Effect of Detergent and Antibiotic Residuals on Proteolytic Activity of Plasmin on Beta Casein

Nahed Soliman and Eman El Dakhakhny[1]

ABSTRACT

Plasmin plays a significant role in cheese ripening and characteristics of dairy products. The antibiotic could be presented in milk as residuals from animal treated by antibiotic, while presence of detergent could be as residuals from cleaning of milking machines, tanks during transpirations, storage tanks and pasteurization equipments etc. The effect of detergents and antibiotic residuals on proteolytic action of plasmin on β-casein in model solution was determined by Urea-polyacrylamide gel electrophoresis. The results suggested that plasmin followed the proteolytic pathway on cow β-casein to produce γ_1, γ_2 and γ_3 as the degradation fragments. The proteolytic pathway has been affected by presence of sodium hydroxide and nitric acid, while was not affected by presence of antibiotic. The results also indicated that the presence of sodium hydrioxid increased the plasmin activity, while plasmin hydrolysate of B-casein slightly inhibited by nitric acid. The proteolytic activity of plasmin on β-casein was broader and higher at the end of incubation period, to yield low-sized fragments as evidenced with polyacrylamide gel electrophoresis. Increased plasmin activity by sodium hydroxide residual could affect cheese making characteristics such as the rennet coagulation time, curd strength, and curd syneresis, produce off-flavor and bitterness in cheese. Since the plasmin is heat stable enzyme, increasing its activity in UHT milk could case gelatoin and produce off-flavor and bitterness.

Key Words: plasmin, β-casein, detergent and antibiotic residuals.

INTRODUCTION

Plasmin (fibrinolysin E.C.3.4.21.7), the principle indigenous proteinase in milk, is a serine proteinase with trypsin– like activity with pH optimum of ~ 7.5, it cleaves bonds of the type Lys – X and to a lesser extent, Arg– X. The properties and significance of plasmin have been extensively reviewed (Bastian and Brown, 1996). In milk, α_{s1} and β – casein are hydrolysed rapidly by plasmin (Andrews and Alichanidis, 1983). α_{s1} – casein is degraded more slowly, while κ-casein is very resistant (Eigel, 1977). Plasmin activity results in the formation of γ-casein, λ-casein and proteose peptone. γ–casein results from the hydrolysis of β–casein by plasmin (Swaisgood, 1982), while λ–casein fraction consists of peptides produced from α_{s1} – casein (Aimutis and Eigel, 1982). The proteose peptone fraction consists mainly of peptides produced from casein by the action of plasmin (Andrews and Alichanidis, 1983). The specificity of plasmin on β–casein is well documented (Eigel, 1977 and 1981). As well as producing γ_1 – (B-CNF29-209), γ_2– (B-CNF-106-209), γ_3–(B-CNF-108-209) casein and their complementary N – terminal peptides, plasmin can also cleave bonds Lys_{113} – Lys_{114} and Arg_{183} – Asp_{184} in β– casein fairly rapidly (Le Bars and Gripon, 1993). Enzyme activity increased in milk with stage of lactation, severity of mastitis infection and lactation number (Eric and Rodney, 1995). Since plasmin is strongly associated with the casein micelles in milk, it is incorporated into the curd during cheese – making and is active in cheese during ripening. The level of plasmin activity depends on the cheese variety (Cait and Patrick, 1999). In ripened cheese, such as cheddar, plasmin activity is evident by the limited hydrolysis of β–casein with a concomitant increase in the γ–casein. The extent to which plasmin contributes to proteolysis during ripening is well defined and it was thought for a long time to be limited to the slow hydrolysis of β–casein. However, Farkye and Fox (1991 and 1992) found that plasmin contributes to both the level and type of water soluble peptides formed from casein during cheddar cheese ripening. The enzyme is heat resistant and survives many UHT treatments, but its role in the gelation of UHT – treated milk is not fully understood (Eric and Rodney, 1995).

Proteolysis of proteins is the major activity attributed to plasmin in the milk. Casein is most susceptible to breakdown by this enzyme. Among the various classes of casein, ß-casein is more susceptible than αs-casein, and k-casein is somewhat resistant to breakdown by the plasmin. Breakdown fragments of casein can produce off-flavor and bitterness in milk. In contrast, milk whey proteins such as α-lactalbumin and ß-lactoglobulin are fairly resistant to the action of plasmin (Bastian and Brown 1996).

Increase plasmin activity decreases the viscosity of a caseinate solution. This is especially noticed in milk from older cows which has higher plasmin activity. Plasmin affects cheese making characteristics of milk such as the rennet coagulation time, curd strength, and curd syneresis (Sousa et al., 2001). Mozzarella cheese made from late lactation milk, which contains higher amounts of plasmin, has inferior stretchability and melting ability. Proteolysis of casein by plasmin also

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increases the moisture content in cheddar cheese. Swiss cheese contains higher plasmin activity than cheddar cheese because the higher cooking temperature required for Swiss cheese destroys the inhibitors of plasmin and plasminogen activators. As a result, more breakdown of casein occurs during ripening of Swiss cheese as compared to cheddar cheese (Bastian and Brown 1996).

The dirt on tanks, pipes, cheese vats and other surfaces is normally clean with alkaline and acid detergents. The milk may contain residues of antibiotics emanating from treatment of cows suffering from mastitis; the most commonly occurring one is penicillin.

No data are available from studing the effecting of detergents and antibiotic residuals on proteolytic activity of plasmin in milk and cheese. The objective of the present work was to determine the effect of detergents and antibiotic residuals on proteolytic action of plasmin on β-casein in model solution by analysing the degradation products using urea-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Materials

Bovine plasmin (fibrinolysin, E.C.3.4.21.7, from bovine plasma) was obtained from Sigma chemicals. It was dissolved in distilled water (34.46 mg/L); this solution had 1.3 units of plasmin activity, where 1 unit is the amount of enzyme that will produce a ΔA_{275} of 10 from α_{s1} – casein in 20 min at pH 7.5 and 37 °C.

β–casein **was** obtained from Sigma chemicals. It was assessed by urea– polyacrylamide gel electrophoresis (PAGE).

Detergents: Sodium hydroxide and Nitric acid were added individually at concentrations of 20 and 50 mg/L, respectively.

Antibiotics: Draxin (antibiotic1) and Marbocyl(antibiotic2) were added individually at concentrations of 50 and 100 mg/L, respectively.

Preparation of β–casein solution

β-casein (5 mg/L) was dissolved in 0.1 M sodium citrate buffer at pH 8. Sodium azide (NaN_3; 0.02 %, w/v) was added to the solution (and all other solutions) to inhibit bacterial growth. The solution was stirred for 1 h (pH 7.6) followed by the addition of 4 – 5 drops of 0.1 or 1 N HCl to adjust the pH at 7.0 by stirring for 15 min. Such solution was transferred into the refrigerator and incubated overnight. After that, it was heated at 78 °C for 15 min followed by cooling at room temperature.

Hydrolysis conditions

1- Effect of antibiotics on the proteolysis of β–casein by bovine plasmin:

Solution of β–casein (5mg/L) containing 0, 50 or 100 mg/L of Draxin and Marbocyl individually prepared. Bovine plasmin (0.25 U/L) was added at 37 °C. Samples were taken after 0, 6, 12 and 24 h and heated at 100 °C for 15 min to inactivate plasmin.

2- Effect of detergent on the proteolysis of β–casein by bovine plasmin:

Solution of β–casein (5 mg/L) containing 0, 20 or 50 mg/L Sodium hydroxide and Nitric acid were individually. Bovine plasmin (0.25 U/L) was added and the solution was incubated at 37 °C. Samples were taken after 0, 6, 12, and 24 h and heated at 100 °C for 15 min to inactivate plasmin.

Urea – polyacrylamide gel electrophoresis (Urea – PAGE)

Gel electrophoresis was performed according to the method of Andrews (1983) with separation gels of T = 12.5 %, C = 4 % and 4.5 M Urea (separation gels buffer was composed of 4.6 % Tris and its pH was adjusted to 8.9 by the addition of HCl). The running buffer was composed of 15 g Tris and 73 g Glycine and was dissolved in 5 L of distilled water and gels were run at approximately 25 V/cm for about 75 – 90 min until Bromophenol blue tracking dye was close to the bottom of the slab. Staining was carried out for 1 h in 0.25 % (w/v) Coomassie brilliant blue G-250 dissolved in 50 % methanol and 12.5 % TCA, while destaining was carried out in a solution of 7 % acetic acid. Sample buffer was 10 % stacking gel buffer containing 8 M Urea and 2 % 2 – mercaptoethanol, and 0.01% Bromophenol. 200 µl of sample was dissolved in 1 ml sample buffer and centrifuged at 5000 xg for 30 min at 4 °C. 5 µl of each sample was applied to the gel.

Gel scanning

Band scanning was carried out by Toshiba Scanner. Quantitative determination of the fragments was made by Molecular Dynamic Image Quant v 5 software.

RESULTS AND DISCUSSION

The hydrolysis of β-casein by the plasmin during incubation time up to 24 h is shown in Figures 1, 2,3 and 4. Electrophoretic chromatogram shows that the action of plasmin on β-caseins released small molecular weight three peptides as evidenced by the relative electrophoretic mobility, since the most sensitive peptide bonds to plasmin are Lys_{28}-Lys_{29}, Lys_{105}-His_{106}, and Lys_{107}-Glu_{108} in cow β-Casein (Andrews,1978 a,b).

The results showed that B-casein was degraded more rapidly by plasmin and high mobility peptides (peptide1, peptid2 and peptid3) were produced after 1hr.

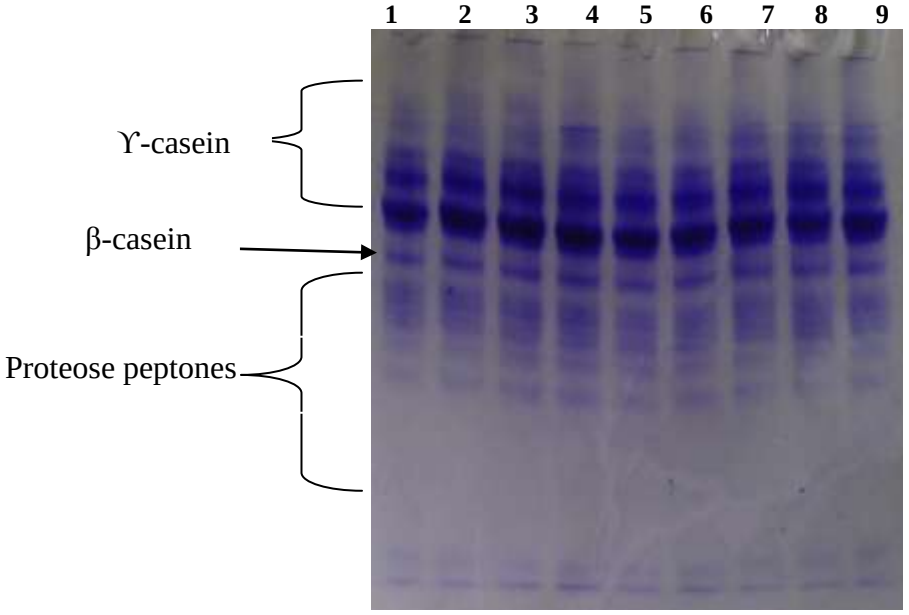

Figure 1. Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin at 1h in the presence of detergentand antibiotic residuals
Lane 1: control; lanes 2-3: antibiotic 1 at 100 and 50 mg/lrespectively
Lanes 4- 5: antibiotic 2 at 100 and 50 mg/l respectively
Lanes 6-7: NaOH at 50 and 20 mg/l respectively
Lanes 8-9: HNo_3 at 50 and 20 mg/l respectively

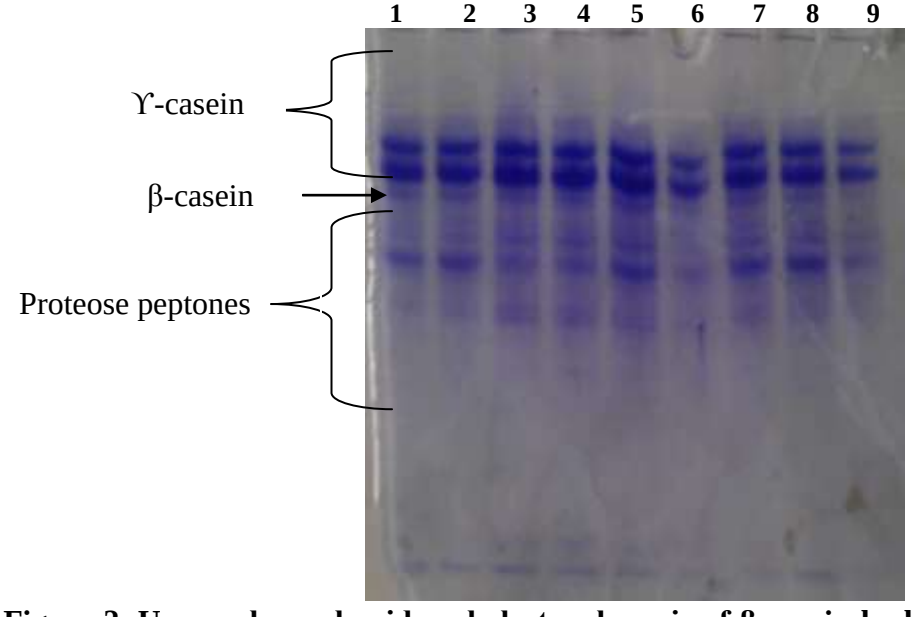

Figure 2. Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin at 6 h in the presence of detergent and antibiotic residuals
Lane 1: control; lanes 2-3: antibiotic 1 at 100 and 50 mg/l respectively
Lanes 4- 5: antibiotic 2 at 100 and 50 mg/l respectively
Lanes 6-7: NaOH at 50 and 20 mg/l respectively
Lanes 8-9: HNo_3 at 50 and 20 mg/l respectively

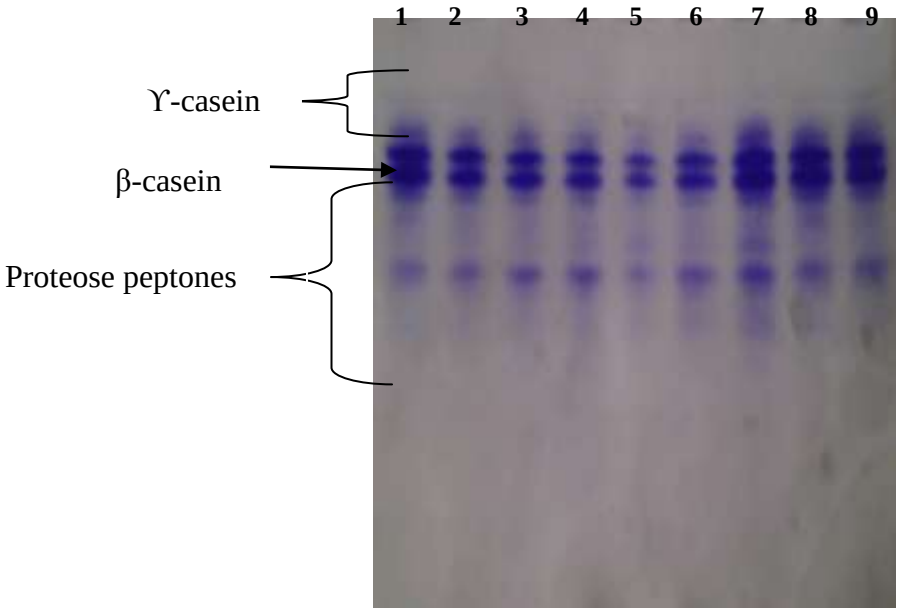

Figure 3. Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin at 12h in the presence of detergent and antibiotic residuals

Lane 1: control; lanes 2-3: antibiotic 1 at 100 and 50 mg/l respectively

Lanes 4- 5: antibiotic 2 at 100 and 50 mg/l respectively

Lanes 6-7: NaOH at 50 and 20 mg/l respectively

Lanes 8-9: HNo_3 at 50 and 20 mg/l respectively

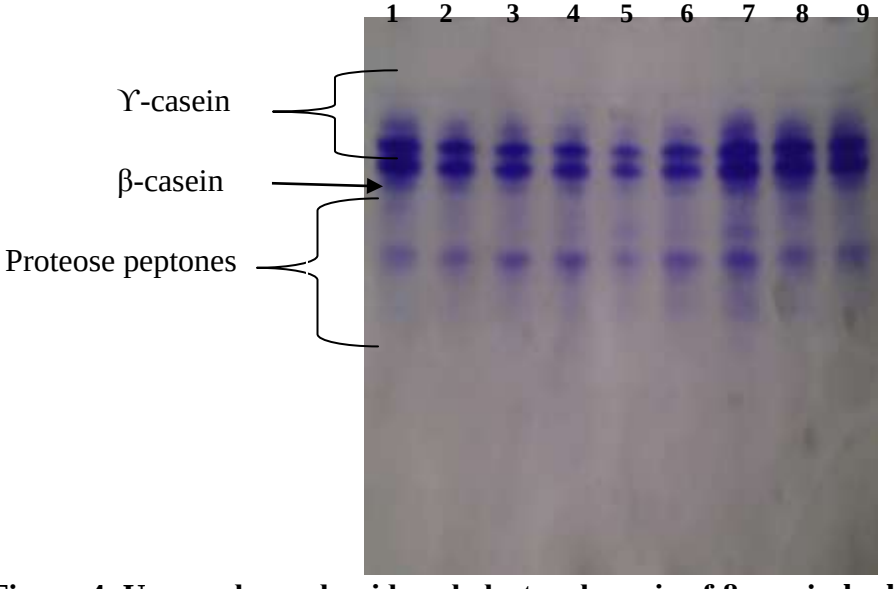

Figure 4. Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin at 24h in the presence of detergent and antibiotic residuals

Lane 1: control; lanes 2-3: antibiotic 1 at 100 and 50 mg/l respectively

Lanes 4- 5: antibiotic 2 at 100 and 50 mg/l respectively

Lanes 6-7: NaOH at 50 and 20 mg/l respectively

Lanes 8-9: HNo_3 at 50 and 20 mg/l respectively

of incubation and their corresponding proteose pepton. Peptide1 was further hydrolysate and disappeared at the end of incubation time, but peptide2 was gradually increased and not degraded during incubation period. This peptide is seen in all electrophoretic profile of all treatment. While peptide3 was not clear during 24hr. of incubation (figure 5) .Low migration peptide which are known γ-casein on urea-PAEE (Awad 2002) were produced from B-casein after 1hr. up to 6hr. of incubation and degraded thereafter (Figures 1 and 5).The hydrolysate products of B-casein were previously isolated and identified by cait et al (1999).It should be noted that some of the small peptides produced by action of plasmin on B-casein or sodium caseimate.

The hydrolysis of B-casein by plasmin in presence antibiotics during 24hr. incubation shown in (figures 1, 2, 3 and 4) There was no different between control and treatment of antibiotics (Draxin and Marbocyl) at both concentration used in this study (50 and 100 ml/l) during 6hr. of incubation .The hydrolysis of B-casein by plasmin was slightly increased during 12hr. of incubation up to the end of incubation period specially in presence Marbocyl (antibiotic2) (figure 6 and 7). Seaman et al (1988), measured somatic cell count , total proteolytic activity and plasmin activity in cow milk during pre-infection, infection and post infection by mastitis. All parameters increased significantly during infection, they concluded that even after curing mastitis, plasmin activity does not return to its pre-level

The electrophoretogram of plasmin hydrolysat of B-casein shows that there was a high molecular weight of band corresponding to γ- casein was seen during 1hr.

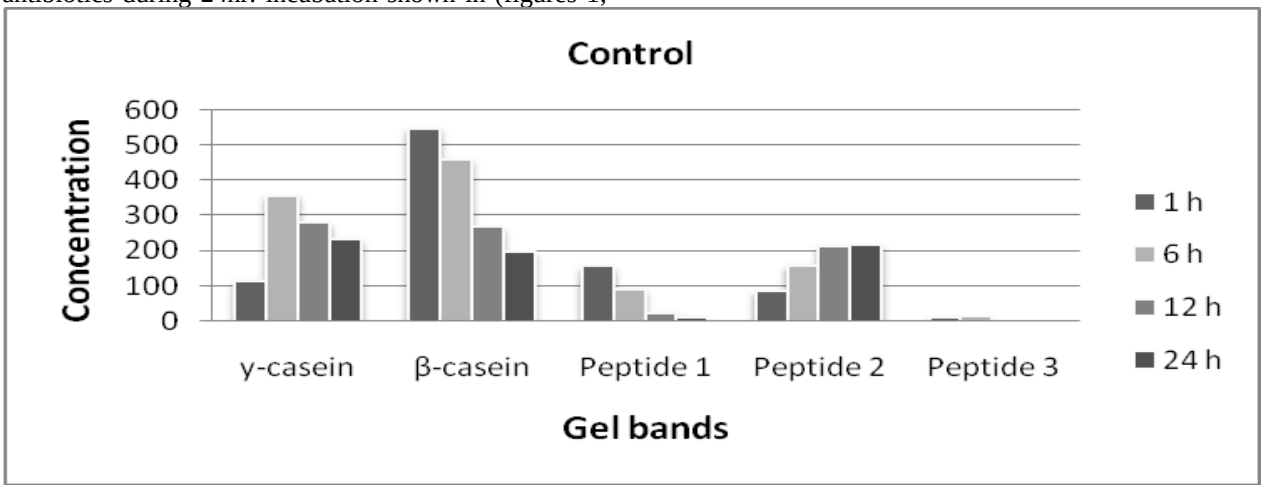

Figure 5. Scanning of Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin at 1, 6, 12 and 24 h

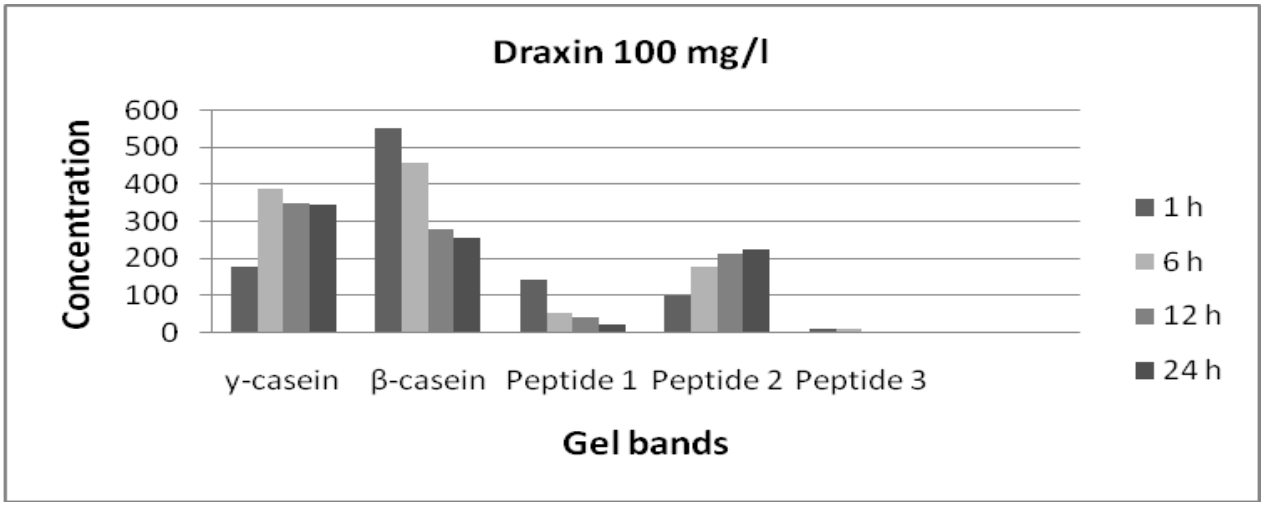

Figure 6. Scanning of Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin in the presence of Draxin antibiotic (100 mg/l) at 1, 6, 12 and 24 h

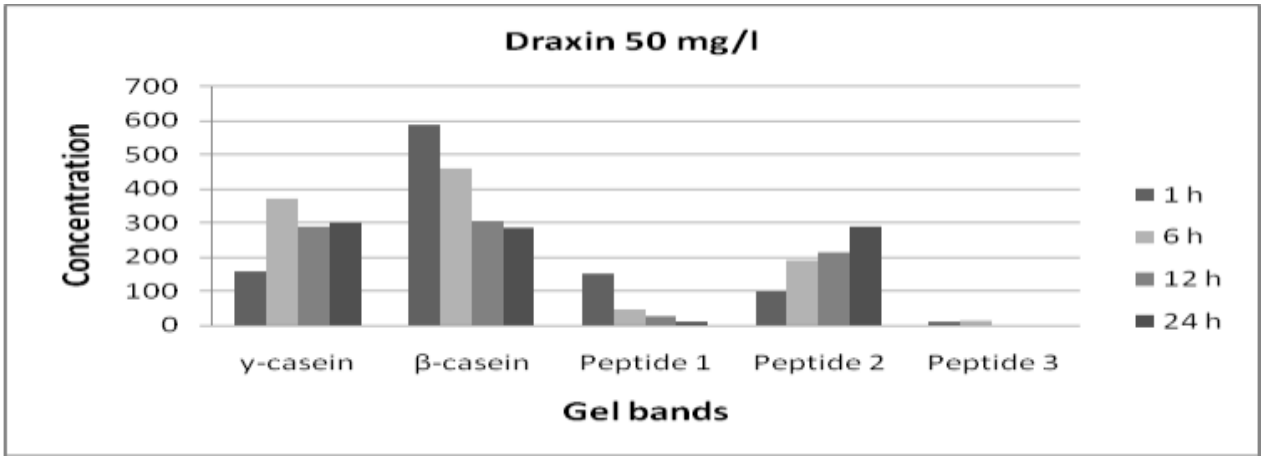

Figure7. Scanning of Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin in the presence of Draxin antibiotic (50 mg/l) at 1, 6, 12 and 24 h

The electrophoretogram of plasmin hydrolysat of B-casein shows that there was a high molecular weight of band corresponding to γ- casein was seen during 1hr.up to 6hr. of incubation and not degraded thereafter, while peptide 1 and peptide 2 were similar with control at all stage of incubation in both two antibiotics (fig 5,6,7,8 and 9) indicating that the specificity of plasmin was not influenced by antibiotics.

The rate of B-casein degradation by plasmin with added sodium hydroxide (NaOH) at 20 and 50 mg/l during 24hr.of incubation shown in (figures 10 and 11) .Results indicated that the plasmin activity was increased by added NaOH. B-casein was degraded by plasmin in presence of NaOH to high molecular weight peptides (γ-casein, peptide 1,peptide 2) with electrophoretic mobilities faster than B- casein specially at 50 mg/l con.(figure10) and further more hydrolysed with increasing incubation time .All B-casein peptides were hydrolysed at faster rate than control and more degraded after 6hr. of incubation and disappeared at the end of incubation, indicating that B-casein and its peptides were more susceptible to the enzyme at high pH values (Fox and stepaniak ,1993) suggested that β-casein is hydrolysed more quickly at high pH by indigenous or added plasmin. It is hydrolysed to γ-caseins and certain proteose peptones (PP). The γ1, γ2 and γ3-caseins correspond to residues 29-209, 106-209 and 108-209 of β-casein, respectively. PP5 consists of two peptides corresponding to β-casein (f1-105) and (f1-107), PP8-fast is β-casein (f1-28) and PP8-slow may be β-casein (f29-105/107).

Also results are shown some of high mobility peptide (peptide 3) that was detected at 1hr. of incubation, then further hydrolysed and disappeared at the end of incubation in control while this peptide was not detected in presence of sodium hydroxide. Peptide2 was decreased with increasing the incubation time to reversely trend the control.

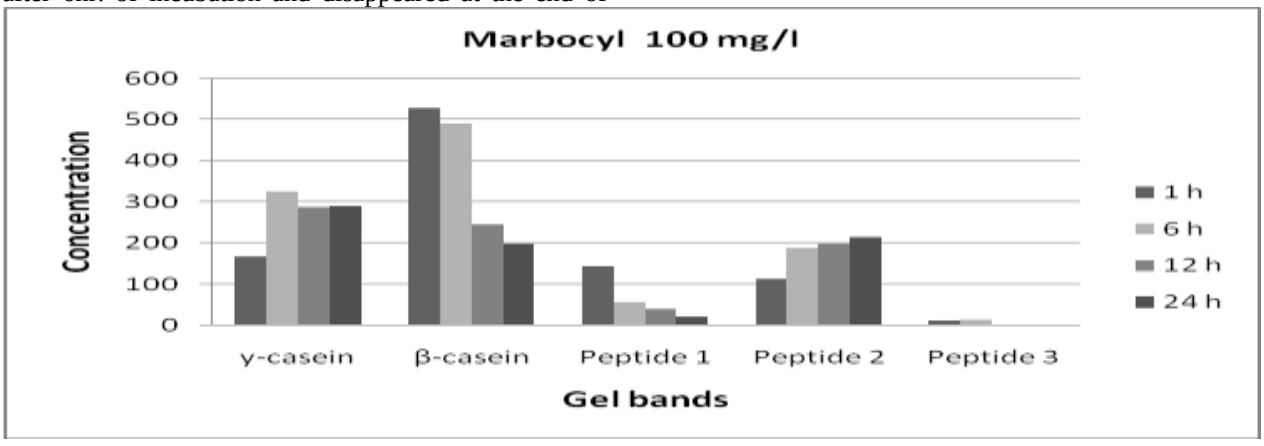

Figure 8. Scanning of Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin in the presence of Marbocyl antibiotic (100 mg/l) at 1, 6,12 and 24 h

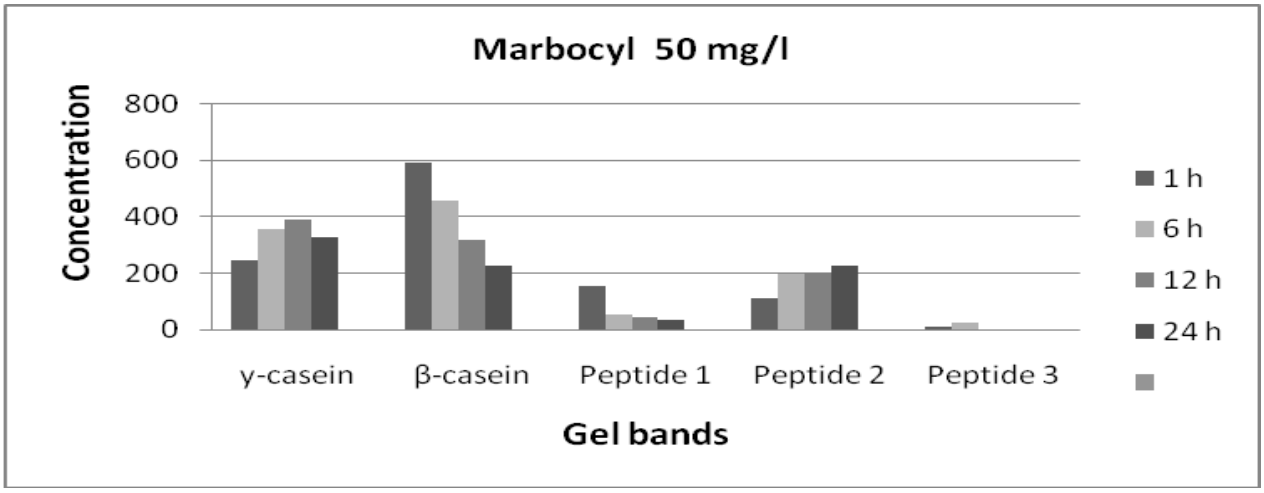

Figure 9. Scanning of Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin in the presence of Marbocyl antibiotic (50 mg/l) at 1, 6, 12 and 24 h

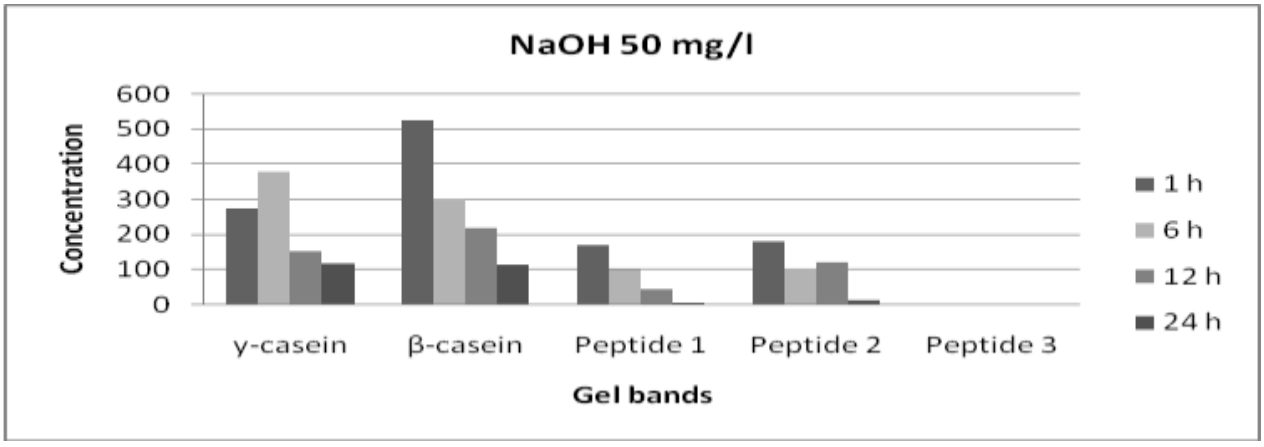

Figure 10. Scanning of Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin in the presence of NaOH (50 mg/l) at 1, 6, 12 and 24 h

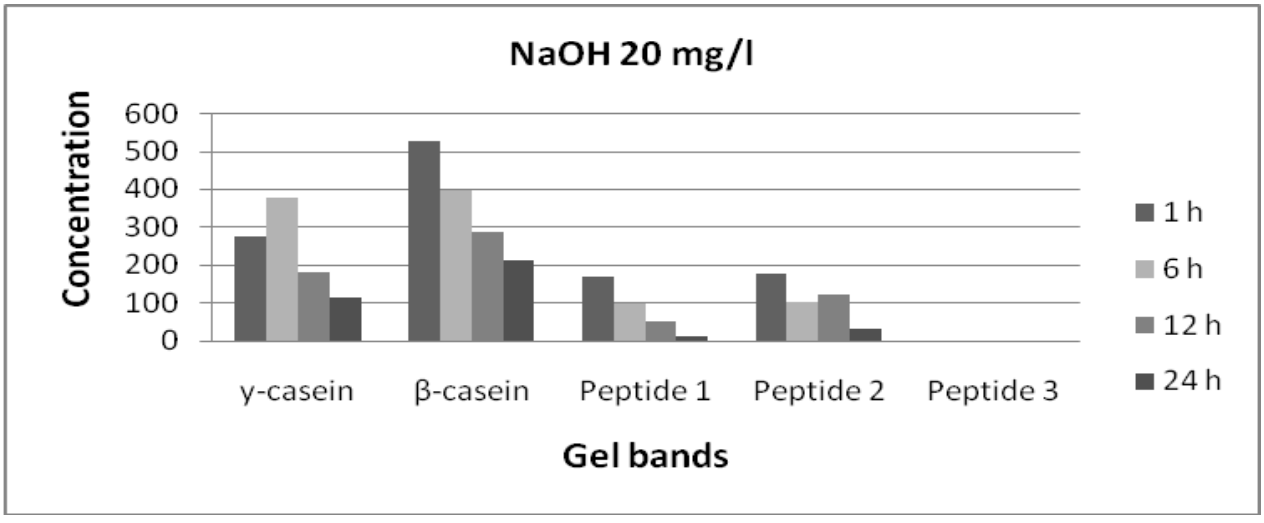

Figure11. Scanning of Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin in the presence of NaOH (20 mg/l) at 1, 6, 12 and 24 h

The effect of Nitric acid on the hydrolysis of B-casein by plasmin illustrated in (figure 12 and 13). The electrophoretograme of plasmin hydrolysat of B-casein was inhibited slightly by Nitric acid and more affected at high concentration (5o mgl/L). The bands of electrophorelogram corresponding to γ-casein, peptide 1 and peptide 2 were seen at low concentration in B-casein hydrolysate after 6hr. of incubation, these three bands migrated faster than B-casein could be seen after 1hr. of incubation in control (figure 5). The γ-casein and proteose peptone(peptide 1 and peptide 2) in the electrophoretic profile of hydrolysate after 6hr. incubation was not hydrolysate during 24hr.of incubation (figure 4,12and 13).Lan and Fox (1999) showed that the activity of plasmin on B-casein was influenced by pH, the rat of hydrolysis increased with increasing pH value from pH 6.5 to 8.4.

CONCLUSION

Plasmin plays a significant role in cheese ripening and characteristics of dairy products. The antibiotic could be presented in milk as residuals from animal treated by antibiotic. While presence of detergent could be as residuals from cleaning of milking machines, tanks during transpirations, soil tanks, pasteurization equipments etc. Activity of plasmin is increased in present of small amounts of sodium hydroxide, this can increase degradation rate of casein and affect the processing of dairy products. Breakdown of milk proteins by proteases affects milk clotting, cheese ripening, and flavor and texture of dairy products. Breakdown fragments of casein by plasmin can produce off-flavor and bitterness in milk. Increased plasmin activity decreases the viscosity of a caseinate solution. Thickening, gelation, and coagulation of milk occurs during storage. This is also attributed to the proteolytic activity either from milk proteases such as plasmin or proteases of bacterial origin. Proteolytic activity caused by plasmin has a particular effect on the taste of milk (appearance of bitter peptides). Bitter peptides produced by the action of proteases influence the flavor of UHT milk, UHT cream, and cheese.

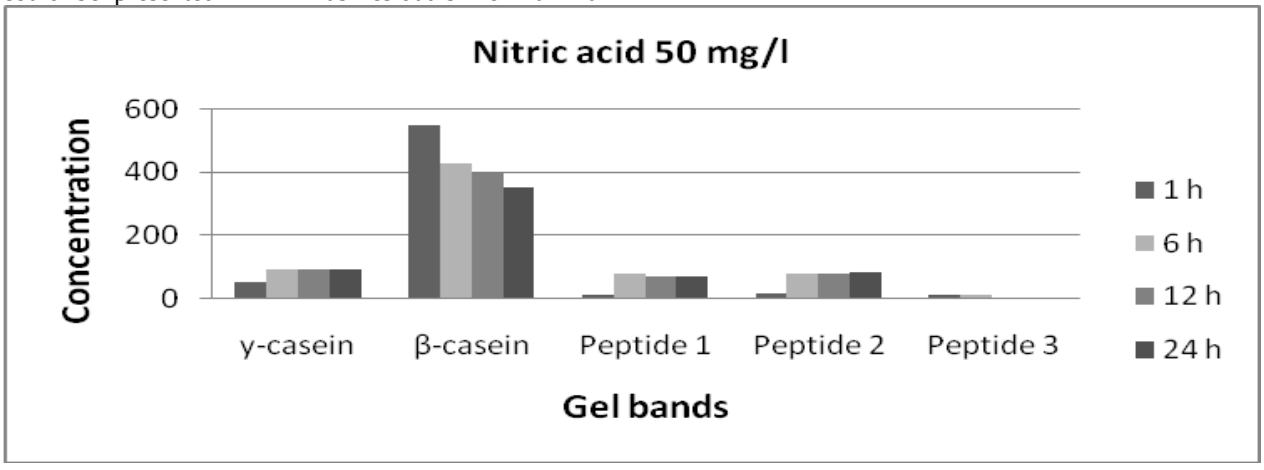

Figure 12. Scanning of Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin in the presence of HNo_3 (50 mg/l) at 1, 6, 12 and 24 h

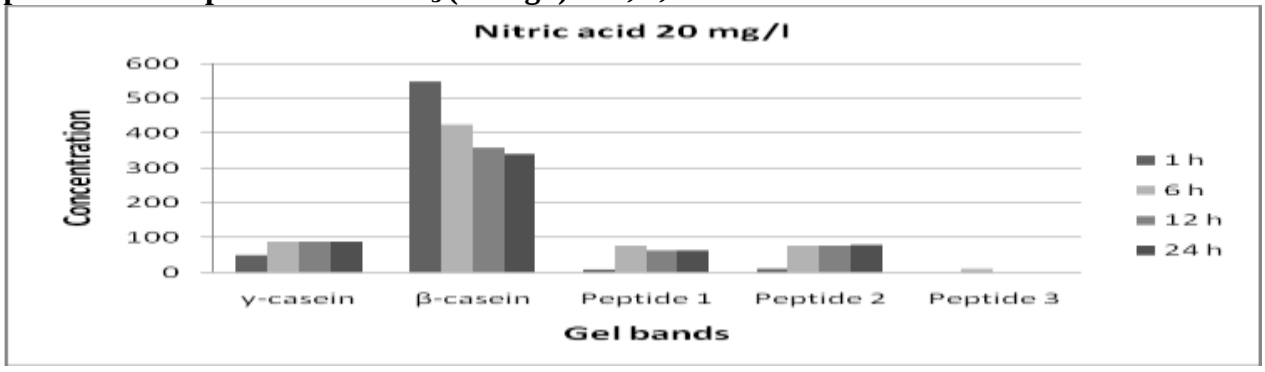

Figure 13. Scanning of Urea-polyacrylamide gel electrophoresis of β-casein hydrolysed by plasmin in the presence of HNo_3 at (20 mg/l) at 1, 6, 12 and 24 h

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الملخص العربي

تأثير متبقيات المضادات الحيوية والمنظفات علي النشاط التحللى لانزيم البلازمين على البيتا كازين

ايمان الدخاخني وناهد السيد سليمان

يلعب انزيم البلازمين دورا معنويا في تسوية الجبن وخواص المنتجات اللبنية. توجد المضادات الحيوية في اللبن كمتبقيات من معالجة الحيوانات بينما وجود متبقيات المنظفات يرجع اليي عمليات التنظيف لماكينات الحلابة والتنكات اثناء عمليات النقل والتخزين واجهزة البسترة. تم دراسة تاثير متبقيات المضادات الحيوية والمنظفات على النشاط التحللي للبيتا كازين في نظام المحاليل المقدرة بواسطة الهجرة في المجال الكهربي.

أثبتت النتائج ان البلازمين يحلل البيتا كازين الى ببتيدات صغيرة متحللة (γ1- γ 2 and γ3) وتاثر هذا التحلل في وجود كلا من هيدروكسيد الصوديوم وحامض النيتريك ولكن لم يتاثرفي وجود المضادات الحيوية.

كما اشارت النتائج ان نشاط البلازمين زاد في وجود هيدروكسيد الصوديوم وكان ذلك واضحا في نهاية فترة التحضين منتجا جزيئات صغيرة الحجم ظاهرة في جيل الأكريلاميد المبلمر بواسطة الهجرة الكهربية ولكن وجود حامض النيتريك ادى الى تثبيط بطيئ لنشاط الانزيم. زيادة نشاط انزيم البلازمين في وجود متبقيات هيدروكسيد الصوديوم قد يؤثر على خواص عملية التجبن مثل وقت التجبن وقوة وتشريش الخثرة كذلك ظهور مرارة وقصور في طعم الجبن. وبما ان البلازمين ثابت حراريا فان زيادة نشاطه في اللبن المعامل بالتسخين الفوق عالي يسبب تكوين الجيل وقصور ومرارة في الطعم.