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Analysis of Some Biochemical Properties of Recombinant Siberian Roe Deer (*Capreolus pygargus*) Chymosin Obtained in the Mammalian Cell Culture (CHO-K1)

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Abstract—Structure of the chymosin gene of Siberian roe deer (*Capreolus pygargus*) was established for the first time and its exon/intron organization was determined. Coding part of the chymosin gene of *C. pygargus* was reconstructed by the Golden Gate method and obtained as a DNA clone. Comparative sequence analysis of the roe deer, cow, and one-humped camel prochymosins revealed a number of amino acid substitutions at the sites forming the substrate-binding cavity of the enzyme and affecting the S4 and S1' + S3' specificity subsites. Integration vector pIP1 was used to construct a plasmid pIP1-Cap in order to express recombinant roe deer prochymosin gene in CHO-K1 cells. CHO-K1-CYM-Cap pool cells were obtained, allowing synthesis and secretion of recombinant prochymosin into the culture fluid. As a result of zymogen activation, a recombinant roe deer chymosin was obtained and its total milk-clotting activity was estimated to be 468.4 ± 11.1 IMCU/ml. Yield of the recombinant roe deer chymosin was 500 mg/liter or ≈468,000 IMCU/liter, which exceeds the yields of genetically engineered chymosins in most of the expression systems used. Basic biochemical properties of the obtained enzyme were compared with the commercial preparations of recombinant chymosins from one-humped camel (*Camelus dromedarius*) and cow (*Bos taurus*). Specific milk-clotting activity of the recombinant chymosin of *C. pygargus* was 938 ± 22 IMCU/mg, which was comparable to that of the reference enzymes. Non-specific proteolytic activity of the recombinant roe deer chymosin was 1.4-4.5 times higher than that of the cow and camel enzymes. In terms of coagulation specificity, recombinant chymosin of *C. pygargus* occupied an intermediate position between the genetically engineered analogs of *B. taurus* and *C. dromedarius* chymosins. Thermostability threshold of the recombinant roe deer chymosin was 55°C. At 60°C, the enzyme retained <1% of its initial milk-clotting activity, and its complete thermal inactivation was observed at 65°C.

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INTRODUCTION

Chymosin is a milk-clotting enzyme (EC 3.4.23.4) belonging to the family of gastral aspartic proteases. Its active center carries two asparagine amino acid residues at positions 32 and 215 [1]. Aspartate proteinases belong to the group of endopeptidases, which are en zymes that hydrolyze intramolecular peptide bonds [2, 3]. Chymosin (Chn) hydrolyzes the F105-M106 bond in

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the k-casein (k-CN) molecule selectively and at a high rate, which causes milk clotting. Natural Chn, derived from the stomachs of newborn farm animals, as well as its genetically engineered analogs are widely used in cheese production.

Chymosin is a convenient model for developing and analyzing efficiency of both pro- and eukaryotic expression systems. Its enzymatic (milk-clotting) activity depends on the correctness of synthesis and posttranslational processing. Milk-clotting activity (MCA) of Chn is determined using simple clotting tests that do not require expensive reagents and equipment.

Different production systems have been used to generate recombinant prochymosin (rProChn). In prokaryotes (mainly *Escherichia coli*), despite a sufficiently high level of synthesis, the target protein accumulates in insoluble form in inclusion bodies, which requires the use of refolding procedure that has low efficiency in the case of prochymosin [4-7].

Bacillus subtilis cells exhibit an extremely low level of prochymosin synthesis [8]. Apparently, this is why this production system is not used to obtain rChn.

A number of works describing production of pProChn in plant expression systems have been published [9-11]. Disadvantages of these systems include the unsolved problem of plant-type protein glycosylation [12].

The most significant results have been achieved by expression of the ProChn gene in fungal (*Trichoderma reesei*, *Aspergillus niger*) and yeast (*Komagataella* (formerly *Pichia pastoris*), *Kluyveromyces lactis*, *Saccharomyces cerevisiae*) cells. In these systems, the target protein is secreted into culture fluid (c.f.) of the producer and, upon activation, exhibits high enzymatic activity [13-17]. Currently, fungal expression systems are widely used in the industrial production of cow (*Bos taurus*) and one-humped camel (*Camelus dromedarius*) rChn [18].

Expression systems based on mammalian cells are best suited for obtaining recombinant proteins of *Mammalia* class representatives, especially in those cases when a certain type of posttranslational modifications (PTM) of the target product is essential. Surprisingly, only two examples of expression of the cow ProChn gene in mammalian cells have been found in the scientific periodicals [19, 20].

Expression systems based on the cells of warm-blooded animals (CHO, NS0, Sp2/0, HEK 293, BHK-21, Per.C6) can provide highly efficient synthesis, PTM, and secretion of recombinant proteins. However, expensive nutrient media and equipment are required for culturing mammalian cells, which increases price of the final products. It is generally believed that the use of these systems is justified only for production of particularly important proteins and enzymes [21]. Therefore, the main application of mammalian production systems is production of monoclonal antibodies and other therapeutic proteins [22, 23].

More than 30 years have passed since the first attempts to produce Chn in mammalian cells. During this time, serious progress has been made in the technologies of constructing producers, considerable experience in the use of highly efficient promoters has been accumulated, and new nutrient media have been developed. All this increase potential for the use of mammalian expression systems, and allows us to consider them as promising tools for obtaining the widest range of recombinant proteins for scientific, technological, and therapeutic purposes [21].

Compared to other mammalian expression systems, the use of Chinese hamster ovary (CHO) cells has a number of advantages, including high viability, ability to achieve high cell densities in suspension culture, long use practice, and highly optimized culturing conditions. Availability of a wide range of specialized nutrient media facilitates continuous maintenance of CHO cell viability [24].

Earlier we have obtained and studied Chns from the representatives of Deer family (*Cervidae*) – rChn of red deer [25-27] and rChn of moose [7], which demonstrated biochemical properties promising for technological use of these enzymes. This suggests that other species belonging to the family Cervidae may serve as sources of Chn with unusual enzymatic properties. Therefore, the ProChn gene of the Siberian roe deer (*Capreolus pygargus* Pallas, 1771) was chosen as an object for expression in mammalian cells.

The aim of this work was partial biochemical characterization of the recombinant roe deer Chn (rChn-Cap) produced in the CHO-K1 production system.

MATERIALS AND METHODS

Identification of the roe deer chymosin gene. Nucleotide sequence of the roe deer chymosin gene (CYM) was obtained as DNA of several overlapping PCR products and sequenced both directly and as clones as part of the pJET1.2 vector. For this purpose, we used total genomic DNA isolated from the horn samples of a three-year-old male *C. pygargus*, kindly provided by the Laboratory of Comparative Genomics of IMCB SB RAS. The obtained roe deer genomic DNA was further purified and concentrated on Amicon 100 kDa columns (Millipore, FRG).

Abbreviations: a.a., amino acid; c.f., culture fluid; Chn, chymosin; CHO, Chinese hamster ovary; CN, casein; MCA, milk-clotting activity; PA, proteolytic activity; ProChn, prochymosin; rChn-Cap, recombinant roe deer chymosin; rChn-Bos, recombinant cow chymosin; rChn-Cam, recombinant one-humped camel chymosin; TS, thermostability.

On average, size of the CYM gene in mammals is approximately 13,000 bp. The most conserved regions of the CYM gene were identified using the previously obtained CYM gene sequences of red deer (*Cervus elaphus*) and moose (*Alces alces*), as well as CYM sequences of cow (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*), and one-humped camel (*C. dromedarius*) from the GenBank database. This allowed to design CYM-specific oligonucleotide primers. Several pairs yielded overlapping PCR fragments of the roe deer CYM gene (CYM-Cap) in amplification (Table 1).

Amplification was performed using a Hot Start Q5 polymerase (New England Biolabs, USA). The obtained amplicons were purified from PCR components by sorption on Speed Beads magnetic particles (GE Healthcare, USA) and sequenced directly using 0.25-0.30 pmol of PCR fragment as DNA template for Sanger reaction. Next the obtained amplicons were cloned into a pJET1.2 vector. Plasmid DNA of the selected transformant clones was produced in preparative quantities and sequenced using the Sanger method with an automatic gene analyzer ABI3130XL or ABI3500XL (Applied Biosystems, USA). The obtained CYM-Cap sequences were analyzed and assembled into contigs using an academic version of Vector NTI 10 program (Invitrogen, Sweden). The sequences were next aligned with the CYM gene structures known for other *Cetartiodactyla* species from the GenBank database in order to confirm belonging to the chymosin gene and to design primers for the next sequencing step. As a result, full-length nucleotide sequence of the CYM gene of *C. pygargus* was obtained (GenBank registration number – OQ427063).

Confirmation of the species affiliation was obtained by direct sequencing of the amplification product of the mitochondrial cytochrome B gene fragment using the same genomic DNA as for the CYM gene.

Construction of recombinant integration vector for expression of the roe deer CYM gene. The following approaches and procedures were used to construct a plasmid vector for synthesis and secretion of the roe deer ProChn in a eukaryotic expression system. Based on the comparative analysis of the CYM gene, the intron/exon structure was established and coding sequence including pre- and pro-fragments was determined. To obtain a single reading frame containing only exons, primers were designed for amplification of each exon followed by seamless assembly using the Golden Gate method [28] (Table 2).

The obtained fragments were cloned into an expression plasmid pIP1. The pIP1 vector has the following main genetic elements: CMV promoter, SV40 polyadenylation signal, polylinker for cloning using the Golden Gate method, IRES sequence of encephalomyocarditis virus, and puromycin resistance gene for selection of transgenic cells.

Table 1. Oligonucleotide primers used for PCR-amplification of DNA fragments of the roe deer *Capreolus pygargus* chymosin gene and their sequencing (SEQ) directly or as clones within the pJET1.2 vector

Primer	Sequence 5'-3'	Couple
Cap-2614F	ggcttcttaaccattgcacca	PCR-2
Cap-3839R	gctctctgggcagcctaaca	SEQ
Cap-6633R	ctgagtcccactaaccctgagc	SEQ
Cap-6830R	aacacgtattgggcacttactacatg	SEQ
Cap-6932R	cagaccccaggaagaactcaag	PCR-3, PCR-9
Cap-7552F	aaaccacggcacagagctgaa	SEQ
Cap-7877F	ctcggtttacatggacaggtagg	SEQ
Cap-9554F	gagcttgcagcaaggacatgg	SEQ
Cap-9724F	agtctccctcacatccatggtc	PCR-1, PCR-9
Cap-10189F	cctgcatctctcttattktttggagc	SEQ
Cap-11696R	gagaaaacaggaagacaagacgga	SEQ
Cap-11745R	gttaagtgtctggcatctagtagca	SEQ
Cap-11862R	tccttgtgaaggtctccacctatc	SEQ
Cap-12127R	accccaggaccctcagacctt	PCR-7
Cap-12311R	caggaatgcagccctaaacg	SEQ
Cap-12624R	tactggggacaagactggctga	PCR-8
Cap-14050F	ctgaccaactacctggatgtgagt	PCR-3
Cap-15290F	catgccagtcggttctaagagaat	PCR-4
Cap-15300R	cctctcactttattctcttagaaccg	SEQ
Cap-15475R	gccatctctatggtttccagagactag	PCR-1
Cap-16024R	gctggtggttttctggaacac	PCR-2
Cap-16798F	atcagatgagctgataacttgctt	PCR-6, PCR-8
Cap-17961R	tgcttagatgctggtgtccttg	SEQ
Cap-19778R	cgctcgtcacatccaagtctg	SEQ
Cap-20433F	tcccttgagatccaagaacttcc	PCR-5, PCR-7
Cap-20856R	ggctcctctctggagatgcatac	PCR-4, PCR-6
Cap-22011R	cattctctactcaattccacctcaag	PCR-5

Table 2. Oligonucleotide primers used for seamless assembly by the Golden Gate method

Primer	Sequence 5'-3'	Purpose
CYM_FWD_(1)	ttacgtctccccatgaggtgtcttgtggtgctac	exon 1
CYM_FWD_(2)	ttacgtctcgagatcaccaggatccctctgtacaaaggc	exon 2
CYM_FWD_(3)	ttacgtctcggtcagtactttgggaagatctacctcg	exon 3
CYM_FWD_(4)	ttacgtctccaaaaccaccagcgcttcg	exon 4
CYM_FWD_(5)	ttacgtctccctccgacattgtggatgtcc	exon 5
CYM_FWD_(6)	ttacgtctcggaatggccaggggagc	exon 6
CYM_FWD_(7)	ttacgtctccagtgtcaccatcagcggtatg	exon 7
CYM_FWD_(8)	ttacgtctccggtatggcgagtttgacatcgactgtgacagc	exon 8
CYM_FWD_(9)	ttacgtctcccaggatgagggcttctgtacc	exon 9
CYM_REV_(1)	ttacgtctccatctcggcgccctggg	exon 1
CYM_REV_(2)	ttacgtctcgtgactatccaggtagttggtcaggg	exon 2
CYM_REV_(3)	ttacgtctcgtttttgcaggcattgctcttgc	exon 3
CYM_REV_(4)	ttacgtctcaggagacagtgacagtgtcgtagcc	exon 4
CYM_REV_(5)	ttacgtctccattcctgtccatgtaaaccgagaacac	exon 5
CYM_REV_(6)	ttacgtctcgcactgtccacagtgaactgcca	exon 6
CYM_REV_(7)	ttacgtctcctaccggttctgggtggct	exon 7
CYM_REV_(8)	ttacgtctcccctggttggtataggcggag	exon 8
CYM_REV_(9)	ttacgtctcccttacacggctttggccagc	exon 9

The expression cassette as part of the pIP1 vector was flanked on both sides by Sleeping Beauty transposon integration arms. Exon amplification was performed using a Q5 HotStart polymerase (NEB, USA) according to the manufacturer's recommendations. Next the amplicons were purified from the reaction mixture using a Cleanup S-Cap reagent kit (Evrogen JSC, Russia). After DNA concentration measurement with a NanoDrop 2000C spectrophotometer (Thermo Scientific, USA), the reaction mixture was prepared according to recommendations of the manufacturer of NEBridge® Golden Gate Assembly Kit BsaI-HF®v2 (NEB, USA). The reaction mixture was next incubated in a thermocycler using the following program: (37°C, 5 min → 16°C, 5 min) × 30 cycles → 60°C, 5 min. After completion of incubation, transformation of the NEB-Stable competent cells with 10 µl of the reaction mixture was performed.

Colonies were screened using a pair of primers complementary to the 3'-end of the nontranslated region of the expression vector and to CYM_REV_(9).

After screening of the transformant clones, plasmid DNA of the positive clones was isolated. Correctness of incorporation was confirmed by Sanger sequencing. As a result, an integration expression vector pIP1-Cap was obtained.

Production of recombinant roe deer prochymosin in CHO-K1 cell culture. CHO-K1 cells were transfected with the obtained expression vector pIP1-Cap together with a pCMV(CAT)T7-SB100 plasmid in a HyClone HyCell TransFx-C medium (Cytiva, Sweden) according to the described protocol [29]. The pCMV(CAT)T7-SB100 plasmid contains sequence of the SB100 transposase facilitating insertion of the target expression cassette flanked by integration arms into the cell genome. Selection was performed in the presence

of puromycin at concentration of 50 μg/ml. After addition of an antibiotic, cell viability dropped to 69% by the fifth day of selection. On the tenth day, cell viability reached the value of 95% and selection was stopped. The resulting pool of producers was washed twice with a phosphate-salt buffer, transferred to 600 ml of HyClone™ HyCell™ CHO medium (Cytiva) and cultivated at 37°C and 5% CO_2 with shaking (185 rpm) until a viable cell concentration of 4×10^6 cells/ml was reached. Temperature was next lowered to 31°C and the cells were cultivated further with a nutrient supplemented HyClone™ Cell Boost™ Kit (Cytiva) for 14 days, until percentage of viable cells decreased <75%. After centrifugation of producer cells at 8000*g* for 10 min, a clarified c.f. was obtained. The c.f. was filtered through a filter with a pore diameter of 0.2 μm (Thermo Fisher Scientific, USA).

Activation of recombinant roe deer prochymosin. Activation of the roe deer rProChn was carried out directly in the c.f. by stepwise pH change [17]. 2.0 M HCl was added gradually to the c.f. containing rProChn under constant stirring to adjust pH to the required level of 3.0. The mixture was incubated at pH 3.0 for 2 h. After incubation, pH of the sample was adjusted to 5.8 using 1.0 M NaOH.

Protein composition of culture fluids and enzyme-substrate mixtures were analyzed with the help of electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) according to the Laemmli protocol [30]. LMW-SDS Marker Kit (GE Healthcare) was used as molecular weight (MW) marker.

Commercial rChn of cow (CHY-MAX® Powder Extra, dry form) and rChn of one-humped camel (CHY-MAX® M 1000, liquid form) produced by Chr Hansen (Denmark) were used as reference samples. Undiluted camel rChn preparation and 1% solution of cow rChn in 20 mM Na-acetate buffer (pH 5.8) were used to determine protein concentration and calculate specific MCA. The enzymes were diluted with 20 mM Na-acetate buffer (pH 5.8) to reach MCA values of 8-12 IMCU/ml for examination of their thermostability (TS) and proteolytic activity (PA). During determination of biochemical properties, concentration of the preparations of roe deer rChn (rChn-Cap), cow rChn (rChn-Bos), and one-humped camel rChn (rChn-Cam) were normalized according to their MCA levels.

Protein concentration in rChn preparations was determined according to the Bradford protocol [31].

Determination of total and specific milk-clotting activity. Collected unpasteurized cow's milk was used as a substrate, in which NaN_3 was added up to 0.02% and pH was adjusted to 6.5. The substrate (1.25 ml) was warmed in a water bath set at 35°C for at least 10 min and rapidly mixed with 0.1 ml of the tested rChn. Time of formation of first flakes of coagulum was recorded. Aqueous solution of dry commercial cow rChn "Chy-Max" (0.5%, Chr. Hansen, Denmark) with a specified MCA of 2201 IMCU/g (IMCU – International Milk Clotting Units) was used as a standard of coagulation activity. All measurements were repeated at least three times ($n \geqslant 3$). Total MCA was expressed in IMCU/ml, which was calculated using the following formula (1):

$$MCA = MCAst/200 \times T1/T2, \quad (1)$$

where: MCAst – reported MCA of the standard in IMCU/g; 200 – dilution factor (ml/g); T1 – time (s) of substrate coagulation by the standard; T2 – time (s) of substrate coagulation by the solution of the tested enzyme.

Specific MCA of rChn was calculated after determination of total MCA and protein concentration and expressed in IMCU/mg.

Determination of total proteolytic activity (PA) and specificity. A 1.0% solution of casein according to Gammersten (MP Biomedicals, France) in 20 mM Na-phosphate buffer (pH 5.65) was used as a substrate. Substrate aliquots (2.0 ml) were placed in a water bath (35°C), heated for 15 min, and 0.5 ml of the tested rChn solution was added. The enzyme-substrate mixtures were mixed thoroughly, and time of the start of incubation was noted. After 30, 90, and 180 min of incubation, proteolysis was stopped by adding 2.5 ml of 5% trichloroacetic acid (TCA) to the enzyme-substrate mixtures. Contents of each tube were stirred, left for 30 min at room temperature, and filtered through a paper filter ("white tape"). Absorbance at 280 nm (A_{280}) was determined in the clear filtrate. In order to prepare a spectrophotometric control, components of the enzyme-substrate mixture were added directly to 5% TCA, incubated for 30 min and filtered through a paper filter. The value of A_{280} after 180 min of incubation was taken as the total (nonspecific) PA. Dependence of A_{280} on duration of incubation was plotted. Specificity was defined as a ratio of specific MCA to total PA (MCA/PA).

Determination of proteolytic specificity by electrophoresis. Whole unpasteurized cow's milk was used for substrate preparation via addition of NaN_3 up to 0.01% and dilution with a 20 mM Na-acetate buffer (pH 5.65) at a ratio 1 : 4. The substrate was used on the day of preparation. Five microliters of the tested rChn with activity of ≈8 IMCU/ml were added to 250 μl of substrate and mixed thoroughly. The resulting enzyme-substrate mixtures were incubated at 35°C for 1 h. After completion of incubation, the enzyme-substrate mixtures were mixed with a Sample buffer for SDS-PAGE (Serva, FRG) at a 1 : 1 ratio and heated in a boiling water bath for 90 s. The samples prepared in this way were analyzed using SDS-PAGE according to Laemmli [30]. In the control samples, 5 μl of 20 mM Na-acetate buffer (pH 5.65) were added instead of rChn solution.

Thermostability determination. rChn solutions were heated in a water bath set at temperatures in the range of 30-70°C for 30 min, quickly cooled to room temperature, and residual MCA was determined in them. Initial MCA values obtained for the samples heated at 30°C were taken as 100%. Graphs of dependence of residual MCA on heating temperature were plotted. Temperature of heating, at which the studied rChn retained not less than 80% of the initial MCA was considered as a threshold of thermal inactivation (thermostability).

Statistical processing of the obtained data was carried out with the help of Excel spreadsheet processor (Microsoft Corporation, USA). The results of quantitative variables were presented as an arithmetic mean (M) with indication of standard deviation (± SD).

RESULTS AND DISCUSSION

Identification of the roe deer CYM gene. Based on the analysis of nucleotide sequences of CYM genes presented in the GenBank database, length of the target gene was assumed to be about 13,000 bp. The CYM-Cap gene boundary region was shown to have high homology with the similar regions of the known chymosins. Therefore, based on the consensus sequence, two pairs of oligonucleotide primers necessary to obtain left and right overlapping fragments were designed.

DNA of the obtained gene fragments prepared in preparative amounts and purified were next used for direct sequencing in both direction using the Sanger method. Multiple overlapping sequences were assembled into a contig including both gene fragments. In order to assure correctness of the assembly, central region of the gene was amplified and sequenced directly. Analysis of the CYM-Cap structure revealed high degree of homology with the previously studied CYM genes of representatives of the *Cervidae* family: red deer (*C. elaphus*), moose (*A. alces*), and reindeer (*Rangifer tarandus*).

Determination of nucleotide and amino acid sequence of the roe deer CYM gene. Comparison of the obtained sequence of the CYM gene of *C. pygargus* with the known mRNAs encoding Chn in mammals allowed us to identify boundaries of the exons/introns of the studied gene and to deduce structure of the CYM-Cap mRNA.

After establishing the intron/exon structure of CYM-Cap, its coding sequence including pre- and pro-fragments was determined. The derived amino acid (a.a.) sequence of the roe deer ProChn was also analyzed and compared with the cow and one-humped camel ProChn sequences.

As a result, a number of remarkable substitutions were found in the deduced a.a. sequence of the *C. pygargus* Chn that distinguish this enzyme from the reference Chn of *B. taurus* and *C. dromedarius*. These a.a. substitutions are located at the sites forming substrate-binding

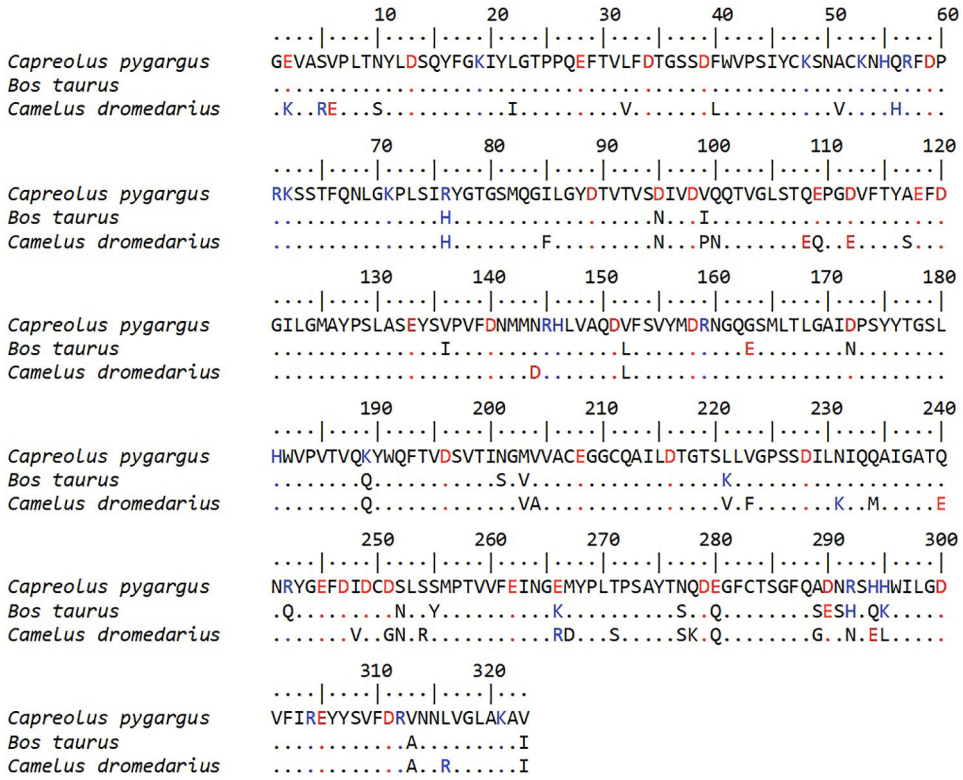

Fig. 1. Alignment of amino acid sequences of the roe deer, cow, and camel chymosins. Positively charged amino acid residues are highlighted in blue, negatively charged amino acid residues in red. Identical amino acid residues are marked with dots.

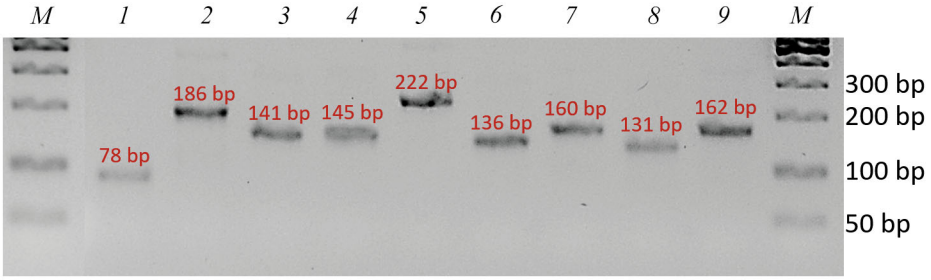

Fig. 2. Electrophoregram of PCR products of the roe deer ProChn gene exon sequences: *1-9*) exons 1-9; *M*) molecular mass markers 100 + 50 bp; SE-M33, SibEnzyme, Russia.

cavity and, theoretically, may affect properties of the enzyme. In particular, the a.a. residue at position 221 in the roe deer Chn, which forms the S4 subsite, is L, whereas we observe K in the cow and V in the camel enzymes. In the roe deer enzyme H is in the position 294 belonging to the S1' + S3' subsites, whereas in the cow enzyme it is Q, and in the camel enzyme – E. Position 295 in the roe deer enzyme is occupied by H, in contrast to K in the cow and L in the camel enzymes (Fig. 1).

Construction of a recombinant vector ensuring expression of the CYM-Cap gene in CHO-K1 cells. Golden Gate assembly was used to seamlessly assemble an expression cassette containing only coding regions of the CYM-Cap gene. The primers were designed so that the 5'-end of each primer contains restriction site IIs for the endonuclease Esp3I. It recognizes the CGTCTC sequence and catalyzes DNA hydrolysis at one nucleotide after the recognition site to form 4-nucleotide "sticky" ends. "Sticky" ends were selected so that they were non-palindromic and each was complementary to only one sticky end. As a result of amplification with the template of the CYM-Cap gene using the designed oligonucleotide primers, 9 fragments encoding exons of the ProChn gene of *C. pygargus* were obtained (Fig. 2).

The expression cassette was cloned into the pIP1 vector developed in the immunochemistry laboratory of State Research Center of Virology and Biotechnology VECTOR. The resulting vector had Sleeping Beauty transposon integration arms flanking the expression cassette and a puromycin resistance gene for integration into the CHO cell genome and further selection of transfected cells.

Preparation of the recombinant roe deer prochymosin. The cell line was chosen based on the following characteristics of CHO-K1: well-developed cultivation methods, resistance to changes in oxygen concentration, pH, temperature, and easy adaptation to composition of the medium. In addition, metabolism of the CHO-K1 cells, compared to other frequently used cell lines (CHO-S and CHO-DG44), is more focused on protein expression than on biomass production [32].

After transfection of the cells with pIP1-Cap plasmid and their selection in the presence of puromycin for 10 days, a polyclonal cell culture (CHO-K1-CYM-Cap) was obtained. Further semi-preparative cultivation was performed according to the above-described methodology for 14 days, until the proportion of viable cells decreased to <75%. The obtained polyclonal cell culture of CHO-K1-CYM-Cap cells provided synthesis and secretion of the target protein into c.f. Electrophoretic analysis of the culture medium during cultivation indicated high (about 0.4-0.6 μg/μl) content of a polypeptide component with MW of about 45 kDa, which corresponds to that calculated for the recombinant roe deer prochymosin (rProChn).

Zymogen activation and production of recombinant roe deer chymosin. Before activation, coagulation activity of the c.f. containing *C. pygargus* rProChn was <0.1 IMCU/ml. After subjecting the c.f. to gradual stepwise decrease and increase of pH, total MCA of the preparation was about 468 IMCU/ml. As a result of activation, the MCA of c.f. increased more than 4600-fold, indicating high efficiency of conversion of the rProChn into the active roe deer rChn.

Concentration of the recombinant roe deer chymosin in the culture fluid. After zymogen activation, concentration of rChn-Cap in c.f. CHO-K1 was 0.50 ± 0.03 mg/ml.

Yield of the recombinant roe deer chymosin. The possibility of expression of the cow prochymosin gene in mammalian cells was first shown in 1986 [19]. The authors used prochymosin transgenic cells of dog kidneys of MDCK (Madin–Darby Canine Kidney) line and shown secretion of the target protein into the culture medium. Activation of zymogen was not performed, so it was impossible to estimate the yield of Chn in the MDCK expression system.

Production of the bovine rChn in the HeLa cell production system was also reported [20]. Selected producer clones secreted ProChn into the culture medium. Yield of the target protein reached 10-20 mg/liter.

In this work, the yield of roe deer ProChn in the CHO-K1 production system was 500 mg/liter or ≈468000 IMCU/liter, which exceeds more than 2-fold the yield of genetically engineered ProChn even in the case of the most efficient producers of these enzymes based on methylotrophic yeast (*Komagataella* (*Pichia*) *pastoris*) [33-35]. Productivity of the polyclonal CHO-K1-CYM-Cap system obtained in this study was

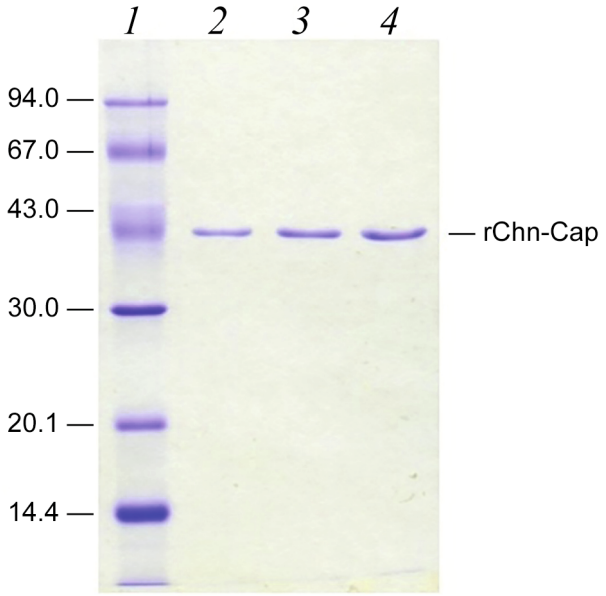

Fig. 3. Electrophoresis of the CHO-K1-CYM-Cap culture fluid preparations after roe deer rChn zymogen activation. Designations: *1*) molecular mass markers; *2*) 0.5 µl of CHO-K1-CYM-Cap culture fluid preparation; *3*) 0.75 µl of CHO-K1-CYM-Cap culture fluid preparation; *4*) 1.0 µl of CHO-K1-CYM-Cap culture fluid preparation. Position of rChn-Cap is indicated on the right. Molecular masses of the markers in kDa are shown on the left.

about 2.6 times lower than the productivity (mg/liter) of the chlorate-resistant mutant of *Aspergillus niger* var. *Awamori* (strain GC1HF1-3; dgr246 ChR 25), which was created to produce the rChn of cow [36]. It should be noted that the MCA yield of cow rChn obtained using the strain GC1HF1-3; dgr246 ChR 25 was ≈90000 IMCU/liter [37], which is 5.2 times less than in the case of roe deer rChn obtained in the CHO-K1 cells in this study.

Analysis of protein composition of the culture fluid. According to electrophoretic data, rChn-Cap was the dominant polypeptide component of the CHO-K1 c.f. (Fig. 3). In SDS-PAGE, the rChn-Cap band migrated as a polypeptide with MW of ≈39 kDa. Thus, conditions of the *C. pygargus* Chn gene expression in the CHO-K1 cells ensured highly efficient synthesis and secretion of the target protein. After zymogen activation, electrophoretic purity of the rChn in the CHO-K1 culture medium was ⩾95%.

Specific milk coagulation activity. When determining parameters of coagulation activity of the roe deer rChn and reference enzymes, activity of the cow rChn was taken as 100% (Table 3). In terms of specific MCA, the roe deer rChn occupied an intermediate position in the series of enzymes studied: it was ~1.4 times higher than the cow rChn, but ~1.2 times lower than the one-humped camel rChn. The highest coagulation efficiency was demonstrated by the rChn-Cam, whose specific MCA was higher than that of the cow and roe deer rChn by ~1.7- and ~1.2-fold, respectively. This is consistent with the previous data [13] showing that the ratio of specific MCA of the *B. taurus* rChn and *C. dromedarius* rChn is 1 : 1.7.

Thus, the *C. pygargus* rChn produced in the CHO-K1 expression system is a highly effective cow's milk coagulant comparable to the high-quality commercially available rChn preparations in terms of specific MCA.

Proteolytic activity and coagulation specificity. Major milk proteins are α-, β-, and κ-caseins (α-CN, β-CN, and κ-CN). In native milk, interaction of all groups of caseins and colloidal calcium phosphate leads to formation of stable protein aggregates called casein micelles [38, 39]. One of the primary physiologic functions of Chn is its ability to coagulate milk in the stomach of newborns. As a result, proteins and other milk nutrients are retained longer in gastrointestinal tract, which ensures their complete digestion [40]. Since the MCA of aspartate proteinases is based on their ability to hydrolyze certain peptide bonds, biochemical characterization of any new milk-clotting enzyme (ME) includes assessment of its PA.

Conventionally, PA of milk coagulants is categorized as specific and nonspecific. Specific or milk coagulation activity is aimed at hydrolyzing only one (key) bond in the κ-CN molecule. Proteolysis of this single bond destabilizes casein micelles, which allows them to converge forming a three-dimensional mesh-like structure, milk clot. In the case of bovine κ-CN, the key bond is the F105-M106. Nonspecific or total PA characterizes ability of ME to hydrolyze a wide range of peptide bonds, with exception of the F105-M106 bond or its analog. The ratio of MCA to total PA (MCA/PA) is called specificity and is used to compare coagulation efficiency of the milk-clotting proteinases [41].

Nonspecific PA of the roe deer rChn was 1.4-4.5 times higher than that of the reference rChns (Fig. 4).

Table 3. Total and specific milk-clotting activity of rChn preparations

Preparation	Total MA (IMCU/ml)	Protein concentration (mg/ml)	Specific MCA (IMCU/mg)	Specific MCA (%)
rChn-Cap	468.4 ± 11.1	0.500 ± 0.030	938 ± 22	141
rChn-Bos	22.0 ± 0.7	0.033 ± 0.005	667 ± 21	100
rChn-Cam	1037.3 ± 13.0	0.936 ± 0.023	1108 ± 14	166

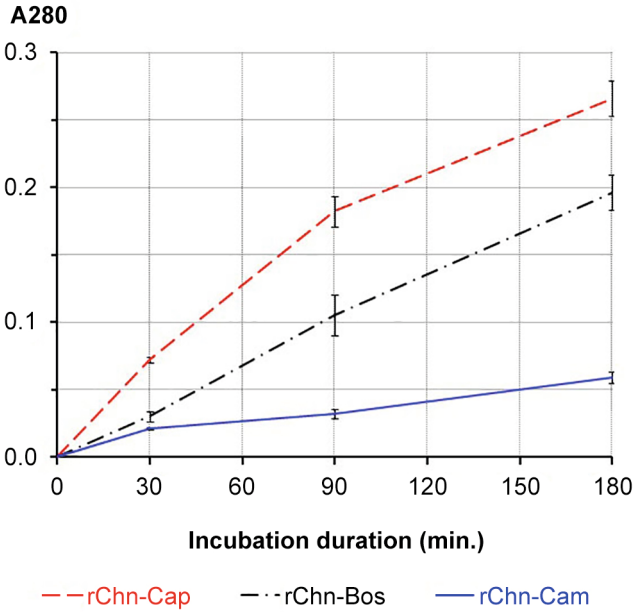

Fig. 4. Total proteolytic activity (A_{280}) of recombinant chymosins. Notation: rChn-Cap – rChn of roe deer; rChn-Bos – rChn of cow; rChn-Cam – rChn of one-humped camel.

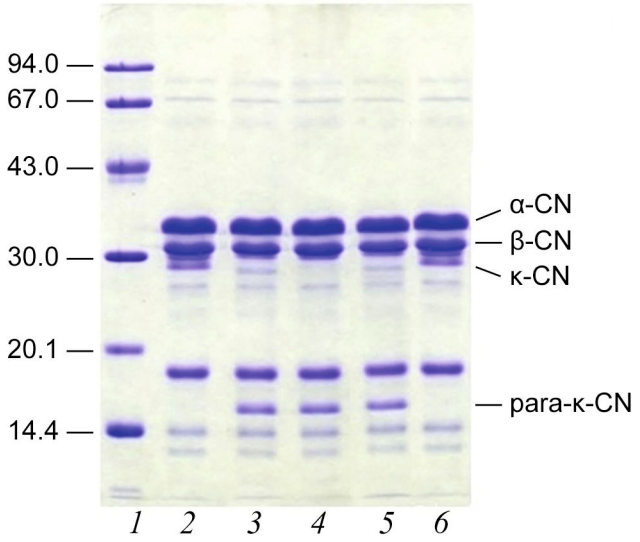

Fig. 5. Proteolytic activity of recombinant chymosins towards the proteins of whole cow's milk at pH = 5.65. Symbols: *1*) molecular mass markers; *2*) milk + 20 mM Na-acetate buffer, pH = 5.65, not heated (control 1); *3*) milk + rChn-Bos; *4*) milk + rChn-Cam; *5*) milk + rChn-Cap; *6*) milk + 20 mM Na-acetate buffer, pH = 5.65, heated as the experimental samples – 60 min, 35°C (control 2). Molecular masses of the markers in kDa are indicated on the left. The bands of α-, β-, κ-casein and para-κ-casein (MW ≈ 16 kDa, on tracks *3-5*) are indicated on the right.

In terms of dynamics of accumulation of the casein (CN) proteolysis products, the roe deer rChn is similar to the cow rChn and differs markedly from the camel rChn. Especially these differences are manifested after 60 min of incubation. Low values of PA obtained in this study are characteristic for the single-humped camel rChn and are confirmed by the previous data [13], in which it was shown that the camel rChn has four times less nonspecific PA than the cow rChn. It has also been shown [6] that, with the same MCA, total PA of the alpaca (*Vicugna pacos*) rChn (the closest phylogenetic relative of the one-humped camel) is about 3 times lower than that of the cow rChn. Apparently, low total PA and high specificity are characteristic of the chymosins of representatives of the *Camelidae* family.

To compare specificity of the roe deer rChn and commercial genetically engineered rChns, data on their specific MCA (%) and total PA (%) were used, with the cow rChn values taken as 100% (Table 4). In terms of the MCA/PA ratio, the roe deer and cow enzymes were almost indistinguishable and were about 5.5 times lower in comparison with the rChn of one-humped camel.

High coagulation specificity (MCA/PA) serves as one of the main criteria for efficiency and selection of ME for cheesemaking [41, 42]. The most of the known milk-clotting asparagine proteinases of different genesis, with the exception of Chn of the one-humped camel, alpaca, and pig [6, 13], cannot be compared with the cow Chn [18]. Therefore, the value of MCA/PA = 1.04 obtained for the rChn-Cap synthesized in the CHO-K1 expression system is of interest in terms of the prospects for practical application of this enzyme.

Proteolytic specificity of the rChn towards proteins of whole cow's milk was investigated by the SDS-PAGE method (Fig. 5). Incubation of milk with the tested rChns resulted in accumulation of para-κ-CN (MW ≈ 16 kDa) in the enzyme-substrate mixtures, which is the result of specific PA aimed at hydrolyzing the F105-M106 bond in the κ-CN molecule (Fig. 5, lanes *3-5*). The recombinant roe deer and cow Chns have similar proteolytic specificity and are able to hydrolyze not only κ-CN but also α- and β-CN during

Table 4. Total and specific milk-clotting activity of rChn preparations

Preparation	Specific MCA (%)	Total PA (A_{280})	Total PA (%)	Specificity (MCA/PA)
rChn-Cap	141	0.266 ± 0.013	136	1.04
rChn-Bos	100	0.196 ± 0.013	100	1.00
rChn-Cam	166	0.059 ± 0.004	30	5.53

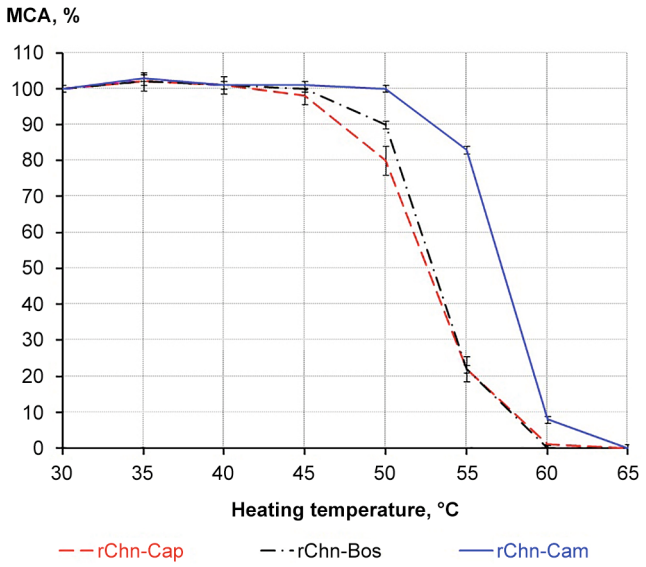

Fig. 6. Dependence of residual milk-clotting activity of the recombinant chymosins on heating temperature (thermostability). Symbols: rChn-Cap – rChn of roe deer; rChn-Bos – rChn of cow; rChn-Cam – rChn of one-humped camel.

the assay time. As a result, electropherograms obtained with these enzymes show weak protein bands with MW 28-29 kDa and low molecular weight polypeptide components with MW ≪ 14 kDa that migrate in the leading dye zone (Fig. 5, lanes *3*, *5*). In the case of camel rChn, only the κ-CN band disappears from the polypeptide profile of milk proteins (Fig. 5, track *4*), reflecting low nonspecific PA of this enzyme.

Thermostability (TS) is defined as ability of a protein to resist denaturation at increasing temperature and serves as an important biochemical characteristic of any enzyme. The results of the study of TS of the roe deer, cow, and one-humped camel rChns are presented in Fig. 6.

Threshold of thermal inactivation of the tested rChn was considered to be a temperature (T, °C) at which MF retained ⩾80% of the initial coagulation activity measured at 30°C. According to this criterion, the TS thresholds of the cow and roe deer rChns were similar reaching 55°C. Although the TS profiles of these enzymes are similar, complete thermal inactivation of the cow rChn occurred at 60°C, whereas the roe deer rChn still retained 0.8% of the original MCA at this temperature.

Threshold of thermal inactivation of the one-humped camel rChn was higher and equal to 60°C.

Thermal inactivation profiles of the cow and camel rChns obtained in this study are confirmed by the results of calorimetric studies, according to which melting points Tm of the *C. dromedarius* rChn is higher than that of *B. taurus* rChn: melting points of these enzymes differ by 3°C and are equal to 57.7 and 60.7°C, respectively [41]. Significance of such differences is demonstrated by the results of application of these enzymes in cheesemaking. It has been shown that the increase of the processing temperature of milk clot from 50 to 56°C leads to the decrease in the intensity of nonspecific proteolysis in the cheeses produced with cow and camel rChn. However, higher concentration of the products of α-CN proteolytic degradation was detected in the cheeses produced with the clot heating temperature of 56°C when the camel rChn was used in comparison with the case when the cow rChn was used. Based on these data, it was concluded that the camel rChn, whose total PA is 75% lower than the PA of cow rChn and whose Tm is 3°C higher, causes more intense proteolysis of α-CN in the ripening and stored cheeses [43].

CONCLUSION

As a result of this study, structure of the Siberian roe deer chymosin gene and its exon/intron organization were determined for the first time. Comparative analysis of the roe deer, cow, and one-humped camel ProChn sequences was carried out, which revealed a number of a.a. substitutions at the sites forming substrate-binding cavity of the enzyme and affecting the subsites S4 and S1' + S3'.

A recombinant plasmid pIP1-Cap was constructed for expression of the roe CYM gene in the CHO-K1 cells using pIP1 integration vector. A polyclonal culture of CHO-K1-CYM-Cap cells was obtained, enabling synthesis and secretion of the roe deer rProChn into the c.f.

After zymogen activation, a preparation of the roe deer rChn was obtained with total MCA of 468.4 ± 11.1 IMCU/ml. Yield of the roe deer rChn in the CHO-K1 production system was 500 mg/liter or ≈468,000 IMCU/liter, which is 2-5 times higher than that of the genetically engineered rChn in yeast and mold fungi expression systems.

Main biochemical properties of the obtained enzyme were determined. Specific MCA of the *C. pygargus* rChn was 938 ± 22 IMCU/mg protein and was comparable to that of the commercial cow and camel rChns. Nonspecific PA of the roe deer rChn was 1.4-4.5 times higher than that of the reference rChns. Nevertheless, due to the high specific MCA, in terms of coagulation specificity (MCA/PA ratio) the rChn-Cap occupied an intermediate position between the genetically engineered cow and one-humped camel chymosins. TS threshold of the roe deer rChn-Cap was 55°C. At 60°C, the enzyme retained <1% of the original MCA, and its complete thermal inactivation was observed at 65°C.

The exceptionally high level of production of the roe deer rChn in CHO-K1 cells is the main result of this work, which indicates the need for further study of the possibilities of using mammalian expression systems for obtaining recombinant milk-clotting proteinases.

Contributions. Murashkin and Belenkaya wrote the manuscript; Bondar created the figures; Elchaninov and Shcherbakov edited the text of the article.

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Ethics declarations. The authors declare no conflict of interest in financial or any other sphere. This article does not contain any studies with human participants or animals performed by any of the authors.

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