.5 µL

Chromatography results

Reversed-phase Rapid resolution liquid chromatography was performed for this study, using the optimal conditions (temperature, flow, mobile phase) for the samples which were previously determined. The results from the RRLC method are shown in Figure 5.

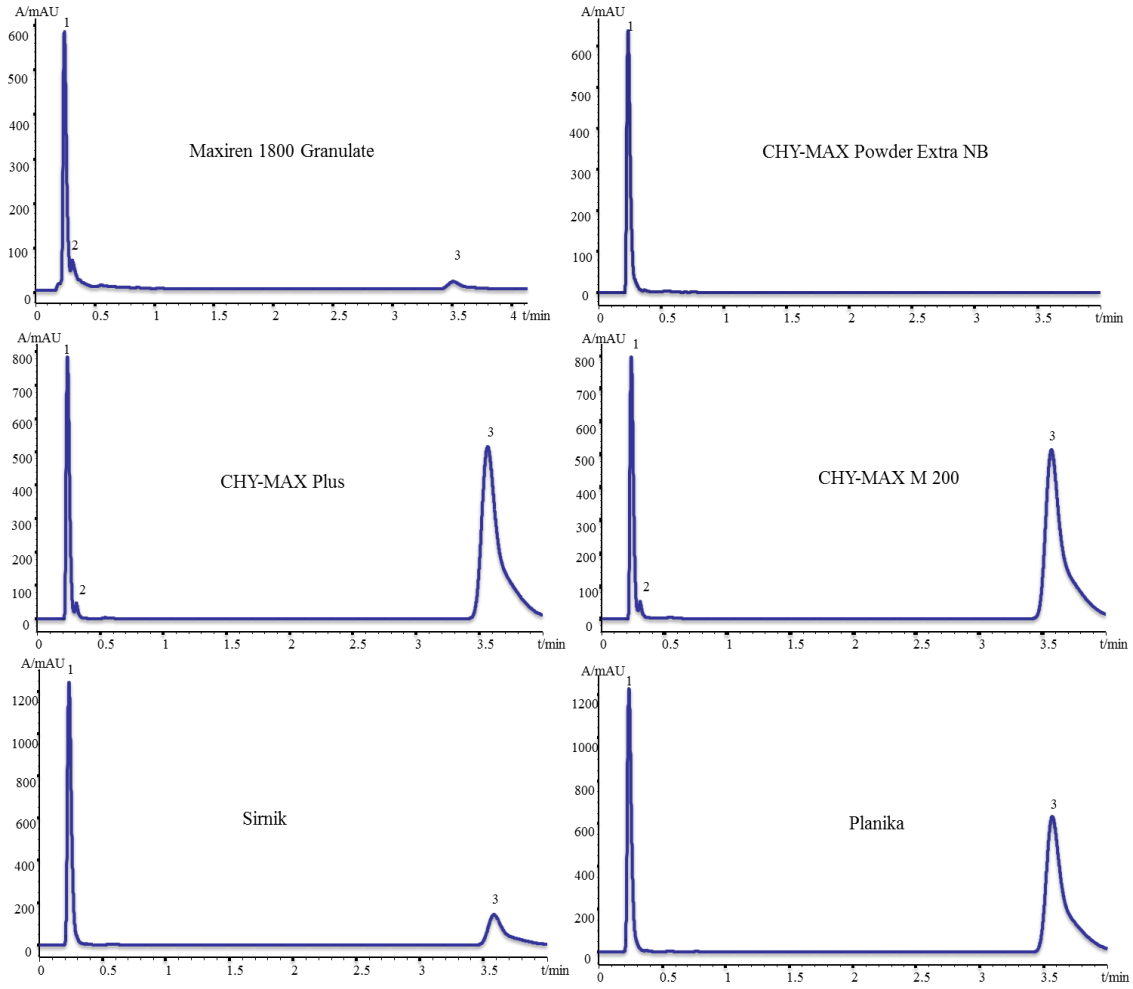

Figure 5. Chromatograms of the analyzed samples using Poroshell 120 EC-C18 (50 mm x 3 mm; 2,7µm) column, mobile phase 0.01 % HCOOH with water/CH_3CN (90/10;*V/V*), flow rate of 1 mL/min, constant column temperature of 25 °C, UV-detection at 195 nm

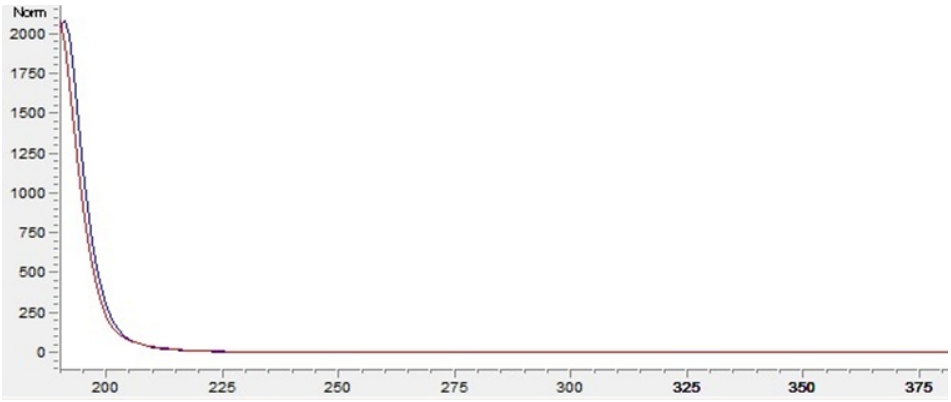

Figure 6. Overlaid spectra of peek 1 from all samples

DISCUSSION

The electrophoretic analyzes (Figure 1, 2, 3 and 4) showed that bovine chymosin presence (36 kDa) is evident in the samples from CHY-MAX Powder Extra NB, CHY-MAX Plus and Maxiren 1800 Granulate when compared to the 35 kDa fraction found in the full range protein standard. The samples from the commercial enzyme CHY-MAX M 200 contain two protein fractions with similar molecular weight, both larger than the bovine chymosin. In order to get better visualization (resolution) of the two fractions, the CHY-MAX M 200 samples were applied in lower concentrations which achieved the wanted effect (Figure

4). Findings by other authors such as Attalah (2007), show the presence of more than one protein fractions in samples containing bovine chymosin. The samples from Sirnik and Planika contain the largest protein fraction among all commercial enzymes, which is a less specific protease from the fungus *R. miehei* (Figure 1, 2 and 3). Due to the lower specificity of the enzyme found in the samples from Sirnik and Planika its concentration is lower compared to the other commercial enzymes in order to compensate the unwanted effect it can have on the cheese making and maturing process.

The chromatographic results (Figure 5) show that all commercial enzymes contain one well formed, symmetrical, narrow and high peak with retention time of 0.238 min (peak 1). Overlapping of the UV spectra of peak 1 and the obtained value for the match factor value (> 990) shown in Figure 6, confirmed the presence of a same component, which matches the active component on all the enzyme declarations. The chromatographic results showed the presence of the preservative sodium benzoate (peak 3) in CHY-MAX Plus, CHY-MAX M 200, Planika, Sirnik and Maxiren 1800 Granulate. The only enzyme that did not have sodium benzoate presence declared was Maxiren 1800 Granulate, most likely due to its low concentration. The highest concentration of sodium benzoate was found in Planika, while the lowest in Maxiren 1800 Granulate. Sodium benzoate was completely absent in CHY-MAX Powder Extra NB.

CONCLUSIONS

The commercial enzymes used in this study did not contain any undeclared protein fractions that could be result of inadequate purification process, with exception of the camel chymosin (CHY-MAX M 200). It contained two protein fractions most likely as a result of a polymorphism taken the protein size differences in account.

Related to the active component in the commercial enzymes, the presence of chymosin (36 kDa) was confirmed in CHY-MAX Powder Extra NB, Maxiren 1800 Granulate, CHY-MAX Plus and CHY-MAX M 200, while the samples Sirnik and Planika contained a protease with a larger molecular size that originates from the fungus *R. miehei*.

All the commercial protein samples contained the preservative sodium benzoate in varying concentrations, except CHY-MAX Powder Extra NB which has a declared absence of them. Sodium benzoate was not declared only in Maxiren 1800 granulate, probably due to its low concentration.

In this study, the results from the electrophoretic and chromatographic methods gave matching results in relation of the enzyme purity, *i.e.* the enzyme declared purity was confirmed.

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