STUDY OF TECHNOLOGICAL PROPERTIES OF MILK-CLOTTING ENZYME FROM IRPEX LACTEUS (*Irpex lacteus* (Fr.) Fr.)

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Abstract: Due to the scarcity of natural rennet, in this study we have considered an option of using in cheesemaking a milk-clotting enzyme produced by the *Irpex lacteus* fungus. We describe main properties of *I. lacteus* coagulant: milk-clotting activity (MA), overall proteolytic activity (PA), thermal stability, MA dependence on pH level and calcium ions content. Partially purified preparations of *I. lacteus* milk-clotting enzyme was obtained by salting out and gel-filtration. Technological properties of the *I. lacteus* coagulant were compared to natural calf rennet and cow pepsin (CP). MA of *I. lacteus* enzyme amounted to 29.1 ± 0.7 RU/ml, with protein content of 23 mg/ml. The coagulant was completely inactivated at 60°C. Thermal stability of *I. lacteus* milk-clotting enzyme, MA sensitivity to pH variations and Ca^{2+} content were comparable to respective parameters of calf rennet and CP. Overall PA of the *I. lacteus* coagulant exceeded CP and calf rennet activity by 33 and 220 times, respectively. As for enzymatic specificity, the following order was observed: calf rennet (100%) > CP (14.9%) > *I. lacteus* coagulant (0.5%). These findings suggest that there is a need to increase the MA of *I. lacteus* coagulant in order to be able to use it in cheesemaking. We have considered chemical, biochemical, and genetic corrective actions applicable to technological properties of microfungal milk coagulants.

Keywords: milk-clotting enzymes, rennet, rennet substitutes, microfungal coagulants, milk-clotting activity, proteolytic activity, thermal stability, cheesemaking, mucorpepsins

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INTRODUCTION

A key step in cheesemaking is milk coagulation and obtaining a milk clot. This was conventionally achieved with rennet: a complex preparation from abomasums of nursing calves and lambs. Rennet contains two milk-clotting enzymes (ME): chymosin (EC 3.4.23.4) and pepsin (EC 3.4.23.1). Chymosin is considered to be the standard enzyme for cheesemaking, due to the combination of its process-related properties: high specificity for cleavage the Phe_{105}-Met_{106} bond in κ-casein molecules, milk-clotting activity (MA) within the mild acidic pH range, low overall proteolytic activity (PA), and thermal lability. Many prokaryotic and eukaryotic proteases demonstrate MA, however, due to the high level of nonspecific proteolysis and thermal stability, that reduce product output and result in cheese taste and texture defects, these are not used in cheesemaking, or else their application is very limited.

In the second part of the XX century, cheesemaking industry faced a challenge of rennet deficit [1], as volumes of rennet-based cheese production kept growing, causing a severe shortage of calf and lamb abomasums [2–4]. This deficit initiated a search for rennet substitutes, but a valid equivalent of cow chymosin was not to be found. In 1990–2006, the problem of rennet shortage was partially resolved when recombinant chymosin (rCh) of cow [5] and camel [6] were developed, however, there are some factors that prevent their wide application. In a number of countries, use of rCh is limited in production of cheese subject to national certification of origin. Besides, a certain customer segment is opting for cheeses produced with natural rennet, not a recombinant one. In the end of the 20th century, the problem of rennet deficiency was exacerbated due to a scrapie epidemic: a prion cattle disease [7]. This epidemic devastated the feedstock of natural ME and triggered a new – and

currently ongoing – phase in the search for rennet substitutes among animal, plant and microbial milk-clotting proteases [2, 3, 8–14]. This search is aimed at the diversification of feedstock in natural milk coagulants, and the selection of enzymes that might be adapted for cheesemaking after certain chemical, biochemical, or genetic "adjustment".

Due to the rennet shortage, starting from the 1970s, modern cheesemaking uses microfungal coagulants: mucorpepsin (E.C.3.4.23.23) and endotiapepsin (EC 3.4.23.22): aspartic proteinases *Rhizomucor miehei* (Cooney & R. Emers.) Schipper (*less often Rhizomucor pusillus* (Lindt) Schipper) and *Cryphonectria parasitica* (Murrill) M.E. Barr (synonym: *Endothia parasitica* (Murrill) P.J. Anderson & H.W. Anderson). Microfungal coagulants have the following advantages: low cost of production, compliance with natural origin criteria and with vegetarian requirements, conformity with kosher and halal eating principle. Their main disadvantages are: low specificity (MA/PA ratio), and high thermal stability [2].

Object of this study is a fungus *Irpex lacteus* (synonym: *Polyporus tulipiferae* (Schwein.) Overh.), which belongs to higher basidiomycetes and is known to produce milk-clotting protease [15]. Milk coagulant, produced by *I. lacteus*, is registered in the IUBMB enzyme nomenclature database as polyporopepsin (EC 3.4.23.29), which is related to the aspartic endopeptidases family (the family of chymosin and pepsin A).

Data on process-related properties of *I. lacteus* coagulant are scarce and fragmented. We should mention a series of studies by K. Murakami et al. [15–18] that presented a partial description of the enzyme and obtained its kDNA sequence, and a number of works carried out in the Donetsk National University (Ukraine) on some aspects of the *I. lacteus* strain productivity, and studies of thermal stability of crude milk-clotting preparations of this fungus [19–21]. Nowadays, *I. lacteus* coagulant is not used in dairy industry as a rennet substitute. Meanwhile, biochemical properties of polyporopepsin indicate that its derivatives might be successfully used in cheesemaking.

The objective of this work is to diversify the feedstock of natural milk-clotting enzymes (ME). The ensuing task of this study is to assess process-related properties of the milk-clotting enzyme derived from the *I. lacteus* basidial fungus, from the point of view of potential applicability in cheesemaking.

OBJECTS AND METHODS OF STUDY

A pure culture of the *I. lacteus* fungus was derived from a spore print in the Republic of Altai (Russian Federation) in 2008. The fungus strain No. 2265 is stored in the Collection of Basidiomycetes Cultures in the Botanical institute named after V.L. Komarov (Saint-Petersburg), and in the Mycology Laboratory of the State Research Center of Virology and Biotechnology VECTOR, where the fungal biomass for this study was produced. Cultivation was performed in 0.75 l flasks, with 0.15 l of tryptone glucose nutrient medium (pH 6.0), for 7 days, in temperature-controlled shaking baths at $26 \pm 2^{\circ}$C. Liquid culture used for inoculation was obtained by fungal cultivation on a liquid medium inoculated with culture blocks grown on agar medium. Fungal biomass suspension was frozen at -30 ± 2°C [22].

Samples of milk-clotting enzyme were obtained in the nucleic acids and recombinant proteins laboratory in the Medical Biotechnology Institute of the State Research Center of Virology and Biotechnology VECTOR. Frozen *I. lacteus* biomass suspension (~850 ml) was thawed in a funnel with gauze filter. The filtrate was made up with dry ammonium sulfate (AS) to the final 70% saturation, stirred, and then left to sediment at 4°C overnight. The mixture was centrifuged at 8000g and 4°C for 15 minutes. The supernatant was rejected, and the sediment dissolved in approx. 25 ml of distilled water, and then desalted by gel-filtration on Sephadex G-25 column (gel volume – 60 ml.). Six fractions of desalted protein material (15 ml each) was obtained, these were combined into two samples: A (fr. 1–3) and B (fr. 4–6). A and B samples were sedimented again with AS at 70% saturation, precipitate dissolved in 20 ml of distilled water, and dialysed for 8 hours against two 1 l charges of 20 mM Tris-HCl, 50mM NaCl (pH 7.5). Total protein level in A and B samples was determined by the Lowry method. A and B samples were poured in 1 ml microflasks and lyophilized.

Technological properties of A and B samples were assessed in the biochemical laboratory of the FSBSI Siberian Scientific Research Institute of Cheesemaking. Milk-clotting activity, overall PA, thermal stability, MA dependence on pH and Ca^{2+} concentration were assessed in accordance with the previously published techniques [14, 23]. Along with the *I. lacteus* coagulant samples, we also studied natural ME of animal origin: an industrial control sample of calf rennet (ICS CR) with approx. 80% of chymosin content, and 100% cow pepsin (CP). Standard milk substrate, ICS CR, and CP were provided as a courtesy of the "Moscow Rennet Plant" JSC.

Milk clotting activity was assessed by mixing solutions of dry standard substrate (DSS) and test ME heated up to 35°C, mix ratio 10 : 1. The result was expressed in reference units (RU), with MA-certified ICS CR used as reference.

For assessment the overall PA, we used Hammersten grade casein solution (1%) as a substrate in 20 mM sodium phosphate buffer (pH = 5.6). ICS CR and CP activities were normalized as per MA demonstrated by the *I. lacteus* coagulant. Test ME were introduced in the substrate solution at 1 : 4 mix ratio and incubated at 35°C for 6, 60, 90, 180 minutes. The reaction was interrupted by adding trichroloacetic acid (5%), then the samples were filtered, and the resulting filtrate was assessed for optical density at $\lambda = 280$ nm (D_{280}). Specificity was determined as MA/PA ratio. PA was assumed as D_{280} of samples incubated for 180 minutes.

Thermal stability. Aliquots of milk-clotting enzymes were heated up for 30 minutes within the temperature range of 30–70°C, and then assessed for

residual MA. MA values obtained in the samples heated at 30°C were assumed to be 100%.

DSS samples with pH 5.5, 6.0, 6.5, 7.0 were prepared, and their coagulum formation time was measured, in order to determine MA dependence on the pH level. Clotting time at pH 5.5 was assumed to be 100%.

MA dependence on Ca^{2+} content was determined by introducing $CaCl_2$ into the DSS samples, making up to the 1–5 mM concentration; clot formation time was then measured. Values obtained in $CaCl_2$-free samples were assumed as 100%.

RESULTS AND DISCUSSION

Protein concentration in A and B samples amounted to 23.6 mg/ml and 29.0 mg/ml, respectively.

Milk-clotting activity. Specific MA of A sample was 29.1 ± 0.7 RU/ml, which amounted to 1230 ± 30 RU/g in protein equivalent. Sample B demonstrated residual MA (< 1 RU/ml) and was removed from further assessment.

Activity of dry commercial milk-clotting enzymes used in cheesemaking amounts to 100–200x10^3 RU/g, of liquid agents, 50–100x10^3 RU/ml. Thus, MA found in sample A is about 10^3 times lower than the required value for commercial ME. Low MA values might result from low productivity of the *I. lacteus* strain 2265, suboptimal cultivation conditions, inadequate purification of the milk-clotting enzyme, and/or presence of inhibitors.

Yet another possible explanation is partial inactivation of the coagulant in the very process of production, for example, due to the pH level of the final buffer (pH 7.5), which exceeds the pH stability limits for polyporopepsin (pH 3–6). Pepsin A, belonging to the same aspartic proteinases family as polyporopepsin, is known to be irreversibly inactivated at pH > 7.0.

MA efficiency is considerably affected by *I. lacteus* strain selection and its cultivation conditions [20]. S.M. Boyko and I.N. Ivanov used a specially selected and highly productive strain of *I. lacteus* Fr., BN-3 (stored in a specialized collection of cultures at the Institute of Botany named after N.G. Kholodniy NAN of Ukraine, Kiev) and, as they modified the surface conditions for fungus cultivation, obtained a cultural filtrate with overall MA of approx. 600 RU/ml, and with specific activity of 186 RU/mg. In deep cultivation of the BN-3 strain, activity of the cultural filtrate increased up to 900 RU/ml. After partial purification of the coagulant, the authors obtained a preparation with specific MA of 1463 RU per 1 mg of protein [21], which fully complies with the activity requirements for dry commercial ME.

Overall proteolytic activity. High overall proteolytic activity of a ME is an extremely negative factor for cheesemaking. Non-specific proteolytic activity of a ME results in considerable losses of proteolysis products with whey, and cheese output is significantly reduced. Coagulants of *R. miehei*, *R. pusillus* and *C. parasitica* might result in 0.5–1.2% decrease in cheese output in comparison to calf rennet [9]. If the remaining within granular curds milk-clotting enzyme is not inactivated by heat treatment, then cheese with a long ripening process and shelf life shall develop texture and taste defects (bitterness). An active proteolytic enzyme with low specificity affects process-related properties of cheese whey, which is used as feedstock for various dairy products and derivatives [3, 13].

The *I. lacteus* milk coagulant has high PA (Fig. 1a). Overall PA of the *I. lacteus* coagulant exceeds this parameter in ICS CR by approx. 220 times, and in CP, by approx. 33 times. Proteolytic activity of the *I. lacteus* coagulant is so much above the control enzymes values that within the scale of Fig. 1a, the curves built for ICS CR and CP almost merge with the horizontal axis. In order to demonstrate differences between ICS CR and CP, we provided a bigger scale graph (Fig. 1b).

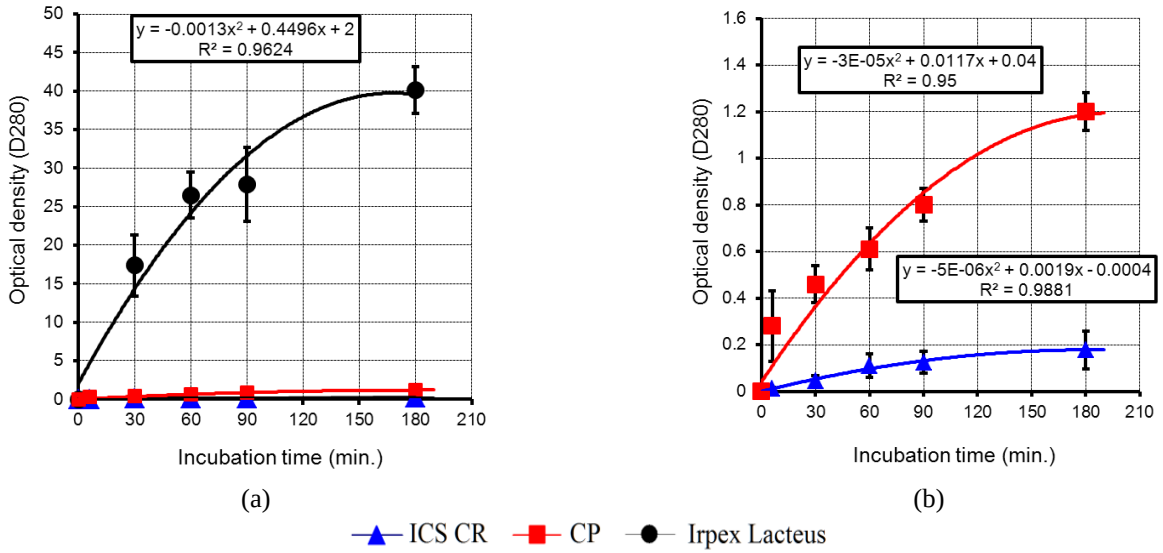

Fig. 1. Assessment of overall proteolytic activity in milk-clotting enzymes: (a) Overall proteolytic activity of *I. lacteus* coagulant, ICS CR, and CP; (b) Overall proteolytic activity of ICS CR and CP. (Please note: in Fig. a and b, Y-axis scales are different). Text boxes show equations for polynomial trend lines (with factor 2) and approximation probability (R^2).

Position of microfungal coagulants among ME for commercial use is determined by M. Harboe et al. [3] classification, which suggests that, along with the specificity decrease (calculated as MA/PA ratio), milk-clotting enzymes are arranges as follows: rCh (camel) > rCh (cow), calf chymosin > cow pepsin > mucorpepsin XL > mucorpepsin L > endotiapepsin. We have performed a similar arrangement, assuming the MA/PA ratio of calf rennet as 100%. We therefore obtained the following sequence aligned with specificity decrease: ICS CR (100.0%) > CP (14.9%) > *I. lacteus* coagulant (0.5%).

One of the possible causes accounting for low specificity of the *I. lacteus* coagulant might be its insufficient purification. Ammonium sulfate in 70% saturation sediments the milk-clotting enzyme together with most proteins present in the cultural liquid. "Ballast" proteolytic enzymes without MA or inhibitors of specific clotting activity are also likely to be present. Desalting allows to eliminate electrolytes and low molecular weight organics, however, it does not provide considerable purification of the resulting enzyme. MA of the *I. lacteus* might have probably been affected by a high pH level of the final buffer. We suggest that, once the purification procedure is improved and more homogeneous products are obtained, we might achieve certain increase in MA and specificity of the *I. lacteus* coagulant, which would allow for its application in cheesemaking. This hypothesis is based on data reported in literature. In 1988–1989, K. Murakami et al. reported a series of experiments on using the *I. lacteus* milk-clotting enzyme in production of soft fibrous cheese without ripening, and also such varieties as Gouda and Cheddar [16–18]. However, these works were discontinued, and the practical results of using the *I. lacteus* coagulant in cheesemaking were never confirmed by other studies.

Based on the collected data, we can conclude that cheesemaking applications for the *Irpex lacteus* milk-clotting enzyme preparations seems unlikely, unless its MA and MA/PA ratio are increased.

MA dependence on calcium ions content in the substrate. In native milk, due to exposed carboxyl and phosphate groups, the surface of casein micelles is negatively charged, which prevents them from binding and provides for aggregation stability of the colloidal system. During pasteurization process, calcium salts present in milk partially convert into insoluble state. This prolongs the duration of rennet coagulation, and results in formation of a flaccid milk clot. In order to compensate for the reduced Ca^{2+} content after pasteurization, $CaCl_2$ in concentration of 0.2–0.5 g/l is introduced into the milk mixture prepared for coagulation. Introduced $CaCl_2$ produces a two-fold effect. First, in presence of Ca^{2+}, negative charge from the surface of casein micelles is partially removed, and K-caseins, located in the "hairy" layer become more available for attacks of milk-clotting enzymes. Second, Ca^{2+} participates in formation of ion "bridges" between micelles during their aggregation phase. As a result, milk clotting time decreases.

Increased $CaCl_2$ content does not only lead to a higher MA, but to a higher overall PA of the enzyme as well. Excessive addition of $CaCl_2$ to the milk mixture might develop in cheese certain texture and taste defects. Therefore, cheesemakers try to stick to the minimum $CaCl_2$ concentration. Low sensitivity to Ca^{2+} content in the milk mixture is a valuable factor, because it provides an opportunity to vary the introduced $CaCl_2$ concentration, without worrying too much about significant MA and PA changes. Whenever preparations with high sensitivity to Ca^{2+} are used, the effect of MA and PA increase should be accounted for.

In comparison to natural ME of animal origin, clotting activity of the *I. lacteus* preparation is the least dependent on the Ca^{2+} content in the DSS (Fig. 2).

At $CaCl_2$ 3 mM (most common concentration in cheesemaking), clotting activity of the *I. lacteus* preparation is increased by 27%, and respective activities of ICS CR and CP, by 55% and 70%, respectively. The biggest differences in clotting time decrease are observed when $CaCl_2$ is increased from 0 to 3 mM. Within the range of $CaCl_2$ 3–5 mM, MA dynamics (inclination angles of all curves) is similar in all tested enzymes.

It should be remembered that ICS CR sensitivity to Ca^{2+} content is partially determined by 20% pepsin admixture. In modern high-quality natural rennet preparations for commercial use, chymosin content reaches up to 96%. Along with the pepsin percentage decrease, rennet sensitivity to Ca^{2+} shall diminish as well and approach the respective values of the *I. lacteus* coagulant.

These findings allow us to conclude that the *I. lacteus* coagulant sensitivity to Ca^{2+} ions content is in conformity with the cheesemaking requirements.

Thermal stability. In cheesemaking, thermal stability of the milk-clotting enzyme is one of the regulating factors for proteolysis intensity and specificity during cheese ripening. The substrate for ME remaining in cheese mass are α- and β-caseins. Degree of enzymatic hydrolysis of these proteins determines the level of non-specific proteolysis which, in turn, influences the ripening time, physical, chemical and organoleptic properties of cheese. All this determines the tactics for practical use of ME with different thermal stability.

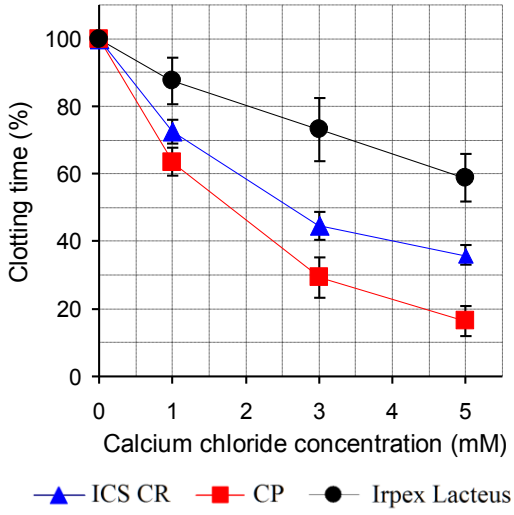

Fig. 2. Dependence of clotting time on $CaCl_2$ content.

Thermolabile enzymes are preferably used in production of hard and semi-hard cheese varieties, with high second heating temperature (52–58°C), and lengthy ripening duration and extended storage time. According to a widely applied cheesemaking practice, cheese is produced in summer, in presence of a thermolabile ME which is completely inactivated by high temperature during the second heating. This leads to a reduced proteolysis rate during cheese ripening and storage. Products made in summer with the help of a thermolabile ME are set for a lengthy storage and distributed in autumn and in winter, when cheese output drops.

Thermostable milk-clotting enzymes that remain proteolytically active at high temperatures (60–70°C) are used for production of soft cheese varieties with short ripening and storage time, or for cheese varieties that do not require ripening. In complex MEAs that consist of milk-clotting enzymes with high and low thermal stability, milk coagulation (at T~32°C) occurs through the joint activity of participating enzymes, while after heat treatment (72–74°C), the required proteolysis level in ripening cheese is ensured by thermostable component only.

Information on polyporopepsin thermal stability presented on the BRENDA enzyme portal [brenda-enzymes.org/enzyme.php?ecno=3.4.23.29] is contradictory: some data suggest that the coagulant is stable at 30°C (pH 4.6) and is completely inactivated at 45°C (pH 4.6), while according to other data, the enzyme can resist heating for 15 minutes at 50°C (pH 4.5), and all activity stops altogether at 60°C (pH 4.5).

E. Kikuchi et al. [16] conducted a comparative study of thermal stability in calf rennet ("Chr. Hansen"), mucorpepsin *R. pusillus* ("Meito"), and *I. lacteus* ME. It was shown that the *I. lacteus* milk-clotting enzyme lost approx. 40% of the baseline MA at 45°C already, while at approx. 55°C it became completely inactive. Control enzymes, calf rennet and mucorpepsin, were inactivated at approx. 60°C and approx. 70°C, respectively. Yu.P. Zagnitko [19] studied thermal stability of milk clotting preparations obtained by SA sedimentation from cultural liquid of *I. lacteus* (strain B-02), and noticed that the coagulant retained its high MA upon incubation at 25–40°C, with maximum MA (230 RU/ml) at 30°C. However, at heating temperatures above 45°C, inactivation of enzyme was observed. In the range of 45–60○C the inactivation degree of *I. lacteus* preparations (B-02 strain) was directly proportional to the exposure.

Results of comparative thermal stability study contrasting *I. lacteus* coagulant, ICS CR, and CP are presented in Fig. 3. At 40°C, all the test enzymes retained their MA around 100%. *I. lacteus* coagulant and ICS CR were almost completely inactivated at 60°C, residual MA amounting to 0.8% and 5.1% of the baseline value. However, the dynamics of inactivation for these enzymes within the range of 40–60°C is different. At 50°C, the *I. lacteus* coagulant was inactivated by 55%, while ICS CR retained almost 90% of the baseline MA. The highest thermal stability was demonstrated by CP, as its activity was reduced by 96% only after heating at 70°C.

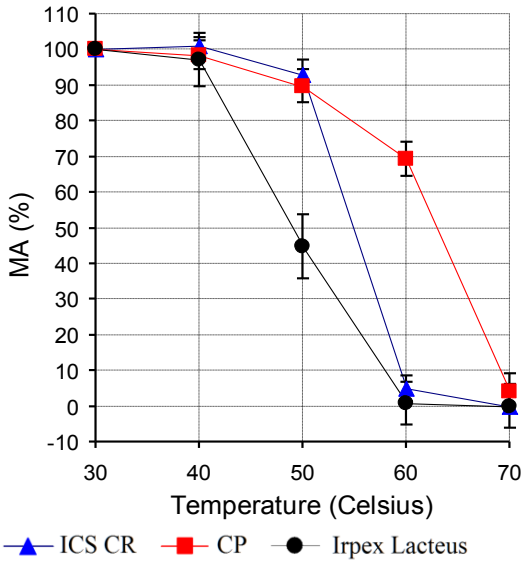

Fig. 3. Milk-clotting activity dependence on enzyme heating temperature (thermal stability).

According to the classification elaborated by "Chr. Hansen" company, microfungal milk coagulants used in cheesemaking are subdivided into several types: L type, natural (thermostable) enzymes; XL type, thermolabile coagulants obtained by oxidation; XP type and XLG type, these are chromatographically purified XL preparations, free from amylase/lipase/cellulase admixtures [2]. In accordance with this classification, the *I. lacteus* coagulant should be classified as XL type, albeit its thermal lability is a natural property of this enzyme, and not a result of chemical modification.

Thus, as far as thermal lability is concerned, the *I. lacteus* milk-clotting enzyme is comparable to ICS CR, and, consequently, complies with the requirements of the cheesemaking industry.

MA dependence on pH level of the substrate. An enzyme used for production of most varieties of cheese shall prove efficient in clotting milk within the pH range of 6.4–6.7.

Variations of pH level of the milk produce certain changes in electrostatic and hydrophobic properties of the milk casein micelle. As pH level grows and is distanced from the casein pI, their cumulative negative charges increase. As a result, forces of intermicellar electrostatic repulsion are also increased. At the same time, casein-casein hydrophobic interactions are diminished, which increases clot formation time during rennet coagulation of the milk. Therefore, whenever pH level of the milk mixture increases within the range of 5.0–7.0, rennet clotting time becomes slower.

Yet another factor affecting the MA of an enzyme is the optimum pH which depends on the substrate nature. Polyporopepsin, chymosin, and pepsin are acid proteinases. That is why, as the substrate is alkalized and its pH is distanced from the pH optimum of the test enzymes, clotting duration should increase.

I. lacteus coagulant and ICS CR are similarly dependant on pH changes in their MA (Fig. 4).

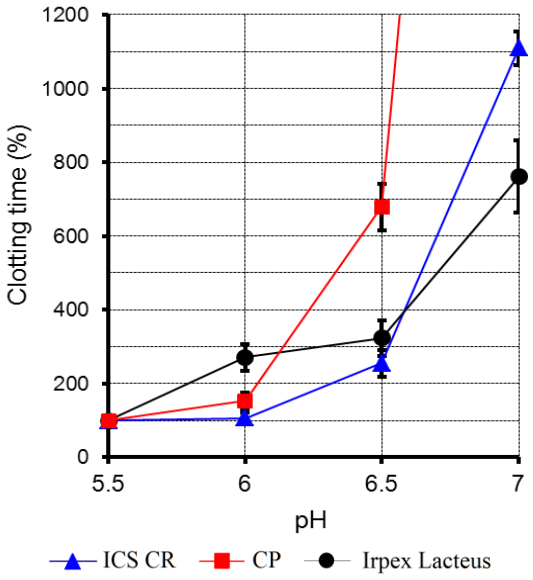

Fig. 4. Dependence of clotting time on pH.

Both preparations demonstrate high clotting activity within the pH range of 6.0–6.5, and gradually become inactivated at pH 6.5–7.0. Such results seem coherent with the BRENDA [brenda-enzymes.org/enzyme.php?ecno=3.4.23.29] data, suggesting that the optimum pH of polyporopepsin lies within the range of 2.5–4.0, and partially coincides with the chymosin optimum of 3.7–4.9. The optimum of pepsin enzymatic activity lies within a more acid range of pH 1.8–2.2. Consequently, CP preparation starts losing its activity earlier: at pH 6.5, activity loss is considerable, while at pH 7.0 the enzyme is almost completely inactivated (at pH 7.0, clotting duration is increased by 46 times).

Thus, MA of *I. lacteus* coagulant dependence on pH within the range of 5.5–7.0 is similar to that of the reference milk milk-clotting enzyme, - calf rennet.

Methods for correcting the technological properties of microfungal milk-clotting enzymes. There are several methods for correcting MA, specificity, and thermal stability of microfungal enzymes in cheesemaking. An attempt to influence one technological parameter usually results in modification of the others. Thus, although thermal stability of *I. lacteus* coagulant does not require any adjustment, we shall consider main methods and examples of changing the technological parameters of a microfungal milk-clotting enzymes in a complex.

Thermal stability of mucorpepsins is known to be positively related to the degree of glycosylation. In order to reduce the glycolysation of coagulants belonging to the *Rhizomucor* genus, several methods of chemical modification were suggested: oxidation, nitrification, acylation, carbamylation, periodate-induced and enzymatic deglycolysation [2]. D.A. Cornelius [24] introduced a method for reducing thermal stability of *R. miehei* and *R. pusillus* proteinases by oxidation run with hydrogen peroxide or photosensibilized oxidation in presence of a colorant. Oxidation does not lead to considerable loss of MA, and is currently widely used in order to reduce thermal stability of *R. miehei* coagulants [2].

Acylation by acid anhydrides significantly enhances MA of mucorpepsin produced by *R. pusillus* [24]. Maximum effect was observed during exposure of *R. pusillus* coagulant to maleic anhydride, however, that agent also inhibited MA in *R. miehei* mucorpepsin. T. Higashi et al. [26] treated *R.* pusillus coagulant with succinic anhydride, and achieved an increase in its specificity due to increased MA and MA/PA ratio. Oxidation by hydrogen peroxide run in presence of maleic anhydrid is used in order to reduced thermal stability and to prevent MA loss in *R. pusillus* mucorpepsin [27].

Methods for enzymic modification of microfungal coagulants have also been reported. Endoglycosidase H treatment for native mucorpepsin *R. miehei* ("Hannilase"), its partially oxydized version ("Modilase S") and *R. miehei* coagulant preparation ("Novoren XL"), derived from a heterologous producer (*Aspergillus oryzae* (Ahlb.) Cohn), allowed to obtain deglycosylated proteases with enhanced cheesemaking properties. Milk-clotting activity of Hannilase and Modilase S would increase by over 30%, and in case of recombinant mucorpepsin *R. miehei* produced by *A. oryzae* ("Novoren XL"), increase in specific activity amounted to approx. 45%. Besides, overall PA would decrease by ~10%, specificity (MA/PA) would increase, thermal stability would go down, pH range for MA would be more extensive, and cohesion of coagulants with milk clot would improve [28, 29].

Another potential method for influencing technological properties of microfungal coagulants is induced mutagenesis. Several focal substitutions can be singled out that might positively influence the technological properties of mucorpepsins. A101T or G186D replacement in a molecule of *R. pusillus* mucorpesin and mutant expression in *S. cerevisiae* allows to enhance significantly thermal stability of the coagulant. Curiously, the same result is ensured by any amino acid substitution in G186 position. Mutant enzyme with two simultaneous substitutions, A101T and G186D, proved to be even less thermostable than mucorpepsins with single replacements, and at the same time, did not show any decrease in MA/PA ratio. Focal substitutions of E19V and Q266E in a mucorpepsin derived from a wild strain of *R. miehei* (CBS 182-67) result in a 4.6 times specificity increase, and a 1.51% growth in cheese output, which is a very significant achievement in cheesemaking [2]. Site-targeted mutagenesis method allowed to obtain a mucorpepsin (the producer being *M. pusillus*) with two simultaneous amino acids substitutions, G186D and E13D, which led to an increased enzymatic specificity due to a higher MA [13].

One of the above listed methods for reducing high non-specific PA in mucorpepsin might lead to a higher specificity (MA/PA) of the *I. lacteus* coagulant, which in future might allow for its application in cheesemaking.

Results. We carried out a complex study of technological properties of a milk coagulant produced by a higher basidial fungus, Irpex lacteus (*I. lacteus)*, and evaluated each parameter in accordance with its suitability for cheesemaking industry application.

I. lacteus coagulant is shown to be on par with natural ME of animal origin used in dairy industry in a series of technological parameters, such as thermal stability, MA dependence on pH level, and Ca^{2+} ions content. Practical application of the milk-clotting enzyme produced by *I. lacteus* is considerably challenged by its low MA and high overall PA. We need to achieve a considerable increase in MA of the coagulant in order to remove this obstacle. There are a number of strategies to be used in order to obtain preparations with a high specific activity: selecting highly productive strains, perfecting cultivation methods, achieving optimal purification, and/or using methods of chemical, biochemical, and genetic modification.

The data we obtained might be used in further work aimed at studying and improving technological properties of a milk-clotting enzyme produced by the *I. lacteus* fungus.

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