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Cloning and Expression of Camel Pro-Chymosin Encoding Gene in *E. coli* and Characterization of the Obtained Active Enzyme

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ABSTRACT

Chymosin is considered the main enzyme in milk industry since it is used for commercial production of cheese. Conventional animal chymosin production losses large numbers of unweaned calves which affect on the animal wealth. In order to reduce the manufacturing cost, recombinant production of chymosin is the good choice. In the current study, camel pro-chymosin gene (1101 bp) was *in vitro* synthesized and cloned into *pASG*-vector. The obtained plasmid *pASG_pro.chym* was transformed into the bacteria *E. coli* BL21(DE3). The bacterial system expressed pro-chymosin under control with tet-promoter and it was able to produce approximately 260 mg/L of recombinant enzyme under lab scale. The SDS-PAGE showed that the zymogen protein (367 amino acids residues long) has a molecular weight of 40.6 kDa, while the active form (323 amino acids residues long) has a molecular weight of 35.6 kDa. The recombinant pro-chymosin is presented in inclusion bodies and it is solubilized in 4-8 M urea. After solubilization and renaturation, recombinant pro-chymosin was subjected to a low pH and it was converted into mature active chymosin. The optimum milk clotting conditions were a pH of 5.75, temperature of 50-55 ℃, and 15 mM of $CaCl_2$. It can be concluded that the obtained recombinant chymosin from *E. coli* is suitable for commercial cheese production.

Keywords: Camel chymosin- gene cloning- expression in *E. coli*- solubilization and renaturation- optimum clotting conditions.

INTRODUCTION

Chymosin (EC 3.4.23.4), an aspartyl protease, is found in mucosal cells of the fourth stomach of unweaned calves (Williams *et al.*, 1997). The enzyme is responsible for partial proteolysis of κ-casein and it attacks the peptide bonds between the amino acids Phe_{105} and Met_{106} which destabilized casein micelles resulting in clotting milk (Albert *et al.*, 1998). So, it is the main additives in cheese manufacturing. Many microorganisms such as *Rhizomucor miehei (Gray et al., 1986)* and *R. Pusillus (Baudys et al., 1988*) produce rennet-like proteases that can replace the calf rennet. However, to produce high quality cheese, animal rennet enzyme (chymosin) is considered to be better choice than plant and microbial rennet which characterized by their un-specific hydrolysis of casein producing un-favourable flavour and taste as well as reducing the cheese yield (Kumar *et al.*, 2010). For that, animal chymosin, especially bovine chymosin, is widely used in dairy industries for cheese production (Mohanty *et al.*, 1999). However, traditional preparation of bovine chymosin requires slaughter of large number of unweaned calves with high price which lead to affect on national economy. Also, the extraction and purification is time consuming and its expose for deterioration agents is high (Starovoitova *et al.*, 2006). So, biologists solve this problem by recombinant producing mammalian chymosin to provide the enzyme requirement in cheese industries. Currently, recombinant chymosin is used for one-third of the cheese production over the world (Starovoitova *et al.*, 2006 and El-Sohaimy *et al.*, 2010).

The calf chymosin gene was among the first mammalian genes that were cloned in different microorganism. Using recombinant DNA technology, calf chymosin encoding gene was expressed in GRAS (Generally Recognized As Safe) microorganisms as bacteria (Nishimori *et al.*, 1982; Emtage *et al.*, 1983; El-Sohaimy *et al.*, 2010; Menzella, 2011 and Noseda *et al.*, 2016), yeast (Mohanty *et al.*, 1999; Starovoitova *et al.*, 2006; Zhang *et al.*, 2009; Noseda *et al.*, 2013; Wang *et al.*, 2015 and Noseda *et al.*, 2016), filamentous fungus (Cardoza *et al.*, 2003 and Sharma *et al.*, 2009), and plant (van Rooijen *et al.*, 2008).

First approach was made by cloning of full-length cDNA of the mRNA and their sequence has been determined (Nishimori *et al.*, 1982). The precursor protein, pre-pro-chymosin, has a 16 amino acids signal peptide that was corresponding on transfer the zymogen pro-chymosin into stomach which is further activated by removal of the pro-peptide, the 42-amino acids from the NH_2-terminus (Emtage *et al.*, 1983). Expression of the pro-chymosin in yeast or fungus resulting on production of the enzyme in an active form. While, expression of the pro-enzyme in *E. coli* exit as insoluble form which needs further solubilization and renaturation of the enzyme from the inclusion bodies (Mule *et al.*, 2009 and El-Sohaimy *et al.*, 2010). The major benefit of this insoluble form that it makes purification and activation could be achieved since most of *E. coli* proteins (about 90%) are precipitated under the acidic condition which is used to activate the pro-chymosin (Rodríguez-Carmona, 2010). Therefore, several approaches have been developed for optimizing the solubilization and renaturation process to reduce the process cost as well as increase the yield of the mature active chymosin (Koritzinsky *et al.*, 2013). The enzymatic characteristics of *E. coli* recombinant chymosin are indistinguishable from those of native one (Starovoitova *et al.*, 2006).

Recently, recombinant camel chymosin attracts great interest in cheese industries because it produces cheese with good flavour and extended cheese shelf life (Langholm Jensen *et al.*, 2013). It has the ability to clot both camel's and cow's milk. In addition, it has 70% higher clotting activity, exhibits higher level of thermostability, and exhibits sevenfold higher clotting activity/proteolytic activity than bovine chymosin (Bansal *et al.*, 2009 and Sorensen *et al.*, 2011).

Camel chymosin has been cloned and expressed firstly in *Aspergillus niger* (Kappeler *et al.*, 2006). The second group was expressed it in yeast *Pichia pastoris* with almost similar properties like the one produce by fungi (Wang *et al.*, 2015). However, expression of camel chymosin in *E. coli* has not been yet reported till now. In the current work, a full-length camel pro-chymosin gene

was *in vitro* synthesized, introduced into *E. coli* BL21(DE3) strain via pASG-plasmid, and expressed under tet-promoter. The insoluble form of the pro-chymosin is further renaturation and activation. Also, the optimum clotting condition for the active camel chymosin was studied.

MATERIALS AND METHODS

Materials

The chemicals used in the current work were purchased from the following company, Sigma-Aldrich, IBA-lifesciences, and Takara. Oligonucleotides were purchased from Tsingke (China). The DNA purification kit, Phusion™ DNA polymerase, and restriction enzyme were purchased from Fermentas-GmbH (Fisher scientific, Schwerte, Germany).

Strains, plasmids and primers.

Bacterial strains, empty vector, and constructed plasmids that were used in this work are presented in table (1).

Table 1. Bacterial strains and Plasmids used in the current study:

Strains	Genotype / relevant characteristics	Source
E. coli, DH5α-cells (Cloning strain)	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
E. coli BL21(DE3)	F⁻ *ompT hsdS$_B$*($r_B^-m_B^-$) *gal dcm rne131*(*DE3*)	Invitrogen
Original vectors		
pE / pENTRY	Cloning & compatible expression vector; Kan^R; ColE1 ori; lacP/Z	
pASG.wt-(blue)	std. expression vector; pseudo-wt; Amp^R; lacP/Z	T. Selmer
pFusion (blue)	fusion vector; Amp^R; lacP/Z	
prepared plasmids		
pE-chym.P.1	Kan^R; ColE1 ori; Chym (part1)	
pE-chym.P.2	Kan^R; ColE1 ori; Chym (part2)	
pF-pro.chym	Amp^R; ColE1 ori; pro-Chym	This study
pE-pro.chym	Kan^R; ColE1 ori; pro-Chym	
pASG-pro.chym	tetP; *Pro.chym*; f1ori; Amp^R; ColE1 ori	

Amp^R, Ampicillin resistance gene; Kan^R, Kanamycin resistance gene

Construction of the chymosin gene.

Camelus dromedaries pro-chymosin protein (367 amino acid, with calculated molecular weight of 40.6 KD) was converted into its coding gene sequence (1101bp) taken in consideration codon optimization for enhancing optimum protein expression in *E. coli*. The pro-chymosin gene (1101 bp) was artificial synthesized by overlapping PCR technique (Bryksin and Matsumura, 2010) in KanR-vector (pEntry) using the oligonucleotides presented in table (2).

Table 2. List of all oligonucleotides used throughout this study.

	Name	Sequence	bP
Oligonucleotides to synthesize the first part of chymosin gene	P1.F1	CATGATGGACCGCCACCTGGTGGCGCGCGACCTGTTCAGCGTGGAGACGATCCAAAGGC	59
	P1.F2	ACCCGTCCCTGGCCAGCGAGTACTCGGTGCCGGTGTTCGACAACATGATGGACCGCCAC	59
	P1.F3	GGTGTTCACCTACTCGGAGTTCGACGGCATCCTGGGCCTGGCCTACCCGTCCCTGGCCA	59
	P1.F4	CGAACCAGACCGTGGGCCTGAGCACGGAGCAGCCGGGCGAGGTGTTCACCTACTCGG	57
	P1.F5	CTTCCTGGGCTACGACACGGTGACCGTGAGCAACATCGTCGACCCGAACCAGACCGTGG	59
	P1.F6	CAAGCCGCTGAGCATCCATTACGGCACCGGCAGCATGGAGGGCTTCCTGGGCTACGAC	55
	P1.F7	AAGCTCTTCATCCCCGCAAGAGCTCGACGTTCCGCAACCTGGGCAAGCCGCTGAGCATC	59
	P1.R1	CTTGTGCAGCGGGATGCGGGTGATGCCCGAGGCCATTGGAGACGTGGCAC	50
	P1.R2	CAGGCCGCGTTCCTTCAGCGCCTTGCGCAGGGTCTTGCCCTTGTGCAGCGGGATG	55
	P1.R3	GCTGCTCACGGCGTACTGCTGGCGCTGCAGGAAGTCCTCCAGCAGGCCGCGTTCCTTC	58
	P1.R4	TGGTCAGCGGTTCGCGGGCCACCTTGCCCAGCGAGCTGTACTTGCTGCTCACGGCGTA	58
	P1.R5	GGTGCCGATGTAGATCTTGCCGAAGTACTGGCTGTCCAGGTAGCTGGTCAGCGGTTCGC	59
	P1.R6	GCTACCGGTGTCGAACACGACCGTGAACTCCTGCGGGGGGGTGCCGATGTAGATCTTG	58
	P1.R7	CACGTTGCTCTTGCAGTAGATGCTCGGGACCCACAGGTCCGAGCTACCGGTGTCGAAC	58
	P1.R8	AAGCTCTTCTGGATCGAAGCGATGGTGGTTCTTGCACACGTTGCTCTTGCAGTAG	55
Oligonucleotides to synthesize the first part of chymosin gene	P2.F1	ACCGCGCGAACAATCGCGTGGGCCTGGCGAAGGCGATCTAATAGGGAGGAGACGATCCA	59
	P2.F2	CCTGGGCGACGTCTTCATCCGCGAGTACTATAGCGTGTTCGACCGCGCGAACAATCG	57
	P2.F3	GCACCAGCGGTTTCCAGGGCGACAACAATAGCGAGCTGTGGATCCTGGGCGACGTCTTC	59
	P2.F4	TCCCCTGAGCCCGAGCGCGTACACCAGCAAGGACCAGGGCTTCTGCACCAGCGGTTTCC	59
	P2.F5	CGCTCCATGCCCACCGTGGTGTTCGAGATCAACGGCCGCGACTATCCCCTGAGCCCGAG	59
	P2.F6	CCGAGAACCGCTACGGCGAGTTCGACGTGAACTGCGGCAGCCTGCGCTCCATGCCCACC	59
	P2.F7	AAGCTCTTCATCGGTGCCACCGAGAACCGCTACGG	35
	P2.R1	GTCAGCATGCTGCCCTGGCCGTTGCGGTCCATGTACACGCGGAGACGTGGCACTTTTCG	59
	P2.R2	CAGGCTACCGGTGTAGTACGACGGGTCGATCGCGCCCAGGGTCAGCATGCTGCCC	55
	P2.R3	CGGTGAACTGCCAGTACTGCTGGACGGTCACCGGCACCCAGTGCAGGCTACCGGTGTAG	59
	P2.R4	GACGGCCACACCGTTGATCGTCACGCTGTCCACGGTGAACTGCCAGTAC	49
	P2.R5	GCCGGTGTCCAGGATCGCCTGGCAACCACCGACGCAGGCGACGGCCACACCGTTG	55
	P2.R6	CTTCAGGATGTCGCTGCTCGGGCCGAACAGCACGCTCGTGCCGGTGTCCAGGATC	55
	P2.R7	AAGCTCTTCTCGATCGCCATCTGGATCTTCAGGATGTCGCTGC	43

Restriction site for the *LguI* restriction enzyme is: GCTCTTC,

The gene was divided into two fragments (Chym.P.1 and chyms.P.2) for accurate assembly. After assembly, the PCR products was cut with *lguI* and ligated with *T4-ligase* then it was transferred into *E. coli* DH5α with the general protocol (Chung *et al.*, 1989). The correct two plasmids (pE-chym.P.1 and pE-chym.P.2) were extracted and identified by cutting with restriction enzyme before sending for sequencing. Afterwards, the two plasmids were cut with *Esp3I* restriction enzyme and the two fragments were fused together and inserted in AmpR vector (pFusion). Then, the assembled fragment (1101 bp) was cloned in pEntry vector to produce *pE-pro.chym* and then sub-cloned into the expression vector (pASG.wt). The expression plasmid (*pASG-pro.chym*) was used for protein production.

Bacteria cultivation:

Escherichia coli DH5α and *E. coli* BL21 (DE3) cells were cultivated at 37°C on lysogeny broth (LB) media supplemented with the appropriate antibiotics according to the demand (ampicillin 100 µg/ml or kanamycin 50 µg/ml). The required plasmids were transferred into chemically competent *E. coli* BL21 (DE3) or DH5α cells following the standard protocol (Chung *et al.*, 1989).

Gene expression and biomass production:

Pro-chymosin gene was expressed in *E. coli* BL21(DE3). Freshly transformed cells were plated on LB-agar medium supplemented with (100 µg/mL Ampicillin) and one correct colony was expanded step-wise to yield final culture volume of 500 mL in LB-Medium (100 µg/mL Ampicillin). When the cells reached an OD_{578} nm of about 0.4, the gene was expressed by adding the inducer (anhydrotetracycline, 200 ng/mL) for 2 hr at 37 °C. The induced cells were harvested by centrifugation at 6,000 g for 15 min, washed with Tris-buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0) and stored at -80°C until used (Aboulnaga *et al.*, 2013).

Inclusion bodies purification:

Inclusion bodies purification was started with about one gram of wet cells. Cells were resuspended in 20 ml of lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mg/ml lysozyme, pH 8.0) and incubated at 37 °C for 30 min. Then, the suspension was sonicated in an ice-water bath using a Branson Sonifier with a duty cycle of 50% at 60% power for 10 min. The inclusion bodies were isolated from the mixture by centrifugation at 10,000 g for 20 min at room temperature. Clear supernatant was discarded and the pellet was washed twice with 50 ml of 10 mM EDTA, once with 0.5% triton X-100, and once with 20 mM phosphate buffer pH 7.5 (Rodríguez-Carmona, 2010).

Solubilization and renaturation of recombinant pro-chymosin:

Solubilization and renaturation has been done according to Menzella (2011) protocol with the following modification. Cleaned inclusion bodies were dissolved in solubilization buffer (50 mM KH_2PO_4, 8 M urea, pH 10.5). The solution was incubated at 30°C for 2 hr with continuous stirring, and then centrifuged at 10,000 g for 10 min to remove the insoluble molecules. The clear preparation was diluted with the renaturation buffer (50 mM KH_2PO_4, 0.5 M arginine, 10 µM $CuSO_4$, pH 10.5) to have a final protein concentration of 1 mg/ml (dilution factor between 20-30 times) and incubated for 12 hr at 4 °C. The correctly folding pro-chymosin was activated by acidifying the solution to pH 2.0 with 1.0 M HCl and incubated for 6-8 hr at 20 °C for digesting the pro-peptide. Then, the pH was adjusted to 6.3 by adding 1 M NaOH and it was incubated for second time for an additional 2 hr and the active chymosin was stored at 4 °C.

Enzyme activity measurements:

The milk-clotting activity of the chymosin was determined based on the end-point dilution methods (Emtage *et al.*, 1983). The substrate was prepared by reconstitute 10 g skim milk in 100 ml phosphate buffer (20 mM KH_2PO_4, 10 mM $CaCl_2$, pH 5.5) at room temperature (25 °C)and then the skim milk solution was preincubated at 35 °C for 30 min before measurement. The international milk clotting units (IMCU) was calculated by using commercial calf chymosin (1500 IMCU) as standard. One unit was defined as the amount of enzyme necessary to clot 1.0 ml milk at 35 °C in 40 min.

Determination of optimal pH for recombinant chymosin activity:

To identify the optimum pH for recombinant chymosin activity, skim milk powder was reconstitute in either 50 mM acetate buffer (pH 5.0- 5.5) or 50 mM potassium phosphate (pH 5.5–6.5) buffer. The clotting activity was measured as mentioned above and the relative activity was plotted against pH.

Determination of optimal temperature for recombinant chymosin activity:

The optimum temperature was determined after incubating both enzyme and substrate solutions (skim milk powder 10% in 20 mM KH_2PO_4, 10 mM $CaCl_2$, pH 5.5) at different temperature ranging from 40 °C to 65 °C for 10 min. Afterwards, the enzyme solution was added and gently mixed with substrate and incubated at the selected temperature to measure the clotting activity.

SDS-PAGE analysis:

SDS-PAGE was performed with 12% polyacrylamide gel to separate proteins of the whole cell extract, cell free extract, and inclusion bodies in the presence of pre-stained marker (10- 250 kDa). To visualize the protein bands, Coomassie brilliant blue (G-250) was used to stain the gels (Laemmli, 1970).

RESULTS AND DISCUSSION

Construction of the camel pro-chymosin gene and expression plasmid:

The amino acids sequence of camel pro-chymosin protein (Fig.1) was obtained from the online database. The encoding codon for this protein was optimized to facilitate maximum expression in *E. coli*. The optimum codon encoding for camel pro-chymosin gene (pro.chym, 1101 base pair) was *in vitro* synthesized and inserted in a bacterial cloning vector (*pE-Entry*). After plasmid extraction, the *pE-pro.chym* plasmid was sequenced to identify the inserted fragment (Fig. 2). The correct plasmid was used further for sub-cloning into expression vector directly downstream *E. coli* strong *tet*-promoter. The resulting plasmid, *pASG-pro.chym*, had a 4263 bp (Fig.3.A) which was appeared as a strong band on gel

electrophoresis after digestion with *HindIII*/ or *SacI* restriction enzyme (Fig.3.B). The transformed *E. coli* BL21 (DE3) with *pASG-pro.chym* plasmid was further analyzed with colonies PCR-screening using internal vector-based-primers (Seq.F and Seq.R) which produced a 1304 bp fragment (Fig.3.C). The correct colony carrying the *pASG-pro.chym* plasmid should produce a 1304 bp fragment (Fig.3.D).

>Camel chymosin Amino acid sequence

MASGITRIPLHKGKTLRKALKERGLLEDFLQRQQYAVSSKYSSLGKVAREPLTSYLDSQYFG
KIYIGTPPQEFTVVFDTGSSDLWVPSIYCKSNVCKNHHRFDPRKSSTFRNLGKPLSIHYGTG
SMEGFLGYDTVTVSNIVDPNQTVGLSTEQPGEVFTYSEFDGILGLAYPSLASEYSVPVFDNM
MDRHLVARDLFSVYMDRNGQGSMLTLGAIDPSYYTGSLHWVPVTVQQYWQFTVDSVTINGVA
VACVGGCQAILDTGTSVLFGPSSDILKIQMAIGATENRYGEFDVNCGSLRSMPTVVFEINGR
DYPLSPSAYTSKDQGFCTSGFQGDNNSELWILGDVFIREYYSVFDRANNRVGLAKAI

Fig. 1. Complete Amino acids sequence of camel chymosin protein. The first 44 amino acids which present the pro-sequence are written in bold red italic line.

>Camel chymosin DNA sequence

ATGGCCTCGG GCATCACCCG CATCCCGCTG CACAAGGGCA AGACCCTGCG
CAAGGCGCTG AAGGAACGCG GCCTGCTGGA GGACTTCCTG CAGCGCCAGC
AGTACGCCGT GAGCAGCAAG TACAGCTCGC TGGGCAAGGT GGCCCGCGAA
CCGCTGACCA GCTACCTGGA CAGCCAGTAC TTCGGCAAGA TCTACATCGG
CACCCCCCCG CAGGAGTTCA CGGTCGTGTT CGACACCGGT AGCTCGGACC
TGTGGGTCCC GAGCATCTAC TGCAAGAGCA ACGTGTGCAA GAACCACCAT
CGCTTCGATC CCCGCAAGAG CTCGACGTTC CGCAACCTGG GCAAGCCGCT
GAGCATCCAT TACGGCACCG GCAGCATGGA GGGCTTCCTG GGCTACGACA
CGGTGACCGT GAGCAACATC GTCGACCCGA ACCAGACCGT GGGCCTGAGC
ACGGAGCAGC CGGGCGAGGT GTTCACCTAC TCGGAGTTCG ACGGCATCCT
GGGCCTGGCC TACCCGTCCC TGGCCAGCGA GTACTCGGTG CCGGTGTTCG
ACAACATGAT GGACCGCCAC CTGGTGGCGC GCGACCTGTT CAGCGTGTAC
ATGGACCGCA ACGGCCAGGG CAGCATGCTG ACCCTGGGCG CGATCGACCC
GTCGTACTAC ACCGGTAGCC TGCACTGGGT GCCGGTGACC GTCCAGCAGT
ACTGGCAGTT CACCGTGGAC AGCGTGACGA TCAACGGTGT GGCCGTCGCC
TGCGTCGGTG GTTGCCAGGC GATCCTGGAC ACCGGCACGA GCGTGCTGTT
CGGCCCGAGC AGCGACATCC TGAAGATCCA GATGGCGATC GGTGCCACCG
AGAACCGCTA CGGCGAGTTC GACGTGAACT GCGGCAGCCT GCGCTCCATG
CCCACCGTGG TGTTCGAGAT CAACGGCCGC GACTATCCCC TGAGCCCGAG
CGCGTACACC AGCAAGGACC AGGGCTTCTG CACCAGCGGT TTCCAGGGCG
ACAACAATAG CGAGCTGTGG ATCCTGGGCG ACGTCTTCAT CCGCGAGTAC
TATAGCGTGT TCGACCGCGC GAACAATCGC GTGGGCCTGG CGAAGGCGAT
CTAA

Fig. 2. Complete DNA sequence of codon optimized camel chymosin gene

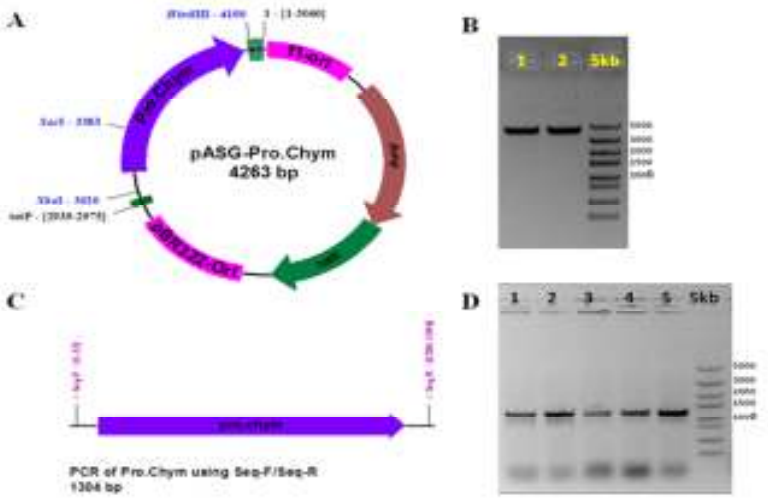

Fig. 3.Construction of the camel pro-chymosin gene and expression plasmid. **(A) Schematic representation of the *pASG-pro.chym* plasmid. The sequence of encoding camel pro-chymosin was expressed under tet-promoter. (B) DNA electrophoresis for the *pASG-pro.chym* plasmid after digestion with *HindIII* (lane 1) and *SacI* (lane 2) restriction enzymes; (C) Schematic representation of DNA-fragment (1,304 bp) containing *pro.chym* gene after amplification with the seq.F and seq.R primers. (D) DNA-electrophoresis of colonies PCR with seq.F and seq.R primers for 5 different colonies.**

Heterologous expression of the pro-chymosin gene in *E. coli*:

Correct *E. coli* BL21 (DE3) colonies carrying *pASG-pro.chym* plasmid were tested for pro-chymosin expression. Cells were grown in LB-media supplemented with ampicillin (100 µg/mL) and induced with 200 ng/mL anhydrotetracycline when the OD_{578} was 0.4. Biomass was harvested before induction and 2 hr post-induction. Cellular extracts were subjected to electrophoresis on 12% SDS- polyacrylamide gel (Fig.4). Obtained result from the analysis of pro-chymosin by SDS-PAGE (Fig.4) revealed that pro-chymosin (367 amino acid residue) was visualized as a sick band. Also its protein had a molecular weight with about 41 kDa, which it is almost similar to the calculated molecular weight (40.6 kDa) as well as the previously reported molecular weight (Kappeler *et al.*, 2007; Langholm Jensen *et al.*, 2013 and Wang *et al.*, 2015). Further analysis of the produced pro-chymosin band using LabWork3.0 indicating that the amount of recombinant protein was 250 mg/L and it was comparable with that reported before for recombinant bovine chymosin (Menzella, 2011). The reported expression level of *E. coli* BL21 (DE3) for recombinant bovine chymosin after 5 hr post-induction was ranged from 260 for the wild type sequence to 450 mg/L after codon optimization (Menzella, 2011). Since the sequence was optimized, it should produce the highest level but the presented data (250 mg/L) was only after 2 hr post-induction which make it comparable with the previously reported data (Menzella, 2011).

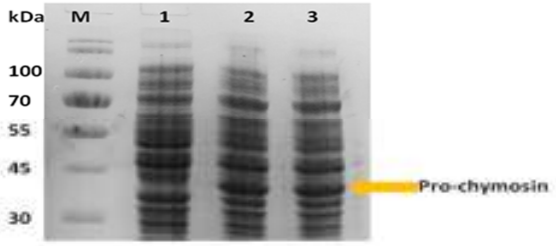

Fig. 4. Expression analysis of the pro-chymosin by SDS-PAGE. M, is molecular weight ladder. Lane 1, lysate of *E. coli* BL21 harbouring *pASG-pro.chym* expression plasmid without induction. Lane 2 and 3, lysate of two different colonies of *E. coli* BL21 harbouring *pASG-pro.chym* expression plasmid after 2hr induction.

Solubilization and renaturation of recombinant pro-chymosin:

Recombinant pro-chymosin is expressed in bacteria in insoluble form and it is presented in the inclusion bodies. This finding has been reported for the production of recombinant bovine chymosin in *E. coli* (Mule *et al.*, 2009; El-Sohaimy *et al.*, 2010 and Menzella, 2011). The recombinant camel chymosin behaves like the bovine one that is presented in inclusion bodies and is not presented in the cell free extract (Fig. 5). The protein is completely soluble in the solubilizing solution which contains 8.0 M urea. After renaturation and subsequently activation, the mature active form of chymosin shows a molecular weight of 35.6 kDa (Fig.5, lane 4 and 5). It should be noticed that, recombinant camel chymosin expressed in fungus or yeast is glycosylated and the apparent molecular weight is between 38-40 kDa, while after treatment with endoglycosidase, the protein show a band corresponding to a molecular weight of 35-37kDa (Kappeler, 2006; Langholm Jensen *et al.*, 2013 and Wang *et al.*, 2015). Since bacteria system does not glycosylate protein which is only happen in eukaryote systems, the apparent molecular weight of protein is almost similar to the calculated one.

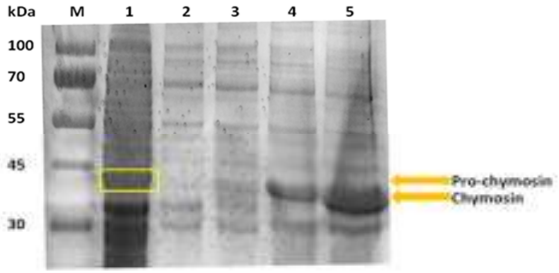

Fig. 5. SDS-PAGE analysis of the recombinant pro-chymosin after Solubilization and renaturation. M, is molecular weight ladder. Lane 1, whole cell lysate of *E. coli* BL21 harbouring *pASG-pro.chym* expression plasmid after 2 hr post-induction. Lane 2 and 3, cell free extract of the lysate *E. coli*. Lane 4 and 5, the active chymosin after activation and precipitation with Ammonium sulphate.

Activity measurement of recombinant camel chymosin:

The clotting activity of recombinant camel chymosin obtained from *E. coli* was compared with commercial calf chymosin in Fig. (6). Recombinant chymosin without renaturation is not active and required renaturation process for the generation of active clotting enzyme. Furthermore, the active ammonium sulphate precipitated recombinant camel chymosin has doubled the clotting activity of commercial calf chymosin. This indicates that, the enzyme has a 300 IMCU/ ml. Therefore, it could be concluded that biologically active chymosin expressed in fungi (Kappeler, 2006) or yeast (Wang *et al.*, 2015) can be produced in *E. coli* after solubilization, renaturation, and acidic activation. Although, calf chymosin has been expressed in *E. coli* and it was active after solubilization, renaturation, and acidic treatment (El-Sohaimy *et al.*, 2010 and Menzella, 2011).

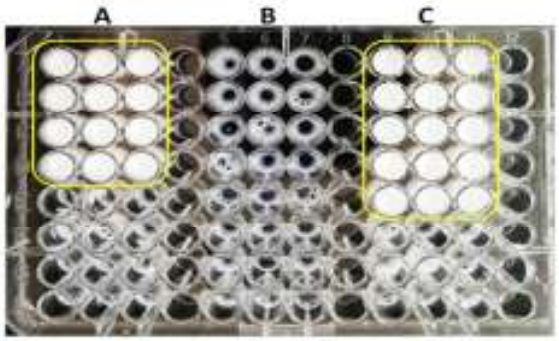

Fig. 6. Milk clotting activity of commercial calf chymosin and the recombinant camel chymosin obtained from *E. coli*. Serial dilutions were prepared in microtiter plate and the clotted milk in the wells is shown. A (column 1-3): Serial dilution of the standard calf chymosin (150 IMU/ ml) was prepared. B (column 5-7): Presented serial dilution of the recombinant camel chymosin derived from *E. coli* before renaturation. C (column 9-11): Presented serial dilution of the recombinant camel chymosin after renaturation and activation.

Optimal pH for the produced recombinant camel chymosin:

The effect of the substrate pH on the clotting activity of recombinant chymosin was evaluated and the results are presented in Fig. (7). The recombinant enzyme has a narrow pH-range for its activity. The enzyme shows more than 80% of its relative activity with the pH value of 5.25 and more than 90% of its relative activity when the pH values were 5.5 or 6.0. The highest level of recombinant camel chymosin activity was observed at pH between 5.5 and 6.0 which is also the range of the animal's milk pH. Two different optimum pH values have been reported before for recombinant camel chymosin. The recombinantly produced camel chymosin in *Asperigillus niger* had an optimum pH of 6.0 (Kappeler, 2006), while the produced enzyme in *Pichia pastoris* had an optimum pH value of 5.04 (Wang *et al.*, 2015). In contrast, recombinant bovine chymosin had a narrow pH-range for its activity between 5.4 to 5.6 (Cardoza *et al.*, 2003; Starovoitova *et al.*, 2006 and Noseda *et al.*, 2013).

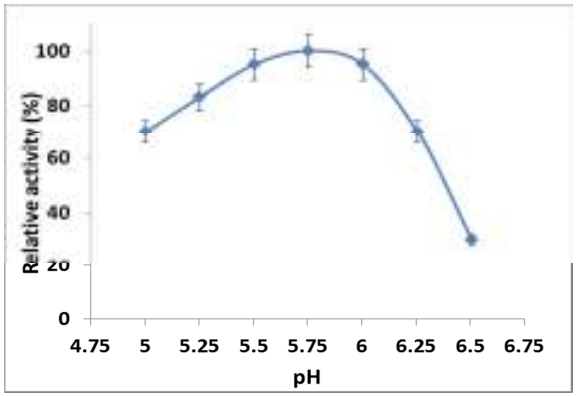

Fig. 7. Optimum substrate pH for the recombinant camel chymosin activity. The effect of pH values on the milk clotting activity of recombinant camel chymosin was determined and it was plotted against the relative activity.

Optimal temperature for the produced recombinant camel chymosin:

Figure (8) show the effect of temperature on the clotting activity of the recombinant camel chymosin. There was a significant effect of the temperature on the milk clotting activity. The enzyme show maximum activity (100%) when the temperature was in the range of 50-55 °C. When the temperature was changed within 5 °C down or above the optimum range, a significant decrease of the activity was observed and it was reached of 70 and 60% relative activity, respectively. Further increment of temperature had a significant inhibition effect on the activity due to denature the proteins (Wang *et al.*, 2015). A further decrease in relative activity to reach about 35 and 5% was observed when the temperature was 65 and 70 °C, respectively. These data are in agreement with the previously published data, in which camel chymosin had an optimum temperature of 45-55 (Kappeler, 2006 and Wang *et al.*, 2015). It is known that, animal source chymosin is temperature sensitive opposite to plant source rennin which is more resistance for high temperature (Gama Salgado *et al.*, 2013 and Li Yuqiu and Qiyun, 2015).

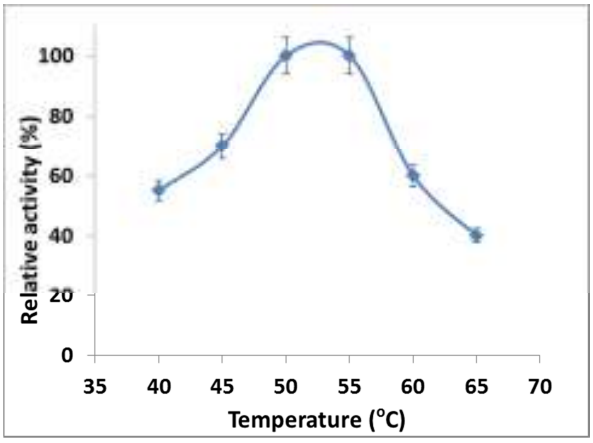

Fig. 8. Optimum temperature for the recombinant camel chymosin clotting activity. The effect of temperature on the milk clotting activity of recombinant camel chymosin was determined and it was plotted against the relative activity.

Optimal concentration of $CaCl_2$:

The effect of $CaCl_2$ on the clotting activity of the recombinant camel chymosin was studied and the data were plotted in Figure (9). Calcium ions has high impact in milk clotting in which it bind with the insoluble peptide derived (Para-casein) from casein hydrolysis and form clot.

The data in Fig. (9) show that, the enzyme required narrow concentration of $CaCl_2$ for optimum activation. The maximum activity was observed when $CaCl_2$ concentration was 15- 30 mM and less than 90% relative activity was detected when $CaCl_2$ concentration was 10 or 45 mM. These data were closed with the reported results for recombinant camel chymosin (Wang *et al.*, 2015) and also with the microbial rennin that has optimum $CaCl_2$ concentration of 50 mM (Foda, 2012).

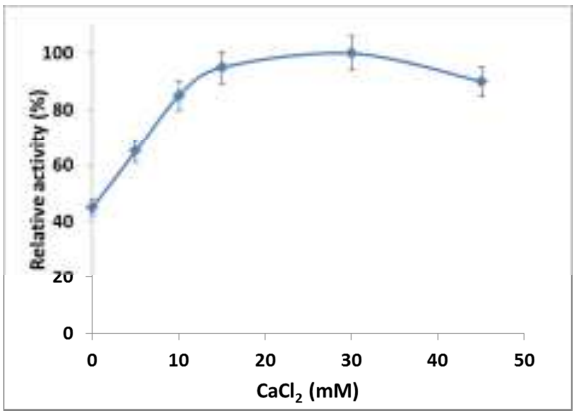

Fig. 9. Optimum concentration of $CaCl_2$. The effect of $CaCl_2$ on the milk clotting activity of recombinant camel chymosin was determined and it was plotted against the relative activity.

CONCLUSION

In this work, recombinant camel pro-chymosin was successfully constructed and expressed in *E. coli* BL21 under tet-promoter. The protein was produced in an insoluble form. It was *in vitro* solubilized, re-folded from the inclusion bodies, and activated under acidic conditions.

The optimum milk-clotting activity conditions for mature camel chymosin were pH of 5.75, temperature between 50-55 °C, and 15 mM of $CaCl_2$. The produced recombinant chymosin could serve as an economic alternative source to bovine chymosin in cheese manufacturing. This study provide that, it is possible to obtain an active mature camel chymosin from *E. coli* as a first step for large-scale production of recombinant enzyme to meet the needs of local market and maintain the foreign currency.

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الاستنساخ والتعبير الجيني لمولد- الكيموزين الجملي في خلايا بكتيريا الـ *E. coli* وتوصيف الانزيم النشط المتحصل عليه

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يعتبر الكيموزين من أهم الانزيمات في صناعة منتجات الالبان حيث انه يستخدم في الانتاج التجاري للجبن. لكن الانتاج التقليدي للكيموزين من الحيوانات يؤدي الي فقد عدد كبير من العجول الرضيعة مما يؤثر بالسلب علي الثروة الحيوانية. ومن اجل تقليل تكاليف الانتاج، يعتبر الانتاج المعدل للكيموزين في الخلايا الميكروبية هو الاختيار الامثل. في هذه الدراسة تم التخليق المعملي لجين مولد-الكيموزين الجملي (طوله 1101 زوج من القواعد) وتم استنساخة في البلازميد *pASG*-vector. وبعد ذلك تم نقل البلازميد *pASG_pro.chym* المتحصل علية الي خلايا بكتريا *E. coli* BL21(DE3). ثم تم التعبير الجيني لمولد-الكيموزين الجملي بهذا النظام البكتيري تحت التحكم بواسطة tet-promoter وكان هذا النظام قادر علي انتاج حوالي 260 مجم/ للتر من الكيموزين المعدل علي النطاق المعملي. وقد اظهرت الهجرة الكهربائية SDS-PAGE ان البروتين الغير نشط (367 متبقي حامض اميني) له وزن جزيئ 40.6 كيلو دالتون، بينما الشكل النشط للانزيم (323 متبقي حامض اميني) له وزن جزيئ 35.6 كيلو دالتون. وظهر الكيموزين الجملي المعدل في الاجسام المترسبة وأمكن اذابته في محلول يحتوي علي اليوريا بتركيز 4-8 مول/لتر. بعد الاذابة واعادة التشكيل الطبيعي للبروتين، تم تعريض الانزيم لـ pH منخفض (وسط حامضي) لتحويله الي كيموزين نشط. وأظهرت النتائج ان الظروف المثلي لعمل الانزيم المعدل هي كالتالي: قيمه الـ pH = 5.75 ، درجة حرارة من 50 الي 55 ْم، وتركيز 15 مليمول/لتر من كلوريد الكالسيوم. من هذه النتائج يمكن استنتاج ان الكيموزين الجملي المعدل المتحصل عليه من خلايا بكتيريا الـ *E. coli* مناسب لـ الانتاج التجاري للجبن.

الكلمات الدالة : الكيموزين الجملي – الاستنساخ والعبور الجيني الي خلايا بكتيريا *E. coli* –الاذابة واعادة التنشيط– الظروف المثلي للتجبن.