Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2013, 3(6):489-494

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Effect of plasmin digest of κ-casein on survival of some pathogenic and probiotic bacteria

Marjaneh Sedaghati[1*], Hamid Ezzatpanah[1], Massod Mashhadi Akbar Boojar[1], Maryam Tajabadi Ebrahimi[1] and Mehdi Aminafshar[3]

[1]*Department of Food Science and Technology, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2]*Department of Biochemistry, University of Kharazmi, Tehran, Iran*
[3]*Department of Science, Faculty of Biology, Islamic Azad University, Tehran Central Branch, Iran*
[4]*Department of Animal Science and Technology, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran*

ABSTRACT

The aim of this work was to evaluate the presence of antibacterial properties in a plasmin digest of bovine κ-casein. Although native bovine κ-caseinis resistant to plasmin, extensive hydrolysis of this milk protein was observed applying a long incubation period. The antibacterial potential of κ-casein, plasmin, PDκ was evaluated against pathogenic (Escherichia coli and Staphylococcus aureus) and probiotic (Lactobacillus casei and Lactobacillus acidophilus) bacteria in vitro. Although κ-casein and plasmin had no antibacterial activity, PDκ showed antibacterial property against the tested bacteria. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of PDκ was determined for the target bacteria. The MIC and MBC of PDκ against Escherichia coli was considerably higher than Staphylococcus aureus, Lactobacillus casei and Lactobacillus acidophilus bacteria. The growth curves alterations of target bacteria in the presence of PDκ were monitored by turbidimetry in broth culture. The effect of PDκ on lag time and maximum absorbance was more significant than slope of tested bacteria. In addition of growth curves, PDκ has inhibitory effects on the plate count confirmation of tested bacteria. The maximum inhibitory effect of PDκ was created in MIC concentration.

Keywords: Bovine; K-casein; Antibacterial; Plasmin; Growth curve

---

INTRODUCTION

Milk is naturally antimicrobial. As milk is formed in the mammary glands, it contains immunity factors, such as immunoglobulins, from the mother's blood [1,2]. These immunoglobulins and non-immunoglobulins proteins affect the neonates fight against microbial infection [3,4]. In addition to naturally occurring antimicrobial proteins present in milk, a variety of antibacterial peptides can be released from their parent molecules such as caseins and whey proteins by hydrolysis of them[5,6,7].

κ-casein consists of a single chain of 169 amino acids and has a theoretical molecular weight of 18 974 Da and a theoretical pI of 5.93. It is amphipathic with very specific hydrophobic and polar domains. This casein plays an important role in the formation and stabilization between caseins in sub-micelles and in the complete micelle[1,8].

Enzymatic hydrolysis of κ-casein may affect human health by improving some of their biological properties, including antithrombotic function, opioid activity and protective properties against different microorganisms and

viruses[9,10,11].Much attention has been paid to the antimicrobial activity of κ-casein. The antimicrobial activities of κ-casein and its hydrolysate offer the potential application of this protein for increasing food products stability by stopping their microbial damage[12, 13, 14, 15]. Since these antimicrobial peptides are generally recognized as safe (GRAS),κ-casein hydrolysate have attracted particular interest [16].

The antimicrobial peptides produced from κ-casein by pepsin, trypsin, chymotrypsin and chymosin, has already been studied [14, 15], but to our knowledge, no study has been carried out on the antimicrobial activities of plasmin digest of κ-casein (PDκ). Plasmin is by far the predominant and most completely studied endogenous protease in bovine milk[17, 18].

Therefore, the present work was undertaken to study the antimicrobial properties of PDκ. This study also want to determine the changes in the growth curves and plate count confirmations of pathogenic and probiotic bacteria in the presence of PDκ.

MATERIALS AND METHODS

Materials

Bovine κ-casein and bovine plasmin (EC Number3.4.21.7) were supplied from Sigma-Aldrich Chemie GmbH (Munich, Germany).Brain-Heart Infusion Agar (**BHIA**), Brain-Heart Infusion Broth (**BHIB**), MRS Agar (Man, Rogosa and Sharpe Agar), and MRS Broth (Man, Rogosa and Sharpe Broth) were obtained from Merck (Darmstadt, Germany). Cultures of *Escherichia coli* (PTCC 1399*)* and *Staphylococcus aureus* (PTCC 1431) came from the Iranian Research Organization for Science and Technology Company (IROST) in Tehran. Cultures of *Lactobacillus acidophilus* (DSMZ 1643*)* and *Lactobacillus casei*(DSMZ 1608) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen Germany company.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed by the method of Dalasgaard et al., (2008) [19]. The bovine κ-casein with concentration of 3 $mgml^{-1}$ was prepared in 10 mM phosphate buffer pH 6.8. Then bovine plasmin was added to the aliquots of bovine κ-casein substrate proteins at an enzyme: substrate ratio of 1:150 (wt/wt). Enzymatic hydrolysis was implemented by incubating at 30°C for 44 h.

Antibacterial assay

K-casein, plasmin and PDκ were tested for antibacterial activity against pathogenic as well as probiotic bacteria. For assaying the overnight of every bacteria culture was diluted to approximately 10^6 $cellml^{-1}$. To each sterile eppendorf vial BHI broth or MRS broth, antibacterial compound, and bacteria culture was added. Control experiment was contained no antibacterial compound. All vials were incubated at 37°C for 18 h (36 h for probiotic bacteria). The optical density was measured at 620 nm using Cecil Spectrophotometers (Cecile 7400 UV-Visible, Cambridge, England) for all samples. The experiments were repeated three times for each sample.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of PDκ

MIC assays were done such as antibacterial assay but different concentration of PDκ was used. The MIC of PDκ was defined as the lowest concentration of this compound that resulted in no increase of absorbance at 620 nm after incubation. The experiments were repeated three times for each sample.MBC assays were performed for all vials in which no bacterial growth was observed. Selected samples were seeded on BHIA for pathogenic bacteria and MRSA for probiotic bacteria. Samples were incubated for 24 h or 48 h at 37 °C.MBC was defined as the lowest concentration of the PDκ that prevented colony formation after subculture on agar medium. MBC assay were tested three replicates for each sample.

Effect of PDκ on growth curves and plate count confirmation of bacteria

For assaying the overnight of every bacteria culture was diluted to approximately 10^6 cell ml^{-1}. BHI broth or MRS broth, and 10µl of overnight cultured bacteria were added to each sterile vial. PDκ was added in different concentrations (MIC concentrations, 0.5 MIC concentrations and 0.25 MIC concentrations). All vials were incubated at 37°C.The optical density was measured at 620 nm every two hours over 24 hours for pathogenic bacteria and every two hour over 48 hours for probiotic bacteria. The lag time (time from beginning of incubation until the time-point when absorbance began to increase), slope (slope of the growth curve in logarithmic growth phase), and maximum absorbance (highest absorbance value measured during log phase) were used as variables describing the bacterial growth.

For plate count test, at each incubation period,1 ml sample was collected, diluted, and plated on to BHI agar or MRS agar. These plates were incubated at 37°C for 24 or 48 h and were read by the colony counter (Colony Star Funke Gerber, Germany).

Statistical methods

In this study the significance effect of different concentrations of PDκ on lag time, slope, and maximum absorbance was determined statistically with the Duncan's New Multiple Range Test, p value < 0.05 using SPSS for Windows, version 19.

RESULTS AND DISCUSSION

Evaluation of antimicrobial activity

In the current study the antibacterial activity of κ-casein, plasmin and PDκ were tested against *E. coli*, *S.aureus*, *L. case*i and *L. acidophilus* bacteria. Although κ-casein and plasmin (10-200 unitml^{-1}) had no antibacterial effect on pathogenic and probiotic bacteria, plasmin digeste of κ-casein (PDκ) showed antimicrobial activity against all target bacteria. PDκ which contained hydrolyzed polypeptides revealed high antibacterial properties against all target bacteria. The optical density of *E. coli*, *S. aureus*, *L. case*i, and *L. acidophilus* samples after exposure to 150 µgml^{-1} of PDκ was shown no turbidity compared with the respective controls. Our findings were similar to Matin et al., (2000); Malkoski et al.,(2001), López-Expósito et al.,(2006); who reported that peptides produced from proteolytic digestion of κ-casein liberated antibacterial activity against tested bacteria[12,14,15].

The results of the antibacterial assay revealed that plasmin had no antibacterial activity on bacteria tested at 10 to 200 unitml^{-1} concentrations. To our knowledge, no study has been carried out on the antimicrobial activities of plasmin.

Determination of minimum inhibitory concentration (MIC)and minimum bactericidal concentration (MBC) of PDκ

The ability of the PDκ to show antimicrobial activity against tested bacteria was assessed and the minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC)was evaluated. Against the Gram-positive bacteria(*S. aureus*, *L. acidophilus*, and *L. case*i), PDκ had MIC and MBC ranging from 20 to 25 µg ml^{-1}.The MIC and MBC of PDκ against the Gram-negative bacteria, *E.coli* (60and75µg ml^{-1}) were indicated that this compound was active against all tested bacteria and the effective MIC was not high (Table 1). Our finding is not consistent with the study of McCannet al.,(2005) who demonstrated the MIC of the chymosin digest of sodium caseinate (CrMIX) was very high against all tested bacteria [20].

PDκ had higher MIC values for Gram-negative bacteria than Gram-positive bacteria (about three times).This is consistent with the findings of Pellegrini, et al., (1999); McCannet al.,(2005), López-Expósito et al.,(2006),who reported that Gram-positive bacteria were more susceptible to the action of antibacterial peptides than Gram-negative bacteria [15,20,21].

In the present study Gram-positive bacteria (*S. aureus*, *L. acidophilus*, and *L. case*i) were more sensitive to the action of the fore mentioned antibacterial component than Gram-negative bacteria *(E.coli)*. The higher resistance of Gram-negative bacteria might relate to the complexity of their cell membrane structure compared with Gram-positive bacteria [22].

Table 1 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of PDκ against tested bacteria (µg.ml^{-1} unite)

Bacteria	MIC (µg.ml^{-1})	MBC (µg.ml^{-1})
E.coli	60	75
S. aureus	20	20
L. casei	25	25
L.acidophilus	20	20

MIC = Minimum inhibitory concentration
MBC= Minimum bactericidal concentration

Effect of PDκ on the growth curves of bacteria

The growth curves of pathogenic (*E. coli* and *S.aureus*) and probiotic bacteria (*L. caseiand L. acidophilus*) were recorded by measuring the optical density over 24 h or 48 h. The effect of different concentrations of PDκ on maximum absorbance, lag phase and the slope of tested bacteria are presented in Table 1 and Figure 1. Although control had a lag phase in the first around 2 h for pathogenic and 4 h for probiotic bacteria, in the presence of PDκa longer lag phase was recorded. In the presence of 0.5 MIC concentrations PDκ, pathogenic bacteria was shown a lag phase in the first 8 h, while probiotic bacteria had 15 h lag phase.

After 2 h, maximum absorbance for the control increased fast and then kept stable. In the log phase, control samples of *E. coli*, *S. aureus*, *L. casei* and *L. acidophilus* could be reached to maximum absorbance of 0.96, 1.89, 2.35 and 3

respectively. In the presence of different concentrations PDκ, maximum absorbance decreased significantly. These reductions in bacteria growth were concentration-dependent. Our result showed that the effect of different concentrations PDκ on the maximum absorbance, lag phase and slope of *E. coli* and *L. case*i was statistically significant(p=0.0). Our findings also cleared that the maximum absorbance, slop and lag phase of *S.aureus* and *L.a cidophilus* in the presence of different concentrations PDκ was statistically significant(p = 0.0).

Although the growth curve of *E.coli* showed similar patterns in the presence of PDκ, these alterations happened at higher PDκ concentrations compared with *S. aureus, L. acidophilus* and *L. casei.*

Our findings were similar to Kutila et al.,(2003)who reported that Lf had inhibitory activity against udder pathogens and growth inhibition by Lf was concentration-dependent. The effect of Lf on the maximum absorbance and slope was significant where as the effect on the lag time was not significant [23]. According to Sekse et al.,(2012) the growth rate of most *Escherichia coli* strains declined when increasing concentrations of lactoferrin were added [24].

Table 2Effect of PDκ on bacterial growth in broth culture, measured by lag time and slop

	E. coli		*S. aureus*		*L. casei*		*L.acidophilus*	
Samples	Lag time (h)	Slop	Lag time (h)	Slop	Lag time (h)	Slop	Lag time (h)	Slop
Control	2 ±0.5	0.068	1.5 ±0.7	0.143	4.5 ±1.5	0.075	4 ±1	0.97
0.25 MIC	5 ±1	0.033	4 ±1.4	0.072	9 ±2.6	0.049	7.5 ±2.5	0.074
0.5 MIC	8 ±1.3	0.019	7 ±0.5	0.042	15 ±3.2	0.021	14 ±3.5	0.021
MIC	ND	0	ND	0	ND	0	ND	0

Lag time = Time from the beginning of incubation until the time-point when the absorbance began to increase
Slope=Slope of the growthcurve in logarithmic growth phase
ND=Not determined as no growth was observed following incubation

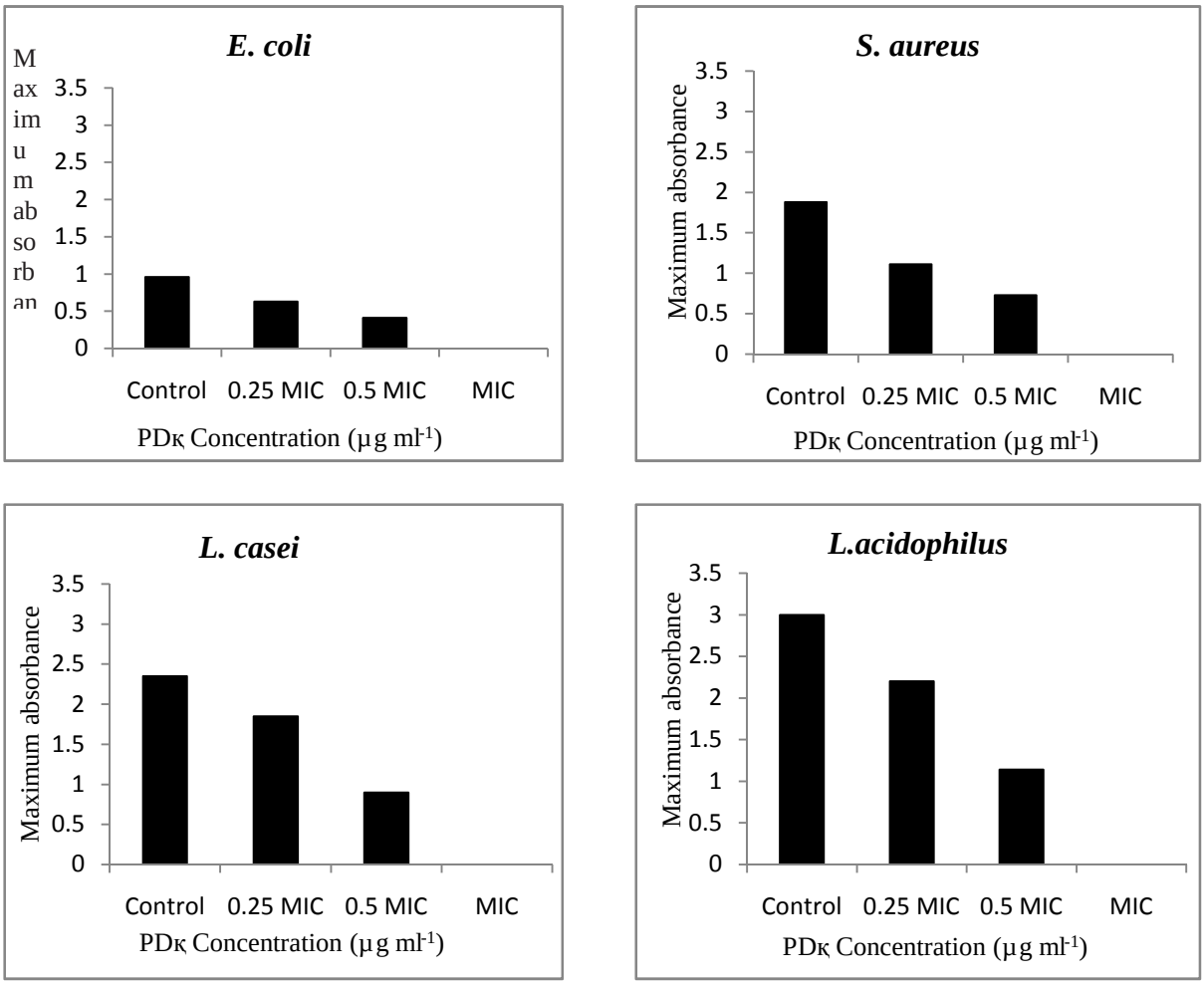

Figure1.Maximum absorbance of tested bacteria in broth culture with different concentrations of PDκ and without PDκ

Plate count for bacteria in the presence of PDκ

As shown in Figure 2 the plate counts of *E. coli* and *S. Aureus* was affected by PDκ with 3.19 and 3.97 log cfuml^{-1}maximum differences over the control after 16 h, respectively (MIC concentration). However, *L. casei* and *L. acidophilus* showed 5.1 and 4.86 log cfuml^{-1}maximum differences between the control and treated samples after 36 h (MIC concentration) respectively. These results showed that maximum difference in log cfuml^{-1}between treated samples and control happened during log phase and for probiotic bacteria was higher than pathogenic bacteria.

Although pathogenic and probiotic bacteria in the presence of PDκ had longer lag phases than the control, the number of bacteria in the lag phases showed limited changes. In the log phase, the plate count of *E. coli*, *S. aureus*, *L. casei* and *L. acidophilus* in the presence of PDκ showed differences of 2.28, 2.61, 3.88 and 3.46over the controls respectively (less than MIC concentration). These differences in the stationary phase for treated samples were less than for the log phase. The death phase had the least difference in the number of bacteria over the control. This is consistent with the findings of McDonnellet al.,(2012), who reported that the number of surviving cells following exposure to different concentrations of antibacterial peptides reduced significantly ($p<0.05$) for *Escherichia coli*[25].

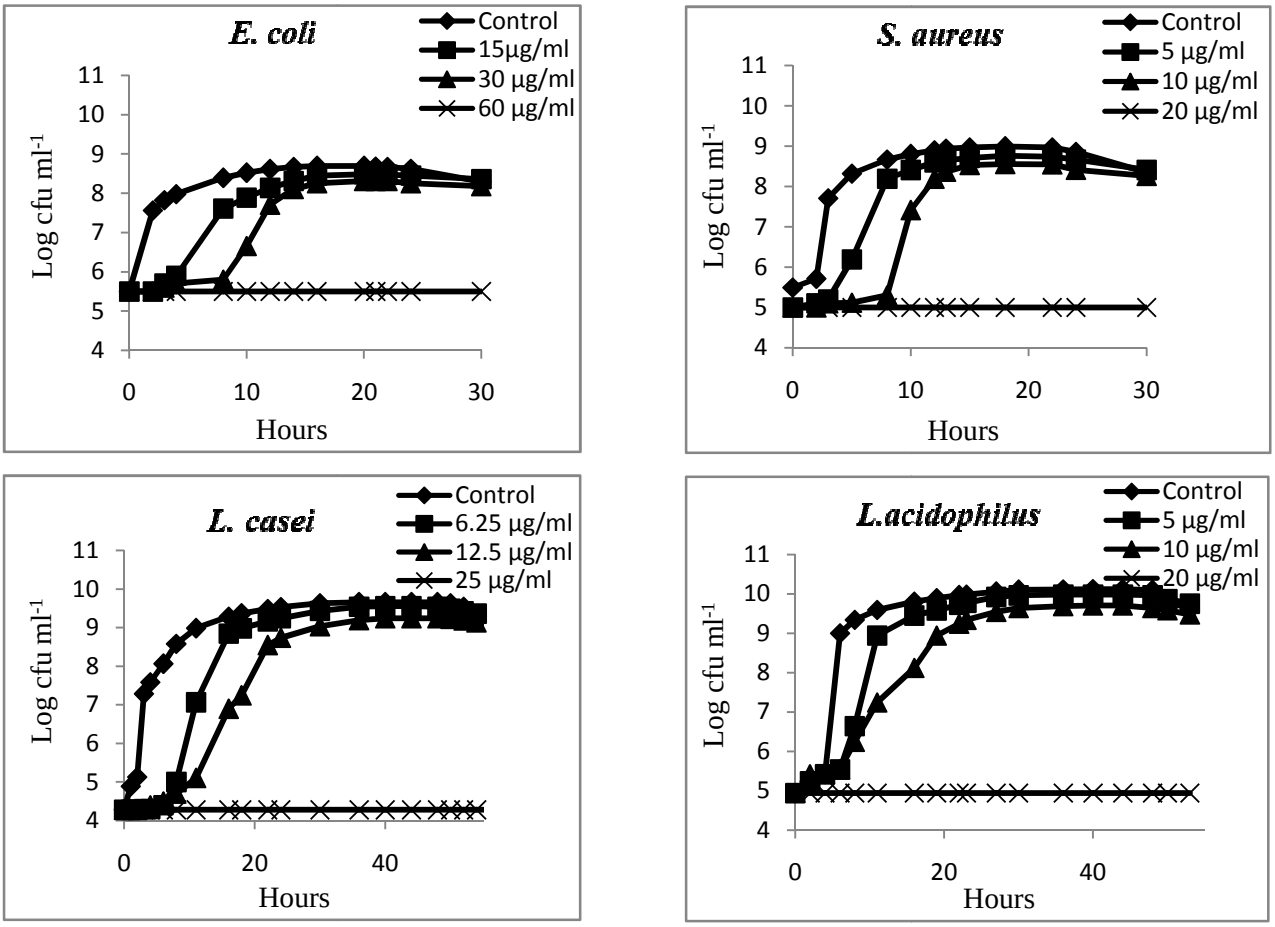

Figure 2.Effect of different concentrations PDκ on colony forming units (cfu) of tested bacteria

Also Christman(2010) reported that trypsin-casein hydrolysate (TCH) and pepsin-casein hydrolysate (PCH) suppressed growth log cfuml^{-1}of *Listeria monocytogenes* and *Escherichia coli* O157:H7over a 24 hour incubation period [26]. In addition Vongsawasdi et al., (2012) revealed that total plate counts of target bacteria (log cfuml^{-1}) decreased when the concentration of nisin increased [27].

The important result from this study proved that all tested bacteria had the most sensitivity to PDκ in the log phase. As a result, we should use them in the log phase for stopping pathogenic bacteria survival in a medium. The effect of PDκ on tested bacteria revealed that this compound has a good potential for increasing food microbial safety. Therefore, PDκ has great potential as natural food additives in food chain. The potential health benefit of PDκ has been a subject of growing commercial interest in the context of health-promoting functional foods.

CONCLUSION

Results of the present study show that antimicrobial activity can be influenced by PDκ. This result increases understandings of κ-casein and reveals that bactericidal peptides can be produced by proteolytic digestion of κ-casein with milk protease plasmin. In the present study, we identified PDκ with a potent inhibition against both Gram-positive and Gram-negative bacteria. All target bacteria had the most sensitivity to PDκ in the log phase. As a result, we should use them in the log phase for stopping pathogenic bacteria survival in a medium. Thus PDκ might have a potentially valuable role as food additives as well as instrengthening the immune system of the host.

Acknowledgments
The support of Islamic Azad University, Science and Research Branch of Tehran, Department of Biochemistry research, University of Kharazmi branch of Tehran, Department of Microbiology related to Islamic Azad University Central of Tehran Branch, is gratefully acknowledged.

REFERENCES

[1]Swaisgood HE. In:Fennema OR. (Ed.), Food Chemistry (Marcel Dekker Inc, NewYork, **1996**), 841–878.
[2]McCann KB,ShiellBJ, MichalskiWP, Lee A, Wan J,Roginski H, CoventryMJ,*IntDairy J*,**2006**,16 (4), 316–323.
[3] ClareDA, CatignaniGL, Swaisgood HE,*Curr Pharm Design*,**2003**, 9, 1239–255.
[4]SilvaSV, MalcataFX,*Int Dairy J*,**2005**, 15, 1–15.
[5]KorhonenH, Pihlanto-LeppäläA.In: ShorttC, BrienJO. (Ed.), Handbook of Functional Dairy Products (CRC Press, Boca Raton, **2004**), 109 – 124.
[6] MichaelidouAM,*Small Ruminant Res*,**2008**,79, 42-50.
[7] ParkYW, In: ParkYW.(Ed.),Bioactive components in milk and dairy products(Wiley-Blackwell, USA, **2009**), 3-5.
[8] ThompsonA, BolandJM, Singh H,*Milk Proteins: from Expression to Food*, Elsevier Academic Press, New York,**2009**.
[9]Jolles P, Levy-ToledanoS, Fiat AM, SoriaC,GillesenD, ThomaidisA, DunnFW, CaenJ;*Eur J Biochem*,**1986,** 158, 379–384.
[10] ChibaH, TaniF, YoshikawaM,*J Dairy Res*,**1989**, 56, 363–366.
[11] QianZY, JollesP, Migliore-SamourD, SchoentgenF, FiatAM,*BiochimBiophys*Acta,**1995**, 1244, 411–417.
[12]MatinMA,MonnaiM,OtaniH,*AnimSci J*,**2000,** 71, 197-207.
[13]LiepkeC,ZuchtHD,ForsmannWG,StandkerL,*JChromatogr*,**2001**,752, 369–377.
[14]MalkoskiM,DashperSG, O'Brien-SimpsonNM,TalboGH,MacrisM, CrossKJ, ReynoldsEC,*Antimicrob Agents Ch*,**2001**,45, 2309–2315.
[15] Lopez-ExpositoI, RecioI,*Int Dairy J*,**2006**,16, 1294–1305.
[16]KorhonenH,Pihlanto-LeppalaA,*Int Dairy J*,**2006**,16 (9), 945–960.
[17] FoxPF,*Food enzymology*, Elsevier Applied Science, New York, **1991**.
[18] IsmailB, NielsenSS,*J Dairy Sci*,**2010**, 93 (11), 4999-5009.
[19]DalasgaardTK, HeegaardCW, LarsenLB,*J Dairy Sci*,**2008**,91, 2175- 2183.
[20]McCannKB, ShiellBJ,MichalskiWP, LeeA, WanJ,RoginskiH,ConventryMJ,*Int Dairy J*,**2005**,15, 133–143.
[21]PellegriniA, ThomasU, BramazN, HunzikerP, Von FellenbergR,*BiochimBiophysActa*,**1999**,1426, 439–448.
[22] HancockREW, LehrerR,*TrendsBiotechnol*,**1998**,16, 82-88.
[23]Kutila1T, Pyörälä1S, Saloniemi1H, KaartinenL,*ActaVet Scand*,**2003**, 44, 35-42.
[24]SekseC, BohlinJ, SkjerveE, VegarudGE,*MicrobInform Exp*,**2012**, 2, 5.
[25] McDonnellMJ, RivasL, BurgessCM, FanningS, DuffyG,*Int J Food Microb*,**2012**, 153, 260–268.
[26] ChristmanJM, Masters theses, Tennessee University (Tennes, USA, 2010)
[27]VongsawasdiP, NopharatanaM, SupanivatinP,Matthana P,*Asian J Food Agro Ind*,**2012**, 5, 52-60.